Transcription factor-specific reporter constructs - basis for the functional identification and isolation of subpopulations of human mesenchymal stem cells and tool for live cell approaches

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TRANSCRIPTION FACTOR-SPECIFIC REPORTER CONSTRUCTS –
BASIS FOR THE FUNCTIONAL IDENTIFICATION AND ISOLATION
OF SUBPOPULATIONS OF HUMAN MESENCHYMAL STEM CELLS
AND TOOL FOR LIVE CELL APPROACHES

A thesis submitted to attain the degree of
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Summary

Cell-based bone tissue engineering emerged as a promising strategy for bone tissue regeneration to overcome the limitations of bone grafting and bone graft alternatives. From the currently available clinical data of cell-based bone tissue engineering, it has become clear that more reproducible osteogenic cell populations for improved bone formation, as well as cell populations with endothelial potential to account for sufficient vascularization, harbor the potential to improve bone tissue engineering. However, adult stem and progenitor cell isolation is hampered by the lack of specific and robust cell surface markers required to reliably perform cell characterization and isolation. Since the resulting heterogeneity of adult stem and progenitor cell populations represents a major obstacle for the progress of adult stem cell biology and applied disciplines, such as bone tissue engineering, the present work was aimed at establishing a novel approach for the identification and isolation of osteoprogenitor and endothelial progenitor cell (EPC) populations from human bone marrow stromal cells (BMSC) and mononuclear cells, respectively. Functional isolation was approached by coupling the expression of a key transcription factor, defining the subpopulation of interest, to the expression of green fluorescent protein (GFP) reporter gene. This reporter system was used to discriminate and isolate cell populations based on fluorescence by means of fluorescence activated cell sorting (FACS).

Several non-viral and viral reporter constructs were generated, each of which was responsive to a particular transcription factor relevant for osteogenesis or endothelial biology.

As regards osteoprogenitors, the well-documented master transcriptional regulator of osteoblast differentiation, RUNX2, accounted for the specificity of the adenoviral reporter Ad.Runx2. Osteogenically induced human BMSCs transduced with Ad.Runx2 were subdivided and the reporter-positive cell population exhibited characteristics appropriate for osteoprogenitors. The pattern of in vitro osteogenic differentiation capability amongst the resulting cell populations in comparison with the original BMSC population indicated a crosstalk between cell populations. Inconsistent results initiated a re-evaluation phase that indicated the original hypothesis, claiming that a single reporter responsive to an appropriate transcription factor is sufficient to reproducibly isolate the progenitor cell population of interest, was incorrect. Thus a new hypothesis, namely that dual reporter systems are required, was proposed.
Extension of the adenoviral GFP reporter system to a lentiviral luciferase reporter system circumvents transient reporter expression and low signal sensitivity. Combined with a standardized cell population, these new cell lines are aimed at additionally utilizing the reporter system for biomaterials testing or drug screening. We performed a candidate drug testing approach to investigate the natural compound resveratrol that had been reported to upregulate Runx2 gene expression for its ability to induce a detectable increase in reporter expression in the self-generated lentivirally transduced stable Runx2 reporter hTERT-MSC system. Stable Runx2 reporter hTERT-MSCs were generated by transduction of human MSCs immortalized through expression of human telomerase reverse transcriptase (hTERT) (hTERT-MSC) with lentiviral Runx2-specific luciferase reporter constructs. The experiment revealed insignificant differences in reporter expression upon resveratrol treatment, indicating that the stable Runx2 reporter hTERT-MSC system is insufficiently sensitive in the performed drug testing approach.

To produce the EPC-specific adenoviral reporters, the transcription factors HOXA9 and VEZF1 were investigated, based on published data present at that time. In vitro validation of these adenoviral reporters revealed that neither of the transcription factors are EPC-/endothelial-specific, which prompted us to not perform EPC isolation experiments.

Collectively, we have established the first step in a novel and versatile approach to functionally identify and isolate progenitor cell populations. Our novel approach fills a niche in the spectrum of possibilities to isolate more homogeneous stem and progenitor populations. Transcription factor-specific reporters not only serve as basis to functionally subdivide and isolate committed subpopulations of stem cell populations, but also as tool for applied approaches such as drug screening and biomaterials testing.

Our results contributed two major pieces of insight to the usage of transcription factor-specific reporters. First, transcription factors need to be considered as players within a regulatory network, which renders single transcription factor-specific reporters prone to inconsistency and lack of robustness. Instead, multiple reporters may serve as more reliable and satisfying indicators of cell lineage specification processes. Second, transcription factor-specific reporters are not necessarily dependent on a transcription factor that is cell type- and tissue-specifically expressed, but need to meet the criterion for specificity only in the range of cell fates the starting cell population can give rise to. These findings have implications both reaching basic research applications and touching clinical applications in the field of stem and progenitor cell biology.
Zusammenfassung


Mehrere nicht-virale und virale Reporterkonstrukte wurden hergestellt, von denen jedes einzelne auf einen bestimmten für die Knochen- oder Blutgefässbildung relevanten Transkriptionsfaktor anspricht.

Ergebnisse erfolgte eine Neubewertung, welche zeigte, dass die ursprüngliche Hypothese wonach ein einzelner Reporter, der auf einen geeigneten Transkriptionsfaktor anspricht, ausreicht, um die untersuchte Vorläuferzell-Population reproduzierbar zu isolieren, inkorrekt war. Es wurde daher die neue Hypothese, gemäß welcher ein doppelter Reporter dazu notwendig ist, aufgestellt.


Für die Erzeugung von für endotheliale Vorläuferzellen spezifischen adenoviralen Reportern wurden die Transkriptionsfaktoren HOX A9 und VEZF1, auf der Basis publizierter Daten, untersucht. Die In-vitro-Validierung der beiden adenoviralen Reporter ergab, dass keiner der Transkriptionsfaktoren spezifisch für endotheliale Vorläuferzellen bzw. Endothelzellen ist. Dies veranlasste uns, die Experimente zur Isolation von endothelialen Vorläuferzellen nicht durchzuführen.

Scope of the thesis

Motivation

Bone tissue engineering approaches unify three key factors, namely a biocompatible, three-dimensional scaffold, osteogenic cells which are seeded onto the scaffold, and growth factors that are applied in a controlled manner. Although all three building blocks are indispensable for potent bone tissue engineering, clinical data revealed application of a more reproducible osteogenic cell population can potentially yield more pronounced bone formation and improve bone tissue engineering. In addition, sufficient vascularization within the newly formed bone represents the determining factor for the volume of vital bone that can be obtained. Vascularization of the bone construct can be stimulated by co-seeding of cells with osteogenic as well as endothelial potential. Stem and progenitor cell populations with osteogenic and endothelial potential exhibit a larger potential for bone formation and vascularization due to their larger proliferative capacity compared with the mature and fully differentiated counterparts.

However, adult stem and progenitor cell isolation is hampered by the lack of specific and robust cell surface markers required to reliably perform cell characterization and isolation. In fact, significant research has been focused on CD marker-based attempts to isolate more homogeneous human MSC subpopulations that are able to discriminate MSCs and subpopulations thereof. To date, no CD marker has been reported that can be used to specifically isolate MSCs. This circumstance implicates that MSCs as starting population for adult stem cell biology, as well as bone tissue engineering, need to be considered as heterogeneous, which in turn provides inherent inconsistency of the experimental outcomes. Similar to MSCs, there also exists a lack of specific markers to differentiate EPCs from endothelial cells and hematopoietic cells, respectively.

Hence, there is a critical need for more reproducible identification and isolation of MSCs and EPCs. Not only is it exciting to investigate whether the obtained cell populations indeed show a more homogeneous in vitro behavior than the initial cell populations or cell populations isolated by means of CD markers, but also to find out how the cell populations perform in an in vivo situation.
Specific aims

The overall aim of this thesis was to establish a novel approach for the identification and isolation of osteoprogenitors and EPCs from human bone marrow stromal cells and mononuclear cells, respectively based on transcription factor-responsive reporter constructs that allow the isolation of a cell population of interest based on a functional, lineage relevant protein, and not based on CD markers or mRNA expression. The work is divided into four specific aims outlined below.

Part 1 of this work (chapter 3) encompassed different establishment and optimization experiments, which ultimately aimed at establishing a basis for the future cell culture experiments to isolate osteoprogenitors by means of the previously generated adenoviral GFP reporter responsive to functionally active RUNX2 protein, Ad.Runx2. Due to the fact that the promoter of Ad.Runx2 is tailor-made, a first aim was to investigate the responsiveness of Ad.Runx2 to RUNX2 protein. Additional aims were to determine and optimize the conditions for the planned FACS experiments, encompassing (1) a cell seeding density that allows for sufficient persistence of reporter expression, (2) determination of the optimal ratio of infectious viral particles to target cells required to obtain a detectable reporter expression while at the same time keeping the cytotoxic effect of adenoviral transduction low, (3) the optimal time point to perform cell sorting of Ad.Runx2-transduced BMSCs, but also (4) the establishment of the cell preparation protocol for cell sorting and cell viability of sorted cells.

In part 2 of this work (chapter 4), we aimed at performing FACS-mediated isolation of osteoprogenitors from human BMSCs transduced with Ad.Runx2, followed by the comparative in vitro characterization of the reporter-positive, reporter-negative, and original cell population. Once this is repeatedly confirmed, the cell populations were aimed at being investigated for their in vivo bone forming capability in a nude mouse model.

In part 3 of this work (chapter 5), the transcription factor-specific adenoviral GFP reporter system was extended to a lentiviral luciferase reporter system. A lentiviral luciferase reporter has two advantages over an adenoviral GFP reporter. First, lentiviral transduction of the reporter constructs allows for stable reporter expression due to integration of the lentiviral genome into the host cells’ genome, and second, luciferase reporter represents a more sensitive reporter compared with GFP. This new reporter system was introduced into
immortalized hTERT-MSCs for the generation of stable Runx2 reporter hTERT-MSCs. These novel self-generated cells were employed for a candidate drug testing approach using resveratrol as a natural compound reported to upregulate Runx2 gene expression, aiming at detecting a Runx2-mediated increase in reporter expression caused by the candidate drug resveratrol. We hypothesized that a stable Runx2 reporter system allows for detection of biomaterial- or drug-mediated differences in the RUNX2 protein activity, which could be linked to the osteoinductive capability of the corresponding biomaterial or drug. This way, the stable Runx2 reporter system might in future serve as a platform for high-throughput procedures to identify new bone forming drug candidates or osteoinductive biomaterials.

Part 4 of this work (chapter 6) aimed at the generation of adenoviral reporter constructs driving GFP expression controlled by the presence of putative EPC-specific transcription factors HOXA9 and VEZF1, respectively. These HoxA9- and Vezf1-responsive adenoviral reporters were originally aimed at being utilized for FACS-mediated isolation of EPCs from mononuclear cells; however, due to the findings in the in vitro validation that neither of the transcription factors are EPC-/endothelial-specific, we did not perform EPC isolation experiments.
1 General introduction

Part of chapter 1 (specifically, the knowledge about RUNX2) has been submitted for publication as a review article to European Cells and Materials.

Role and Regulation of Runx2 in osteogenesis
Marco Bruderer, R. Geoff Richards, Mauro Alini, Martin J. Stoddart

Marco Bruderer: manuscript writing. R. Geoff Richards, Mauro Alini and Martin J. Stoddart: manuscript writing, final approval of manuscript.
This chapter provides background information on bone biology and bone tissue engineering as well as two cell populations which are used in bone tissue engineering approaches, namely mesenchymal stem cells and endothelial progenitor cells. The process of bone formation is complex and tightly regulated. Elucidation of bone formation processes contributed a large body of knowledge to be applied to bone tissue engineering strategies. Due to the complexity of bone as a biologic system, it helps to consider each constituent of bone tissue engineering separately: the scaffolds, the cells, and the growth factors applied. As regards the cells, MSCs are potentially considered as the most potent cell source for bone tissue engineering. Although MSCs had been discovered several decades ago, their identification and isolation is still aimed at being optimized by searching for robust cell surface markers for isolation of MSCs. To date, CD markers are not specific enough to discriminate MSCs from other cells. As some CD marker proteins do not play a role in cell signaling, but rather are involved in cell adhesion, and not even a large set of CD markers is sufficient to reliably identify and isolate MSCs, future identification approaches may be based on characteristics that better and more specifically reflect MSC function. Characteristics that deserve consideration may encompass markers indicative of MSC propensity to differentiate or of MSC ageing which indirectly correlates with MSC multipotency (for review see [Boxall and Jones, 2012]).

Transcription factors can function as integrators for multiple signaling pathways, each of which can be stimulated by a variety of signals including those from ECM, growth factors, and mechanical signals. By regulation of specific target genes, transcription factors play crucial roles in initiating global changes in gene expression that ultimately direct cell differentiation. Hence, certain transcription factors have the status of master regulators of cellular differentiation. However, it is speculative whether the activity of a master transcription factor indeed correlates with long-term cellular differentiation and serves as a reliable indicator for complete cellular differentiation. Furthermore, the simplistic view of a transcription factor to be considered as a converging point of multiple signaling pathways is in stark contrast to the complexity of gene regulatory network, of which transcription factors are players. For example, individual transcription factors are regulated by protein-protein interactions. In addition, biologic activity of transcription factors is regulated by post-translational modifications including phosphorylation, which can have either stimulatory or inhibitory effects on the activity of the transcription factor. This complexity of regulatory mechanisms of transcription factors is exemplified by the runt-related transcription factor 2, referred to as RUNX2. A large body of research has
demonstrated and substantiated it is essential in osteoblast differentiation. In fact, RUNX2 is a protein that amongst mesenchymal lineages is specifically expressed during osteoblast differentiation. The convincing results of Runx2 overexpression and loss of function approaches performed mainly in rodent systems may even justify considering RUNX2 isolated as an indicator of osteogenesis. However, in human systems, a discrepancy between Runx2 mRNA and protein level was reported (Shui et al., 2003). Osteoblast differentiation in human MSCs has been demonstrated to be primarily associated with increases in RUNX2 protein activity, and not with increased Runx2 mRNA or protein levels (Shui et al., 2003). Because of the commonly accepted unique role for RUNX2 in osteogenesis, and especially due to the activity of RUNX2 protein as indicative of osteogenic differentiation in human systems, RUNX2 protein, or rather the level of RUNX2 protein activity, arises as a promising marker to be used for isolation purposes of human osteogenically committed mesenchymal stem/progenitor cell populations. These circumstances would render assessment of multiple genes to obtain a broader picture of gene expression behavior unnecessary, and instead could be potentially reduced to a method to track functionally active RUNX2 protein. This could be approached by a reporter assay which is responsive to functionally active RUNX2 protein, which then would render the system accessible for cell sorting by means of FACS if a suitable fluorescent reporter such as GFP is chosen.

Reporter gene assay technology sheds light on biological processes such as regulation of a gene of interest, signaling pathways, promoter activity, but also presence of individual gene regulatory proteins (Liu et al., 2009). A reporter gene assay is designed by cloning regulatory elements of interest upstream of a reporter gene. Depending on the research question, regulatory elements encompass potential or known promoters, but also response or enhancer elements. Depending on how active the regulatory element or how strong the promoter is in the cell system analyzed, little or much reporter gene mRNA is transcribed. Reporter mRNA is translated into active protein. Ultimately, the amount of reporter protein which correlates with the activity of the promoter or regulatory element being studied can be measured using an appropriate assay, resulting in a comparative estimation of the activity of the promoter or regulatory element of interest.

While single reporters track only one biological variable at a time, multiple reporter systems allow monitoring of multiple biological variables simultaneously. Although a single reporter sounds simple and practical and is frequently used, its usage is not without problems. Namely, a single reporter does not reflect the reality and complexity of the cells
and their environment they are exposed to. It is a simplistic system which nowhere near enough reflects the totality of cellular events. Nevertheless, in situations where the transcription factor, to which the single reporter is responsive, possesses a unique role in a particular biological process, like RUNX2 in the case of osteogenesis, a single reporter may be predictive enough.

Besides using the reporter assay for identification and isolation purposes, a GFP reporter assay would allow to be utilized for different possible applications such as testing of biomaterials for their osteoinductive capability or chemical library screening to identify new bone forming drug candidates. If the hypothesis that biomaterial- or drug-mediated differences in the RUNX2 protein activity levels can be correlated to the osteoinductive capability of the corresponding biomaterial or drug is true, RUNX2 protein activity would become an indicator of osteoinductive capacity of biomaterials or drugs. This would represent a major step towards an earlier reliable indicator of osteoinductive capacity compared with the current standard being calcification, assessed at late time points (Declercq et al., 2005). This finding would appear as even more valuable when considering that in vitro, calcification does not appear to represent an ideal predictive marker for in vivo bone forming capability.

1.1 Bone biology, bone tissue engineering

Bone is unique in its capability to heal without leaving scar tissue. One of the reasons why bone is able to heal itself is its high extent of vascularization. Vascularization of the bone graft is essential, as already proven by Cutting and colleagues when they showed that vascularized autologous bone grafts were superior to unvascularized ones during repair (Cutting and McCarthy, 1983). Later, the important role of the vasculature for bone integrity was further underlined by the findings that bone vascularization is not only important for calcium homeostasis, but also plays a crucial role during bone regeneration by providing the damaged tissue with nutrients, oxygen, growth factors, and also precursor cells (Trueta and MORGAN, 1960; Trueta and LITTLE, 1960).

1.1.1 Bone formation/ossification

The skeletal system is an organ derived from mesoderm and its development starts with mesenchymal condensations, in which skeletal progenitor cells either give rise to
chondrocytes of cartilage or osteoblasts of bone. Skeletal cells are derived from three different embryonic cell lineages: cranial neural crest cells give rise to the craniofacial skeleton, sclerotome cells from somites contribute to the axial skeleton, and lateral plate mesodermal cells generate the limb skeleton (for review see (Olsen et al., 2000)). Bone formation (osteogenesis) starts during prenatal development and persists throughout adulthood. There are two distinct developmental modes of osteogenesis: intramembranous ossification, that is, bone formation that takes place directly, and endochondral ossification, that is, bone formation that takes place indirectly via a cartilaginous template (Hall, 2005; Kini and Nandeesh, 2012).

Intramembranous ossification differs from endochondral ossification by the lack of a cartilaginous template from which the bone is formed. In fact, intramembranous bone is directly formed from undifferentiated mesenchymal cells which condense at ossification zones, and which are directly converted into osteoblasts. Bone formation emanating from a cartilaginous template is referred to as endochondral ossification. It represents a multi-level process in which sequential formation and degradation of cartilaginous structures serving as templates for the development of bone take place, whereby the cartilaginous template is replaced by bone. In both modes, osteoblasts secrete bone matrix and at the end of the bone formation phase, osteoblasts either become entrapped in the bone matrix as osteocytes (the mature bone cells), get inactive osteoblasts or bone lining cells, or undergo apoptosis.

Intramembranous ossification is the characteristic mode of flat bone formation and gives rise to most of the vertebrate skull (the cranial vault, some facial bones, and parts of the mandible and clavicle) (Figure 1.1) (Kini and Nandeesh, 2012). Mesenchymal stem cells (MSC) within the mesenchyme during development initiate intramembranous ossification. The process of intramembranous ossification starts at certain sites referred to as centers of ossification. At such a center of ossification, first, small groups of adjacent MSCs proliferate and condense to form small, dense aggregations referred to as nodules. Hereupon, MSCs stop proliferating and become osteoprogenitor cells. Then, osteoprogenitor cells differentiate into osteoblasts which produce the bone matrix, also called osteoid, which is composed mainly of collagen type I. While osteoblasts at the periphery of the nodule continue to produce osteoid in the center of the nodule, a few osteoblasts get entrapped into the bone matrix and become osteocytes. At this point, the osteoid becomes calcified by osteoblasts through the deposition of hydroxyapatite around the collagen fibers. As a result, the original aggregation of MSCs has become rudimentary bone tissue.
The first crystals of bone that form are needle-like structures called bone spicules. As the spicules grow into different directions, they fuse to form trabeculae. The increasing growth of trabeculae results in interconnection. The resulting network is referred to as woven bone.

The process of entrapping osteoblasts continues, the trabeculae thicken, and the intervening space becomes progressively narrowed. Where the bone persists as cancellous bone, the process slows down (Kini and Nandeesh, 2012). Spaces between trabeculae of cancellous bone are typically filled with bone marrow.

Eventually, woven bone is replaced by lamellar bone, i.e. the pattern of collagen forming the osteoid is replaced by more resilient lamellar bone.

**Figure 1.1:** Intramembranous ossification. A) Schematic overview. Intramembranous ossification starts in mesenchymal condensations. Mesenchymal condensations differentiate into osteoprogenitor cells, which in turn generate the osteoblasts which produce the bone matrix. Osteoblasts differentiate into osteocytes which are entrapped within the bone matrix. In mice, formation of frontal bones begins in mesenchymal condensations on the lateral side of the head. The mesenchymal condensations spread upward towards the top of
the skull. The ends of the condensed masses (osteogenic front) meet at the midline where a suture is formed. (Nakashima and de Crombrugghe, 2003), edited. B) Early events of intramembranous ossification. The first bone structures formed in intramembranous ossification are bone spicules. Condensed mesenchyme transforms into osteoprogenitor cells, which in turn differentiate into osteoblasts, located on the outer surface of the spicules. When osteoblasts are entrapped by the calcified osteoid, they become osteocytes. Near the spicule, a region of condensed mesenchyme can be found. (Source: http://microanatomy.net/bone/devbone2_lab.htm)

Endochondral ossification is responsible for the rest of the skeleton including the bones of the limbs and the vertebrae (Figure 1.2) (Kini and Nandeesh, 2012). The process of endochondral ossification starts with the formation of mesenchymal condensations. Cells in the mesenchymal condensations differentiate into chondrocytes. Chondrocytes in the center of the cartilaginous template actively proliferate before differentiating into hypertrophic chondrocytes, which mineralize their ECM. Calcified cartilage is impermeable to the diffusion of nutrients, which causes chondrocytes to die and leave small cavities. These small cavities are invaded by blood vessels, induced by vascular endothelial growth factor the hypertrophic chondrocytes secreted before apoptosis. This allows not only nutrients, but also MSCs, osteoblasts, and osteoclasts to enter into the cavities. Osteoblasts and MSCs differentiated into osteoblasts, start producing and depositing a bone matrix onto the cartilaginous matrix template which is degraded by osteoclasts. Chondrocyte hypertrophy and apoptosis, followed by bone formation start from the center of cartilaginous template and spreads outwards, which leads to an ordered cellular arrangement of proliferating, hypertrophic, and mineralizing chondrocytes. The first site of ossification is in the middle of the diaphysis, which is why this location is referred to as primary ossification center. After the nutrient source has been located to the center of the bone where the feeding blood vessels entered, the diaphysis starts elongating due to cells dividing in the primary ossification center. Just about the time of birth, a secondary ossification center appears in each epiphysis of long bones. They undergo the same process as the primary ossification center. The cartilage between the primary and secondary ossification centers is called epiphyseal plate (also called growth plate). It continues to form new cartilage which is subsequently replaced by bone. This leads to a constant growth of bone in length until the child reaches skeletal maturity (18-25 years of age).
In contrast, the growth in diameter of bones around the diaphysis takes place by deposition of osteoid onto the existing surface (periosteum). This process is referred to as appositional growth, i.e. growth on a pre-existing surface (Jayakumar and Di Silvio, 2010).

Besides the difference in the modes of ossification in the skeleton of vertebrates, bones also differ with regard to the germ layers they originate from. Whereas most of the bones present in the vertebrate skull derive from the neural crest, most bones of the axial and appendicular skeleton are mesoderm-derived (for review see (Franz-Odendaal, 2011)).

Overall, ossification can be divided into the following main phases: induction of cells to the skeletogenic lineage which normally occurs via an epithelial-mesenchymal interaction, condensation formation, and differentiation of the cells. The process is completed by the
actual ossification, i.e. the production of bone matrix (for review see (Franz-Odendaal, 2011)).

Once the mesenchymal cells are induced towards the osteogenic lineage, cells aggregate to form a so-called skeletogenic condensation. The condensation needs to reach a critical size so that the transition to differentiation can take place.

Osteoblasts are commonly known as the actual bone forming cells and, along with bone lining cells, osteocytes, osteoclasts, and cells of periosteum, endosteum, and bone marrow, are the cell types involved in bone biology as well as bone repair (Jayakumar and Di Silvio, 2010). Osteoblasts are derived from mesenchymal stem cells, and are secretory cells, around 15-30 µm in size, which produce the unmineralized osteoid containing collagen type I and non-collagenous proteins such as osteopontin, osteonectin, osteoproptiserin, osteocalcin, and bone morphogenetic proteins (for review see (Rupani et al., 2012)).

1.1.2 Bone types

There are two types of bone with regard to the morphology of the bone tissue: cancellous and cortical bone (Figure 1.3) (Jayakumar and Di Silvio, 2010).

Figure 1.3: Schematic image of a cross-section through a layer of bone showing both cortical and cancellous bone. (O’Toole, 2009), edited

Cancellous bone, also referred to as trabecular bone or spongy bone, is less dense, softer, weaker, and less stiff, and has a higher surface area compared to cortical bone. Cancellous bone is typically found at the ends of long bones, but also within vertebrae. Cancellous bone is highly vascular and contains the red bone marrow where hematopoiesis takes
place (for review see (Bohner, 2010)). The primary anatomical and functional unit of cancellous bone is the trabecula.

Cortical bone, also referred to as compact bone, forms the hard outer layer of most bones. It is much denser, harder, stronger and stiffer than cancellous bone. The basic structural unit of cortical bone is referred to as osteon. Each osteon is composed of concentric layers, referred to as lamellae, of compact bone tissue which surround a central canal, the Haversian canal. It contains blood vessels and nerves. The outer delimitation of an osteon is referred to as the cement line. Besides the concentric lamellae, there are two other kinds of lamellae: interstitial and circumferential lamellae. Interstitial lamellae present between neighboring osteons are remainders of old osteons that had been partially resorbed during the process of bone remodeling. At near-surface spots of compact bone, the lamellae are arranged parallel to the surface and referred to as circumferential lamellae. Within a particular osteon, osteocytes are found in small spaces called lacuna. Osteocytes make contact with each other through cytoplasmic processes via small transverse channels referred to as canaliculi. This network of channels allows the exchange of nutrients and metabolites. Interconnection between osteons as well as connection of osteons to the periosteum is ensured by transverse channels referred to as Volkmann's canals.

During bone formation, the first bone that forms is always cancellous. Where compact bone is needed, cancellous bone forms first and later is remodeled into the denser, compact bone.

Bone is also classified microscopically into lamellar and woven bone, respectively, based on the pattern and alignment of collagen fibers (Jayakumar and Di Silvio, 2010). Lamellar bone is mechanically stronger than woven bone due to its regular parallel alignment of collagen fibers. Normal, living bone is classified as lamellar bone, and is distinguished into either cancellous or cortical bone, based on morphological criteria mentioned above (Jayakumar and Di Silvio, 2010).

In contrast, immature or pathological bone is classified as woven bone (Jayakumar and Di Silvio, 2010). Woven bone exhibits randomly arranged collagen fibers, does not show stress orientation of its collagen fibers, and is present in young fetal bone and in adult bone during remodeling and fracture repair (Jayakumar and Di Silvio, 2010).
1.1.3 Clinical aspects, treatments of bone defects

The bone healing process which takes place e.g. upon a bone fracture, is a complex process. Generally, two mechanisms of bone healing can be distinguished: primary bone healing and secondary bone healing (for review see (Shapiro, 2008)). Primary bone healing occurs when absolute rigidity, not even micromovement, is present at the fracture site. ‘Contact healing’ and ‘gap healing’ represent two types of primary bone healing (for review see (Shapiro, 2008)). Primary bone healing represents the direct formation of new bone across a fracture from the adjacent bone without callus formation. Most fractures, however, involve a certain extent of motion and therefore heal by secondary bone healing (for review see (Shapiro, 2008)). Secondary bone healing occurs when motion is present, even if only micromovement. Generally, internal or external fixation serves to minimize motion; however, micromovement is still present. The formation of a callus is characteristic for this type of bone healing. Secondary bone healing which involves both intramembranous and endochondral ossification encompasses three distinct but overlapping phases: 1) the early inflammatory phase, 2) the repair phase, and 3) the late remodeling phase (Kalfas, 2001).

The hallmark of secondary bone healing is the fibrocartilage callus which is formed at the site of fracture after an inflammation phase. This soft callus eventually hardens into a hard callus. In the final step of the repair process which is referred to as remodeling phase, the callus starts to remodel itself. Woven bone is remodeled into stronger lamellar bone through the action of osteoblasts which form the bone and osteoclasts which resorb it.

The bone healing mentioned above makes use of the capability of bone to restore itself. However, in certain clinical situations accompanied by great bone loss, the natural bone repair is inadequate, e.g. large bone defects after multi-fragmentary fracture or bone tumor resection. These defects require bony tissue to fill the gap. Several types of bone grafting materials are used to fill the gap: bone autografts, bone allografts, as well as bone graft substitutes (Bauer and Muschler, 2000; Betz, 2002) (for review see (Perry, 1999)). How promising a bone grafting material is considered to be is judged based on the following properties: osteogenesis, osteoinduction, and osteoconduction (Kalfas, 2001). Osteogenesis is referred to as the capability of the graft to form new bone originating from living bone cells present within the graft (Kalfas, 2001; Albrektsson and Johansson, 2001). Osteoinduction is the ability to stimulate primitive, undifferentiated cells to differentiate into mature bone cells that then begin new bone formation (Kalfas, 2001; Albrektsson and
Johansson, 2001). Osteoconduction is the property of the bone grafting material to serve as scaffold for new bone growth (Kalfas, 2001; Albrektsson and Johansson, 2001). The current ways for intervention to fill large gaps are autologous or allogenic bone grafting (Bauer and Muschler, 2000; Betz, 2002). Whereas bone autografts incorporate all three properties mentioned above (osteogenesis, osteoinduction, and osteoconduction), bone allografts are deficient in osteogenesis. Since allografts bring about mainly immunological problems, autologous bone grafting is considered the golden standard for treating these kinds of bony defects in orthopedic surgery (Betz, 2002). However, despite the obvious advantages of autografts such as capacity for osteogenesis, osteoinduction, and osteoconduction (Glowacki and Mulliken, 1985) as well as limited immune response, there are numerous disadvantages such as donor site morbidity and limited availability (Coventry and Tapper, 1972; Betz, 2002).

The reality shows that the bone grafting procedures performed exceeds the bone tissue donors being available (Greenwald et al., 2001). The limited availability of bone tissue donors, along with the drawbacks associated with bone autografts and allografts, prompted researchers to develop bone graft substitutes, representing bone graft alternatives. Bone graft substitutes are materials developed to mimic the properties of bone, and ideally are biocompatible, bioreabsorbable, osteoinductive, osteoconductive, structurally similar to bone, easy to use and cost-effective (Bauer and Muschler, 2000; Parikh, 2002; Greenwald et al., 2001) (for review see (Perry, 1999)). An increasing number of bone graft alternatives have become commercially available. The two main types are the osteoconductive bone graft substitutes and osteoinductive agents such as growth factors. Commercially available examples of osteoconductive bone graft substitutes include Vitoss® (Stryker) or chronOS® (Synthes), β-tricalcium phosphate products, which have been reported as successful scaffold in repairing bone defects due to its osteoconductive properties in animal and human studies, respectively (Damron, 2007). Another osteoconductive bone graft substitute, Grafton® (Osteotech), demineralized bone matrix combined with glycerol, which is available in multiple forms such as pellets, plugs, but also injectable gels, has proven clinical success (Callan et al., 2000). Commercially available examples of osteoinductive bone graft substitutes are Infuse® (Medtronic), composed of rhBMP-2 protein with absorbable collagen type I sponge, as well as OP-1 Implant (Stryker), rhBMP-7 combined with collagen type I. The latter two examples of bone graft substitutes have combined osteoinductive and osteoconductive properties, whereby the bone initiated to form by the BMP can grow on the osteoconductive collagen scaffold.
Although different bone graft substitutes have been reported to be successful in human studies, they do not meet all of the requirements of an ideal bone graft substitute. Therefore, new strategies are necessary to get closer to the ideal bone graft substitute. In recent years, a promising new strategy to overcome the limitations of the previously mentioned bone graft substitutes has emerged, referred to as bone tissue engineering. Elucidation of the process of bone formation in recent years has considerably contributed to the evolution of bone tissue engineering, if not has rendered it possible at all.

### 1.1.4 Bone tissue engineering

Approaches to bone tissue engineering, or more precisely cell-based bone tissue engineering, unify three key factors, namely a biocompatible, supportive, three-dimensional scaffold; osteogenic cells which are seeded onto the scaffold; and growth factors applied in a controlled manner. After an *in vitro* cultivation lasting a few days, these cell-seeded constructs are then implanted *in vivo* to induce growth of new tissue. Alternatively, the intended tissue is developed *in vitro* over several weeks and subsequently implanted into the patient. In this manner, bone tissue engineering aims at providing ideal bone graft substitutes (Figure 1.4).

**Figure 1.4**: Schematic overview of bone tissue engineering. (1) Mesenchymal stem cells (MSC) are harvested from a bone marrow biopsy. (2) Isolated MSCs are expanded *in vitro*. (3) In vitro expanded MSCs are seeded onto an osteoinductive scaffold and supplemented with
osteoinductive growth factors and biologically active factors. This combination is referred to as a tissue engineering construct. (4) The tissue engineering construct is cultured in vitro for a few days, after which it is implanted back into the patient for bone regeneration (5). (Source: http://biomed.brown.edu/Courses/Bi08/Bi08_2007_Groups/group12/Homepage.html)

Although all three building blocks (scaffolds, cells, and growth factors) are indispensable for potent bone tissue engineering, in this thesis, we mainly focus on the aspect of the cells.

Before discussing the aspect of cells for bone tissue engineering, a brief overview of the scaffolds used for bone tissue engineering is given in the following (for review see (Frohlich et al., 2008)).

The scaffold is a key component of successful bone tissue engineering, since it provides the substrate for the cells to proliferate, differentiate, and eventually form new bone. The demands a suitable scaffold for bone tissue engineering needs to meet encompass biocompatibility, mechanical strength similar to the one of bone, degradation rate similar to bone formation rate, osteoinduction, osteoconduction, porosity enabling cell seeding and infiltration (for review see (Frohlich et al., 2008)).

A variety of materials have been investigated for their performance in bone tissue engineering. Overall, the materials can be grouped into polymers, ceramics and composites (for review see (Frohlich et al., 2008)). Collagen type I, a naturally occurring ECM polymer, has been reported in the context of tissue engineering applications to provide osteoinduction in vitro and osteogenesis in vivo (Mizuno et al., 1997). Natural polymers have the advantage of high biological performance including beneficial effect on cell adhesion. However, natural polymers such as collagen type I as scaffold materials for bone tissue engineering display relatively poor mechanical properties and less controllable biodegradability (Glowacki and Mizuno, 2008). In contrast, synthetic polymers tend to have better mechanical properties than collagen scaffolds and can be reproducibly produced on a large scale (for review see (Liu and Ma, 2004)). However, synthetic polymers have disadvantages such as their hydrophobic behavior leading to reduced proliferation and increased apoptosis rate in osteogenic cells (for review see (Anselme, 2000)). Examples of frequently used synthetic polymers are poly(glycolic acid) (PGA), poly(lactic acid) (PLA), their copolymer poly(lactic-co-glycolic acid) (PLGA), polyanhydrides, polycarbonates, polyphosphazenes, polycaprolactone, polyfumarates (for review see (Liu and Ma, 2004)). Ceramics that have been used for bone tissue engineering applications are either naturally
produced ones such as corals, or synthetic calcium-based ceramics such as tricalcium phosphate and hydroxyapatite. Whereas corals have good biocompatibility, appropriate mechanical properties, and a porous structure similar to that of cancellous bone, synthetic calcium-based ceramics do not combine good mechanical properties and high porosity (for review see (Frohlich et al., 2008)), while both natural and synthetic ceramics exhibit osteoconductive properties.

Development of composite materials by the combination of polymers and ceramics allows the combination of the advantageous properties of each material component. For example, collagen-hydroxyapatite composite scaffolds to combine the beneficial mechanical properties of a hydroxyapatite scaffold with the high biological performance of pure collagen scaffolds have been developed (Al-Munajjed et al., 2009).

In essence, the combination of calcium-based ceramics with polymers improves the mechanical properties of the resulting composite scaffold, but also enhances the osteoconductive properties of the scaffold (for review see (Hutmacher et al., 2007)). Overall, a clear drift towards the development of composites is detectable, which will enter into clinical bone tissue engineering applications as the next-generation scaffolds (for review see (Hutmacher et al., 2007)).

The main task of the cells in bone tissue engineering is to produce a cell distribution and extracellular matrix composition mimicking the \textit{in vivo} situation. The ideal candidate of a cell population should exhibit a number of properties, namely a high osteogenic potential, availability in large numbers (either initially or after culture) as well as a capability for long term \textit{in vitro} propagation, but also non-immunogenic and non-tumorigenic behavior (Lanza et al., 2007) (for review see (Meijer et al., 2007)).

The current literature describes various cells for bone tissue engineering, which includes mesenchymal stem cells as well as primary osteoblasts (for review see (Rupani et al., 2012)). Although differentiated cells such as primary osteoblasts represent good cell candidates for bone tissue engineering, the requirement of availability in large numbers is difficult to achieve with differentiated cells that exhibit only limited population doublings (PD) and short life spans (for review see (Rupani et al., 2012)). This criterion, however, can be fulfilled by stem cells.

Stem cells are good candidate cells for tissue engineering in general, but also for bone tissue engineering in particular, because of their ability to self-renew in an undifferentiated state and to give rise to committed progenitors. These in turn
differentiate into either one lineage (unipotent), into multiple cell lineages from the respective embryonic germ layer (multipotent), or into cells from all three embryonic germ layers (pluripotent). Among the currently identified stem cells, embryonic stem (ES) cells are more broadly applicable for general tissue engineering due to the fact that these cells exhibit extensive self-renewal capability \textit{in vitro} and are pluripotent. However, ES cells are associated with ethical concerns which has been hampered their usage. Potential teratoma formation and immune rejection upon transplantation are further problems that come along with their usage (for review see (Winkler et al., 2005)). Therefore, adult stem cells are an alternative cell type for tissue engineering applications owing to their capability for self-renewal and multipotent nature. Adult stem cells are present in different tissues or organs in the human body. Examples are hematopoietic stem cells (HSC) or cardiac stem cells. Adult stem cells are very rare, and their exact phenotype \textit{in vivo} is unclear, thus they are difficult to isolate.

A suitable adult stem cell population for bone tissue engineering purposes should in addition exhibit osteogenic differentiation potential. Therefore, mesenchymal stem cells (MSC) are potentially the most suitable cell type for these purposes.

\section*{1.2 Mesenchymal stem cells and runt-related transcription factors}

\subsection*{1.2.1 Mesenchymal stem cells}

MSCs constitute a class of adult stem cells that are characterized by their self-renewal potential as well as their multidifferentiation potential into several cell types of the mesenchymal lineage, including bone, cartilage, adipose tissue, and muscle (Caplan, 1991; Caplan, 1994) (Figure 1.5).
General introduction

Figure 1.5: The mesengenic process. MSCs are able to self-renew themselves and to differentiate into different mesenchymal lineages, amongst others bone, cartilage, adipose tissue, and tendon. (Caplan, 2009)

However, MSCs are rare. Human multipotent adult progenitor cells have been estimated to be present at a frequency of $1 \times 10^7$ to $1 \times 10^8$ cells (Reyes and Verfaillie, 2001), whereas others estimate them to be present at a frequency of $1 \times 10^4$ to $1 \times 10^5$ cells (Pittenger et al., 1999). Furthermore, the concentration of MSCs or progenitor cells can highly vary depending on the donor. The number of osteoprogenitors in human bone marrow has been reported to depend on donor age, whereby the frequency decreases from $66.2 \pm 9.6$ per $10^6$ cells in younger donors (3-36 years old) to $14.7 \pm 2.6$ per $10^6$ cells in older donors (41-70 years old) (D’Ippolito et al., 1999). This study corroborated the trend of age-related reduction of osteoprogenitor numbers found in rats and mice (Tsuji et al., 1990; Bergman et al., 1996).

MSCs from human and rat bone marrow have been most investigated and characterized due to the fact they are both relatively easy to isolate based on their capability to adhere to tissue culture plastic and are easy to expand in vitro. However, MSCs from murine bone marrow, in contrast, are more difficult to isolate because hematopoietic cells also adhere
to tissue culture plastic and thus contaminate the isolation (Phinney et al., 1999). This circumstance gives rise to additional processing steps to deplete contaminating cell types from murine MSCs.

Adult human MSCs have also been reported not to elicit an immune response as they express major histocompatibility complex (MHC) class I proteins (MHC I), but not MHC class II proteins (Le and Ringden, 2005). This useful characteristic indicates cell therapies in an allogenic setting may be possible without the need of immunosuppression (Schipani and Kronenberg, 2008).

MSCs were first identified by Friedenstein and colleagues in the bone marrow (Friedenstein et al., 1976). MSCs are sometimes referred to as bone marrow stromal cells (BMSC) because they seem to emerge from the complex system of supporting structures in the marrow (Prockop, 1997). This cell population can be easily isolated from bone marrow and expanded in vitro. Both the colonies derived from single MSCs, and the cells within such a colony, are heterogeneous. Heterogeneity of MSCs concerns various cellular characteristics such as size, morphology, proliferation potential, efficacy with which they differentiate, level of enzyme activity, but also developmental potential (Owen et al., 1987; Muraglia et al., 2000; Pittenger et al., 1999).

However, to address whether the bone marrow contains a definitive stem cell able to differentiate into all three mesenchymal lineages, experiments with clonal populations are required. Taken together, the insight arose that not only are expanded populations of bone marrow stromal cells heterogeneous, but even colony-forming units fibroblasts (CFU-F) are heterogeneous, e. g. with regard to cell morphology as well as differentiation potential (Kuznetsov et al., 1997). As regards the cell morphology, Kuznetsov and colleagues reported the distinction of at least three different cell morphologies of human BMSC colonies: spindle-shaped colonies, flat colonies composed of very flattened BMSCs without bipolar orientation, and intermediate colonies composed of larger BMSCs whose shape is intermediate between spindle and flat (Kuznetsov et al., 1997). Furthermore, their data indicated that human CFU-Fs are heterogeneous with regard to the osteogenic differentiation potential, supporting similar findings with nonhuman-derived cells. Single BMSC-derived colonies were reported to form bone in 20% of the transplants in the case of rabbit (Friedenstein et al., 1987), and 58.8% in the case of human (Kuznetsov et al., 1997).
With respect to cell morphology, four morphologically distinct types of cells can be observed in single cell-derived colonies of human BMSCs: spindle shaped cells representing the most abundant ones, large flattened cells, star-shaped cells (Muraglia et al., 2000), and extremely small round cells that are rapidly self-renewing (RS cells) (Colter et al., 2001). Apart from the bone marrow, MSCs were found in, and can be harvested from, many different tissues such as adipose tissue, skeletal muscle, periosteum, but also placenta (for review see (Barrilleaux et al., 2006)). Irrespective of the source, MSCs are able to give rise to bone, cartilage, adipose tissue, and muscle, and undergo a finite number of PDs in culture before reaching senescence. Bruder and colleagues determined the growth kinetics and self-renewal capacity of human MSCs from iliac crest bone marrow aspirates (Bruder et al., 1997b). They found that MSCs averaged 38 ± 4 PDs before they became senescent, uninfluenced whether the cells were cryopreserved during the cell expansion process or not. Interestingly, the osteogenic potential was maintained throughout every passage as shown by Alkaline Phosphatase (ALP) assay and quantitative calcium determination (Bruder et al., 1997b).

Proliferation potential is influenced by many culturing factors. It has been reported that after MSCs reach their first confluence in vitro, the proliferation rate is considerably decreased and comes along with loss of multipotency (DiGirolamo et al., 1999; Banfi et al., 2000). However, DiGirolamo and colleagues reported that the cells retained their osteogenic differentiation potential after extensive proliferation in vitro, which were confirmed by Muraglia and colleagues (Muraglia et al., 2000). Furthermore, upon in vitro expansion, in vivo bone-forming efficiency of MSCs is reduced (Banfi et al., 2000). This limits the use of MSCs for therapeutic applications. The effects of growth factors on proliferation of MSCs have been investigated by several groups (Pitaru et al., 1993; Robinson et al., 1995; Locklin et al., 1995; Quito et al., 1996). The possibility to increase in vitro proliferation of MSCs is favorable for the progress of cell and gene therapy strategies. Indications have been found that MSC populations can be amplified by serum-derived growth factors (Castro-Malaspina et al., 1980). Namely, mitogens such as epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) (Tamama et al., 2006), and fibroblast growth factor 2 (Bianchi et al., 2003) have been reported to lead to further expansion of MSCs. Supplementation of FGF-2 (also called basic FGF (bFGF) to the culture medium has been reported to prolong the life span of bone marrow-derived MSCs to more than 70 PDs thereby maintaining their differentiation potential until 50 PDs (Bianchi et al., 2003). Furthermore, cell seeding density also plays a role in the self-renewal capacity of
MSCs. Higher expansion yields of MSCs can be achieved when they are plated at low
density (1.5-3 cells/cm²) but not at high density (12 cells/cm²), resulting in an increase in the
fold expansion of total cells (2,000-fold and 60-fold expansion, respectively) (Colter et al.,
2000).
The multilineage differentiation potential of MSCs has been studied extensively in vitro.
Overall, studies have demonstrated that bone marrow-derived MSCs have the capacity to
differentiate into mesenchymal lineages in vitro and in vivo, including bone, cartilage, and
fat. Human bone marrow-derived MSCs have been shown to form bone in vivo upon
subcutaneous implantation of porous calcium phosphate ceramics seeded with MSCs
(Haynesworth et al., 1992). Techniques have been described to direct MSCs into a specific
lineage in vitro. Jaiswal and colleagues established a system for the in vitro osteogenic
differentiation of human MSCs (Jaiswal et al., 1997). Thereby, they reported the ingredients
β-glycerophosphate, ascorbate-2-phosphate, and dexamethasone, which became the
standard ingredients of osteogenic medium.
Furthermore, individual colonies derived from single MSCs have been reported to be
heterogeneous regarding their multilineage differentiation potential. Furthermore,
heterogeneity of the cell population which includes multipotent as well as committed cells
is evidenced by the fact that unipotent osteoprogenitors as well as bi-, tri- (Muraglia et al.,
2000), and quadri- (Dennis et al., 1999) potent progenitors can be distinguished within the
MSC population.
Pittenger and colleagues reported that only one third of the MSC colonies derived from
clonal cells are tripotent (osteo-chondro-adipogenic potential) (Pittenger et al., 1999). In
addition, it has been shown that 30% of in vitro derived non-immortalized MSC clones
exhibited the potential to differentiate into the three main lineages (osteo-, chondro-, and
adipogenic), whereas the rest displayed bi-lineage (osteo-chondrogenic) or uni-lineage
(osteogenic) potential (Muraglia et al., 2000).
Taken together, all these studies show that clonally-derived MSCs are heterogeneous with
respect to their self-renewal capacity as well as their differentiation potential. However, it
is crucial to keep consistency within cell culture protocols in order to get reproducible and
comparable results.

Despite intense research over several years addressing the functional and phenotypical
characterization of MSCs, methods for the distinction and isolation of MSCs, even
knowledge about the MSC phenotype as such, are still rudimentarily present. Current
isolation methods either make use of the fact that MSCs easily adhere to tissue culture plastic initially described by Friedenstein and colleagues (Friedenstein et al., 1970), or are based on cell surface antigen expression. In fact, MSC is the common designation for plastic-adherent cells isolated from bone marrow and other sources. However, the biological properties of such a unfractionated cell population seem not to meet the criteria for being true stem cells, thus rendering the term scientifically imprecise (Horwitz et al., 2005). Certain cell surface markers, e.g. Stro-1, CD105, CD271, or CD34 have been shown to enrich the number of progenitors within the population (Simmons and Torok-Storb, 1991b; Majumdar et al., 2000; Quirici et al., 2002; Simmons and Torok-Storb, 1991a); however, no unique cell surface marker is presently known which is capable of isolating a pure (sub)population of MSCs.

Investigators over the whole world report studies of MSCs using different approaches to characterize the cells. One step to bring clarity into this issue came from The Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy. They proposed minimal criteria to define human MSCs (Dominici et al., 2006). First, MSCs must be plastic-adherent when maintained in standard culture conditions. Secondly, 95% or more of the MSC population must express MSC surface markers CD105, CD73, and CD90, along with 2% or less expression of hematopoietic cell markers CD45, CD34, CD14 or CD11b, CD79α or CD19, and HLA-DR. Thirdly, MSCs must differentiate into osteoblasts, adipocytes and chondroblasts in vitro under appropriate differentiating conditions, demonstrated by staining of in vitro cell culture. Differentiation into osteoblasts can be demonstrated by staining with Alizarin Red or Van Kossa staining. Adipogenic differentiation is most readily shown by Oil Red O staining. Chondrogenic differentiation is demonstrated by Alcian blue staining. However, even if this is currently the best definition of a MSC, in practice this is very difficult and unhandy to deal with.

A few monoclonal antibodies have been raised against cell surface proteins expressed on MSCs, but the isolation of truly homogeneous MSC populations has still not been achieved. In the following, the above-mentioned CD markers are discussed:

CD105 (also known as endoglin) is a transforming growth factor (TGF)-β type III receptor that is present on many cell types other than MSCs including endothelial cells, erythroblasts, and monocytes, and is recognized by the monoclonal antibody SH-2 (Barry et al., 1999). Endoglin on MSCs potentially plays a role in TGF-β signaling in the control of chondrogenic differentiation of MSCs and also in mediating interactions between MSCs and hematopoietic cells in the bone marrow.
CD73 (also known as ecto-5'-nucleotidase) is a cell surface molecule originally recognized by the monoclonal antibodies SH-3 and SH-4, which recognize two different epitopes on CD73 (Barry et al., 2001). Differentiation of MSCs towards either the osteogenic or chondrogenic lineage leads to the disappearance of these mentioned markers, thus these antibodies recognize undifferentiated MSCs.

A further marker is CD90 (also known as Thy-1), a 25-35 kDa protein that is expressed on 1-4% of human fetal liver cells, cord blood cells, and bone marrow cells (Craig et al., 1993). CD90 is reported to be one of the crucial surface molecules expressed on human MSCs from bone marrow and other sources (Pittenger et al., 1999; Lee et al., 2004).

Mareddy and colleagues assessed the expression of MSC markers on single cell-derived clonal MSC cultures by flow cytometry (Mareddy et al., 2007). They distinguished between fast-growing and slow-growing clones and could correlate this characteristic with the differentiation potential: all except one of the fast-growing clones exhibited trilineage potential, whereas slow-growing ones showed limited differentiation potential. However, they did not find an obvious difference in the expression of the MSC surface markers CD29, CD44, CD73, CD90, CD105, and CD166 amongst the different clone types as assessed by flow cytometry, indicating a strong need to investigate novel MSC surface markers to identify multipotent MSCs.

Besides these positive selection markers for MSCs officially accepted as part of the minimal criteria for MSCs, there are many other commonly reported positive MSC markers present in the literature: CD166, STRO-1 antigen, CD271, and STRO-3 antigen.

CD166 (also known as activated leukocyte cell adhesion molecule (ALCAM)) is a cell surface molecule that is recognized by the monoclonal antibody SB-10 (Bruder et al., 1998). ALCAM has been attributed a role as cell adhesion molecule involved in osteogenesis, and acts as a CD6 ligand (Bowen et al., 1995). ALCAM is expressed on the surface of MSCs prior to differentiation, and is lost during lineage progression once the osteoblast marker alkaline phosphatase (ALP) is expressed (Bruder et al., 1997a).

The monoclonal antibody STRO-1 that reacts with an as yet unidentified cell surface antigen has been found to recognize cells of adult bone marrow with CFU-F activity (Simmons and Torok-Storb, 1991b). In fact, CFU-F were exclusively found in the STRO-1+ fraction. The isolated cells were able to differentiate into fibroblasts, adipocytes, and smooth muscle cells (Simmons and Torok-Storb, 1991b), as well as osteoblasts (Gronthos et al., 1994). However, the STRO-1 monoclonal antibody was found to also react with
hematopoietic cells including nucleated erythroid cells and a subset of B lymphocytes, thus the STRO-1+ population still represents a heterogeneous population (Simmons and Torok-Storb, 1991b). When the STRO-1 monoclonal antibody was combined with an antibody against vascular cell adhesion molecule 1 (VCAM-1), also referred to as CD106, a highly purified mesenchymal progenitor population could be isolated (Gronthos et al., 2003). These cells were shown to possess stem cell characteristics such as extensive proliferation potential and the capacity of multi-differentiation for osteogenesis, chondrogenesis, and adipogenesis (Gronthos et al., 2003).

CD271 (also known as low-affinity nerve growth factor receptor (LNGFR) or p75 neurotrophin receptor (NTR)) is a cell surface molecule that was initially described to be widely expressed on human cells types derived from all three germ layers (Thomson et al., 1988). Quirici and colleagues reported that positive selection using CD271 can be used to obtain highly homogeneous MSCs (Quirici et al., 2002).

Another monoclonal antibody, referred to as STRO-3, has recently been characterized and reported to react with a subset of STRO-1+ bone marrow-derived cells (Gronthos et al., 2007). The corresponding STRO-3 antigen has been identified as tissue nonspecific alkaline phosphatase (TNSALP), a cell surface protein that not only is involved in acting as a marker for osteoblasts, but also represents a cell surface protein of immature MSCs.

However, none of these MSC markers can be used alone to reliably isolate MSCs and several MSC markers such as CD105 and CD166 are expressed on many other cell types, e.g. STRO-3 on osteoblasts. Therefore, investigators are trying to find a unique cell surface marker that could identify multipotent MSCs or subpopulations thereof. Yet, further studies are required to help to identify ideal cells for tissue regeneration: are multipotent cells more ideal than unipotent progenitors which are only able to generate the intended tissue?

Furthermore, it has to be kept in mind that MSCs are reported to make up only 1/10,000 to 1/100,000 of the bone marrow mononuclear cells. This fact highlights the challenge of identifying MSCs or subpopulations thereof.

One issue in the context of identifying novel markers for isolation and phenotypic characterization of MSCs is the fact that various studies have utilized culture-expanded MSCs, which tend to have different surface markers than non-expanded cells (Katz et al., 2005).

Besides the current isolation methods being simple adherence to tissue culture plastic and antibody selection using CD markers, attempts to isolate more homogeneous MSC
populations on the basis of size and culture conditions as well as further cell surface marker-based isolation approaches have been made (Table 1.1).

<table>
<thead>
<tr>
<th>Nomenclature</th>
<th>Isolation criteria</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mesodermal progenitor cells (MPCs)</td>
<td>CD45⁺GlyA⁻ cells cultured on fibronectin in presence of EGF and PDGF</td>
<td>Bone marrow</td>
</tr>
<tr>
<td>Mesenchymal stem cells</td>
<td>NGFR⁺ cells</td>
<td>Bone marrow</td>
</tr>
<tr>
<td>Mesenchymal stem cells</td>
<td>Density gradient followed by adherence</td>
<td>Bone marrow</td>
</tr>
<tr>
<td>Stromal stem cells</td>
<td>STRO-1Bright/VCAM-1⁺</td>
<td>Bone marrow</td>
</tr>
<tr>
<td>Mesenchymal stem cells</td>
<td>MNCs greater than 3 micron</td>
<td>Bone marrow</td>
</tr>
<tr>
<td>Marrow-isolated adult multilineage inducible (MIAMI) cells</td>
<td>BM cultured on fibronectin under low oxygen conditions</td>
<td>Bone marrow</td>
</tr>
<tr>
<td>Mesenchymal stem cells</td>
<td>FSlowSSlow recycling stem (RS) cells</td>
<td>Bone marrow</td>
</tr>
<tr>
<td>Blood-derived mesenchymal precursor cells (BMPCs)</td>
<td>Centrifugal elutriation of MNCs</td>
<td>Blood</td>
</tr>
<tr>
<td>Stromal stem cell</td>
<td>CD45⁻CD34⁻CD105⁺CD31⁻ cells</td>
<td>Adipose tissue</td>
</tr>
<tr>
<td>Mesenchymal progenitor cells</td>
<td>Collagenase-treated trabecular bone fragments cultured</td>
<td>Trabecular bone</td>
</tr>
<tr>
<td>Periosteum-derived cells (PDCs)</td>
<td>Enzymatic release of cells from periosteum</td>
<td>Periosteum</td>
</tr>
<tr>
<td>Mesenchymal stem cells</td>
<td>Enzymatic release of cells from membrane</td>
<td>Synovial membrane</td>
</tr>
</tbody>
</table>

Table 1.1: Different attempts to isolate more homogeneous human MSC populations, leading to many alternative nomenclatures for the term ‘MSC’. (Satija et al., 2007)

In addition, a population of early progenitors could be identified upon culture of early passage MSCs in the absence of serum. These cells exhibited longer telomeres and showed enhanced expression of ES cell-associated genes such as Oct-4 and TERT, indicating that the selected cells represent a subpopulation of very early progenitor cells (Pochampally et al., 2004).

One of the key hallmarks of human MSCs is their multidifferentiation potential, their ability to differentiate along multiple mesenchymal cell lineages, as evidenced by comparative analysis of the differentiation potential of clonally derived MSCs (Pittenger et al., 1999). The commitment of MSCs and the advancement along certain lineages requires the transcriptional control of specific groups of genes. Transcription factors have been
identified to direct MSC differentiation towards the osteoblast, chondrocyte, or adipocyte lineage: these are Runx-related transcription factor 2 (Runx2) and Osterix (Osx) for osteoblast differentiation (Ducy et al., 1997; Nakashima et al., 2002), SRY (sex determining region Y)-box 9 (Sox9) for chondrocyte lineage (Lefebvre et al., 2001), and peroxisome proliferator-activated receptor gamma 2 (PPARγ2) and CCAAT-enhancer binding protein alpha (C/EBPα) for adipocyte differentiation (Tontonoz et al., 1995; Freytag et al., 1994). Expression of these transcription factors in progenitor cells indicates early differentiation and destines them for a particular lineage commitment. The focus in the following will be particularly on osteoblast differentiation of MSCs.

During embryonic development, skeletal elements are built up from condensations of MSCs. The principal and best characterized transcriptional regulator of osteoblast differentiation is Runx2, underlined by the fact that Runx2-null mice completely lack osteoblastic differentiation (Komori et al., 1997; Otto et al., 1997). However, Runx2 is necessary, but not sufficient for stable osteogenesis.

### 1.2.2 Family of runt-related transcription factor

RUNX2 belongs to the family of runt-related transcription factors, generally agreed to be termed RUNXs (van Wijnen et al., 2004). Mammalian RUNXs encode for the DNA-binding α subunit of the heterodimeric RUNXs. The family of RUNXs encompasses three members, RUNX1, RUNX2, and RUNX3 and are proteins with the common and defining characteristic being a 128-amino acids long ‘Runt domain’ which is responsible for both the binding to DNA (Ogawa et al., 1993b) and the heterodimerization with the non-DNA binding β subunit (Ogawa et al., 1993b; Kagoshima et al., 1993; Golling et al., 1996). The ‘Runt domain’ is an evolutionarily conserved domain located at the N-terminal site which has its name from the fact that the pair rule gene runt in *Drosophila melanogaster* is the founding member of the Runt domain family of transcription factors (Nusslein-Volard and Wieschaus, 1980).

Runt domain proteins exhibit a high homology in amino acids 1-20 at the N-terminus, along with a common 5-amino acids long domain (VWRPY) located at the C-terminus, which was reported to be responsible for the interaction with *Drosophila* Groucho or the mammalian TLE (transducin-like Enhancer of split) homologues, thereby mediating transcriptional repression (Aronson et al., 1997).

Furthermore, Runt domain proteins have in common that they are able to bind DNA as heterodimer with the β subunit. Although Runt domain proteins, i.e. the α subunits, bind to
DNA as monomers, the association with the non-DNA binding β subunit both enhances the DNA binding affinity of Runt domain proteins and stabilizes the interaction between the α subunit and the DNA (Ogawa et al., 1993a; Golling et al., 1996). To date, only one gene has been identified which encodes CBFβ (also referred to as PEBP2β) that acts as non-DNA binding β subunit (Adya et al., 2000). The *Drosophila* homologues of CBFβ are called brother and big brother (Golling et al., 1996). They serve as dimerization partners for Drosophila Runt proteins.

In mammals, three genes (*Cbfa1/Pebp2αA*, *Cbfa2/Pebp2αB*, and *Cbfa3/Pebp2αC*) have been identified which encode the CBFα subunits (Ogawa et al., 1993b; Bae et al., 1993; Bae et al., 1995). On the basis of the function, the genes have been independently identified multiple times in the past, leading to different names for the same gene. Originally, the α subunit was identified as a sequence-specific DNA-binding protein of polyoma virus enhancer (Piette and Yaniv, 1987; Kamachi et al., 1990). Therefore, the protein has been named polyomavirus enhancer-binding protein 2 (PEBP2). PEBP2 was found to be identical to core binding factor (CBF) which binds the conserved core site in enhancers in the Moloney murine leukemia virus (Wang and Speck, 1992). Furthermore, PEBP2αB was demonstrated to be identical to the acute myeloid leukemia 1 protein (AML1) (Bae et al., 1993), whose gene is involved in the chromosomal translocation t(8; 21) associated with acute myeloid leukemia (AML). Due to the mentioned history of the different genes encoding CBFα subunits, the nomenclature has been inconsistent. In the meantime, the proteins have been decided to be referred to as RUNX1-3 according to the introduced standard nomenclature (van Wijnen et al., 2004):

- RUNX1, its synonyms are: AML1, CBFA2, or PEBP2αB
- RUNX2, its synonyms are: AML3, CBFA1, or PEBP2αA
- RUNX3, its synonyms are: AML2, CBFA3, or PEBP2αC

Gene knock out (KO) studies revealed well-defined biological roles of the Runx proteins. Runx1 has been found to be indispensable for definitive hematopoiesis, as demonstrated by the findings that Runx1-deficient mice lack fetal liver-derived definitive hematopoiesis (Wang et al., 1996), although yolk sac-derived primitive hematopoiesis was unaffected (Okuda et al., 1996). Furthermore, Runx1-deficient mice showed hemorrhaging within the central nervous system, indicating a crucial role of Runx1 in blood vessel formation (Okuda et al., 1996; Wang et al., 1996).
A first important role of Runx3 was revealed to be neurogenesis. Runx3 KO mice exhibit loss of proprioceptive neurons in dorsal root ganglia, resulting in the development of severe limb ataxia due to disruption of monosynaptic connectivity between intraspinal afferents and motoneurons (Inoue et al., 2002; Levanon et al., 2002). Further phenotypic defects of Runx3 deficiency are demonstrated in thymopoiesis and in the control of cell proliferation and apoptosis of gastric epithelium (Woolf et al., 2003; Li et al., 2002). Runx3-deficient mice display hyperplastic gastric epithelium owing to increased proliferation and decreased apoptosis of the epithelial cells, and the cells of the gastric epithelium lose responsiveness to anti-proliferative and apoptosis-inducing signals of TGF-β (Li et al., 2002).

Of the three RUNXs, the present thesis only deals with RUNX2. Therefore, RUNX2 will be considered in more detail which includes a more precise description of its structure and function.

**1.2.3 RUNX2**

Remark: If not otherwise stated, all the information that follows about RUNX2 concerns RUNX2 in general and is irrespective of the species, although the references have used a particular model system to base their results on.

**1.2.3.1 Gene, genomic structure/organization**

Human *RUNX2* gene was identified and localized on chromosome 6p21 (Levanon et al., 1994), mouse *Runx2* gene on chromosome 17 (Bae et al., 1994). The chromosomal location of human *RUNX2* indicates an association of the gene to cleidocranial dysplasia (CCD), an autosomal dominant bone disease, which has been mapped to chromosome 6p21 (Mundlos et al., 1995). Further evidence for an association between the Runx2 gene and CCD comes from the phenotype of heterozygous (*Runx2*<sup>+</sup>) mice which exhibit hypoplastic clavicles and nasal bones along with retarded ossification of parietal, interparietal, and supraoccipital bones (Komori et al., 1997). These skeletal changes resemble those of CCD (Komori et al., 1997; Otto et al., 1997). Even more interestingly, there is another mouse model that shows similarities to human CCD (Sillence et al., 1987). The radiation-induced mouse mutant was found to carry the mutation in chromosome 17 in the same region
where the mouse Runx2 gene is located (Mundlos et al., 1996). For all these reasons, Runx2 is commonly considered as the gene that is mutated in human CCD.

CCD is an autosomal, dominantly inherited disorder affecting skeletal ossification and tooth development (Jarvis and Keats, 1974). Typical characteristics include hypoplasia or aplasia of clavicles, patent cranial sutures and fontanelles, and moderately short stature (Jarvis and Keats, 1974; Mundlos et al., 1995).

Runx2 gene expression is transcriptionally regulated by two promoters: the distal promoter P1 and the proximal promoter P2, leading to two different mRNAs differing in the 5' regions: type I Runx2 mRNA by the proximal promoter P2, type II Runx2 mRNA by the distal promoter P1. While the 5' ends of the Runx2 mRNA isoforms differ, their 3' ends are identical.

Type I Runx2 is predicted to encode a 513-amino acid protein, starting with the N-terminal amino acid sequence MRIPVD (Ogawa et al., 1993b; Satake et al., 1995). This isoform was reported to be expressed in only a few tissues and cell lines, including thymus, Ha-ras-transformed NIH3T3 cells, and murine T cell lines (Ogawa et al., 1993b; Satake et al., 1995). Type II Runx2 isoform, starting with the N-terminal amino acid sequence MASNSL, has been found to be expressed in the T47i lymphoma cell line and in osteoblast and osteosarcoma cell lines (Stewart et al., 1997a). This isoform encodes a 528-amino acid protein.

In addition, another isoform has been isolated from a murine osteoblast cDNA library (Ducy et al., 1997). The isoform is referred to as Osf2/Cbfa1 and starts with the N-terminal amino acid sequence MLHSPH (Ducy et al., 1997). Osf2/Cbfa1 transcripts were detected in bone and osteoblasts, but in no other tissues examined (Ducy et al., 1997). Notably, no Osf2/Cbfa1 expression was detected in thymus, where the 'MRIPVD' isoform is expressed (Satake et al., 1995). However, it was found that there is no Osf2/Cbfa1 homologue in human, while being present in mice and rats. Due to the similarity of the amino acid sequence of the 'MASNSL' isoforms in mice, rats and humans and the absence of the Osf2/Cbfa1 isoform in humans, the 'MASNSL' isoform may represent the isoform that controls human osteoblast development (Xiao et al., 1998b).

The human RUNX2 gene spans a region of approximately 200 kb (Levanon et al., 1994). The human Runx2 gene comprises at least eight exons that have been numbered differently
depending on the authors (Geoffroy et al., 1998; Xiao et al., 1998b; Otto et al., 2002); herein the exons are referred to as exon -1 till 7 (Xiao et al., 1998b). Exons 1 till 7 encode the putative ATP binding site, the glutamine/alanine-rich domain, the runt domain region, two proline, serine, threonine-rich regions, and a nuclear matrix target site. The two most upstream exons -1 and 0 are considered to account for the generation of additional isoforms with different N-terminal sequences (Xiao et al., 1998b). A cryptic 3’ splice site in exon 1 of Runx2 gene leads the generation of two transcripts in humans that encode proteins with different N-terminal amino acid sequences (Mundlos et al., 1997). The translation start codon of type I Runx2 (the ‘MRIPVD’ isoform) is located within exon 1 (Mundlos et al., 1997). The second main Runx2 isoform, the ‘MASNSL’ isoform, originates from the alternative translation start codon within exon 0 (Mundlos et al., 1997; Xiao et al., 1998b). The most upstream exon -1 contains the translation start codon for the Osf2/Cbfα isoform, which is absent in human (Xiao et al., 1998b). In sum, several Runx2 isoforms differing in the N-terminal sequences originate from different translation start codons located in exons -1, 0, and 1 of the Runx2 gene (Xiao et al., 1998b).

Expression of the two major Runx2 isoforms results from two different promoters, referred to as P1 and P2 (Drissi et al., 2000; Xiao et al., 2001). The upstream promoter P1 accounts for the expression of the ‘MASNSL’ isoform (type II Runx2 mRNA), which is the most abundant Runx2 protein in osteoblastic cells (Drissi et al., 2000). The downstream promoter P2 regulates the expression of the ‘MRIPVD’ isoform (type I Runx2 mRNA), which is mainly expressed in T cells, but also was found to be expressed in osteoblasts (Harada et al., 1999). Both type I and type II Runx2 mRNAs are expressed in osteoblasts and chondrocytes, whereas type II Runx2 mRNA is mainly expressed in osteoblasts (Enomoto et al., 2000; Banerjee et al., 2001). The two isoforms have similar functions, but differ in their dependency on the co-factor Cbfβ (Kanatani et al., 2006). Further isoforms result from alternative splicing (Geoffroy et al., 1998; Xiao et al., 1998b; Ogawa et al., 2000).

The expression of the different Runx2 isoforms in human vs. rodent is summarized in Figure 1.6.
Different Runx2 isoforms:

- **MLHSPH Osf2/Cbfa1** (Ducy et al., 1997)
  - Bone- and osteoblast-specific – rodent

- **MRIPVD 513 amino acids** (Ogawa et al., 1993b)
  - Type I Runx2 mRNA – P2 promoter
  - Mainly T cells

- **MASNSL 528 amino acids** (Stewart et al., 1997a)
  - Type II Runx2 mRNA – P1 promoter
  - Likely predominant form for osteoblasts in human (Xiao et al., 1998b)

*Figure 1.6: Expression of Runx2 isoforms in human vs. rodent. The principal two Runx2 mRNA types are derived from two different Runx2 promoters, P1 and P2: promoter P2 accounts for the expression of type I mRNA (MRIPVD isoform), while P1 accounts for the expression of type II mRNA (MASNSL isoform). In rodents, an additional Runx2 isoform is expressed, referred to as Osf2/Cbfa1, exhibiting the N-terminal amino acid sequence MLHSPH.*

Both mentioned promoters P1 and P2 are present in the 5’-flanking region of the human Runx2 gene, whereby both promoters are linked by a purine-rich sequence. DNA sequence analyses revealed recognition sites for several transcription factors (Drissi et al., 2000; Tou et al., 2003). Namely, two AP1 and six OSE2 binding sites were identified in the proximal promoter, and three AP1 sites in the distal promoter region (Tou et al., 2003). Additionally, the distal promoter was revealed to contain a single OSE1 binding site, a single C/EBP binding site, and a consensus Smad binding site (Tou et al., 2003). Interestingly, they demonstrated that forced expression of Runx2 protein is able to downregulate rat Runx2 promoter activity in NIH3T3, and that a single Runx2 binding site is sufficient for the downregulation of transcription (Drissi et al., 2000). While these findings showed that Runx2 protein mediates autosuppression, others found a positive autoregulation of its own promoter, which was studied in non-osteoblastic COS-7 cells though (Ducy et al.,...
Other studies reported Runx2 autoregulation even in a pre-osteoblast cell line (Tou et al., 2003).

### 1.2.3.2 Protein

RUNX2 is known to act as a transcription factor, i.e. a protein that binds to specific DNA sequences within target genes (often referred to as response elements) and then influences transcription of its target genes either positively or negatively (Latchman, 1997). In fact, transcription factors are frequently classified based on their DNA binding domains. RUNX2 protein contains the highly conserved Runt domain which acts as the DNA binding domain (Ogawa et al., 1993b). In addition, the Runt domain is responsible for the heterodimerization with CBFβ (Kagoshima et al., 1993; Golling et al., 1996). Besides the defining DNA binding domain, transcription factors contain additional protein domains necessary to regulate transcription. There have been identified several more protein domains in RUNX2, and the ones shared by the two major RUNX2 isoforms are described in the following.

N-terminal to the Runt domain, the QA domain consisting of glutamine-alanine repeats is located. This domain is composed of 23 glutamine repeats on the N-terminal side and 17 alanine repeats on the C-terminal side. It was revealed to act as a transactivation domain (Thirunavukkarasu et al., 1998). A more detailed deletion analysis showed that within the QA domain, it is the glutamine stretch that bears the transactivation ability (Thirunavukkarasu et al., 1998). Besides, the QA domain was found to prevent heterodimerization of the ‘MASNSL’ isoform of RUNX2 with CBFβ (Thirunavukkarasu et al., 1998). Another transactivation domain comprising of the first 19 amino acids at the N-terminus could be identified (Thirunavukkarasu et al., 1998).

C-terminal to the Runt domain, the PST domain rich in proline-serine-threonine is located. In general, the PST domain has been attributed the function as transactivation domain (Bae et al., 1994). A more detailed deletion analysis suggested that the N-terminal half of the PST domain has transactivation ability, whereas the C-terminal half of the PST domain bears transcription repression ability. Similarly, solely the last five amino acids at the C-terminus, the VWRPY motif, which are conserved amongst all runt proteins, were found to act as transcriptional repression domain (Thirunavukkarasu et al., 1998). In addition, the VWRPY motif was shown to mediate the interaction with the transcriptional repressor transducin-like Enhancer of split 2 (TLE2) that is expressed in osteoblasts
(Thirunavukkarasu et al., 1998). TLE2 is a mammalian homologue of Groucho, and Groucho has been reported to repress the transactivation ability of Runt domain proteins by means of the VWRPY motif in *Drosophila* (Aronson et al., 1997). Another domain, found at the transition from the Runt domain and the PST domain, which consists of a 9-amino-acid stretch (PRRHRQKLD) was identified to act as nuclear localization signal (NLS) and to be related to the NLS of c-Myc (Thirunavukkarasu et al., 1998). The NLS mediates the signal for the transport of a protein into the nucleus. In RUNX2, the function as NLS could be assigned to the mentioned stretch of 9 amino acids by means of DNA cotransfection experiments using Runx2 cDNA with deleted NLS (Thirunavukkarasu et al., 1998). Runx2 cDNA with deleted NLS showed a loss of transactivation of an OSE2-dependent luciferase reporter construct (p6OSE2luc), which in further experiments could be attributed to the failed translocation of the NLS-deleted Runx2 protein (Thirunavukkarasu et al., 1998). Furthermore, within the PST domain, a 38-amino-acid sequence referred to as nuclear matrix targeting signal (NMTS) could be identified that mediates the targeting of RUNX2 to distinct subnuclear locations which are associated with the nuclear matrix (Zaidi et al., 2001). Besides, the specific targeting of RUNX2 to nuclear matrix-associated regions was revealed to be essential for proper transactivation of the *osteocalcin* gene (Zaidi et al., 2001).

### 1.2.3.3 Expression

Initially, detection of Runx2 expression on the RNA level was reported in Ha-ras-transformed NIH3T3 cells and murine T cell lines, but found to be absent in murine B cell lines, as shown by Northern blot analysis (Ogawa et al., 1993b). It was also found in murine thymus and T cells as well as in testis, whereas other tissues analyzed such as brain, lung, heart, liver, and kidney lacked the expression of Runx2 (Satake et al., 1995). These findings led to the assumption that Runx2 is a T cell-specific transcriptional regulator (Satake et al., 1995). Elucidation of the function of Runx2 *in vivo* which was then reported by several different research groups, resulting in the demonstration of a crucial role of Runx2 in osteoblast differentiation and bone formation (Komori et al., 1997; Otto et al., 1997). Mice with a homozygous mutation in Runx2 died just after birth and showed complete absence of bone formation, whereas the development of cartilage was nearly normal (Komori et al., 1997; Otto et al., 1997). Thorough examination and analysis of the heterozygous and homozygous Runx2 mutant mouse models revealed that Runx2 is crucial for both
intramembranous and endochondral ossification, and that Runx2 plays an essential role in both osteoblast maturation and expression of osteoblast-specific genes (Komori et al., 1997; Otto et al., 1997). Further evidence for the involvement of Runx2 in osteoblast differentiation came from Ducy and colleagues (Ducy and Karsenty, 1995; Ducy et al., 1997). They investigated the mechanisms of osteoblast-specific gene expression by analyzing the cis-acting elements of mouse osteocalcin gene, the most osteoblast-specific gene (Ducy and Karsenty, 1995). In the osteocalcin promoter, they found two osteoblast-specific cis-acting elements, referred to as osteoblast-specific element 1 (OSE1) and OSE2, present in the osteocalcin promoter which are responsible for its osteoblast-specific expression.

Investigation of the OSE2 sequence was identified to be identical to the DNA binding site of the runt-related transcription factors, and one member of the family of runt-related transcription factors was revealed to bind specifically to OSE2 and to be immunologically related to runt-related transcription factors (Geoffroy et al., 1995; Merriman et al., 1995). Eventually, Runx2 was cloned as the factor that bound to OSE2 with the sequence ACCACA, according to (Geoffroy et al., 1995). In that paper, Runx2 was not only identified as the transcriptional activator of the osteoblast-specific gene osteocalcin, but also Runx2 expression was identified to mark cells of the osteoblast lineage (Ducy and Karsenty, 1995; Ducy et al., 1997). Furthermore, a key role of Runx2 in osteoblast differentiation has been substantiated by the findings that Runx2 both regulates the expression of several osteoblast marker genes in osteoblasts and induces expression of osteoblast marker genes osteocalcin, collagen type I alpha 1 (Col\(\alpha_1\)), bone sialoprotein (BSP), and osteopontin in non-osteoblastic cells (Ducy et al., 1997).

Summing up their findings with regard to Runx2 expression during mouse development (Ducy et al., 1997), the earliest occurrence of Runx2 expression is in mesenchymal condensations early during skeletal development. These cells of the mesenchymal condensations represent the common precursors of osteoblasts and chondrocytes. In the course of differentiation of these mesenchymal cells, expression is maintained in those cells giving rise to osteoblasts. In bones that arise through intramembranous ossification, Runx2 expression is detected until the differentiation into osteoblasts. Instead, in bone that arises through endochondral ossification, expression is restricted to those cells located at the periphery of mesenchymal condensations, which differentiate into osteoblasts. The centrally located cells, however, which give rise to chondrocytes, gradually lose Runx2 expression. Whereas Runx2 expression is rather low in proliferating and resting chondrocytes, Runx2 expression becomes restricted to the prehypertrophic and
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hypertrophic chondrocytes. Furthermore, in postnatal stages, Runx2 was demonstrated to also regulate osteoblast function. Thereby, Runx2 is restricted to osteoblasts and cells of the perichondrium (Ducy et al., 1999).

 Taken together, all these findings have led to the generally accepted view that RUNX2 is a master transcription factor of osteoblast differentiation (Schinke and Karsenty, 2008) (for review see (Lian and Stein, 2003)).

Pathways in which RUNX2 protein is involved have started to be elucidated. In the following, pathways that control the expression of RUNX2, pathways that lie downstream of RUNX2, and interacting partners of RUNX2 will be described in detail.

1.2.3.4 Regulation of gene expression

Several pathways have been described which regulate Runx2 gene transcription and RUNX2 activity on a post-translational level, respectively.

Runx2 as target gene – regulation of Runx2 gene transcription

The Runx2 gene is known to be transcribed from two different promoters P1 and P2. The promoter region was revealed to contain binding sites for several transcription factors (Drissi et al., 2000; Tou et al., 2003). Especially, two AP1 and six OSE2 binding sites identified in the proximal promoter along with three AP1 sites in the distal promoter region (Tou et al., 2003), and a NF1 binding site identified in a different study (Zambotti et al., 2002) are of particular importance because direct binding of the respective transcription factors to the binding site and transactivation have been reported (Drissi et al., 2002; Zambotti et al., 2002). Interestingly, Drissi and colleagues demonstrated that forced expression of Runx2 protein is able to downregulate rat Runx2 promoter activity, suggesting Runx2 autosuppression, while others found positive autoregulation of its own promoter both in non-osteoblastic COS-7 cells as well as in a pre-osteoblast cell line (Drissi et al., 2000; Ducy et al., 1999; Tou et al., 2003). AP1 binding site, through binding of JunD/FosB AP1 complex present in osteoblastic cells, has been reported to affect Runx2 promoter activity and thus Runx2 expression in a positive fashion, while NF1 binding site, through binding of NF1-A isoform present in non-osteoblastic cells, acts in an inhibitory way on Runx2 promoter activity (Zambotti et al., 2002). Additionally, several other transcription factors have been reported to regulate Runx2 expression, without evidence of direct
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binding to the Runx2 promoter: the homeobox proteins Hoxa-2 (inhibitory), Bapx1 (stimulatory), and Msx2 (stimulatory), as well as the regulator of adipocyte differentiation peroxisome proliferator-activated receptor γ2 (PPARγ2) (inhibitory) (Kanzler et al., 1998; Tribioli and Lufkin, 1999; Satokata et al., 2000; Lecka-Czernik et al., 1999).

Extracellular signaling by different members of growth factor families are involved in the regulation of osteoblastic differentiation mediated by RUNX2.

RUNX2 is a component of the bone morphogenetic protein/transforming growth factor β (BMP/TGFβ) signaling pathways (for review see (Wharton and Derynck, 2009)). TGFβ and BMPs bind to specific receptors, TGFβ type I and II receptors in the case of TGFβ and BMP type I and II receptors in the case of BMPs. These receptors are serine/threonine kinase receptors. Ligand binding causes receptor phosphorylation and subsequent phosphorylation of Smads, the effectors of the signaling, which translocate into the nucleus and ultimately regulate the transcription of target genes. While Smad2 and Smad3 are activated by TGFβ, BMPs activate Smad1, Smad5, and Smad8. As regards the functions of TGFβ and BMPs in osteogenesis, in general, these factors have been assigned opposed effects (for review see (Bonewald and Dallas, 1994)). BMPs have been reported to act beneficially on the osteoblast phenotype. Recombinant human BMP-2 both induces the osteoblast phenotype in the non-osteogenic mouse pluripotent cell line C3H10T1/2 as well as in C2C12 mesenchymal precursor cells (Katagiri et al., 1990; Lee et al., 2000), and also stimulates osteoblast maturation of rat osteoblast precursor cell line ROB-C26 (Yamaguchi et al., 1991). Instead, TGFβ signaling can inhibit progression of osteoblast differentiation (for review see (Bonewald and Dallas, 1994)). Strictly speaking, TGFβ varies its influence on osteoblast biology depending on the differentiation stage of the cells: TGFβ stimulates proliferation of osteoblasts and early osteoblast differentiation, while it inhibits terminal differentiation (for review see (Bonewald and Dallas, 1994)). This inhibition turned out to involve TGFβ-mediated inhibition of Runx2 and osteocalcin expression (Alliston et al., 2001). Thereby, elucidation of the mechanism showed that Smad3, a known effector of TGFβ signaling, interacts with RUNX2 and represses its transcriptional activity at the OSE2 binding sequence present in the promoters of many osteoblast-specific genes (Alliston et al., 2001). Not only did TGFβ lead to the inhibition of RUNX2 transcriptional activity, but it also inhibited Runx2 transcription, which was shown to require both the presence of RUNX2 and its binding to the Runx2 promoter (Alliston et al., 2001). In brief, these findings provide an explanation for the TGFβ-mediated inhibition of osteoblast differentiation.
(Alliston et al., 2001). In contrast, interaction of Smad3/4 and RUNX2 led to enhanced RUNX2 transcriptional activation of the mouse germline Ig Cα promoter in response to TGFβ (Zhang et al., 2000). These conflicting findings suggest that the effect of TGFβ on Smad3 to either repress or enhance transcriptional activation is dependent, amongst other things, on the promoter sequence (Zhang et al., 2000; Alliston et al., 2001).

Using the C2C12 mesenchymal precursor cell model system, Lee and colleagues identified RUNX2 as a common target that can be induced by both TGFβ1 and BMP-2 signaling (Lee et al., 2000). However, induction of osteoblast-specific gene expression additionally requires BMP-specific Smad5 (Lee et al., 2000). Furthermore, induction of Runx2 transcription by BMP-2 was shown to involve BMP-specific Smads as well (Lee et al., 2000). Besides, another study reported that BMP4/7 also induces Runx2 expression (Tsuji et al., 1998).

The differentiation process follows the activation of expression of a set of bone-specific genes such as alkaline phosphatase and osteocalcin. RUNX2 regulates the expression of both genes and cooperates with BMP-specific R-Smads. Furthermore, BMP transcriptionally activates Runx2 in C2C12 mesenchymal progenitor cells (Lee et al., 2000). Importantly, mutation studies revealed that RUNX2 holds an essential function to transmit the BMP signaling to regulate osteoblast-specific downstream target genes (Zhang et al., 2000). In summary, BMPs and TGFβ exert their effects on Runx2 expression via specific Smad proteins, leading to the inducing effect in the case of BMPs and the inducing or repressing effects in the case of TGFβ.

Another family of growth factors reported to positively regulate Runx2 expression are fibroblast growth factors (FGF) (Zhou et al., 2000). Mice carrying an activating Pro250Arg mutation in Fgf receptor 1 (Fgfr1) showed premature fusion of calvarial sutures due to accelerated bone formation and osteoblast proliferation (Zhou et al., 2000). Moreover, mutated Fgfr1 resulted in increased expression of Runx2 and other osteoblast differentiation-related genes in the sutures compared to those of wild-type mice. In vitro, treatment of C3H10T1/2 cells with by FGF2 and FGF8 was shown to induce Runx2 expression (Zhou et al., 2000). Furthermore, all-trans retinoic acid has been reported to induce Runx2 expression (Jimenez et al., 2001).

Amongst the important negative regulators of Runx2 expression are 1,25(OH)₂-vitamin D₃ and TNF-α (Gilbert et al., 2002). The steroid hormone 1,25(OH)₂-vitamin D₃ has been shown to suppress Runx2 transcription both in mouse MC3T3 osteoblasts and rat ROS 17/2.8 osteosarcoma cells, by binding to the vitamin D₃ responsive element present in the proximal promoter of Runx2 (Drissi et al., 2002). TNF-α has been documented to dose-
dependently suppress Runx2 transcription in MC3T3-E1 clonal pre-osteoblastic cells (Gilbert et al., 2002).

Further important regulators of Runx2 expression are glucocorticoids, although their effects have been shown to differ amongst species (Prince et al., 2001). Glucocorticoid rapidly suppresses functional RUNX2 in nuclear extracts from rat osteoblast cultures (Chang et al., 1998). However, they reported the negative effect of glucocorticoids on Runx2 only at the protein level (Chang et al., 1998). In a human cell model, the synthetic glucocorticoid dexamethasone induced an increase in both protein level and DNA binding activity of RUNX2 in human osteoblast (HOB) cell lines, while the Runx2 mRNA levels stayed unchanged (Prince et al., 2001). In contrast, rodent osteoblasts responded differently upon treatment with dexamethasone: rat osteoblasts showed decreased RUNX2 protein levels, while the RUNX2 protein level in mouse osteoblasts was not affected (Prince et al., 2001).

Consistent with the essential role in osteoblast differentiation, RUNX2 is tightly controlled. Besides the transcriptional regulation of Runx2 expression, regulation of translation and post-translational regulation have been demonstrated as well. Furthermore, RUNX2 participates in many protein-protein interactions. Most of them either activate or repress RUNX2 transactivation capability.

The suggestion of RUNX2 post-translational regulation originated from studies about the osteoblast-specific transcriptional response of MC3T3-E1 preosteoblasts to ECM signals. They found that collagen matrix production, induced by the addition of ascorbic acid, increased OSE2-dependent osteocalcin transcription, and interestingly, the increased transcriptional activity was not associated with changes in Runx2 mRNA or protein levels (Xiao et al., 1997; Xiao et al., 1998a). These findings raised the issue that post-translational modifications may be required for RUNX2 activation (Xiao et al., 1998a).

**Post-transcriptional regulation of Runx2 expression**

*Translational regulation of RUNX2*

Translation has been shown to be another level of regulation of Runx2 gene expression. Studies using human osteoblast (HOB) cell lines which were treated with dexamethasone to induce differentiation revealed discordance between RUNX2 protein and mRNA levels (Prince et al., 2001). These findings set the base for further experiments which essentially showed that while both Runx2 mRNA isoforms were detected in osteoblastic cells,
osteoblast precursors, as well as nonosteoblastic cells of both human and rodent origin, Runx2 mRNA was polysome-associated in differentiated osteoblastic cells, but polysome-free in osteoblast precursors and nonosteoblastic cells (Sudhakar et al., 2001). Accordingly, only osteoblastic cells were found to express RUNX2 protein, where both isoforms were found (Sudhakar et al., 2001). These results provide evidence that Runx2 expression is regulated at the level of translation (Sudhakar et al., 2001).

Regulation of RUNX2 intracellular localization

Protein level can be affected by regulating the protein transport and in this way changing the intracellular localization of the corresponding protein.

RUNX2 exerts its effects as a transcription factor within the nucleus. Transport into the nucleus is mediated by a NLS which is located on the C-terminal side of the ‘Runt domain’ (Thirunavukkarasu et al., 1998). Within the nucleus, RUNX2 has been reported to be targeted to distinct subnuclear regions which are associated with the nuclear matrix (Zaidi et al., 2001). For this nuclear matrix-associated subnuclear localization, a nuclear matrix targeting signal (NMTS) is responsible (Zaidi et al., 2001). Functionally, the NMTS has been demonstrated not only to be essential for RUNX2 transactivation capability in vitro, but also mice lacking NMTS and the remaining C-terminus do not generate bone, owing to maturational arrest of osteoblasts, indicating that this region is required for RUNX2 function in vivo (Zaidi et al., 2001; Choi et al., 2001). In these studies, the lack of the NMTS region left RUNX2 DNA binding ability and nuclear import unaffected.

Post-translational modifications are well documented to alter the activity and function of many proteins including transcription factors. Amongst the most important post-translational regulation mechanisms are phosphorylation, acetylation, and ubiquitination.

Regulation of RUNX2 by phosphorylation

Phosphorylation constitutes an essential mechanism to change the activity of proteins post-translationally. Usually, serine, threonine, and tyrosine residues are the amino acids that undergo phosphorylation.

In human bone marrow stromal cells, RUNX2 activity has been demonstrated to be positively regulated upon phosphorylation, and this increased protein activity in turn is associated with a more advanced stage of osteoblastic differentiation (Shui et al., 2003).
In vitro experiments using MC3T3-E1 preosteoblasts demonstrated that phosphorylation of RUNX2 regulates its transactivation potential of the osteocalcin gene (Xiao et al., 2000). Thereby, RUNX2 phosphorylation was shown to be controlled by the mitogen-activated protein kinase (MAPK) pathway (Xiao et al., 2000). Since, several groups have reported that RUNX2 is phosphorylated via the MAPK pathway, and this pathway mediates the response of osteogenic cells to different external stimuli including ECM signals, osteogenic factors such as FGF-2 and IGF-1, as well as mechanical signals (Xiao et al., 1998a; Xiao et al., 2002; Qiao et al., 2004; Ziros et al., 2002; Kanno et al., 2007). Furthermore, a stimulatory in vivo function in bone development for the MAPK pathway and its involvement in RUNX2 stimulation by phosphorylation has been demonstrated (Ge et al., 2007).

The stimulatory role of MAPK signaling in RUNX2 phosphorylation and transactivation capability has been well documented (Xiao et al., 2000; Ge et al., 2009; Zou et al., 2011; Ge et al., 2012; Li et al., 2012). However, the MAPK signaling has also been attributed an inhibitory effect (Huang et al., 2012). They reported that RUNX2 is negatively regulated upon phosphorylation by c-Jun N-terminal kinase 1 (JNK1), another MAPK, induced by BMP2 treatment (Huang et al., 2012).

In addition, phosphorylation and activation of RUNX2 has been documented to be mediated by other kinases including protein kinase A (PKA), protein kinase C δ (PKCδ), Akt (also referred to as protein kinase B (PKB)), homeodomain-interacting protein kinase 3 (HIPK3), and cyclin-dependent kinase 1 (CDK1) (Selvamurugan et al., 2000; Kim et al., 2006a; Pande et al., 2013; Sierra and Towler, 2010; Qiao et al., 2006; Pierce et al., 2012). In contrast, RUNX2 inhibiting phosphorylation has been reported to be mediated by cyclin D1/cyclin-dependent kinase 4 (CDK4) as well as glycogen synthase kinase-3β (GSK-3β) (Shen et al., 2006; Kugimiya et al., 2007).

RUNX2 comprises multiple phosphorylation sites, and phosphorylation at different sites has either stimulatory or inhibitory effects on RUNX2 activity. In contrast to the stimulatory effects of MAPK-mediated phosphorylation mentioned above, RUNX2 comprises several serine residues that are constitutively phosphorylated and of which two are reported to inhibit RUNX2 activity (Wee et al., 2002). One of these two negatively regulated serine residues is the same one reported by Zou and colleagues, who conversely attributed a stimulatory effect to the phosphorylation of that serine residue (Zou et al., 2011). Additionally, dexamethasone was reported to decrease RUNX2 phosphorylation level on a serine residue in a rat cell model, and in this way, at least partly, induces osteogenesis (Phillips et al., 2006). This residue represents the same one reported by two independent
studies, substantiating the negative effect of phosphorylation of that particular serine residue (Wee et al., 2002; Huang et al., 2012).

Taken together, RUNX2 activity is regulated in opposite ways by phosphorylation of different amino acid residues.

Certain protein domains of RUNX2 could be assigned a function in phosphorylation by means of deletion studies. In this way the PST domain, as well as the Runt domain, have been reported to contain amino acid residues that are phosphorylated upon FGF-2 stimulation and by Akt kinase, respectively (Xiao et al., 2002; Pande et al., 2013). However, the specific amino acid residues being phosphorylated are only incompletely known.

As the phosphorylation of RUNX2 is a central element of this thesis and the base of the adenoviral reporters, the amino acids residues undergoing phosphorylation are listed in Table 1.2.

<table>
<thead>
<tr>
<th>Amino acid residue</th>
<th>Effect of phosphorylation</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>S28</td>
<td>stimulatory</td>
<td>(Selvamurugan et al., 2009; Zou et al., 2011)</td>
</tr>
<tr>
<td>S43</td>
<td>stimulatory</td>
<td>(Ge et al., 2009)</td>
</tr>
<tr>
<td>S118</td>
<td>inhibitory</td>
<td>(Huang et al., 2012; Phillips et al., 2006; Wee et al., 2002)</td>
</tr>
<tr>
<td>S196</td>
<td>stimulatory</td>
<td>(Pande et al., 2013)</td>
</tr>
<tr>
<td>T198</td>
<td>stimulatory</td>
<td>(Pande et al., 2013)</td>
</tr>
<tr>
<td>T200</td>
<td>stimulatory</td>
<td>(Pande et al., 2013)</td>
</tr>
<tr>
<td>S237</td>
<td>stimulatory</td>
<td>(Zou et al., 2011)</td>
</tr>
<tr>
<td>S240</td>
<td>stimulatory</td>
<td>(Kim et al., 2006a)</td>
</tr>
<tr>
<td>S275</td>
<td>stimulatory</td>
<td>(Zou et al., 2011)</td>
</tr>
<tr>
<td>S294</td>
<td>stimulatory</td>
<td>(Zou et al., 2011; Ge et al., 2009; Sierra and Towler, 2010; Li et al., 2012; Park et al., 2010)</td>
</tr>
<tr>
<td>S312</td>
<td>stimulatory</td>
<td>(Zou et al., 2011; Ge et al., 2009; Ge et al., 2012; Li et al., 2012)</td>
</tr>
<tr>
<td>T319</td>
<td>stimulatory</td>
<td>(Sierra and Towler, 2010)</td>
</tr>
<tr>
<td>S347</td>
<td>stimulatory</td>
<td>(Selvamurugan et al., 2009)</td>
</tr>
<tr>
<td>S465</td>
<td>inhibitory, stimulatory</td>
<td>(Pierce et al., 2012; Zou et al., 2011; Qiao et al., 2006; Wee et al., 2002)</td>
</tr>
<tr>
<td>S503</td>
<td>stimulatory</td>
<td>(Ge et al., 2009)</td>
</tr>
</tbody>
</table>
Table 1.2: Published and known phosphorylation sites of human RUNX2. The compilation of RUNX2 phosphorylation sites is based on above-mentioned references as well as the open, web-based bioinformatics database of protein post-translational modifications, PhosphoSitePlus (www.phosphosite.org) (Hornbeck et al., 2012). The amino acid residue numbering is according to human type II RUNX2 isoform with the N-terminus ‘MASNSL’ (521 amino acids, 56.648 kDa), and phosphorylation sites identified in species other than humans are listed in the renumbered form to correspond to the human amino acid numbering for the sake of consistency.

Regulation of RUNX2 by acetylation

Acetylation represents the process of introducing an acetyl group into a compound. Protein acetylation has an important role in the regulation of the chromatin structure and gene expression in general, and it occurs both co-translationally and post-translationally. Whereas co-translational acetylation is an irreversible process (Polevoda and Sherman, 2000), post-translational acetylation of lysines is reversible and has emerged as a significant post-translational regulation mechanism, reported to occur in histones, transcription factors and other proteins (for review see (Yang, 2004)). Lysine acetylation of histones leads to reduction of their DNA affinity within the chromatin structure and in turn makes the DNA more accessible for transcription factors (for review see (Shahbazian and Grunstein, 2007)). The process of histone acetylation is controlled by the activity of histone acetyltransferases (HAT) and histone deacetylases (HDAC), of which the latter remove the acetyl moiety from the histones, leading to transcriptional repression. Together with certain HATs which have been reported to even acetylate non-histone proteins such as transcription factors, acetyltransferases in general which by definition modify non-histone proteins represent a large group of enzymes, categorized into several protein families (for review see (Sterner and Berger, 2000; Kouzarides, 2000; Yang, 2004)). HATs, lysine acetyltransferases in general, as well as HDACs have been documented to interact with and even to acetylate RUNX2. The general conclusion is that acetylation results in a stimulatory effect on RUNX2 stability and transactivation capability.

The p300 protein, also referred to as E1A-associated 300 kDa protein, which functions as a transcriptional co-activator possessing intrinsic HAT activity, is able to acetylate several non-histone proteins (Kouzarides, 2000). Jeon and colleagues reported that p300 mediates RUNX2 acetylation upon BMP-2 signaling, thereby increasing RUNX2 transactivation activity as well as stability (Jeon et al., 2006). Furthermore, inhibition of HDAC4 and -5
which deacetylate RUNX2, enforced BMP-2 stimulated in vitro osteogenic differentiation and bone formation in vivo (Jeon et al., 2006). RUNX2 acetylation and stabilization induced by BMP-2 were shown to depend on MAPK signaling (Jun et al., 2010). Upon PTH treatment, RUNX2 has been reported to recruit p300 to the MMP-13 promoter, both of which are required for acetylation of histones H3 and H4, and led to transcriptional activation of the target gene MMP-13 in rat osteoblastic UMR 106-01 cells (Boumah et al., 2009).

Regulation of RUNX2 by ubiquitination
Protein ubiquitination plays a crucial role in protein degradation by the proteasome (for review see (Hershko and Ciechanover, 1998)). This degradation pathway takes place in a cascade-like manner governed by E1 ubiquitin-activating enzymes, E2 ubiquitin-conjugating enzymes, and E3 ubiquitin ligases (for review see (Pickart, 2001)). E3 ubiquitin ligases account for the specificity of protein ubiquitination, and proteins polyubiquitinated by these enzymes are targeted to degradation by the proteasome (for review see (Hershko and Ciechanover, 1998)).

It has been shown that RUNX2 is degraded through an ubiquitination-dependent pathway by the proteasome (Tintut et al., 1999). An E3 ubiquitin ligase responsible for targeting RUNX2 to proteasomal degradation has been revealed to be Smad ubiquitin regulatory factor 1 (Smurf1) (Zhao et al., 2003). Consistently, the suppressing role of Smurf1 in osteoblast differentiation in vitro and in vivo bone formation has been reported, whereby Smurf1 overexpression had inhibitory effects, whereas Smurf1-deficient mice exhibited increased bone formation through control of proteasomal degradation of MEKK2, also known as MAPK kinase kinase 2, a major upstream kinase of the MAPK pathway (Zhao et al., 2004; Yamashita et al., 2005).

Additional E3 ubiquitin ligases reported to promote RUNX2 ubiquitination and proteasomal degradation as well as to negatively regulate osteoblast differentiation are C terminus of Hsc70-interacting protein (CHIP) as well as WW domain-containing E3 ubiquitin protein ligase 1 (WWP1) together with the adaptor protein Schnurri-3 (Shn3) (Li et al., 2008; Jones et al., 2006). Besides E3 ubiquitin ligase-induced RUNX2 ubiquitination and degradation, another mechanism leading to ubiquitination and subsequent proteasomal degradation has been reported to be induced by cyclin D1/CDK4 and acts phosphorylation-dependently (Shen et al., 2006).
In summary, although the different post-translational regulation mechanisms of RUNX2 have been individually investigated, they are not unconnected by any means, which is exemplified by the following three studies. Jeon and colleagues have found that acetylation protects RUNX2 from Smurf1-mediated degradation, clearly suggesting a molecular link between acetylation and ubiquitination-mediated proteasomal degradation (Jeon et al., 2006).

Furthermore, it is worth mentioning that although many phosphorylation sites and kinases involved have been investigated, how RUNX2 phosphorylation is linked to enhanced transcriptional activity and protein stability is poorly understood. Recently, Park and colleagues concluded that serine phosphorylation, exemplified with one particular serine residue (S294), triggers RUNX2 acetylation, which in turn accounts for RUNX2 transcriptional activity as well as stabilization by inhibiting ubiquitin-dependent degradation (Park et al., 2010). This study indicates an additional link of the different post-translational regulation mechanisms.

Thirdly, cyclin D1/CDK4 has been reported to phosphorylate RUNX2 at S472. However, cyclin D1/CDK4 induced not only RUNX2 phosphorylation, but also triggered subsequent ubiquitination and proteasomal degradation. Thus, this study suggests a phosphorylation-dependent proteasomal degradation of RUNX2, another link between different post-translational regulation mechanisms.

Interaction partners of RUNX2

Activity of RUNX2 is modulated by the interactions with a variety of regulatory proteins. The best known interacting partner of RUNX2 is the non-DNA binding β subunit CBFβ. It interacts with RUNX2 by binding to the Runt domain (Ogawa et al., 1993b; Kagoshima et al., 1993; Golling et al., 1996). The association of RUNX2 with CBFβ both enhances the DNA binding affinity of Runt domain proteins and stabilizes the interaction between RUNX2, the α subunit, and the DNA (Ogawa et al., 1993a; Golling et al., 1996). In Drosophila, it could be shown that the interaction between Runt domain proteins and CBFβ additionally impacts the transactivation potential of Runt domain proteins (Li and Gergen, 1999).

Next, TLE proteins (the mammalian homologues of Drosophila Groucho) interact with the VWRPY motif at the C-terminus of RUNX2 and in this way act as transcriptional corepressors (Thirunavukkarasu et al., 1998; Javed et al., 2000). Osteocalcin is an example of a RUNX2 target gene whose activation by is repressed by TLE proteins (Javed et al., 2000).
Further interacting partners encompass the basic helix-loop-helix protein Hairy and Enhancer of split 1 (HES-1) which is expressed in rat osteoblastic osteosarcoma ROS17/2.8 cells (Matsue et al., 1997). HES-1 was shown to physically interact with RUNX2 and in this way modulates RUNX2 transactivation function (McLaren et al., 2000). Yes-associated protein (YAP) acts as a transcriptional co-activator of RUNX2 (Yagi et al., 1999), and Smads (Hanai et al., 1999; Zhang et al., 2000; Lee et al., 2000).

In addition, CCAAT/enhancer-binding Proteins (C/EBP) were revealed to physically interact with RUNX2 and to synergistically activate osteocalcin gene expression (Gutierrez et al., 2002). Interaction of the homeobox protein Msx2 with RUNX2 leads to the repression of transcriptional activity of RUNX2 (Shirakabe et al., 2001). The repressive activity of Msx2 is counteracted by another homeobox protein Dlx5 (Shirakabe et al., 2001). Furthermore, c-Fos and c-Jun, the protein subunits making up the heterodimeric activator protein (AP-1), were identified as interaction partners of RUNX2 through the Runt domain, and this interaction was demonstrated to be required to activate rat collagenase 3 promoter (D'Alonzo et al., 2002).

In conclusion, the presence of so many co-regulators that govern RUNX2-mediated transcription indicates a complex regulation of gene expression that RUNX2 holds as a master transcription factor of osteogenesis.

**Target genes of RUNX2**

RUNX2 is essential for osteoblast differentiation (Komori et al., 1997; Otto et al., 1997; Ducy et al., 1997; Banerjee et al., 1997). RUNX2 regulates expression of several genes related or specific to osteoblast differentiation. For RUNX2 to be able to regulate the expression of a particular gene, the target genes require binding sites for RUNX2 in their promoter region and regulatory elements, respectively. OSE2, which was originally identified as a cis-acting element present in the mouse osteocalcin promoter accounting for its osteoblast-specific expression (Ducy and Karsenty, 1995), is found in the promoters of many RUNX2 target genes, is recognized by RUNX2 and serves as a Runx2 binding site (Geoffroy et al., 1995). Originally, OSE2 was reported to comprise the sequence ACCACA (Geoffroy et al., 1995). Nucleotide sequence comparison between human, rat, mouse, rabbit collagenase 3 promoter regions and human, rat, mouse osteocalcin promoter regions showed sequence identity in the sequence AACACAA, which in the present thesis is generally considered as the consensus Runx2 binding site (Jimenez et al., 1999). Strictly speaking, the term ‘OSE2’ is designated for the corresponding Runx2 binding site in mice (Ducy and Karsenty, 1995).
Initially, RUNX2 was reported to transactivate the expression of **osteocalcin** (Ducy and Karsenty, 1995; Geoffroy et al., 1995; Merriman et al., 1995). Since, **osteocalcin** as a target gene of RUNX2 has been addressed and documented in more detail by many studies (Banerjee et al., 1997; Ducy et al., 1997; Frendo et al., 1998) (Javed et al., 1999).

Furthermore, RUNX2 was found to both regulate the expression of several osteoblast marker genes in osteoblasts and induce expression of several osteoblast marker genes in non-osteoblastic cells in addition to osteocalcin: collagen type I alpha 1 (Col1α1), bone sialoprotein (BSP), and osteopontin (Ducy et al., 1997). As regards BSP as RUNX2 target gene, conflicting results have been reported (Javed et al., 2001). Javed and colleagues reported that the *Gallus* BSP promoter which contains seven functional Runx2 binding sites is repressed by RUNX2 both in rat and *Gallus* osteoblasts (Javed et al., 2001). They proposed that the repression takes place by a mechanism different from the known transcriptional repression mechanism involving TLE proteins and their interaction with the VWRPY domain at the C-terminus of RUNX2 (Aronson et al., 1997; Thirunavukkarasu et al., 1998).

Collagenase 3, also referred to as matrix metalloproteinase 13 (MMP-13), was revealed as another target of RUNX2, as evidenced by both in vitro and in vivo experiments (Jimenez et al., 1999). Furthermore, the TGFβ type I receptor was revealed as another RUNX2 target gene. At least six Runx2 binding sites were identified in the TGFβ type I receptor promoter and were shown to regulate expression of TGFβ type I receptor, by physically associating with RUNX2 (Ji et al., 1998). Besides, in accordance with (Ducy et al., 1997), the ability of RUNX2 to directly regulate the transcriptional activation of **osteopontin** gene was substantiated by another study (Sato et al., 1998). Transactivation was revealed to be dependent on OSE2; any change in its nucleotide sequence AACCACA abolished its ability for RUNX2 binding (Sato et al., 1998). In short, most of the identified target genes of RUNX2 are regulated in a positive fashion by RUNX2 and are coding for bone ECM proteins.

Another ECM protein RUNX2 target gene is ameloblastin (Dhamija and Krebsbach, 2001). Transcription of the ameloblastin gene, which encodes a tooth-specific ECM protein, has been shown to be regulated in a positive fashion by RUNX2 (Dhamija and Krebsbach, 2001). The ameloblastin promoter region contains Runx2 binding sites, mediating their physical interaction with RUNX2 (Dhamija and Krebsbach, 2001). These findings are in accordance with the phenotypical characteristic of CCD patients showing supernumerary teeth and delayed tooth eruption, indicating a role of RUNX2 in tooth formation (Dhamija and Krebsbach, 2001).
RUNX2 has been documented to regulate the expression of the osteoprotegerin gene whose promoter has been revealed to contain 12 OSE2 elements (Thirunavukkarasu et al., 2000). These findings indicate a molecular connection between osteoblastogenesis and osteoclastogenesis, in which RUNX2, in addition to its role in osteoblast differentiation, inhibits osteoclast formation by positively regulating osteoprotegerin, which in turn inhibits osteoclast differentiation (Thirunavukkarasu et al., 2000).

Another gene involved in osteoclastogenesis was identified as a RUNX2 target gene, namely receptor activator of NF-κB ligand (RANKL) (Geoffroy et al., 2002). This was underlined by the fact that the RANKL promoter exhibits a putative Runx2 binding site (Kitazawa et al., 1999). These findings offer an explanatory approach for the elevated bone resorption rate that exceeds bone formation observed in transgenic mice overexpressing Runx2 (Geoffroy et al., 2002).

During endochondral ossification, hypertrophy of chondrocytes in the cartilaginous template is followed by invasion of blood vessels into cartilage. As a result, osteoblast as well as chondro-/osteoclasts are brought into the cartilaginous template, ultimately remodeling the cartilaginous template into bone. In hypertrophic chondrocytes, RUNX2 was reported to increase the activity of a BMP-responsive region of the promoter of collagen type X (Leboy et al., 2001). Together with the fact the BMP-responsive region of the promoter of collagen type X contains a Runx2 consensus binding site, it is hypothesized that RUNX2 directly regulates expression of the commonly known hypertrophic chondrocyte marker collagen type X (Leboy et al., 2001). Besides, invasion of blood vessels into the cartilage comes along with VEGF upregulation in hypertrophic chondrocytes (Haigh et al., 2000). VEGF was revealed as another gene whose expression is upregulated upon RUNX2 induction in hypertrophic chondrocytes (Zelzer et al., 2001).

Identification of further putative RUNX2 target genes was approached by searching for genes differentially expressed in C3H10T1/2 mesenchymal precursor cells overexpressing Runx2 compared to wildtype cells, using a differential hybridization technique and cDNA microarray analysis (Stock et al., 2004). The candidate target gene with the strongest difference in expression between Runx2-overexpressing and wildtype cells was pituitary tumor-transforming 1 interacting protein (Ptg1ip) (Stock et al., 2004). Furthermore, Ptg1ip was not only shown to be expressed in osteoblast-like MC3T3-E1 cells and in primary mouse calvarial cells, but RUNX2 also binds to the 5′ flanking region of murine Ptg1ip and directly transactivates expression of Ptg1ip (Stock et al., 2004). Additionally, RUNX2 has
been reported to regulate the transcription of galectin-3, whose promoter contains Runx2 binding sites (Stock et al., 2003).

In sum, the opposing regulation of osteoblast marker genes highlights the importance of the promoter context of Runx2 binding sites, making up the transcriptional control of the RUNX2 target genes.

**Biological functions**

RUNX2 is best known as the master regulator of osteoblast differentiation and osteoblast marker gene expression as well as osteoblast function. In fact, the osteogenic activity of bone marrow stromal cells was reported to be enhanced upon Runx2 overexpression, both in vitro and in vivo (Zhao et al., 2005). Primary murine MSCs transduced with Runx2-producing AdRunx2 formed more ectopic bone in vivo than cells transduced with control virus. However, one drawback arose to be the formation of osteosarcoma (Zhao et al., 2005).

A variety of additional biological functions of RUNX2 have been demonstrated, which include:

- antiproliferative role in (pre)osteoblasts (Pratap et al., 2003; Galindo et al., 2005)
- tooth development (D'Souza et al., 1999)
- chondrocyte maturation and hypertrophy (Takeda et al., 2001; Yoshida et al., 2004), as evidenced by the induction of collagen type X (Col10a), a marker specific for hypertrophic chondrocytes (Enomoto et al., 2000)
- tumor metastasis to bone (Pratap et al., 2006)
- inhibition of rRNA transcription (Young et al., 2007)
- endothelial cell biology as well as angiogenesis (Namba et al., 2000; Sun et al., 2001; Sun et al., 2004).

In osteoblast biology, RUNX2 regulates the process of osteoblast differentiation at different stages. Regulation by RUNX2 takes place in a positive manner at early stages of differentiation, while RUNX2 inhibits the process at later stages (Figure 1.7). The whole process from an undifferentiated MSC to an osteoblast occurs in different phases, and each of these phases is characterized by a particular pattern of expressed osteoblast marker genes. RUNX2 controls expression of osteoblast marker genes by binding to OSE2, the RUNX2 binding site, found in the promoter region of all major osteoblast marker genes.
The functions of Runx2 in osteoblast and chondrocyte differentiation are depicted in Figure 1.7.

**Figure 1.7: Regulation of osteoblast and chondrocyte differentiation by Runx2.** During the process of osteoblast differentiation, Runx2 is crucial for the commitment of mesenchymal stem cells to the osteoblast lineage and positively influences early stages of osteoblast differentiation. Osx starts playing an important role in osteoblast differentiation following Runx2-mediated mesenchymal condensation. During the process of osteoblast differentiation, Runx2 is involved in the expression of bone matrix genes Col1, osteopontin, BSP, and osteocalcin and maintains the expression of osteopontin and BSP. For further bone maturation, Runx2 expression has to be downregulated. During the process of chondrocyte differentiation initiated by Sox9-mediated mesenchymal condensation, Runx2 is crucial for chondrocyte maturation from immature to terminal hypertrophic chondrocytes, and inhibits immature chondrocytes from adopting the phenotype of permanent cartilage. Runx2 induces expression of Col10a1 in hypertrophic chondrocytes, is involved in the matrix production of terminal hypertrophic chondrocytes, and plays a crucial role in vascular invasion of cartilage. (Komori, 2003)

RUNX2 has been reported to be involved in osteoblast differentiation. However, in mice, the two major RUNX2 isoforms, type I and II, have been revealed to possess distinct subfunctions within osteoblast biology. First, as regards the regulation of different stages
of osteoblast differentiation, expression of both RUNX2 type I and II isoform have been
detected in osteoblasts. However, RUNX2 type I isoform also existed in osteoprogenitor
cells and preosteoblasts (Choi et al., 2002). Thus, RUNX2 type I has been found to have an
exclusive role in early osteoblastogenesis, while RUNX2 type II is necessary for terminal
stages of osteoblastic maturation (Choi et al., 2002; Xiao et al., 2004).
Second, it has been demonstrated that type I isoform is sufficient for intramembranous
ossification, while the type II isoform is necessary for endochondral ossification (Xiao et al.,
2004).
The knowledge about RUNX2 as detailed in section 1.2.3 has been submitted for publication
as a review article to European Cells and Materials:

Role and Regulation of Runx2 in osteogenesis
Marco Bruderer, R. Geoff Richards, Mauro Alini, Martin J. Stoddart

1.3 Endothelial progenitor cells
It is known that vascularization plays a critical role for bone integrity and healing: its
important role in bone grafts implicates one of the limiting aspects in bone tissue
engineering approaches. The three-dimensional bone construct possesses oxygen and
nutrient supply gradients as the sole driving force for the supply is diffusion. This reduces
efficiency of the bone constructs because the extent of viable cells with osteogenic
potential is the determining factor. One way to overcome this problem is to develop tissue
engineered bone grafts which enable osteogenesis as well as vascularization. The general
approach is to develop a three-dimensional scaffold seeded with both MSCs and
endothelial cells (EC). However, the amount and the quality of ECs gained from veins are
not sufficient to stimulate vascularization in the tissue engineered bone graft.
Furthermore, mature ECs are terminally differentiated cells with a low proliferative
capacity and thus only limited potential to substitute endothelium. Therefore, vascular
repair may be improved by the use of other cell types. An alternative candidate cell source
for the vascularization of the bone graft are endothelial progenitor cells (EPC). EPCs are
progenitor cells that have the capacity to proliferate and differentiate into mature ECs
(Hristov et al., 2003), and recent studies using ischemia animal models showed an effective
participation of EPCs in neovascularization (Hristov et al., 2003; Hristov and Weber, 2004).
EPCs were originally identified and isolated in human adults in 1997 in peripheral blood (Asahara et al., 1997).

Prior to 1997, it was believed that vasculogenesis, the formation of new blood vessels from endothelial progenitor cells (EPC), was restricted to embryogenesis, whereas vessel neoformation in adults exclusively resulted from preexisting vessels, a process referred to as angiogenesis (Folkman, 1984; Folkman and Shing, 1992). The discovery of EPCs in adults, which were shown to be able to differentiate into mature ECs in vitro and to induce vessel neoformation in ischemia animal models in vivo (Asahara et al., 1999), overturned the concept of a clear separation into embryological and adult processes of new blood vessel formation. Thus, the bone marrow was proposed as a source of progenitor cells that are able to differentiate into ECs during postnatal vascular growth. Cells originating from bone marrow having the potential to differentiate into mature ECs were commonly referred to as EPCs.

Since the first report of EPCs more than a decade ago, the term has broadened its meaning and currently includes a set of circulating cells displaying some, or all, characteristics mentioned above (for review see (Richardson and Yoder, 2011)): These are proangiogenic hematopoietic cells and platelets which mainly serve as supporting cells for the endothelial colony forming cells (ECFC), the ECs with proliferative potential, which are also called late outgrowth ECs (OEC). In sum, currently, the term EPC does not define a distinct cell type. Despite the recent insight that the term EPC is misleading, in this thesis we adhere to the term EPC, implying ECFC though. First, we believe maintaining the term EPC is essential. This term indicates a general cell type to many investigators and has become omnipresent in the scientific literature for more than a decade. Hence, we would like to maintain the continuity of scientific discourse.

EPCs are believed to provide a critical contribution to neovascularization of ischemic lesions, to reendothelialization of injured vessels, but also to tumor angiogenesis. In fact, several studies estimated the contribution of EPCs to half of all ECs in tumor neovessels (Garcia-Barros et al., 2003). However, the contribution of EPCs to angiogenesis and tumor growth is still up for debate (Purhonen et al., 2008).

EPCs have been intensively studied and evidence has accumulated that EPCs have an immense and promising potential in cell therapy for cardiovascular diseases and ischemic diseases, in tissue engineering of blood vessels as well as in engineering vascularized tissue such as bone (Rouwkema et al., 2009) or skin (for review see (Hendrickx et al., 2011)). The most frequent application of EPCs in tissue engineering is vascular tissue engineering,
where EPCs (or ECs) are used to tissue engineer vessels destined for vascular grafting. Not only are EPCs used to re-build the actual organ being the blood vasculature, but also to support the regeneration of a biological tissue by providing blood vasculature. For large tissue constructs, insufficient blood supply reduces the delivery of nutrients and oxygen, and represents one of the main limitations in tissue engineering. This challenge can be overcome by EPCs. However, one prerequisite for EPCs to become clinically more relevant is the establishment of safer and more reproducible isolation protocols, leading to more homogeneous EC populations. This is required in order to guarantee consistent outcomes of clinical studies.

Current isolation methods for ECs and their progenitors that are most widely used are antibody selection using cell surface markers, along with selection systems based on promoter-dependent expression of reporter genes. However, the lack of a unique surface marker in the case of the first method, ectopic promoter activity differing from endogenous marker expression in the case of the second method (Hirai et al., 2003) leave these methods unsatisfying.

Many investigators have identified human EPCs with flow cytometry using either a single or various cell surface markers, leading to a complicated list of putative human EPC phenotypes (Figure 1.8) (Timmermans et al., 2009).
To conclude, there are controversial issues in the field of EPCs which are caused by the lack of a unique EPC marker and by the phenotypical as well as functional overlap between EPCs, hematopoietic cells and mature ECs. Thus, these circumstances highlight the need and importance to identify markers specific for ECs or rather their progenitors. Whereas endothelial-/EPC-specific cell surface markers, if any endothelial-specific one exists, are of interest as well, specific endothelial-/EPC-specific transcription factors possibly reflect the cell phenotype better than a CD marker, when keeping in mind that transcription factors are key elements of gene regulatory networks which contribute to all different functions of a particular cell type such as EPCs.

**Figure 1.8: Putative human EPC phenotypes.** Cell surface markers (and combinations thereof) used in published flow cytometry-based studies to identify human EPCs. As the table shows, most of the surface marker combinations used included CD34. (Timmermans et al., 2009)
However, osteoprogenitors and EPCs differ considerably in terms of knowledge about transcription factors that define these two cell populations. Whereas transcription factors as markers of even immature osteoprogenitors such as Runx2 (Huang et al., 2007; Schroeder et al., 2005) are known, the situation in EPCs is to date not known. There are no specific transcription factors known to date that define EPCs.
2 Materials and methods

2.1 Cell Culture

In this thesis, primary human BMSCs, as well as different cell lines were cultured. Each cell type’s particular cell culture conditions are detailed in a separate section.

In each case, cells were cultured either in tissue culture flasks, in tissue culture plates, or in multi-well plates at 37°C in an atmosphere of 5% CO\textsubscript{2} and 95% humidity. All plasticware for cell culture that were used are made of polystyrene and were purchased from TPP (Trasadingen, Switzerland).

Furthermore, irrespective of the cell type, for passaging, cells were trypsinized by incubating with pre-warmed 0.05% trypsin/0.02% EDTA solution (Gibco) in PBS for 5-10 minutes at 37°C in an atmosphere of 5% CO\textsubscript{2} and 95% humidity. Cell detachment was assessed using an Axiovert 25 microscope (Zeiss, Oberkochen, Germany). Thereafter, trypsin was inactivated by the addition of two volumes of the appropriate culture medium containing 10% FBS. Cell suspension was transferred to a Falcon tube and centrifuged at 300 g for 10 minutes. After removal of the supernatant, cells were resuspended in the appropriate culture medium prior to cell counting using the hemocytometer. Afterwards, the cell density of choice was achieved by plating the calculated volume of cell suspension into the appropriate tissue culture flask, tissue culture plate, or multi-well plate.

For the cell culture, the following types of cell culture media were used:

- Alpha Minimal Essential Medium (αMEM) containing 1.0 g/L glucose, 2 mM L-glutamine, 110 mg/L sodium pyruvate (Gibco, Paisley, United Kingdom), supplemented with 2.2 g/L sodium bicarbonate (Sigma, Buchs SG, Switzerland), 0.02 M HEPES (Gibco), and 1% penicillin and streptomycin (Gibco)
- Dulbecco’s Modified Eagle medium (DMEM) high glucose containing 4.5 g/L glucose, 4 mM L-glutamine (Gibco), supplemented with 3.7 g/L sodium bicarbonate, and 110 mg/L sodium pyruvate (Sigma), 0.02 M HEPES, and 1% penicillin and streptomycin
- Dulbecco’s Modified Eagle medium (DMEM) low glucose containing 1.0 g/L glucose, 4 mM L-glutamine, 110 mg/L sodium pyruvate (Gibco), supplemented with 3.7 g/L sodium bicarbonate, 0.02 M HEPES, and 1% penicillin and streptomycin
- Medium 200 (M200), supplemented with Low Serum Growth Supplement (LSGS, 50x; Gibco) and 1% penicillin and streptomycin
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- Roswell Park Memorial Institute (RPMI) 1640 medium containing 4.5 g/L glucose, 2 mM L-glutamine, 110 mg/L sodium pyruvate (Gibco), 1.5 g/L sodium bicarbonate, 0.01 M HEPES, supplemented with 1% penicillin and streptomycin

2.1.1 Human bone marrow-derived stromal cells

The isolation and expansion of human bone marrow-derived stromal cells (BMSC) were conducted according to the method reported by Pittenger and colleagues (Pittenger et al., 1999). For the sake of clarity: The terms 'bone marrow-derived stromal cells' (BMSC) and 'mesenchymal stem cells' (MSC) are used interchangeably.

As regards the isolation of human BMSCs, human bone marrow aspirates were obtained with ethical approval and the written consent of patients undergoing total hip replacement, spinal fusion or vertebral body isolation. Bone marrow aspirates were kindly provided by Dr. Sven Hoppe and Dr. Lorin Benneker (Inselspital Bern, Bern, Switzerland), Dr. Gian Salzmann (Universitätsklinikum Freiburg, Freiburg, Germany), and Dr. Markus Loibl (Universitätsklinikum Regensburg, Regensburg, Germany). Bone marrow was diluted 1:5 with serum-free αMEM. Next, the fat layer was removed by centrifugation at 170 g for 5 minutes, and the resulting cell pellet was resuspended in the same volume of serum-free αMEM used for the initial dilution step. Per 1 ml of undiluted bone marrow aspirate, 2.6 ml of Ficoll (Histopaque-1077; Sigma) which was allowed to adjust to room temperature before use were added to a 15 ml Falcon tube. The diluted bone marrow aspirate was carefully layered over the Ficoll and centrifuged at 800 g for 20 minutes with acceleration and brake on the lowest level. Mononuclear cells were collected from the interphase (Figure 2.1) using a 2 ml pipette.

*Figure 2.1: Isolation of bone marrow-derived stromal cells (BMSC) by means of Ficoll density gradient centrifugation from bone marrow aspirates. After centrifugation at 800 g for 20 minutes of the bone marrow aspirate layered over Ficoll, the mononuclear cells containing*
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the BMSCs form an interphase between the Ficoll and the blood plasma. (Source: standard operation procedure (SOP) PRBC 002/04 (AO Research Institute Davos, Davos, Switzerland))

Thereafter, cells were washed twice with αMEM supplemented with 5% heat-inactivated FBS (Gibco). Between the two washing steps, cells were centrifuged at 400 g for 15 minutes. Afterwards, the cell pellet was resuspended in an appropriate volume of BMSC culture medium to count the cells using a hemocytometer. BMSC culture medium is composed of αMEM, supplemented with 10% heat-inactivated FBS. Mononuclear cells were seeded at a density of 13,333 cells/cm² into cell culture flasks. Cells were cultured and kept untouched for 4 days in BMSC culture medium to allow BMSCs to attach. After 4 days, cells were replenished with new BMSC culture medium to remove non-adherent and dead cells, respectively.

As regards the expansion of human BMSCs, the isolated cells were cultured in BMSC culture medium, the adherent cell fraction was passaged once, grown to confluence, and cryopreserved in cryogenic tubes in 92% heat-inactivated FBS and 8% dimethyl sulfoxide (DMSO; Sigma) at 2.0 x 10⁶ cells/ml for future use. Cryopreserved BMSCs became part of the common stock at the AO Research Institute Davos, Davos, Switzerland, and all BMSCs used for the experiments in this thesis were used from this common BMSC stock. Prior to the experiments, cells were thawed and expanded to the required cell number in BMSC expansion medium at 37°C in an atmosphere of 5% CO₂ and 95% humidity. BMSC expansion medium is composed of BMSC culture medium, supplemented with 5 ng/ml recombinant human basic fibroblast growth factor (bFGF; Fitzgerald Industries International, Concord, MA). Cells were replenished with new medium every second day and passaged when reaching a confluence of 70-80%.

For osteogenic differentiation, BMSCs were trypsinized, seeded in BMSC expansion medium, and subjected to osteogenic differentiation according to one of the following procedures:

- Osteogenic differentiation assay of expanded post-sort BMSCs: cells were seeded in 24-well plates at a cell density of 10,000 cells/cm² in BMSC expansion medium. When the cells reached confluence, medium was switched to BMSC osteogenic medium and control medium, respectively.

- Osteogenic differentiation of BMSCs seeded at low cell density: cells were seeded at a cell density of 15,000 cells/cm² in BMSC expansion medium. Next day, medium was switched to BMSC osteogenic medium and control medium, respectively.
- Osteogenic differentiation of BMSCs seeded at high cell density: cells were seeded at a cell density of 60,000 cells/cm² in BMSC expansion medium. Next day, medium was switched to BMSC osteogenic medium and control medium, respectively.

Irrespective of which osteogenic differentiation procedure was applied, BMSC osteogenic medium is composed of BMSC culture medium, supplemented with 1% non-essential amino acids (Gibco), 10 mM β-glycerophosphate (Sigma), 50 µg/ml ascorbate-2-phosphate (Sigma), and 10⁻⁷ M dexamethasone (Sigma). BMSC culture medium served as BMSC control medium.

Unless otherwise stated, heat-inactivated FBS was used for the cell culture of human BMSCs, and where specifically stated, any of two additional FBS were used for the cell culture of human BMSCs: human MSC-qualified fetal bovine serum (hMSC-FBS; Gibco) and Biochrom FBS Superior (Biochrom AG, Berlin, Germany). The hMSC-FBS was specially tested for the ability to support the expansion and function of human MSCs, whereas the Biochrom FBS Superior represents a standardized serum appropriate for routine cell culture.

### 2.1.2 AD-293 cells, HeLa cells

Human embryonic kidney 293 (HEK293)-derived AD-293 cells (Stratagene, La Jolla, CA) as well as HeLa cells (American Type Culture Collection (ATCC), Manassas, VA) were cultured in tissue culture flasks or multi-well plates in AD-293 culture medium and HeLa culture medium, respectively. Both AD-293 culture medium and HeLa culture medium are composed of DMEM high glucose, supplemented with 10% heat-inactivated FBS. Cells were replenished with new medium every second day and passaged by a ratio 1:10 when reaching a confluence of 50-60% (AD-293) and 80% (HeLa), respectively. 1.0 x 10⁶ cells/ml were cryopreserved in cryogenic tubes in AD-293 and HeLa freezing medium, respectively made up of 50% DMEM high glucose, 40% heat-inactivated FBS, and 10% DMSO for future use.
2.1.3 Lenti-X 293T cells

Lenti-X 293T cells (Clontech, Mountain View, CA) are HEK 293T-derived cells which are optimized for lentivirus production according to the Lenti-X™ Lentiviral Expression Systems instructions (Clontech). Lenti-X 293T cells were cultured in tissue culture flasks or multi-well plates in Lenti-X 293T culture medium. Lenti-X 293T culture medium is composed of DMEM high glucose, supplemented with 10% heat-inactivated FBS. Cells were replenished with new medium every second day, passaged when reaching a confluence of 50-60%, and reseeded at a seeding density of 3,000 cells/cm². 1.0 x 10⁶ cells/ml were cryopreserved in cryogenic tubes in Lenti-X 293T freezing medium, made up of 50% DMEM high glucose, 40% heat-inactivated FBS, and 10% DMSO for future use.

2.1.4 HUVEC

Human umbilical vein endothelial cells (HUVEC) pooled from multiple isolates (HUVECP; Gibco) were cultured in tissue culture flasks or multi-well plates in complete M200 medium (Gibco). Cells were replenished with new medium every second day, passaged when reaching a confluence of 70-80%, and reseeded at a seeding density of 2,500 cells/cm². 1.0 x 10⁶ cells/ml were cryopreserved in cryogenic tubes in 90% heat-inactivated FBS and 10% DMSO for future use.

2.1.5 hTERT-MSCs

Human MSCs immortalized through expression of human telomerase reverse transcriptase (hTERT) (hTERT-MSC) and constitutively expressing GFP were kindly provided by Dr. Dario Campana, St. Jude Children’s Research Hospital, Memphis, TN (Mihara et al., 2003). hTERT-MSCs were cultured in tissue culture flasks or multi-well plates in hTERT-MSC culture medium. hTERT-MSC culture medium is composed of RPMI1640 medium, supplemented with 10% heat-inactivated FBS and 10⁻⁶ M hydrocortisone (Sigma). Cells were replenished with new medium every second day, passaged when reaching a confluence of 70-80%, and reseeded at a seeding density of 3,000 cells/cm². 0.5 x 10⁶ cells/ml were cryopreserved in cryogenic tubes in hTERT-MSC freezing medium made up of 70% hTERT-MSC culture medium, 20% heat-inactivated FBS, and 10% DMSO for future use. For osteogenic differentiation, hTERT-MSCs were trypsinized and seeded at a cell density of 20,000 cells/cm² in hTERT-MSC culture medium. When the cells reached confluence,
medium was switched to hTERT-MSC osteogenic medium and control medium, respectively. hTERT-MSC osteogenic medium is composed of DMEM low glucose, supplemented with 10% heat-inactivated FBS, 1% non-essential amino acids, 10 mM β-glycerophosphate, 50 µg/ml ascorbate-2-phosphate, and 10⁻⁷ M dexamethasone. DMEM low glucose, supplemented with 10% heat-inactivated FBS served as hTERT-MSC control medium.

2.1.6 Rat MSCs

Rat MSCs had been harvested from whole bone marrow plugs obtained from rat femur and tibia and cryopreserved in liquid nitrogen. Cells were cultured in tissue culture flasks or multi-well plates in rat MSC culture medium. Rat MSC culture medium is composed of αMEM, supplemented with 15% heat-inactivated FBS. Cells were replenished with new medium every second day, passaged when reaching a confluence of 70-80%, and reseeded at a seeding density of 4,000-5,000 cells/cm².

For osteogenic differentiation, rat MSCs were trypsinized and seeded at a cell density of choice (either 15,000 or 60,000 cells/cm²) in rat MSC culture medium. Next day, medium was switched to rat MSC osteogenic medium and control medium, respectively. Rat MSC osteogenic medium is composed of rat MSC culture medium, supplemented with 1% non-essential amino acids, 10 mM β-glycerophosphate, 50 µg/ml ascorbate-2-phosphate, and 10⁻⁷ M dexamethasone. Rat MSC culture medium served as rat MSC control medium.

2.1.7 Fluorescence activated cell sorting (FACS)-mediated cell isolation

MSCs osteogenically induced for 3 days after adenoviral transduction (see section 2.3.1) were dissociated into a single cell suspension using Accutase (PAA Laboratories, Pasching, Austria) treatment for 20 minutes, followed by Collagenase type 2 (3,000 units/ml; Worthington Biochemical Corporation, Lakewood, NJ) treatment for 60 minutes and subsequent treatment with 0.05% trypsin/0.02% EDTA solution (Gibco) for 5-10 minutes to detach remaining cells that have reattached during Collagenase type 2 treatment. Total cell suspension was pelleted, and then resuspended at 9 x 10⁶ total cells/ml in FACS buffer composed of PBS containing 1% FBS and 1 mM EDTA (personal communication from Cornelius Fischer, Operator FACS Sorting Service at ETH Hönggerberg, ETH Zurich,
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Switzerland). According to the personal communication, a minimal amount of FBS is crucial to avoid clump formation of the cells, while EDTA serves to chelate Ca\(^{2+}\) ions required for cadherin-mediated cell-cell adhesion and clump formation. An equal volume of AccuMax (PAA Laboratories) was added to the resuspended cell pellet and incubated for 10 minutes at 37°C to prevent cell clump formation. Prior to cell sorting, cells were filtered through a 40 µm cell strainer (BD Biosciences) to remove cell aggregates. To separate the two cell populations Runx2 GFP\(^+\) and Runx2 GFP\(^-\), cells were sorted using a FACSaria cell sorter (BD Biosciences, Franklin Lakes, NJ) and FACSDiva software (BD Biosciences). FACSaria II was equipped with a 488 nm laser and a 100 µm nozzle. Sorting was performed at 20 psi sheath pressure. Debris and dead cells were gated out in the light scatter dot plot and an uninfected control was used to set a threshold to define a positive EGFP signal. The EGFP signal was collected in the fluorescein isothiocyanate (FITC) channel through a 530/30-nm bandpass filter. Post-sorting, the resulting cells were collected in BMSC culture medium and replated into T-75 at a seeding density of approximately 5,000 cells/cm\(^2\) to separately expand the cell populations. Unsorted cell population was taken from the original cell population that was subjected to cell sorting.

Where specifically stated in the experimental chapters, staining with propidium iodide (PI; Miltenyi Biotec, Bergisch Gladbach, Germany) at a final concentration of 1 µg/ml was included to assess cell viability and to exclude dead cells.

2.2 Generation of recombinant adenoviruses

In this thesis, the following two different recombinant adenoviruses had already been generated and were ready to be used:

- Ad.GFP: adenoviral reporter driving EGFP expression controlled by the ubiquitously active cytomegalovirus (CMV) promoter
- Ad.Runx2: adenoviral reporter driving EGFP expression controlled by the presence of functional and active RUNX2 protein

Therefore, their generation is described only briefly. However, in the course of the thesis, new Ad.Runx2 was generated starting from an early plasmid stage, i.e. pAdEasy-1-pShuttle-Runx2-MinPro-EGFP-polyA_clone 8.1 that had been used for the original generation of Ad.Runx2. Therefore, the sections about 'Production of primary adenoviral stock in AD-293 cells' and 'Amplification of primary adenoviral stock in AD-293 cells' are described in more...
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detail even though originally, Ad.Runx2 had already been generated to be used for cell culture experiments.

In this thesis, the following two different recombinant adenoviruses were generated:

- Ad.HoxA9: adenoviral reporter driving EGFP expression controlled by the presence of HOXA9 protein
- Ad.Vezf1: adenoviral reporter driving EGFP expression controlled by the presence of VEZF1 protein

Therefore, their generation is described in detail.

All recombinant adenoviral reporter constructs were generated in a series of steps according to the AdEasy™ XL Adenoviral Vector System (Stratagene), based on the work of He and colleagues (He et al., 1998).

In brief, the DNA of interest is cloned into the shuttle vector pShuttle. Thereafter, the shuttle vector is linearized and then subjected to homologous recombination with the adenoviral backbone plasmid pAdEasy-1 present in recombination proficient BJ5183-AD-1 bacteria. The recombined pAdEasy-1-pShuttle construct is linearized and then transfected into HEK293-derived AD-293 cells as packaging line able to complement the deleted viral assembly gene E1 (Graham et al., 1977), where finally the production of the recombinant adenovirus particles takes place. The production of recombinant adenoviruses follows the procedure schematically shown in Figure 2.2.
Figure 2.2: Schematic overview of the production of recombinant adenovirus according to the pAdEasy™ XL Adenoviral Vector System. The DNA of interest is cloned into the shuttle vector pShuttle. Then, the linearized shuttle vector is subjected to homologous recombination with the adenoviral backbone plasmid pAdEasy-1 present in recombination proficient BJ5183-AD-1 bacteria, and finally production of the recombinant adenovirus particles is performed in AD-293 packaging cells which provide the E1 gene in trans.
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As regards the generation of the reporter constructs by means of classical cloning techniques up to the stage of the recombinant Ad plasmid, all central plasmid intermediate cloning stages were verified by sequencing (Microsynth, Balgach, Switzerland) prior to continuation with cloning and usage for experiments, respectively.

### 2.2.1 Generation of Ad.GFP

In brief, for the construction of the corresponding shuttle vector, pShuttle-CMV-EGFP-polyA, the fragment containing EGFP was excised from pmaxGFP (Amaxa GmbH, Köln, Germany). Kozak consensus translation initiation site with the sequence ACCATGG was introduced into the EGFP fragment by PCR, resulting in EGFPK. All described experiments use the EGFP gene including the Kozak consensus translation initiation site for all subsequent clonings, however the term "EGFP" instead of "EGFPK" in all the construct names is used.

The fragment containing EGFP gene including Kozak consensus translation initiation site was ligated into pShuttle-CMV (7,469 bp), a shuttle vector containing the ubiquitously active CMV promoter as well as a SV40 polyA sequence, which resulted in pShuttle-CMV-EGFP-polyA (8,203 bp), depicted in Figure 2.3. The sequence of the construct is mentioned in appendix 8.1.2.
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**Figure 2.3:** Map of pShuttle-CMV-EGFP-polyA, the shuttle vector containing the EGFP gene including Kozak consensus translation initiation site, whose expression is under the control of the ubiquitously active CMV promoter. Only the features making up the functioning as a reporter construct are shown, however the features crucial for cloning are not depicted.

Once the gene of interest had been cloned into the shuttle vector, the recombinant adenoviral reporter was generated according to the AdEasy™ XL Adenoviral Vector System in the way described below during the generation of Ad.Runx2, Ad.HoxA9, and Ad.Vezf1, respectively. Following the production of primary adenoviral stock and its amplification in AD-293 cells, viral titer was determined to be $0.17 \times 10^{12}$ particles/ml according to optical density (OD$_{260}$); thereby, one OD$_{260}$ corresponds to approximately $10^{11}$ viral particles/ml. The dialyzed adenovirus fraction was aliquoted in 100 µl aliquots into sterile Eppendorf tubes, snap-frozen using liquid nitrogen, and stored at -80°C. The resulting adenovirus was designated Ad.GFP.

### 2.2.2 Generation of Ad.Runx2

The Runx2 reporter construct was generated by means of multiple rounds of classical restriction and ligation cloning, thereby cloning the following features into the shuttle vector pShuttle: a 12x tandemly arranged 7 bp sequence, AACCACA, of the Runx2 binding site in the MMP13 promoter (Jimenez et al., 1999), followed by a 136 bp minimal promoter sequence from the 5'-flanking region of the human MMP13 gene reported to be essential for basic transcription (Tardif et al., 1997), followed by the EGFP gene including Kozak consensus translation initiation site, terminated by the SV40 polyA sequence. Thus, the shuttle vector containing the Runx2 reporter construct is referred to as pShuttle-Runx2-MinPro-EGFP-polyA, depicted in Figure 2.4.
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Figure 2.4: Map of pShuttle-Runx2-MinPro-EGFP-polyA, the shuttle vector containing the Runx2 reporter construct. Only the features making up the functioning as a reporter construct are shown, however the features crucial for cloning are not depicted.

For the construction of the pShuttle-Runx2-MinPro-EGFP-polyA (Figure 2.4), the fragment containing 12xRunx2-MinPro (containing the Runx2 binding site) was excised from pCR-Script-12xRunx2-MMP13 (3,353 bp; constructed by Microsynth, Balgach, Switzerland) with EcoRV and XbaI. The fragment containing the EGFP gene, including Kozak consensus translation initiation site, was excised from pShuttle-CMV-EGFP-polyA (8,203 bp) with XbaI and EcoRV. Thereafter, both fragments were ligated into EcoRV digested pShuttle-polyA (6,854 bp), which resulted in pShuttle-Runx2-MinPro-EGFP-polyA (8,004 bp). The sequence of the construct is mentioned in appendix 8.1.2.

After the gene of interest had been cloned into the shuttle vector, the recombinant adenoviral reporter was generated by means of homologous recombination between two homologous DNA segments of the adenoviral backbone vector, pAdEasy-1, and the linearized shuttle vector containing the gene of interest in E. coli. pShuttle-Runx2-MinPro-EGFP-polyA was digested with PmeI in order to linearize the plasmid. Then, the linearized pShuttle-Runx2-MinPro-EGFP-polyA was transformed into electrocompetent BJ5183-AD-1 bacteria and selected for kanamycin-resistance, which is present on the unstable, linear pShuttle-Runx2-MinPro-EGFP-polyA. BJ5183-AD-1 is a BJ5183 strain pre-transformed with...
the pAdEasy-1 plasmid. The BJ5183-AD-1 strain is recA proficient and provides the machinery required for the recombination event between the shuttle vector and the pAdEasy vector. The resulting recombined plasmid DNA was verified by sequencing of the appropriate regions using pShuttle forward and pShuttle-CMV reverse primers in two different reactions. As a result, pAdEasy-1-pShuttle-Runx2-MinPro-EGFP-polyA_clone 8.1 was used for the production of Ad.Runx2.

2.2.3 Generation of Ad.HoxA9

First generation, E1-, E3-deleted, serotype 5 recombinant adenoviral vector carrying the HoxA9 responsive reporter construct was created according to the AdEasy™ XL Adenoviral Vector System (Stratagene, La Jolla, CA), based on the work of He and colleagues (He et al., 1998). In brief, HoxA9 reporter construct was generated by means of multiple rounds of classical restriction and ligation cloning, thereby cloning the following features into the shuttle vector pShuttle: a 12x tandemly arranged 12 bp sequence, TTAATAAAATTG, of the HoxA9 binding site in the human E-selectin promoter (Bandyopadhyay et al., 2007), followed by a 136 bp minimal promoter sequence from the 5'-flanking region of the human MMP13 gene reported to be essential for basic transcription (Tardif et al., 1997), followed by the EGFP gene including Kozak consensus translation initiation site, terminated by the SV40 polyA sequence. Thus, the shuttle vector containing the HoxA9 reporter construct is referred to as pShuttle-HoxA9-MinPro-EGFP-polyA, depicted in Figure 2.5.
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Figure 2.5: Map of pShuttle-HoxA9-MinPro-EGFP-polyA, the shuttle vector containing the HoxA9 reporter construct. Only the features making up the functioning as a reporter construct are shown, however the features crucial for cloning are not depicted.

In more detail, starting from HoxA9 fragment 1 (HoxA9F1) and 2 (HoxA9F2) making up the whole 12x tandemly arranged 12 bp HoxA9 binding site sequence, second-strand synthesis was performed by PCR using Taq DNA polymerase using specific primers referred to as HoxA9F1 rev and HoxA9F2 rev, respectively. This resulted in double-stranded HoxA9F1 and HoxA9F2, each with 3' A overhangs on both sides of the PCR products. Fragment size (HoxA9F1: 122 bp, HoxA9F2: 124 bp) was confirmed by means of gel electrophoresis using a 2% agarose gel, as detailed in section 2.10.2. The PCR product of HoxA9F1 was ligated into pGEM®-T (Promega, Madison, WI) by ligating the PCR product into pGEM®-T in the presence of T4 DNA ligase (Promega) for 1 hour at room temperature, followed by heat inactivation of T4 DNA ligase for 15 minutes at 65°C. The PCR product of HoxA9F2 was inserted into TOPO vector pCR®II-TOPO (Invitrogen) using the TOPO TA cloning kit (Invitrogen) by incubating the PCR product together with TOPO vector pCR®II-TOPO for 5-10 minutes at room temperature, referred to as TA cloning. Thereafter, the resulting cloning reactions were transformed into One Shot® TOP10 chemically competent cells by means of heat shock transformation (30 minutes on ice, 30 seconds at 42°C, 2 minutes on ice). Transformed cells were plated on Ampicillin-containing agar plates. To find positive
clones, HoxA9F2 clones were screened by PCR screening using M13rev together with HoxA9F1rev and HoxA9F2rev, respectively to test for correct orientation of insert, and HoxA9F1 clones were screened using M13for(-20) together with M13rev for presence of insert. Positive candidate colonies were picked to produce clonal bacteria liquid culture in the presence of Ampicillin. Plasmid DNA was isolated as detailed in section 2.10.1. This resulted in pGEM-T-HoxA9F1 and pCRII-TOPO-HoxA9F2.

The 115 bp HoxA9F1 fragment was excised from pGEM-T-HoxA9F1 with NotI and HindIII, the 167 bp HoxA9F2 fragment was excised from pCRII-TOPO-HoxA9F2 with HindIII and XhoI. After agarose gel electrophoresis to separate the resulting DNA fragments according to the procedure detailed in section 2.10.2, the bands of interest were excised from the low melting agarose gel, and DNA fragments were extracted and purified as detailed in section 2.10.3. Thereafter, HoxA9F1 and HoxA9F2 were both ligated into NotI/XhoI digested pShuttle-polyA (6,854 bp) using T4 DNA ligase, afterwards T4 DNA ligase was heat inactivated for 15 minutes at 65°C, and the resulting cloning reaction, pShuttle-HoxA9-polyA (7,129 bp), was transformed into One Shot® TOP10 electrocompetent cells (Invitrogen, Carlsbad, CA) by means of electroporation using the MicroPulser™ Electroporation Apparatus (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer’s recommendations. Transformed cells were plated on Kanamycin-containing agar plates. Several colonies were picked to produce clonal bacteria liquid culture in the presence of Kanamycin. Plasmid DNA was isolated as detailed in section 2.10.1. Control digests using Clal together with BstXI, and HindIII together with BstXI resulted in the correct band patterns. The resulting clone of pShuttle-HoxA9-polyA was verified by sequencing of the appropriate regions using pShuttle forward and pShuttle-CMV reverse primers in two different reactions.

The region containing the MinPro-EGFP fragment in pShuttle-12xRunx-MinPro-EGFP was amplified by PCR using MinPro for Clal and EGFP rev XhoI primers. Fragment size (MinPro-EGFP: 852 bp) was confirmed by means of gel electrophoresis using a 2% agarose gel, as detailed in section 2.10.2. PCR product was inserted into TOPO vector pCR® II-TOPO (Invitrogen) using the TOPO TA cloning kit (Invitrogen) by incubating the PCR product together with TOPO vector pCR® II-TOPO for 5-10 minutes at room temperature. Thereafter, the resulting cloning reaction was transformed into One Shot® TOP10 chemically competent cells by means of heat shock transformation (30 minutes on ice, 30 seconds at 42°C, 2 minutes on ice). Transformed cells were plated on Kanamycin-containing agar plates. To find positive clones, colonies were screened by PCR screening using M13for(-20)
together with M13rev primers. Positive candidate colonies were picked to produce clonal bacteria liquid culture in the presence of Kanamycin. Plasmid DNA was isolated as detailed in section 2.10.1. This resulted in pCRII-TOPO-MinPro-EGFP.

The 856 bp MinPro-EGFP fragment was excised from pCRII-TOPO-MinPro-EGFP with EcoRI and XhoI. The 252 bp 12xHoxA9 fragment was excised from pShuttle-HoxA9-polyA with KpnI and EcoRI. After agarose gel electrophoresis to separate the resulting DNA fragments according to the procedure detailed in section 2.10.2, the bands of interest were excised from the low melting agarose gel, and DNA fragments were extracted and purified as detailed in section 2.10.3. Thereafter, MinPro-EGFP and 12xHoxA9 fragments were ligated into KpnI/XhoI digested pShuttle-HoxA9-polyA (7,129 bp; SAP treated afterwards for 60 minutes at 37°C, followed by heat inactivation of SAP for 20 minutes at 70°C) using T4 DNA ligase, afterwards T4 DNA ligase was heat inactivated for 15 minutes at 65°C, and the resulting cloning reaction, pShuttle-HoxA9-MinPro-EGFP-polyA (7,952 bp), was transformed into One Shot® TOP10 electrocompetent cells by means of electroporation using the MicroPulser™ Electroporation Apparatus according to the manufacturer’s recommendations. Transformed cells were plated on Kanamycin-containing agar plates. To find positive clones, colonies were screened by PCR screening using pShuttle forward together with pShuttle-CMV reverse primers. Positive candidate colonies were picked to produce clonal bacteria liquid culture in the presence of Kanamycin. Plasmid DNA was isolated as detailed in section 2.10.1. Control digest using HindIII/BamHI resulted in the correct band pattern. The resulting clone of pShuttle-HoxA9-MinPro-EGFP-polyA was verified by sequencing of the appropriate regions using pShuttle forward and pShuttle-CMV reverse primers in two different reactions. As a result, pShuttle-HoxA9-MinPro-EGFP-polyA_clone 2 (7,952 bp) was used for the production of Ad.HoxA9. The sequence of the construct is mentioned in appendix 8.1.2.

After the gene of interest had been cloned into the shuttle vector, pShuttle, the recombinant adenoviral genome is generated by means of homologous recombination between two homologous DNA segments of the adenoviral backbone vector, pAdEasy-1, and the shuttle vector containing the gene of interest in E. coli. This approach has been incorporated into the AdEasy™ system (Stratagene) (He et al., 1998). pShuttle-HoxA9-MinPro-EGFP-polyA_clone2 was digested with Pmel in order to linearize the plasmid. Then, the linearized pShuttle-HoxA9-MinPro-EGFP-polyA_clone2 was transformed into electrocompetent BJ5183-AD-1 bacteria and selected for kanamycin-resistance. BJ5183-AD-1 is a BJ5183 strain pre-transformed with the pAdEasy-1 plasmid. The BJ5183-AD-1 strain is
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recA proficient and provides the machinery required for the recombination event between the shuttle vector and the pAdEasy vector. The resulting transformed bacteria colonies appear as three populations: very large, intermediate-sized, and small-sized colonies. 12 of the smallest colonies representing the most potential recombinants were picked and used to produce liquid culture. Plasmid DNA was isolated as detailed in section 2.10.1. Control digests using Pacl were performed and run on a 0.8% agarose gel, as detailed in section 2.10.2. Restriction digest of recombinant Ad plasmid DNA with Pacl should yield a large fragment of 30 kb, and a smaller fragment of either 3.0 kb or 4.5 kb, depending on where the recombination took place. Often, there are faint background bands in BJ5183-AD-1 plasmid Minipreps. Therefore, to clarify whether the putative recombinants exhibiting the expected bands as predominant bands are indeed recombinants, the plasmids are transformed into One Shot TOP 10 E. coli. Furthermore, another reason for transforming the plasmids into One Shot TOP 10 E. coli is to remove the plasmids from the milieu present in BJ5183-AD-1 that favors further, unwanted recombination events. Plasmid DNA was isolated as detailed in section 2.10.1. Control digests using Pacl were performed and run on a 0.8% agarose gel, as detailed in section 2.10.2. Ideally, the faint background bands present in BJ5183-AD-1 should now be absent. Two recombinants whose band size pattern was confirmed in the second preparation were verified by sequencing of the appropriate regions using pShuttle forward and pShuttle-CMV reverse primers in two different reactions. As a result, pAdEasy-1-pShuttle-HoxA9-MinPro-EGFP-polyA_clone 7 and clone 10 (~40 kb) were used for the production of Ad.HoxA9.

The resulting recombinant Ad plasmid was further amplified in E. coli and plasmid DNA was isolated by means of Midiprep procedure as detailed in section 2.10.1. This resulted in 231.5 µg (pAdEasy-1-pShuttle-HoxA9-MinPro-EGFP-polyA_clone 7) and 117.5 µg (pAdEasy-1-pShuttle-HoxA9-MinPro-EGFP-polyA_clone 10) of plasmid, respectively. 6 µg of recombinant Ad plasmid were digested with Pacl to linearize the recombinant Ad plasmid, followed by heat inactivation of restriction enzyme for 20 minutes at 65°C. Complete digest of the 6 µg of recombinant Ad plasmid were verified on a 0.8% agarose gel, as detailed in section 2.10.2.

2.2.4 Generation of Ad.Vezf1

First generation, E1-, E3-deleted, serotype 5 recombinant adenoviral vector carrying the Vezf1 responsive reporter construct was created according to the AdEasy™ XL Adenoviral
Vector System (Stratagene, La Jolla, CA), based on the work of He and colleagues (He et al., 1998).

In brief, Vezf1 reporter construct was generated by means of multiple rounds of classical restriction and ligation cloning, thereby cloning the following features into the shuttle vector pShuttle: a 12x tandemly arranged 12 bp sequence, TTAACCCCCACTC, of the Vezf1 binding site in the human endothelin-1 promoter (Aitsebaomo et al., 2001), followed by a 136 bp minimal promoter sequence from the 5′-flanking region of the human MMP13 gene reported to be essential for basic transcription (Tardif et al., 1997), followed by the EGFP gene including Kozak consensus translation initiation site, terminated by the SV40 polyA sequence. Thus, the shuttle vector containing the Vezf1 reporter construct is referred to as pShuttle-Vezf1-MinPro-EGFP-polyA, depicted in Figure 2.6.

Figure 2.6: Map of pShuttle-Vezf1-MinPro-EGFP-polyA, the shuttle vector containing the Vezf1 reporter construct. Only the features making up the functioning as a reporter construct are shown, however the features crucial for cloning are not depicted.

Plasmids are transformed and cultured in dam⁻/dcm⁻ E. coli (New England Biolabs GmbH) as the ClaI restriction site required for the cloning of the Vezf1 construct shows dam methylation sensitivity.

In more detail, starting from Vezf1 fragment 1 (Vezf1F1) and 2 (Vezf1F2) making up the whole 12x tandemly arranged 12 bp Vezf1 binding site sequence, second-strand synthesis
was performed by PCR using Taq DNA polymerase using specific primers referred to as VezfiF1 rev and VezfiF2 rev, respectively. This resulted in double-stranded VezfiF1 and VezfiF2, each with 3’ A overhangs on both sides of the PCR products. Fragment size (VezfiF1: 120 bp, VezfiF2: 118 bp) was confirmed by means of gel electrophoresis using a 2% agarose gel, as detailed in section 2.10.2. PCR products of VezfiF1 and VezfiF2 were ligated into pGEM®-T by ligating each PCR product into pGEM®-T in the presence of T4 DNA ligase for 1 hour at room temperature, followed by heat inactivation of T4 DNA ligase for 15 minutes at 65°C. Thereafter, the resulting cloning reactions were transformed into dam
/dcm
 chemically competent E. coli (New England Biolabs, Ipswich, MA) by means of heat shock transformation (30 minutes on ice, 30 seconds at 42°C, 2 minutes on ice). Transformed cells were plated on Ampicillin-containing agar plates. To find positive clones, VezfiF1 and VezfiF2 clones were screened by PCR screening (thermal cycling conditions: initial denaturation for 2 minutes at 95°C; 25 cycles of 20 seconds at 95°C (denaturation), 45 seconds at 56°C (annealing), 45 seconds at 72°C (extension); 5 minutes at 72°C (final extension)) using M13for(-20) and M13rev primers for presence of insert. Two positive candidate colonies each were picked to produce clonal bacteria liquid culture in the presence of Ampicillin. Plasmid DNA was isolated as detailed in section 2.10.1. This resulted in pGEM-T-VezfiF1 and pGEM-T-VezfiF2.
In order to perform a KpnI/ClaI restriction digest of pShuttle-HoxA9-MinPro-EGFP-polyA_clone2 required for the subsequent cloning procedure, the plasmid was transformed into dam
/dcm
 electrocompetent cells by means of electroporation using the MicroPulser™ Electroporation Apparatus according to the manufacturer’s recommendations. Transformed cells were plated on Kanamycin-containing agar plates. Two colonies were picked to produce clonal bacteria liquid culture in the presence of Kanamycin. Plasmid DNA was isolated as detailed in section 2.10.1. The 110 bp VezfiF1 was excised from pGEM-T-VezfiF1 with KpnI and HindIII, the 111 bp VezfiF2 was excised from pGEM-T-VezfiF2 with HindIII and Clal, and the 7,684 bp pShuttle-MinPro-EGFP-polyA was excised from pShuttle-HoxA9-MinPro-EGFP-polyA_clone2 with KpnI and Clal (SAP treated afterwards for 60 minutes at 37°C, followed by heat inactivation of SAP for 20 minutes at 70°C). After agarose gel electrophoresis to separate the resulting DNA fragments according to the procedure detailed in section 2.10.2, the bands of interest were excised from the 2% low melting agarose gel, and DNA fragments were extracted and purified as detailed in section 2.10.3. Thereafter, VezfiF1 and VezfiF2 were both ligated into gel purified KpnI/Clal digested pShuttle-HoxA9-MinPro-EGFP-poly (7,684 bp) using T4
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DNA ligase, afterwards T4 DNA ligase was heat inactivated for 15 minutes at 65°C, and the resulting cloning reaction, pShuttle-Vezf1-MinPro-EGFP-polyA (7,905 bp), was transformed into One Shot® TOP10 electrocompetent cells by means of electroporation using the MicroPulser™ Electroporation Apparatus according to the manufacturer’s recommendations. Transformed cells were plated on Kanamycin-containing agar plates. To find positive clones, pShuttle-Vezf1-MinPro-EGFP-polyA clones were screened by PCR screening (thermal cycling conditions: initial denaturation for 2 minutes at 95°C; 25 cycles of 20 seconds at 95°C (denaturation), 45 seconds at 56°C (annealing), 45 seconds at 72°C (extension); 5 minutes at 72°C (final extension)) using pShuttle for_2 and 12xVezf1-MinPro rev primers for presence of a 423 bp fragment containing the 12xVezf1 binding site (Vezf1F1 and Vezf1F2). One positive candidate colony was picked to produce clonal bacteria liquid culture in the presence of Kanamycin. Plasmid DNA was isolated as detailed in section 2.10.1. Control digests using either SacII or EcoRI resulted in the correct band patterns. The resulting clone of pShuttle-Vezf1-MinPro-EGFP-polyA was verified by sequencing of the appropriate regions using pShuttle forward and pShuttle-CMV reverse primers in two different reactions. As a result, pShuttle-Vezf1-MinPro-EGFP-polyA_clone 24 (7,905 bp) was used for the production of Ad.Vezf1. The sequence of the construct is mentioned in appendix 7.1.2.

After the gene of interest had been cloned into the shuttle vector, pShuttle, the recombinant adenoviral genome is generated by means of homologous recombination between two homologous DNA segments of the adenoviral backbone vector, pAdEasy-1, and the shuttle vector containing the gene of interest in E. coli. This approach has been incorporated into the AdEasy™ system (Stratagene) (He et al., 1998). pShuttle-Vezf1-MinPro-EGFP-polyA_clone24 was digested with PmeI in order to linearize the plasmid, followed by SAP treatment for 60 minutes at 37°C, and rounded off with heat inactivation of SAP for 20 minutes at 70°C. Then, the linearized pShuttle-Vezf1-MinPro-EGFP-polyA_clone24 was transformed into electrocompetent BJ5183-AD-1 bacteria and selected for kanamycin-resistance. The resulting transformed bacteria colonies appear as three populations: very large, intermediate-sized, and small-sized colonies. 14 of the smallest colonies representing the most potential recombinants were picked and used to produce liquid culture. Plasmid DNA was isolated as detailed in section 2.10.1. Control digests using PacI were performed and run on a 0.8% agarose gel, as detailed in section 2.10.2. Restriction digest of recombinant Ad plasmid DNA with PacI should yield a large fragment of 30 kb, and a smaller fragment of either 3.0 kb or 4.5 kb, depending on where the recombination
took place. Two recombinants, whose band size pattern was confirmed, were verified by sequencing of the appropriate regions using pShuttle forward and pShuttle-CMV reverse primers in two different reactions. As a result, pAdEasy-1-pShuttle-Vezf1-MinPro-EGFP-polyA_clone 8_2 and clone 11_2 (~40 kb) were used for the production of Ad.Vezf1.

The resulting recombinant Ad plasmid was further amplified in E. coli and plasmid DNA was isolated by means of Midiprep procedure as detailed in section 2.10.1. This resulted in 65.4 µg (pAdEasy-1-pShuttle-Vezf1-MinPro-EGFP-polyA_clone 8_2) and 91.2 µg (pAdEasy-1-pShuttle-Vezf1-MinPro-EGFP-polyA_clone 11_2) of plasmid, respectively. 6 µg of recombinant Ad plasmid were digested with PacI to linearize the recombinant Ad plasmid, followed by heat inactivation of restriction enzyme for 20 minutes at 65°C. Complete digest of the 6 µg of recombinant Ad plasmid was verified on a 0.8% agarose gel, as detailed in section 2.10.2.

2.2.4.1 Production of primary adenoviral stock in AD-293 cells

Recombinant adenovirus clones were produced in AD-293 cells. For each preparation, 200,000 - 300,000 AD-293 cells were plated in one well of a 6-well plate 16 hours prior to transfection, by which time they reached 90% confluence. For each virus, 2-3 µg of recombinant adenoviral plasmid DNA, digested with PacI, was used to transfect AD-293 cells by means of FuGENE® HD Transfection Reagent (Roche Applied Science). First, plasmid DNA was diluted appropriately by adding 190 µl of serum-free DMEM to 2 µg (~10 µl) of linearized recombinant adenoviral plasmid DNA. The transfection mix was prepared by adding 8 µl of FuGENE® HD Transfection Reagent directly into 200 µl of diluted DNA without allowing contact with the walls of the Eppendorf tube, immediately followed by a vortex for 1-2 seconds. Prior to usage, FuGENE® HD Transfection Reagent, DNA, and serum-free DMEM were allowed to adjust to room temperature, and the FuGENE® HD Transfection Reagent vial was vortexed for one second. After incubation at room temperature for 15 minutes (with an additional vortex after 7 minutes), 100 - 200 µl of transfection mix was added to each of two wells of the 6-well plate covered with AD-293 cells in a drop-wise manner at different places distributed over the whole well. Transfected cells were monitored for cytopathic effects, 1 ml of fresh culture medium was added on day 3 post-transfection, and adenovirus was harvested on day 10 post-transfection, when prominent cytopathic effects were observed throughout the cells, by aspirating the cell suspension and pelleting them at 2,000 g for 10 minutes at 4°C. Thereafter, supernatant
was discarded and cell pellet was resuspended in 0.5 ml of sterile PBS. After three cycles of freezing in liquid nitrogen and rapid thawing at 37°C, cellular debris were collected by centrifugation at 2,000 g for 10 minutes. The supernatant representing the primary adenoviral stock was transferred to a fresh microcentrifuge tube, snap-frozen using liquid nitrogen, and stored at -80°C.

2.2.4.2 Amplification of primary adenoviral stock in AD-293 cells

To generate higher titer viral stocks, for each recombinant adenovirus clone, 4 x 10^7 AD-293 cells per T-300 flask were infected with primary adenoviral stock (30 µl of adenoviral stock per T-300 flask) in a minimal volume of medium (15 ml per T-300 flask) for 2 hours. Thereafter 35 ml of culture medium per T-300 flask were added, and grown for 3-4 days until prominent cytopathic effects were observed, at which time viruses were harvested. Harvested cells were pelleted at 2,000 g for 10 minutes at 4°C, supernatant was discarded, and cell pellet was resuspended in a total of 12 ml of serum-free DMEM. Cell suspensions were snap-frozen once using liquid nitrogen and stored at -80°C until further proceeding. The resultant amplified viruses, after three cycles of freezing in liquid nitrogen and rapid thawing at 37°C followed by Benzonase (50 U/ml; Sigma) treatment at 37°C for 30 minutes were purified over successive cesium chloride (CsCl; Sigma) gradients by two rounds of centrifugation, each at 120,000 g for 1.5 hours. The heavy CsCl solution (density: 1.4 g/ml; in 10 mM Tris-hydrochloric acid, pH 7.9 (Tris-HCl; Sigma)) was added very slowly under the light CsCl solution (density: 1.2 g/ml; in 10 mM Tris-HCl, pH 7.9), followed by adding the amplified virus very gently on top of the CsCl gradient, and finally supplementing with 10 mM Tris-HCl, pH 7.9 to about 2-3 mm below the top of the polycrystalline centrifugation tube (Thermo Scientific, Waltham, MA). After dialysis against 10 mM Tris-HCl, pH 7.5, 200 mM sodium chloride (NaCl; Sigma), 1 mM EDTA (Sigma), and 4% (w/v) sucrose (Sigma) in Float-A-Lyzer® G2 (molecular weight cut off: 50 kDa; Spectrum Laboratories Europe, Breda, The Netherlands), viral particle concentrations were estimated to be between 0.55-1.1 x 10^{12} viral particles/ml according to optical density (OD_{260}); thereby, one OD_{260} corresponds to approximately 10^{12} viral particles/ml. Infectious particles are estimated to be 1:100 of the total. The dialyzed adenovirus fractions were aliquoted in 50 µl aliquots into sterile Eppendorf tubes, snap-frozen using liquid nitrogen, and stored at -80°C. The resulting adenoviruses were designated Ad.Runx2 clone 8.1, Ad.HoxA9 clone 7 and clone 10, Ad.Vezfi
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clone 8 and clone 11. The infectious titer was subsequently determined by performing a standard plaque assay on confluent AD-293 cultures.

In the course of the thesis, several batches successively produced were utilized for cell culture experiments. Production of a fresh batch of the reporter adenovirus was performed by amplifying aliquots in AD-293 cells by infection at a MOI of 1, and thereafter following the same procedure like for the amplification of primary adenoviral stock in AD-293 cells.

2.2.5 Plaque assay

Assays for measuring adenoviral particle concentration can generally be divided into physical and biological assays (Mittereder et al., 1996). Physical methods measure the viral particle concentration without taking biological functionality into account, e.g. by optical absorbance of the viral DNA (Mittereder et al., 1996). In contrast, biological methods such as the plaque assay are based on infection of cultured cells, which in turn can be detected due to aspects of the biological functionality of the virus, e.g. formation of visible plaques in a cell monolayer which support replication of the virus, such as 293 cells (Graham and van der Eb, 1973; Mittereder et al., 1996). In short, the plaque assay is the assay of choice for the determination of viral particle functionality, albeit correct assessment of functional viral particle titer is not error-free due to the complexity of the assay and its dependency on assay conditions such as the time of viral particle adsorption and adsorption volume (Mittereder et al., 1996).

Although the optical absorbance assay is an accurate assay to determine the total viral particle concentration, counts of functional viral particles allow a more precise determination of the multiplicity of infection (MOI) applied for cell culture experiments, and serve to substantiate the OD_{260}^-based viral titer determination. Observation during the course of the plaque assay should be performed daily. Due to cytopathic effects occurring after cells had been infected with adenoviral particles, the infected cells undergo lysis and infect their neighbor cells. After lysis of these cells, released viral particles infect adjacent cells. In this way, the virus spreads and gradually results in a cleared spot which is distinguishable macroscopically, which is referred to as plaque.

Plaque assay was performed according to the AdEasy™ XL Adenoviral Vector System (Stratagene). In detail, AD-293 cells were plated at a seeding density of 500,000 cells per well of a 6-well plate. After incubation of the cells overnight at 37°C, serial 1:10 dilutions
were prepared in AD-293 growth medium in 15 ml Falcon tubes, starting from a $10^{-5}$ dilution of the viral stock. Thus, the dilution factors were $10^{-5}$ to $10^{-9}$, along with a "medium only" control. For transduction, culture medium was aspirated from the AD-293 cells and replaced with 1 ml of each serially diluted viral stock. The infected cells were incubated at 37°C for 2 hours with gentle rocking every 20 minutes. Thereafter, viral stock-containing growth medium was carefully removed and cells were overlaid with 3 ml of 1.25% SeaPlaque low-melting point agarose in growth medium (maintained as a 5% solution in sterile PBS at 45°C immediately prior to dilution to 1.25% with growth medium). The assay was incubated at 37°C. If agarose overlay turned yellow, an additional overlay of 1.5 ml was poured. Plaques were counted within 12-19 days by microscopic inspection. The average of the number of plaques from duplicate wells where isolated plaques were clearly visible and countable was determined, and the infectious viral titer was determined by multiplying this value by the corresponding dilution factor.

### Adenoviral transduction of cells

Two different approaches were utilized to transduce cells with adenovirus:

- Lanthofection was used to efficiently transduced BMSCs and cell lines for actual experiments
- Standard adenoviral transduction was used for the amplification of adenoviral stocks in AD-293 cells

#### 2.3.1 Lanthofection

Cells were plated at a particular seeding density and transduced with the appropriate reporter adenovirus the following day. High efficiency transduction of the cells was achieved using lanthofection (Palmer et al., 2008). Unless otherwise stated, cells were transduced at a multiplicity of infection (MOI) of 100 using any of the transcription factor-specific reporter adenoviruses. In this case, in brief, adenoviral vector stocks were added to serum-free αMEM to achieve a concentration of 100 infectious viral particles/cell based on the determined infectious viral titer, as assessed by plaque assay. Where specifically stated (section 4.2.3.5: 'OD$_{260}$-based viral titer'), adenoviral vector stocks to achieve a concentration of estimated 100 infectious viral particles/ml were added based on the determined viral titer as assessed by OD$_{260}$ determination, considering that infectious viral particles are
estimated to be 1:100 of the total viral particles. Lanthanum chloride (LaCl₃; Sigma) was added to result in a final concentration of 100 µM. The mixture was gently vortexed and incubated for 15 minutes at room temperature. Thereafter, 1 ml of adenovirus suspension per 25 cm² of growth area seeded with cells was added to cells containing the appropriate volume of culture medium for cultivation of the cells. After 4 hours of infection at 37°C, medium was aspirated, cells were washed twice with phosphate buffered saline (PBS; Sigma) and replenished with fresh appropriate culture medium.

### 2.3.2 Standard adenoviral transduction

Passage 3-6 cells were plated at a seeding density of 60,000 cells/cm² and transduced with the Runx2 reporter adenovirus Ad.Runx2 the following day. High efficiency transduction

To generate higher titer viral stocks, for each of the two resulting recombinant Ad clones, a total of $2.4 \times 10^8$ AD-293 cells in 6 x T-300 flasks ($4 \times 10^7$ AD-293 cells per T-300 flask) were infected with primary adenoviral stock (30 µl of adenoviral stock per T-300 flask) in a minimal volume of medium (15 ml per T-300 flask) for 2 hours, thereafter 35 ml of culture medium per T-300 flask were added.

AD-293 cells were plated at a particular seeding density and transduced with the appropriate reporter adenovirus the following day. Cells were transduced at a MOI of 1 using any of the transcription factor-specific reporter adenoviruses to be amplified. In brief, adenoviral vector stocks were dissolved in AD-293 culture medium to achieve a concentration of 100 viral particles/cell (1 in 100 viral particles is considered to be infectious) based on the determined viral titer, assessed by either OD₂₆₀ determination or plaque assay. Alternatively, in case of transduction with primary adenoviral stocks where the viral cannot be determined, an arbitrary volume (30 µl of adenoviral stock per T-300 flask) was applied. Thereafter, culture medium in the flasks was aspirated, and 15 ml of adenovirus suspension per T-300 flask seeded with cells was added. After 2 hours of infection at 37°C, 35 ml of AD-293 culture medium was added. Cells were incubated, inspected daily until complete cytopathic effects were observed. At this time point, the adenovirus was harvested.
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2.4 Generation of recombinant DNA constructs

In this thesis, the following two different recombinant DNA constructs were generated:
- pCBG68-12xRunx2-MinPro-control vector: Runx2-responsive luciferase reporter construct containing the same Runx2 binding sites used in the Runx2 reporter adenovirus
- pCMV-Myc: non-functional Runx2 expressing construct based on pCMV-Runx2-Myc, the functional Runx2 expressing construct

These set of constructs was used for cotransfection experiments to assess the responsiveness of the Runx2 binding sites to human RUNX2 protein.

2.4.1 Generation of Runx2-responsive luciferase reporter

In order to investigate the functionality and specificity of the feature of the Runx2 reporter adenovirus which is responsive to functional Runx2 protein, we cloned a luciferase reporter construct containing the same Runx2 binding sites and Minimal Promoter used in the Runx2 reporter adenovirus, referred to as pCBG68-12xRunx2-MinPro-control vector (Figure 2.7).

The region containing the 12xRunx2-MinPro fragment in pShuttle-12xRunx-MinPro-EGFP was amplified by PCR using 12xRunx2 for and MinPro rev HindIII primers. Fragment size (12xRunx2-MinPro: ~400 bp) was confirmed by means of gel electrophoresis using a 2% agarose gel, as detailed in section 2.10.2. After agarose gel electrophoresis to separate the resulting DNA fragments, the band of interest was excised from the low melting agarose gel, and DNA fragment was extracted and purified as detailed in section 2.10.3. XhoI/HindIII digested PCR product (407 bp) was ligated into XhoI/HindIII digested pCBG68-control vector (5,035 bp; SAP treated afterwards for 60 minutes at 37°C, followed by heat inactivation of SAP for 20 minutes at 70°C) using T4 DNA ligase, afterwards T4 DNA ligase was heat inactivated for 15 minutes at 65°C, and the resulting cloning reaction, pCBG68-12xRunx2-MinPro-control vector (5,429 bp), was transformed into One Shot® TOP10 electrocompetent cells by means of electroporation using the MicroPulser™ Electroporation Apparatus according to the manufacturer’s recommendations. Transformed cells were plated on Ampicillin-containing agar plates. Several colonies were picked to produce clonal bacteria liquid culture in the presence of Ampicillin. Plasmid DNA was isolated as detailed in section 2.10.1. Control digest using XbaI resulted in the correct band patterns. One of the resulting clones, pCBG68-12xRunx2-MinPro-control vector clone
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3, was verified by sequencing of the appropriate regions using 12xRunx2 for and MinPro rev HindIII primers in two different reactions. The sequence of the construct is mentioned in appendix 8.1.2.

Figure 2.7: Map of pCBG68-12xRunx2-MinPro-control vector, the functional Runx2-responsive luciferase reporter construct containing the same Runx2 binding sites used in the Runx2 reporter adenovirus. Only the features making up the functioning as a reporter construct are shown, however the features crucial for cloning are not depicted.

2.4.2 Generation of Runx2 expressing constructs

Furthermore, functional as well as non-functional Runx2 expressing constructs acting as effector plasmids were required in order to investigate the transactivation of the Runx2-responsive luciferase reporter construct in a cotransfection setup. Functional Runx2 expressing construct, pCMV-Runx2-Myc was kindly provided by Akinori Kan, Sensory & Motor System Medicine, Faculty of Medicine, University of Tokyo, Japan.

To generate the non-functional Runx2 expressing construct pCMV-Myc (Figure 2.8), pCMV-Runx2-Myc was transformed into dam/ -dcm/ chemically competent E. coli by means of heat shock transformation (30 minutes on ice, 30 seconds at 42°C, 2 minutes on ice). Transformed cells were plated on Ampicillin-containing agar plates. Several colonies were
picked to produce clonal bacteria liquid culture in the presence of Ampicillin. Plasmid DNA was isolated as detailed in section 2.10.1.

The ~2 kb fragment representing the coding sequence of human Runx2 cDNA was excised from pCMV-Runx2-Myc with XbaI. After 1.0% agarose gel electrophoresis to separate the resulting DNA fragments according to the procedure detailed in section 2.10.2, the band of interest (3.3 kb) was excised from the low melting agarose gel, and the DNA fragment was extracted and purified as detailed in section 2.10.3. Thereafter, XbaI digested pCMV-Runx2-Myc (3,282 bp; SAP treated afterwards for 60 minutes at 37°C, followed by heat inactivation of SAP for 20 minutes at 70°C) was self-ligated using T4 DNA ligase overnight, afterwards T4 DNA ligase was heat inactivated for 15 minutes at 65°C, and the resulting cloning reaction, pCMV-Myc, was transformed into One Shot® TOP10 chemically competent cells by means of heat shock transformation (30 minutes on ice, 30 seconds at 42°C, 2 minutes on ice). Transformed cells were plated on Ampicillin-containing agar plates. To find positive clones, colonies were screened by PCR screening using pCMV-IE forward together with M13 reverse primers. Positive candidate colonies exhibiting a 572 bp band were picked to produce clonal bacteria liquid culture in the presence of Ampicillin. Plasmid DNA was isolated as detailed in section 2.10.1. Control digest using Sacl resulted in the correct band pattern. The resulting clone of pCMV-Myc was verified by sequencing of the appropriate regions using pCMV-IE forward and M13 reverse primers in two different reactions. As a result, pCMV-Myc_clone6 (3,282 bp) was achieved (Figure 2.8). The sequence of the construct is mentioned in appendix 8.1.2.
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Figure 2.8: Map of pCMV-Myc, the non-functional Runx2-expressing construct. Only the feature making up the functioning as a reporter construct is shown, however the features crucial for cloning are not depicted.

2.5 Generation of recombinant lentiviruses

In this thesis, the following two different recombinant lentiviruses were generated, starting from custom-made lentiviral Runx2-specific MetLuc reporter constructs:

- Lv.1xRunx2: lentiviral reporter driving MetLuc expression controlled by the full rodent Runx2 promoter, i.e. the reporter reflects Runx2 promoter activity
- Lv.12xRunx2: lentiviral reporter driving MetLuc expression controlled by the a 12x tandemly arranged 7 bp sequence, AACCACA, of the Runx2 binding site in the MMP13 promoter (Jimenez et al., 1999), followed by a 136 bp minimal promoter sequence from the 5’-flanking region of the human MMP13 gene reported to be essential for basic transcription (Tardif et al., 1997), i.e. the reporter reflects RUNX2 protein activity

The above-mentioned recombinant lentiviral reporter constructs were generated in a series of steps according to the Lenti-X™ Lentiviral Expression System (Clontech, Mountain View, CA).

In brief, the DNA of interest is cloned into a lentiviral vector. Then, the lentiviral expression construct is cotransfected together with a Lenti-X HTX Packaging Mix into Lenti-X 293T cells. The Lenti-X HTX Packaging Mix is an optimized mixture of plasmids that express viral proteins in ratios that have been optimized for high efficiency packaging of lentiviral vector transcripts into lentiviral particles. After the lentiviral particles have been produced by the transfected Lenti-X 293T cells, the lentiviral supernatants are used to transduce the target cells. After the transduced cells have been subjected to antibiotic selection due to an antibiotic selection marker present in the lentiviral constructs, a cell line stably expressing the reporter can be generated. The production of lentiviruses by the Lenti-X™ Lentiviral Expression System is schematically shown in Figure 2.9.
Figure 2.9: Schematic overview of the production of lentiviruses according to the Lenti-X™ Lentiviral Expression System. The DNA of interest is cloned into the lentiviral vector. Then, the lentiviral vector is cotransfected with the Lenti-X HTX Packaging Mix into Lenti-X 293T cells, where the lentiviral particles are assembled, then bud from the cell. Finally, the lentivirus can be harvested from the cell culture supernatant. While the lentiviral particles are infectious, they lack several genes required for subsequent replication and production in any target cells other than Lenti-X 293T cells.

2.5.1 Generation of lentiviral vectors

The lentiviral vectors pLV.ExSi.P/Puro-Runx2-MetLuc for the generation of Lv.1xRunx2 and pLV.ExSi.P/Puro-Runx2_X12-MetLuc for the generation of Lv.12xRunx2 were custom-made
and purchased as plasmid preparations at a concentration of 100 ng/µl (Cyagen, Sunnyvale, CA). The features of the constructs are highlighted in Figure 2.10.

**Figure 2.10:** Map of pLV.ExSi.P/Puro-Runx2-MetLuc and pLV.ExSi.P/Puro-Runx2_X12-MetLuc, the lentiviral MetLuc reporters responding to rodent Runx2 promoter and active RUNX2 protein, respectively. Both the features making up the functioning as a reporter construct as well as the features crucial for lentiviral biology are depicted.
2.5.2 Production of lentivirus from lentiviral vectors

For each of the two lentiviral vectors, $4.5 \times 10^6$ Lenti-X 293T cells (which had already been thawed and expanded in culture for 5 days at this point) were plated in a 100 mm tissue culture plate 24 hours prior to transfection, by which time they reached 80-90% confluence. 7 µg of each lentiviral vector was used to transfect Lenti-X 293T cells by means of Xfect™ HD Transfection Reagent (Clontech). For each transfection, two microcentrifuge tubes were prepared. Into tube 1, 494 µl of Xfect Reaction Buffer were added, followed by 36 µl of Lenti-X HTX Packaging Mix and 70 µl (7 µg) of lentiviral vector, leading to a total volume of 600 µl. Into tube 2, 592.5 µl of Xfect Reaction Buffer were added, followed by 7.5 µl of Xfect Polymer (which had been thoroughly vortex prior to use), leading to a total volume of 600 µl. Each tube was vortexed well to mix and then, the transfection mix was prepared by adding the polymer solution (tube 2) to the DNA solution (tube 1), immediately followed by a vortex at a medium speed for 10 seconds. After incubation of the transfection mixture at room temperature for 10 minutes to allow nanoparticles to form, the entire 1,200 µl of transfection mix was added to an individual 100 tissue culture plate covered with Lenti-X 293T cells in a drop-wise manner at different places distributed over the whole plate. The plate was gently rocked back and forth to mix, and then incubated at 37°C in the incubator. After 8 hours of transfection, the transfection medium was replaced with 10 ml of fresh Lenti-X 293T culture medium, and the transfected cells were incubated at 37°C for an additional 24-48 hours. At 24 and 48 hours post-transfection, respectively, 2 ml of each lentiviral supernatant was harvested, and thereafter, the plate was supplemented with 2 ml of Lenti-X 293T culture medium and further cultured. Lentiviral supernatants were centrifuged at 500 g for 10 minutes to remove cellular debris and used directly to transduce hTERT-MSC target cells.

2.6 Generation of stable Runx2 reporter hTERT-MSCs

2.6.1 Transduction of hTERT-MSC target cells with lentiviruses

To transduce hTERT-MSCs, the protocol from Lenti-X™ Lentiviral Expression System was used, which represents a general method for transduction of adherent cell lines using Polybrene. Polybrene represents a polycation that reduces charge repulsion between the lentivirus and the cell membrane and in this way increases transduction efficiency.
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The day before transduction, for each of the two lentiviruses, hTERT-MSCs (which had already been thawed and expanded in culture for 5 days at this point) were plated in a 100 mm tissue culture plate at a cell seeding density of both 500 cells/cm² and 1,000 cells/cm², respectively. 1 ml of lentiviral supernatant per transduction of each lentivirus was used to transduce hTERT-MSCs. For each transduction, 1 ml of lentiviral supernatant was supplemented with 2 µl of Polybrene (Millipore, Billerica, MA; 10 mg/ml), and the mixture was added to 9 ml of culture medium present in the tissue culture plates of hTERT-MSCs. This led to a final concentration of 2 µg/ml of Polybrene. The entire transduction mix was added to an individual 100 tissue culture plate covered with hTERT-MSCs in a drop-wise manner at different places distributed over the whole plate. The transduction was performed for 8 hours at 37°C in the incubator. Thereafter, the lentivirus-containing transduction medium was removed and replenished with fresh hTERT-MSC culture medium. To improve transduction efficiency, hTERT-MSCs were re-transduced with fresh corresponding lentiviral supernatant (corresponding to 72 hours post-transduction) for 8 hours of transfection, and in turn replenished with fresh hTERT-MSC culture medium. The incubation of the cells was continued for 2 more days to allow the cells to express the antibiotic resistance gene.

2.6.2 Killing curve analysis of hTERT-MSCs

Untransduced hTERT-MSCs were cultured in hTERT-MSC culture medium in 6-well plates at a cell seeding density of 1,000 cells/cm². After 24 hours, the cells of different wells were treated or not with three different concentrations of Puromycin (Puromycin dihydrochloride; Carl Roth GmbH, Karlsruhe, Germany) (2 µg/ml, 6 µg/ml, or 10 µg/ml). hTERT-MSCs were subjected to antibiotic selection for 15 days, changing the medium every 2-3 days.

2.6.3 Antibiotic selection and expansion of stably transduced hTERT-MSCs

After allowing the re-transduced hTERT-MSCs to grow for 2 more days in hTERT-MSC culture medium, 2 µg/ml of Puromycin was utilized for antibiotic selection of lentivirally transduced hTERT-MSCs. The effective selection concentration of Puromycin was determined from killing curve analysis. Untransduced cells were subjected to antibiotic
selection as well to confirm whether the conditions of antibiotic selection were sufficiently stringent to eliminate cells not expressing the resistance gene.

The selection was performed for 12 days changing the medium containing selection antibiotic every 2-3 days, at which time drug-resistance clones appeared. The resulting cell lines were referred to as Lv.1xRunx2-hTERT-MSC and Lv.12xRunx2-hTERT-MSC, whereby at this point in time, for each cell line, a 500 cells/cm² and a 1,000 cells/cm² condition were present, each in an individual T-25 cell culture flask.

For expansion and establishment of stably transduced hTERT-MSC cell lines, Lv.1xRunx2-hTERT-MSC as well as Lv.12xRunx2-hTERT-MSC, each at both cell seeding density conditions, were trypsinized and transferred into an individual T-75 cell culture flask. Thereby, for the further expansion of the selected cells, the Puromycin concentration was reduced from the selection concentration to half the selection concentration, referred to as the expansion concentration.

At the time point when the different cell lines became confluent in the T-75 cell culture flasks, the cells were trypsinized, cells of both cell seeding density conditions of an individual lentivirally transduced hTERT-MSC cell line were pooled, and transferred into an individual T-300 cell culture flask. From this point on, expanded cells were either further expanded while sticking to cell seeding density of 3,000 cells/cm², used for validation experiments, or frozen in hTERT-MSC freezing medium and stored in the liquid nitrogen.

2.7 Analyses of reporter expression

2.7.1 Fluorescence microscopy, fluorescence microscopy image analysis

Fluorescence microscopy was performed using an Olympus CK40 inverted microscope (Olympus, Volketswil, Switzerland) equipped with a mercury lamp. Microscopic images were captured with an Olympus DP71 microscope digital camera (Olympus) using the manufacturer's DP Controller software version 3.1.1.267 (Olympus).

The total area of green fluorescent cells was quantified by measuring the cumulative pixel area that was considered as green, as schematically shown in Figure 2.11. To exclude green fluorescent debris, only structures larger than 50 pixels were included. The percentaged pixel area of a particular image was then reported as the percentage of cumulative pixel
area divided by the total 2040 x 1536 pixel image area. All the image analysis steps were performed using KS400 image analysis software (Zeiss, Göttingen, Germany). First, the captured red-green-blue (RGB) color image was converted into a gray scale image: the brighter a particular green pixel, the higher the resulting gray scale value in the converted image. The gray scale histogram of an image represents the distribution of the pixels over the gray level scale in the image. The image is then analyzed based on this histogram by means of the thresholding technique. Thereby, all pixels above a threshold gray level (individually determined for each image) are considered to belong to the object (i.e. green cell), whereas all pixels below that threshold are considered to be outside the object. While determining the threshold, a visual display reflecting those pixels that meet the threshold requirements served to visually find the best match with the actual fluorescent image. Once the optimal threshold was set, the KS400 image analysis software calculated the cumulative pixel area belonging to the object. The resulting value served as starting point for subsequent calculations, as stated in the corresponding results sections.

**Figure 2.11:** Assessment of the pixel area of green fluorescent cells. In the left panel, a sample fluorescent image of MSCs transduced with AdRunx2 is shown which was imaged by means of an Olympus CK40 inverted microscope (equipped with a mercury lamp). Original RGB fluorescent images were converted into a gray scale image, of which a histogram showing the gray scale values profile was created (right panel). As the user adjusted the threshold for the gray scale value, a visual display was updated to reflect those pixels that meet the threshold requirements. That threshold that led to the most accurate reflection of the actual fluorescent image was chosen to determine the cumulative pixel area. The areas composed of pixels with grey scale values above the threshold were bordered to highlight what was included in the cumulative pixel area count (left panel), clearly showing that the thresholding technique identifies the objects of interest in the image.
2.7.2 Luciferase assays

Overall, two different luciferase assays were performed:
- Chroma-Glo™ Luciferase Assay System (Promega) for the generation of luminescence from firefly luciferase, a non-secretory enzyme
- Renilla Luciferase Assay System (Promega) for the generation of luminescence from Metridia luciferase (MetLuc). MetLuc belongs to the more recently identified luciferases that, unlike most of the luciferases, are naturally secreted molecules.

2.7.2.1 Chroma-Glo Luciferase Assay

The Chroma-Glo Reagent was prepared by transferring the content of one bottle of Chroma-Glo Assay Buffer to one bottle of Chroma-Glo Assay Substrate, followed by mixing until the substrate was thoroughly dissolved. Aliquots were stored at -80°C. On the day of measurement, Chroma-Glo Reagent and cells were equilibrated to room temperature for 30 minutes. 250 µl of Chroma-Glo Reagent were added to the same volume of culture medium present in individual wells of a 48-well plate. After addition, cell lysis was allowed to be completed for at least 5 minutes. 5, 30, and 60 minutes after the onset of cell lysis at room temperature while the plate was wrapped in aluminum foil to protect from light, 100 µl of the samples were transferred to separate wells of a 96-well white plate. Luminescence was measured using a Victor3™ 1420 Multilabel Reader (without emission filter; measurement time: 1 second). Luminescence was expressed in the unit 'counts per second (CPS)'.

2.7.2.2 Renilla Luciferase Assay System

The Renilla Luciferase Assay Reagent was prepared by adding the content of one vial of Renilla Assay Substrate to one bottle of Renilla Assay Buffer. Aliquots were stored at -80°C. On the day of measurement, Renilla Assay Reagent were equilibrated to room temperature for 45 minutes. 100 µl of Renilla Assay Reagent were added to 20 µl of conditioned medium containing MetLuc which had been transferred into individual wells of a 96-well white plate. Directly after the addition of the Renilla Assay Reagent, luminescence was measured using a Victor3™ 1420 Multilabel Reader (without emission filter; measurement time: 1 second). Luminescence was expressed in the unit 'counts per second (CPS)'.

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2.8 Determination of cell numbers and proliferation

2.8.1 Quantification of cellular DNA

DNA content of cell samples was quantified by means of the Hoechst 33258 (bisbenzimide) method as described by Labarca and Paigen (Labarca and Paigen, 1980). The assay is based on the binding of Hoechst 33258 to the minor groove of DNA, thereby leading to an enhancement of the fluorescence of the dye. The fluorescence of Hoechst 33258 is related to the AT content of a DNA sample, so it is very important to use a standard similar to the sample under investigation. Calf thymus DNA standard is double-stranded, highly polymerized, and it has an approximate content of 60% AT (40% GC).

For this purpose, cell monolayers (samples in quadruplicates) were washed once with PBS and digested with 0.5 mg/ml recombinant Proteinase K (Roche, Rotkreuz, Switzerland) solution at 56°C for approximately 16 hours. Thereafter, samples were transferred into individual 1.5 ml Eppendorf tubes, Proteinase K was heat-inactivated at 95°C for 10 minutes on a thermostat 5320 (Eppendorf, Hamburg, Germany), and samples were stored at -20°C until further analysis. To quantify DNA, 40 µl of Proteinase K sample was transferred into individual wells of a 96-well white plate (Becton Dickinson, Franklin Lakes, NY), and 160 µl of a 1 µg/ml Hoechst 33258 assay solution was added to each well. After incubation for 20 minutes at room temperature while the plate was wrapped in aluminum foil to protect from light, and immediately afterwards, fluorescence intensity was measured using a Victor3™ 1420 Multilabel Reader (Perkin Elmer Life and Analytical Sciences, Schwerzenbach, Switzerland) at 360 nm (excitation) and 465 nm (fluorescence emission). DNA content was calculated based on a standard curve covering a range of 62.5 to 2,000 ng of DNA in 40 µl per well generated with calf thymus DNA (Invitrogen) dissolved in DPBS, which is composed of PBS, pH 7.4 containing 2 M NaCl (Sigma). DNA amount was expressed in the unit 'ng DNA/well'.

2.8.2 Cell Titer Blue assay

The assessment of cell proliferation and cell viability, respectively was performed using the Cell Titer Blue Viability Assay (Promega). This method is based on the ability of viable cells to metabolically reduce weakly fluorescent indicator dye resazurin into highly fluorescent resorufin (Czekanska, 2011). This reaction is mediated by different enzymes present in mitochondria, and the reaction product resorufin is excreted outside the cells to the
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medium. The rate of reduction and accumulation of highly fluorescent resorufin can be quantified and serves as a measure of the number of cells and their metabolic activity, respectively.

In this thesis, only the relative number of live cells using Cell Titer Blue assay was assessed. The appropriate number of cells (mentioned in the respective experimental chapters) was seeded into multi-well plates. At each time point when Cell Titer Blue assay was performed, the medium was aspirated and replenished with fresh culture medium containing a 10% solution of Cell Titer Blue. Three no-cell control wells were included. For 48-well plate format, 200 µl were used per well; for 96-well plate format, 100 µl were used per well. Multi-well plates were wrapped in aluminum foil and incubated – independent of the cell type investigated – for 2 hours at 37°C.

After incubation, 100 µl of each sample and no-cell control were transferred to a white opaque 96-well plate in duplicates. Fluorescence was measured using a Victor3™ 1420 Multilabel Reader at 530-570 nm (excitation) and 580-620 nm (fluorescence emission). Blank corrected results were calculated by averaging fluorescence values of the samples, followed by subtraction of the average fluorescence value of no-cell controls. Relative numbers of live cells were determined based on the linear relationship between fluorescence values and cell numbers. Relative number of live cells was expressed either in ‘%’ or as the measured raw value in ‘a.u.’.

2.8.3 Colony forming unit assay for bone marrow-derived mesenchymal stem cells

Colony forming unit (CFU) assay was performed as described elsewhere (Pochampally, 2008), by plating the different human BMSC populations in 100 mm tissue culture plates in triplicate at a seeding density of 100 cells/plate. BMSC expansion medium was changed twice a week. After 14 days of culture, cultures were washed twice with ice-cold PBS, fixed with methanol (Sigma) for 10 minutes and stained with 0.5% Crystal Violet (Sigma) in 25% methanol for 10 minutes, and finally air-dried. Colonies with diameter larger than 5 mm were referred to as fast-growing CFU, distinguishing them from slow-growing CFU. This was used in order to estimate proliferation. Colonies were counted microscopically and distinction of fast-growing and small-growing CFUs was conducted using AxioVision software (Zeiss).
2.9 Characterization of in vitro osteogenic differentiation

2.9.1 Alkaline phosphatase staining

Qualitative alkaline phosphatase (ALP) measurement was performed using Leukocyte Alkaline Phosphate Kit No. 85 (Sigma) according to the manufacturer’s recommendations, both for ALP staining in multi-well plates as well as for colony-forming unit ALP (CFU-ALP) assay.

The procedure is based on the fact that in the presence of ALP, naphthol AS-MX is liberated from naphthol AS-MX phosphate, which in turn is immediately coupled with the diazonium salt (fast blue RR salt), forming an insoluble, visible blue pigment at sites of ALP activity. The intensity of this pigment reflects the activity of ALP.

The medium was aspirated and cells were washed twice with PBS. Then, cells were fixed with citrate buffered acetone 60% for 30 seconds at room temperature. Fixed cells were rinsed briefly using MilliQ water, and then stained with ALP staining solution for 30 minutes at room temperature while shielded from light. ALP staining solution was prepared by dissolving the content of a fast blue RR salt capsule in 48 ml of MilliQ water, followed by the addition of 2 ml of naphthol AS-MX phosphate alkaline solution immediately before use. After staining for 30 minutes, staining solution was aspirated, samples were rinsed briefly using MilliQ water, and allowed to air-dry. Samples were either imaged using an Axiovert 25 microscope in the case of ALP stainings performed in multi-well plates, or stained colonies were counted manually in the case of CFU-ALP.

2.9.2 Quantification of ALP activity

In brief, ALP activity was measured using the Kit No. 104 (Sigma) for colorimetric ALP quantification which depends on the hydrolysis of p-nitrophenyl phosphate by ALP, yielding p-nitrophenol (p-Np) and inorganic phosphate. When made alkaline, p-nitrophenol is converted into a yellow complex which can be measured at 400-420 nm. Thereby, the intensity of the color formed in directly proportional to the amount of ALP activity.

For the determination of alkaline phosphatase (ALP) activity, cell monolayer samples (n=2 pooled samples from each 2 wells of 24-well plate per time point) were washed three times with cold PBS and extracted with 200 µl/well of 0.1% TritonX-100 (Sigma) in 10 mM Tris-HCl, pH 7.4 at 4°C for 2 hours on a gyratory shaker as previously described (Alini et al., 1994).
The extracts were frozen at -20°C until assayed. At the day of analysis, once the samples had been thawed while being stored at 4°C for 30 minutes, 50 µl of alkaline buffer (2-amino-2-methylpropanol, 1.9 M, pH 10.3), 50 µl of substrate solution (4-nitrophenyl phosphate disodium salt hexahydrate (Sigma) in 1 M diethanolamine (pH 9.8; Sigma) containing 0.5 mM MgCl₂ (Sigma)) were added into an 1.5 ml Eppendorf tube. Then, 400 µl of cell lysate (2 pooled samples) were added into each tube. The mixture was vortexed and incubated for 15 minutes at 37°C in a heated block. The reaction was stopped by adding 50 µl of 0.55 M NaOH. The standard curve covering a range of 10 to 70 nM p-Np was prepared by using serial dilutions of a 550 nM p-Np solution in 0.1% Triton-X in 10mM Tris-HCl that were incubated for 15 minutes at 37°C like the unknown samples. The absorbance of 200 µl of standard and samples were measured at 405 nm in duplicates on a Victor3™ 1420 Multilabel Reader (Perkin Elmer), and compared with the p-Np standard curve. ALP activity was expressed as nmol of p-Np formed per min, and the resulting values were related to the DNA amount in µg of parallel cultures, resulting in the ALP unit 'nmol/min/µg DNA'.

2.9.3 Determination of calcification

2.9.3.1 ⁴⁵Ca incorporation

Matrix mineralization was determined by the incorporation of radioactive calcium chloride (⁴⁵CaCl₂; Perkin Elmer Life and Analytical Sciences) into newly mineralized matrix. For this purpose, medium of samples in 24-well plates was aspirated and replenished with 300 µl/well of the appropriate culture medium supplemented with ⁴⁵CaCl₂ to achieve an activity of 1.25 µCi/ml. Cell cultures wrapped with aluminum foil were incubated at 37°C in the incubator overnight, and rinsed once with PBS the following day. Afterwards, 500 µl/well of 70% formic acid (Sigma) was added, the multi-well plate was wrapped in Parafilm and aluminum foil, and incubated at 65°C for 1 hour to solubilize the radioactivity, as previously described (Alini et al., 1994). The multi-well plates were put into the radioactivity fume hood to let them cool down. Then, samples were transferred into individual scintillation tubes, and 3.5 ml of scintillation fluid (OptiPhase HiSafe 3; Perkin Elmer Life and Analytical Sciences) was added to each sample. The radioactivity was measured using a 1414 WinSpectral Liquid Scintillation Counter (Perkin Elmer Life and Analytical Sciences), and the resulting values were related to the DNA amount in µg of parallel cultures, resulting in the ⁴⁵Ca incorporation unit 'counts per min (CPM)/µg DNA'.

2.9.3.2 Alizarin Red S staining

Qualitative assessment of mineralization was performed by means of Alizarin Red S staining. Cells were cultured in 24-well plates and at each time point, samples were washed three times with ice-cold PBS and fixed with 70% ethanol (Sigma) for 1 hour at 4°C. Once fixed, cells were washed three times with MilliQ water for 5 minutes each. Subsequently, 0.3 ml of 40 mM Alizarin Red S (Sigma) in MilliQ water, pH 4.2 was added into individual wells of a 24-well plate and the multi-well plate was covered in aluminum foil. Samples were stained for 1 hour at room temperature shaking 30 rpm on an orbital shaker. After incubation, Alizarin Red S staining solution was aspirated, samples were rinsed five times with MilliQ water for 10 seconds each, washed once with MilliQ water for 15 minutes shaking 30 rpm on an orbital shaker, left in MilliQ water and stored at 4°C until images were taken using an Axiovert 25 microscope.

2.10 Molecular biology techniques

2.10.1 Plasmid DNA isolation from recombinant bacteria

Depending on the amount of required recombinant plasmid DNA, either DNA isolation Mini- or Midiprep procedure was utilized to extract plasmid DNA from bacterial overnight liquid cultures.

For small scale plasmid DNA preparation using GenElute™ HP Plasmid Miniprep Kit (Sigma), single bacteria colony was inoculated in 10 ml of LB medium containing the appropriate antibiotic and grown overnight at 37°C shaking 220 rpm. The next day, cells were harvested by centrifugation of 1-5 ml of liquid culture at 12,000 g for 1 minute. Depending on whether the plasmid to be isolated is a high or low copy number plasmid, 1 and 5 ml of liquid culture, respectively were used. Thereafter, cell pellet was resuspended in 200 µl of Resuspension Solution (stored at 4°C; previously supplemented with RNase A) by repetitive pipetting up and down. For lysis, 200 µl of Lysis Buffer was added and inverted gently 6-8 times to mix, and then, the cell lysate was incubated for 3-5 minutes at room temperature. For neutralization of the cell lysate, 350 µl of Neutralization Buffer was added, inverted gently 4-6 times to mix. Separation of cell debris from plasmid DNA was achieved by centrifugation at 12,000 g for 10 minutes. After centrifugation, the cleared lysate was transferred into a Miniprep Binding Column (placed in a collection tube), which
had been pre-incubated with 500 µl of Column Preparation Solution and thereafter been removed by centrifugation at 12,000 g for 1 minute. The column loaded with the cleared lysate was incubated for 2 minutes at room temperature, and thereafter centrifuged at 12,000 g for 1 minute. The column was washed once with 500 µl of Washing Solution 1 and once with 750 µl of Washing Solution 2 by centrifugation at 12,000 g for 1 minute each. Thereafter, an additional centrifugation step was performed at 12,000 g for 1 minute to remove all traces of ethanol derived from the Washing Solution 2. Finally, to elute the plasmid DNA, columns were transferred into a fresh 1.5 ml Eppendorf tube, and 50 µl of Elution Buffer which had been warmed up for 5 seconds in the microwave was applied directly to the center of the column. Following centrifugation at 12,000 g for 1 minute, the DNA concentration of the Miniprep was determined by $OD_{260}$ measurement using Nanodrop. Thereby, an $OD_{260}$ value of 1.0 corresponds to 50 ng/µl of double-stranded DNA. The microcentrifuge containing the DNA was stored at -20°C.

For larger scale plasmid DNA preparation using PureYield™ Plasmid Midiprep System (Promega), single bacteria colony-derived small volume liquid culture was further inoculated in 250 ml of LB medium containing the appropriate antibiotic for an additional overnight period at 37°C shaking 220 rpm. The next day, cells were subjected to the Midiprep procedure which represents an upscaled Miniprep procedure with the difference being that the procedure we utilized was based on a vacuum method, and not based on a centrifugation method like the Miniprep procedure.

### 2.10.2 Agarose gel electrophoresis

Agarose gel electrophoresis was used to separate DNA according to its molecular weight. Depending of the DNA fragment size of interest to be separated, a 0.8-2% agarose gel was run. The gel was prepared by dissolving the appropriate amount of agarose in 30 ml of 1x TAE buffer (Tris-acetate-EDTA buffer; 40 mM Tris, 20 mM acetid acid, 1 mM EDTA, pH 8.0) per gel, followed by microwaving the agarose solution for 1-3 minutes until the agarose was completely dissolved. Prior to casting the gel, ethidium bromide was added to a final concentration of approximately 0.2-0.5 µg/ml. Ethidium bromide binds to the DNA and allows visualization of the DNA under UV light. The agarose gel was poured into a gel tray with the well comb in place, and the gel was allowed to solidify for 1 hour at room temperature. Thereafter, the gel was submerged in 1x TAE buffer serving as running buffer,
the comb was removed, and samples were prepared for gel loading. Samples were mixed with 1/6 volume of 6x loading dye (Promega). Depending on the comb size, samples at a volume of either 12 µl for the narrow comb and 24 µl for the wide comb were loaded into individual wells of the gel. Gel was run at 50 V until the dye front was approximately 80% on the way down the gel. To estimate the size of the DNA in the applied samples, a marker with defined fragment sizes was run in parallel. DNA fragments, referred to as bands, were visualized by UV light using a Bio-Rad Chemi Doc and UV Transilluminator 2000 System (Bio-Rad).

2.10.3 DNA extraction and purification from agarose gel

Agarose gels destined for subsequent DNA extraction and purification were prepared with low melting agarose (Promega) instead of standard agarose (Sigma). To extract DNA from agarose gels for further cloning procedure (e.g. after restriction enzyme digestion), the desired band was cut out from the ethidium bromide stained low melting agarose gel under UV light with a clean, sharp scalpel blade. The obtained agarose gel slice was weighed and DNA was extracted according to the Wizard® SV Gel and PCR Clean-Up System (Promega). For this purpose, Membrane Binding Solution was added at a ratio of 10 µl of solution per 10 mg of agarose gel slice. The mixture was vortexed and incubated at 56°C for 10 minutes until the gel slice was completely dissolved. DNA purification was performed according to the centrifugation procedure. For each dissolved gel slice, dissolved gel mixture was transferred to a SV Minicolumn (placed in a collection tube) and incubated for 1 minute at room temperature. Thereafter, the column was centrifuged at 16,000 g for 1 minute and washed with 700 µl of Membrane Wash Solution (previously diluted with 95% ethanol) by centrifugation at 16,000 g for 1 minute. Wash step was repeated with 500 µl of Membrane Wash Solution by centrifugation at 16,000 g for 5 minutes. Thereafter, an additional centrifugation step was performed at 16,000 g for 1 minute to remove all traces of ethanol derived from the Membrane Wash Solution. Finally, to elute the DNA, columns were transferred into a fresh 1.5 ml Eppendorf tube, and 50 µl of Nuclease-Free Water was applied directly to the center of the column. Following incubation at room temperature for 1 minute, the column was centrifuged at 16,000 g for 1 minute. Concentration of the eluted DNA was determined by OD$_{260}$ measurement using Nanodrop. Thereby, an OD$_{260}$ value of 1.0 corresponds to 50 ng/µl of double-stranded DNA. The microcentrifuge containing the DNA was stored at -20°C.
2.11 Gene expression analyses

2.11.1 RNA isolation

At specific time points, cell monolayer samples were lysed by adding 0.5 ml of TRI reagent (Molecular Research Center, Cincinnati, OH), supplemented with 1:200 Polyacryl Carrier (Molecular Research Center), to the first of two wells of a 24-well plate, letting the TRI reagent act for 5-10 minutes at room temperature, and transferring the cell lysate to the duplicate well for the purpose of pooling. After again 5-10 minutes of letting the TRI reagent act on the cell monolayer, the cell lysis was completed by repetitive pipetting up and down. Then, the cell lysate was transferred to a 1.5 ml RNase-free Eppendorf tube and stored at -80°C until RNA isolation was performed. After having thawed the samples at room temperature, 0.1 ml of 1-bromo-3-chloropropane (Sigma) per 1 ml of TRI reagent originally used was added to the samples and they were shaked vigorously for 15 s, followed by incubation for 15 minutes at room temperature shaking 20-25 rpm on an orbital shaker (Heidolph Instruments, Schwabach, Germany) for the purpose of phase separation. Then, samples were centrifuged using the 5417R centrifuge (Eppendorf, Hamburg, Germany) at 12,000 g for 15 minutes at 4°C. Following centrifugation, the mixture separated into three phases: a lower phenol-chloroform phase containing proteins, an interphase containing DNA, and an upper aqueous phase containing RNA. The aqueous phase of each sample was carefully transferred into a fresh 1.5 ml RNase-free Eppendorf tube. Thereafter, 0.5 ml of isopropanol (Sigma) per 1 ml of TRI reagent originally used was added to the samples and they were incubated for 10 minutes at room temperature shaking 20-25 rpm on an orbital shaker for the purpose of RNA precipitation. RNA was precipitated by centrifugation of the samples at 12,000 g for 8 minutes at 4°C. Following centrifugation, the supernatant was removed and the RNA pellet was washed by adding 1 ml of 75% ethanol (Sigma) in diethylpyrocarbonate (DEPC; Sigma)-treated water per 1 ml of TRI reagent originally used. The RNA was re-pelleted by centrifugation at 12,000 g for 5 minutes at 4°C. To solubilize the RNA, the ethanol supernatant was removed and the pellets were allowed to air-dry for 30 minutes. Thereafter, RNA pellets were dissolved in 20 of DEPC-treated water by repetitive pipetting up and down, followed by incubation for 15 minutes at 56°C to achieve complete solubilization. RNA quantity and purity was assessed using a Nanodrop ND-1000 spectrophotometer (NanoDrop, Wilmington, DE). To determine the RNA quantity, the optical density (also called absorbance) at 260 nm (OD$_{260}$) is used since RNA has its absorption maximum at 260 nm: An OD$_{260}$ value of 1.0 is
equivalent to 40 µg/ml of RNA. The assessment of 260/280 ratio and 260/230 ratios gives an indication of the purity. The 260/280 ratio is an indication of protein contamination in the sample (proteins have their absorption maximum at 280 nm), whereas the 260/230 ratio is an indication of contamination with chaotropic salts or rests of phenol. For pure RNA, both ratios were expected to be around 1.9. Samples were then stored at -80°C for further use.

2.11.2 Reverse transcription

A particular reverse transcription reaction was performed with 500 ng of isolated RNA in a total reaction volume of 20 µl using TaqMan Reverse Transcription Reagents (Applied Biosystems, Carlsbad, CA). First, the reaction mix was prepared by adding the following non-enzymatic components to a 1.5 ml RNase-free Eppendorf tube: 2.0 µl of 10x PCR Buffer II, 4.4 µl of 25 mM magnesium chloride, 4.0 µl of deoxynucleotide triphosphates (dNTP) mixture (2.5 mM of each dATP, dCTP, dGTP, dTTP), and 1.0 µl of random hexamers (50 µM). After having briefly vortexed the mixture, 0.4 µl of RNase inhibitor (20 U/µl; resulting in 8 U) and 0.5 µl of MultiScribe reverse transcriptase (50 U/µl; resulting in 25 U) were added. Then, 12.3 µl of the mixture were distributed into single 200 µl thermowell tubes (Corning Incorporated, Corning, NY), and the appropriate volume of total RNA sample (corresponding to 500 ng RNA) was added, and DEPC-treated water was added to bring the final volume to 20 µl. The reaction was performed in a GeneAmp 5700 SDS instrument (Applied Biosystems) according to the following conditions: 10 minutes at 25°C for primer incubation, 30 minutes at 48°C for reverse transcription, and 5 minutes at 95°C for reverse transcriptase inactivation. After the reaction was completed, cDNA samples were diluted with 80 µl of Tris-EDTA (TE) buffer and stored at -20°C. If less than 500 ng of RNA had to be used for the reverse transcription, the volume of TE buffer was adjusted accordingly to keep the final dilution of 500 ng of input RNA diluted in a final volume of 100 µl constant.

2.11.3 Real time polymerase chain reaction

Real time polymerase chain reaction (PCR) is a method which is used to quantify a target DNA of interest. In contrast to standard PCR where the DNA amplified by PCR is detected at the end of the reaction, real time PCR is characterized by the fact that the DNA is detected in 'real time' while the PCR progresses. Real time PCR reactions are characterized by the
time point during cycling at which the amplified DNA reaches a certain threshold fluorescence. The number of cycles at which the fluorescence exceeds the threshold is referred to as the threshold cycle (C_t). The higher the starting copy number of the target DNA, the earlier the threshold in fluorescence is reached, i.e. the lower the C_t.

Detection of PCR products is commonly performed by either TaqMan® chemistry or by SYBR® Green chemistry. TaqMan® chemistry uses a fluorescently-labelled probe that binds to the specific PCR product, SYBR® Green chemistry makes use of a highly specific double-stranded DNA binding dye to detect the PCR product. In this thesis, TaqMan® chemistry was applied.

To perform real time PCR, either self-designed primers and probes synthesized by Microsynth and validated by with our group were used, or TaqMan Gene Expression Assays purchased from Applied Biosystems were used. Self-designed primers and probe, as well as TaqMan Gene Expression Assays that were used are listed in Table 2.1.

For real time PCR, a master mix was prepared containing 10 µl TaqMan Universal PCR master mix without AmpErase UNG (Applied Biosystems), either 1 µl of TaqMan Gene Expression Assay or 0.4 µl of self-designed forward and reverse primer each (final concentration: 900 nM) and 0.4 µl of self-designed probe (final concentration: 250 nM), and 6.8 µl (in the case of self-designed primers and probe) and 7 µl (in the case of TaqMan Gene Expression Assay) of DEPC-treated water, respectively to fill up to 18 µl of master mix per reaction. 18 µl of the appropriate master mix was distributed into individual wells of a thermo-fast 96 PCR detection plate (Thermo Scientific), and afterwards, 2 µl of cDNA per reaction was added, resulting in the final reaction volume of 20 µl. The plate was covered with absolute QPCR seal (Thermo Scientific) and briefly centrifuged (impulse at 1,000 g) using a Hettich Rotanta 46 centrifuge (Hettich, Tuttlingen, Germany). Real time PCR was performed on a GeneAmp 7500 Real Time PCR System (Applied Biosystems). For each sample, reaction was carried out in duplicates. PCR conditions were 95°C for 10 minutes to activate DNA polymerase, followed by 40 cycles of amplification at 95°C for 15 seconds and 60°C for 1 minute.

For the analysis, C_t values were automatically derived using the 7500 system software (Applied Biosystems). Gene expression analysis was performed using the comparative C_t method. Ribosomal protein L13A (RPL13A) served as housekeeping gene (Quiroz et al., 2010). The gene expression analysis consisted of four steps: First, the average C_t was determined from duplicate wells. Secondly, the ΔC_t value was calculated by subtracting the C_t value of the housekeeping gene from the C_t value of the gene of interest. Thirdly, the ΔΔC_t value
was calculated by subtracting the ∆Ct value of the reference sample (referred to as calibrator) from the ∆Ct value of the sample. Finally, the relative gene expression, i.e. the amount of mRNA relative to the calibrator used as the basis for comparative results, was calculated as $2^{\Delta\Delta Ct}$.

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Sequence/Applied Biosystems ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>human RPL13A</td>
<td>Hs01926559_ gi</td>
</tr>
<tr>
<td>human SOX9</td>
<td>Hs00165814_m1</td>
</tr>
</tbody>
</table>
| human RUNX2 osteo (detects only osteogenic variant) | forward primer: 5'-AAGCAGTATTTACACGAGGCTACAAG-3'  
reverse primer: 5'-GGTGCTCGGGATCCCAA-3'  
probe: 5'(FAM)-CATAAAACAGGCTCTCTCAGCAGTGACAC-3'(TAMRA) |
| human RUNX2 (detects all variants) | forward primer: 5'-AGCAAGGTTTACAACAGAGGGTACAAG-3'  
reverse primer: 5'-TTTGTGAAGACGGTTATGGTCAA-3'  
probe: 5'(FAM)-TGAAAATTTGCTCGGTCACTCCG-3'(TAMRA) |

Table 2.1: Self-designed primers and probe as well as TaqMan Gene Expression Assays used for real time PCR. For the TaqMan Gene Expression Assays, no sequences get provided from the supplier.

2.12 Protein biochemical methods

2.12.1 Preparation of nuclear extracts from cells

To prepare nuclear extracts from cells cultured as monolayer culture in 100 tissue culture plates, cells were washed twice with PBS, and then 0.5 ml of lysis buffer A were added directly to each plate and incubated at room temperature for 10 minutes. Lysis buffer A is composed of 10 mM HEPES pH 7.9 (Gibco), 10 mM KCl (Sigma), 0.1 mM EDTA (Sigma), 0.4% IGEPAL CA-630 (Sigma), supplemented just before use with the appropriate volume of Protease Inhibitor Cocktail (Sigma; 1 ml of cocktail for a total of $10^9$ cells). Thereafter, cells were scraped with a new sterile scraper (pre-cooled at -20°C), cell clumps were disrupted by repetitive pipetting up and down, and the cell lysate was then transferred into a 1.5 ml Eppendorf tube and placed on ice. Next, the cell lysate was centrifuged at 15,000 g for 3 minutes at 4°C. The supernatant representing the cytosolic fraction was kept on ice while the procedure to prepare the nuclear extract continued.

To prepare the nuclear extract, 0.15 ml of lysis buffer B per pellet resulting from a 100 mm tissue culture plate were added to each Eppendorf tube containing the pellet, and the pellet was resuspended by repetitive pipetting up and down, and then the Eppendorf tube was placed on ice. Lysis buffer B is composed of 20 mM HEPES pH 7.9, 0.4 mM NaCl (Sigma), 1 mM EDTA, 10% Glycerol (Sigma), supplemented just before use with the
appropriate volume of Protease Inhibitor Cocktail (1 ml of cocktail for a total of 10⁹ cells). The Eppendorf tube was shaken vigorously at 4°C for 2 hours on a horizontal shaker at 250 rpm. Thereafter, the tube was centrifuged at 15,000 g for 5 minutes at 4°C. The supernatant representing the nuclear fraction was kept on ice. Protein concentrations of cytosolic and nuclear extracts were determined by means of Bradford Assay using BSA as standard. Then, cytosolic and nuclear extracts were aliquoted into sterile 0.5 ml Eppendorf tubes, snap-frozen using liquid nitrogen, and stored at -80°C.

### 2.12.2 Bradford assay

The protein content was assessed by means of Quick Start™ Bradford Protein Assay (Bio-Rad). The Bradford assay is based on the binding of Coomassie Brilliant Blue G-250 dye to proteins. The dye exists in three forms: cationic (red), neutral (green) and anionic (blue) which absorbs light at different wavelengths. Whereas under acidic conditions, the dye is present predominantly in the cationic red form, upon binding to protein, the dye is converted to a stable unprotonated blue form with an absorbance maximum at 595 nm ($A_{\text{max}} = 595 \text{ nm}$). The blue dye form which is generated proportionally to the concentration of protein present in the sample is measured using a spectrophotometer or microplate reader, and the protein concentration of interest can be assessed relatively to the standard curve.

For the assay, 4-5 µl of cell extract samples were filled up to 200 µl with Bradford Reagent. The Bradford Reagent was incubated for 10 minutes at room temperature, and then 200 µl of incubated samples were distributed in duplicates into individual wells of a transparent 96-well flat bottom plate. The standard curve covering a range of 5 to 25 µg/ml bovine serum albumin (BSA) was prepared from a BSA standard stock (Bio-Rad; 2 mg/ml). The absorbance of 200 µl of standard and samples were measured at 595 nm in duplicates on a Victor3™ 1420 Multilabel Reader, and compared with the BSA standard curve. Protein content was expressed as 'µg/ml'; if samples were diluted, the final protein concentration was adjusted by multiplication with the dilution factor used.
2.12.3 Protein separation by means of sodium dodecylsulfate polyacrylamide gel electrophoresis

To separate proteins according to their size, denaturing sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed. The anionic detergent SDS denatures proteins, covers the intrinsic charge of the proteins, and overall provides the proteins with a negative net charge. In order to completely denature the secondary and tertiary structure of the proteins, the sample buffer contains reducing agents such as dithiothreitol (DTT) or β-mercaptoethanol. Samples are also boiled at 95°C for 5 minutes for complete denaturation. The uniform mass to charge ratio and the complete denaturation of the proteins ensures that the proteins are exclusively separated based on their molecular weight.

Separating gels were prepared using Acrylamide 4K 30% solution (29:1 acrylamide:bisacrylamide mixture), stacking gels were prepared using Acrylamide 4K 40% solution (37.5:1 mix acrylamide:bisacrylamide mixture), and the gels were poured using the Mini-PROTEAN 3 Electrophoresis Cell (Bio-Rad). Thereby, ammonium persulfate (APS; Sigma) initiates the formation of radicals, while N,N,N',N'-tetramethylethylenediamine (TEMED; Bio-Rad) serves as a catalyst. The stacking gel which constituted a 4.3% acrylamide gel in 0.125 M Tris-HCl (Sigma) pH 6.8, 0.1% SDS was poured on top of the separating gel which constituted a 10% acrylamide gel in 0.375 M Tris-HCl pH 8.8, 0.1% SDS. Gels were left for polymerization for at least 1 hour and used on the same day. After the polymerization of the gel, samples containing a total of 6 µg of protein were mixed 1:1 with 2x Laemmli buffer (Bio-Rad) supplemented with 1:20 of β-mercaptoethanol (Sigma), and boiled at 95°C for 5 minutes. Thereafter, samples at a volume of 16 µl and 5 µl of Precision Plus Protein WesternC Standard (Bio-Rad) were loaded, and the gel was run at 100 V constant until the dye front reached the resolving gel. The gel run was finished with 80 V constant for 1.5 hours. Running buffer was composed of 25 mM Tris, 192 mM glycine (Sigma), according to the manufacturer’s recommendations.

2.12.4 Western Blotting

Following protein separation by means of SDS-PAGE, proteins were transferred onto polyvinylidene difluoride (PVDF) membranes (Immobilon-P transfer membrane; Millipore) in a Mini Trans-Blot® Electrophoretic Transfer Cell (Bio-Rad). Per gel, one PVDF membrane,
two fiber pads and four filter papers were used. The PVDF membrane which was cut to the size of the gel was first activated in 100% methanol at room temperature for 2-3 minutes, thereafter soaked in MilliQ water for 10 minutes, and finally soaked in transfer buffer for 15 minutes. Transfer buffer was composed of 25 mM Tris pH 8.3, 192 mM glycine, 0.1% SDS (Sigma), and 20% methanol (Sigma). The gel, the fiber pads and the filter papers were soaked only in transfer buffer for 15 minutes. Then, the soaked gel and PVDF membrane were placed between a stack of two soaked filter papers and one fiber pad on each side, and air bubbles were removed by rolling a pipette over the filter papers. This stack was then placed into the holding cassette of the blotting chamber, both holding cassettes were put into the electrode module, and the whole electrode module was put into the blotting tank. Transfer was performed at 100 V constant (leading to an amperage of 360 mA) for 1 hour at 4°C to avoid heating in transfer buffer.

After the transfer, the blotting stacks were disassembled and the membrane was subjected to the blocking step according to the antibody's specific datasheet.

Western Blot analysis of HOXA9 expression was performed based on the antibody's specific datasheet. After the transfer, the blotted membrane was washed twice water. Blotted PVDF membrane was blocked in freshly prepared PBS containing 3% nonfat dry milk for 1 hour at room temperature shaking 30 rpm on a horizontal shaker. Blocked PVDF membrane was incubated with 1.0 µg/ml of rabbit anti-human/mouse HOXA9 antibody (Millipore) diluted in freshly prepared PBS containing 3% nonfat dry milk overnight at 4°C shaking 4 rpm on an orbital shaker. Membrane was washed twice with water for 45 seconds each shaking 15 rpm on an orbital shaker. Then, membrane was incubated with PBS containing 3% nonfat dry milk, a 1:10,000 dilution of a horseradish peroxidase (HRP)-conjugated donkey anti-rabbit antibody (GE Healthcare, Buckinghamshire, United Kingdom), and a 1:10,000 dilution of Precision Protein StrepTactin-HRP Conjugate (Bio-Rad) to detect the bands of the Precision Plus Protein WesternC standards for 30 minutes at room temperature shaking 4 rpm on an orbital shaker. Membrane was washed twice as described above, washed once in PBS containing 0.05% Tween-20 (Sigma) for 3-5 minutes shaking 15 rpm on an orbital shaker and rinsed four to five times with water for 5 seconds each. Chemiluminescent detection was performed with Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare) according to the manufacturer's recommendations. Bands were visualized with a Syngene ChemiGenius Bioimaging System (Syngene Europe, Cambridge, United Kingdom).
Western Blot analysis of VEZF1 expression was performed based on the antibody’s specific datasheet. After the transfer, the blotted PVDF membrane was blocked in Tris-buffered saline (TBS) containing 0.1% Tween-20 and 5% nonfat dry milk for 1 hour at room temperature shaking 30 rpm on a horizontal shaker. Blocked PVDF membrane was incubated with 0.5 µg/ml of mouse anti-human VEZF1 antibody (GenWay Biotech, San Diego, CA) diluted in TBS containing 0.1% Tween-20 and 1% nonfat dry milk for 1 hour at room temperature shaking 4 rpm on an orbital shaker. Membrane was washed three times with TBS containing 0.1% Tween-20 for 5 minutes each shaking 15 rpm on an orbital shaker. Then, membrane was incubated with TBS containing 0.1% Tween-20, 1% nonfat dry milk, a 1:10,000 dilution of a horseradish peroxidase (HRP)-conjugated donkey anti-mouse antibody (Abcam, Cambridge, United Kingdom), and a 1:10,000 dilution of Precision Protein StrepTactin-HRP Conjugate to detect the bands of the Precision Plus Protein WesternC standards for 30 minutes at room temperature shaking 4 rpm on an orbital shaker. Membrane was washed four times as described above. Chemiluminescent detection was performed with Amersham ECL Prime Western Blotting Detection Reagent according to the manufacturer’s recommendations. Bands were visualized with a Syngene ChemiGenius Bioimaging System.

Western Blot analysis of Lamin B1 expression which served as nuclear loading control was performed based on the antibody’s specific datasheet. After the transfer, the blotted PVDF membrane was blocked in TBS containing 5% nonfat dry milk overnight at 4°C shaking 30 rpm on a horizontal shaker. Blocked PVDF membrane was incubated with 1.0 µg/ml of rabbit anti-human Lamin B1 antibody (Thermo Scientific) diluted in TBS containing 0.05% Tween-20 and 5% nonfat dry milk for 1 hour at room temperature shaking 4 rpm on an orbital shaker. Membrane was rinsed once with TBS containing 0.05% Tween-20 for 5 seconds, washed once with TBS containing 0.05% Tween-20 for 10 minutes shaking 15 rpm on an orbital shaker and washed twice with TBS containing 0.05% Tween-20 and 0.5% normal donkey serum (Millipore) for 10 minutes each shaking 15 rpm on an orbital shaker. Then, membrane was incubated with TBS containing 0.05% Tween-20, 5% nonfat dry milk, a 1:10,000 dilution of a HRP-conjugated donkey anti-rabbit antibody, and a 1:10,000 dilution of Precision Protein StrepTactin-HRP Conjugate to detect the bands of the Precision Plus Protein WesternC standards for 1 hour at room temperature shaking 4 rpm on an orbital shaker. Membrane was rinsed once with TBS containing 0.05% Tween-20 for
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5 seconds and washed three times with TBS containing 0.05% Tween-20 for 10 minutes each shaking 15 rpm on an orbital shaker. Chemiluminescent detection was performed with Amersham ECL Prime Western Blotting Detection Reagent according to the manufacturer's recommendations. Bands were visualized with a Syngene ChemiGenius Bioimaging System.

2.13 Statistical Analysis

Statistical analysis was performed with the SPSS software package V.19 (IBM, Armonk, NY). The significance of differences between the groups was determined with a general linear model and multivariate analysis with a Tukey's honestly significant difference post hoc test. A $p<0.01$ and $p<0.05$ were considered highly significant and significant, respectively.
3 Establishment and optimization of methodology

3.1 Introduction

In human systems, functionally active RUNX2 protein harbors the potential to be a criterion for specific identification of osteoprogenitors. In contrast, CD markers and mRNA expression levels which are commonly used to subdivide MSCs and to characterize MSCs and subpopulations thereof are not unique or reliable enough. MSCs can only be defined by a large set of CD markers which for practical reasons is unmanageable, whereas mRNA expression levels of genes associated with osteoblast differentiation do not necessarily correlate with the relevance on the protein level. However, RUNX2 as the principal transcriptional regulator of osteoblast differentiation is a protein that amongst mesenchymal lineages is specifically expressed during osteoblast differentiation. In human systems, a discrepancy between Runx2 mRNA and protein level was reported (Shui et al., 2003). Osteoblast differentiation in human BMSCs has been demonstrated to be primarily associated with increases in RUNX2 protein activity, and not with increased Runx2 mRNA or protein levels. These findings render even Runx2 mRNA expression levels useless as criterion for identification of osteoprogenitors.

However, whereas expression of CD markers in general, but also at which extent a CD marker is expressed, can be easily assessed by means of flow cytometry using fluorescently labeled antibodies against one or several CD markers of interest present in the cell membrane, presence of a functionally active transcription factor is more challenging to be determined, or even to be quantified. Whereas protocols exist to label intracellular transcription factors by means of specific fluorescently labeled antibodies and to quantify them by means of flow cytometry, these protocols require destructive fixation steps of the cells which makes sorting and further culture of the cells impossible.

A method to track functionally active RUNX2 protein was developed in this thesis. This approach relies on the usage of a reporter which is responsive to functionally active RUNX2 protein. Thereby, the expression of RUNX2 is coupled to the expression of the reporter EGFP via the reporter system, and the EGFP expression in turn is then used to identify and isolate the cells by means of FACS. The basic principle to account for specificity to RUNX2 of the reporter consists in its construction such that the EGFP reporter expression is dependent on Runx2 binding sites present in RUNX2 target genes such as MMP13 which need to be bound by RUNX2 to activate EGFP expression. Introduction of the reporter into human BMSCs is performed by means of a viral method since non-viral transfection
methods exhibit very poor efficiency in primary cells such as human BMSCs. Since an adenoviral system fulfills all criteria to be met for the experimental setup of the planned isolation procedure (Figure 3.1) and further to this brings increased safety when compared with integrating viruses such as retroviruses, the Runx2-responsive reporter has been generated as an adenoviral reporter referred to as Ad.Runx2. Thereby, responsiveness and specificity of Ad.Runx2 are crucial characteristics which need to be fulfilled prior to performing and interpreting subsequent FACS experiments.

**Figure 3.1:** Overall experimental procedure for FACS-mediated isolation of osteoprogenitors. Human BMSCs are obtained from bone marrow aspirates, plating onto tissue culture plastic, and allowing adherent cells to attach. BMSCs are expanded in culture in presence of bFGF. Reseeded cells are transduced with Ad.Runx2 and thereafter stimulated towards the osteoblast phenotype. Cells induced to the osteogenic lineage will express RUNX2 and cause the expression of EGFP, thus the cell becomes green. The fluorescing cells can be identified and sorted by means of FACS. Reporter positive (i.e. green), negative, and unsorted cells are thereafter separately expanded in monolayer culture thereby losing the adenovirus. Once the required cell number is achieved, the different cell populations are subjected to in vitro osteogenic differentiation assay, and comparative investigation of osteogenic differentiation potential is performed.
3.1.1 Adenovirus

3.1.1.1 Adenovirus biology

Adenoviruses were first discovered and isolated in 1953 in adenoid tissue (ROWE et al., 1953). Since then, more than 50 serotypes of human adenoviruses have been reported to infect humans. Adenoviruses cause respiratory illness, gastrointestinal and ocular infections in humans, depending on the serotype.

Adenoviruses are non-enveloped, double-stranded DNA viruses 70-100 nm in diameter consisting of an outer icosahedral protein capsid and an inner nucleoprotein core (Shenk, 2001) (Figure 3.2). The capsid is mainly composed of pentons (penton base and fiber) and hexons.

![Figure 3.2: Structure of an adenovirus. Schematic showing the main parts of the adenoviral capsid and core. The black line represents the adenovirus genome. (Shenk, 2001) (modified)](image)

The adenovirus genome is a linear, approximately 36 kb long double-stranded DNA molecule that is tightly coiled around histone-like proteins V and VII, forming a chromatin-like structure (Shenk, 2001). Each end of the genome has an inverted terminal repeat (ITR) of 100-140 nucleotides to which the terminal protein is covalently linked. At the left end of the adenoviral genome, there is the packaging sequence of approximately 200 nucleotides which is required for encapsulation of viral DNA (Hearing et al., 1987). The adenovirus genome comprises early (E1-E4), intermediate and late genes (L1-L5).
The adenoviral infectious cycle starts when the virion binds to the cell surface (for review see (McConnell and Imperiale, 2004)). Initial attachment of the virion particle to the target cell surface occurs through binding of the fiber knob to the extracellular domain of coxsackievirus and adenovirus receptor (CAR) (Bergelson et al., 1997). After initial attachment to the cell surface, an arginine-glycine-aspartate (RGD) sequence exposed on the penton base interacts with $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins on the cell surface, which promotes virion entry into the host cell (Stewart et al., 1997b). After clathrin-dependent, receptor-mediated endocytosis, the virion escapes from the endosomal compartment to the cytosol induced by the acidic environment, and gets transported towards the nucleus along microtubules (Leopold et al., 2000). Disassembly of the viral capsid which starts at the cell surface does not get completed until it reaches the cell nucleus. Upon docking to the nuclear pore complex (NPC), the viral genome is released and imported into the nucleus via the NPC (Greber et al., 1997). The covalently bound terminal protein which stays associated with the DNA contains a NLS and is therefore considered to lead the DNA through the nuclear pore (Shenk, 2001). Once in the nucleus, viral replication and transcription begins by utilization of the cellular machinery, thereby generating virus-specific mRNAs (Shenk, 2001). First, the early events such as transcription and translation of E1 proteins are initiated, followed by the onset of the expression of the adenoviral late genes. The late genes initiate the assembly of new viral particles. Completion of the adenoviral cycle triggers cell death and the release of the new viral particle progeny into the extracellular environment. The adenoviral life cycle lasts approximately 3 days, schematically shown in Figure 3.3.
Adenoviral transduction of target cells leads to the transport of the adenovirus into the nucleus; however, it does not integrate into the host genome. Therefore, expression of the gene of interest in recombinant adenoviruses is transient and persists only as long as the adenoviral genome is present in the target cell. Factors that influence the persistence of the adenovirus encompass amongst others the doubling time of the target cells. In actively dividing cells, the adenovirus genome is diluted out step by step as cell division is ongoing. This leads to a decrease in transgene expression, finally back to background levels within 1-2 weeks post-transduction. In non-dividing cells, transgene expression is more persistent and can last up to 6 months post-transduction. Correspondingly, maximal transgene expression in actively dividing cells is assignable to a precise time, after which the expression level starts to decline, whereas in more slowly dividing or non-dividing cells, maximal transgene expression levels persist for a longer time.

CAR has been reported to be involved in the entry pathway of adenoviruses by mediating the initial attachment, leading to their uptake into cells such as the 293 cell line, HeLa cells, and cells of the airways. The cellular function of CAR is still elusive and CAR is only expressed in some cell types. There are examples of cells that are not efficiently transduced by adenoviruses. Mesenchymal cells such as primary fibroblasts are an example. Whereas fibroblasts express $\alpha_v$ integrins (Gailit and Clark, 1996), they do not express CAR and therefore are not efficiently transduced by adenoviruses. Besides transduction with an adenovirus expressing CAR, different modifications of the adenoviral fiber such as addition of a RGD sequence to the fiber end have been reported to enhance gene transfer into fibroblasts (Hidaka et al., 1999). These findings indicate that CAR deficiency can be rescued by either supplying CAR or by altering the fiber of the adenovirus to allow attachment to other receptors. Consequently, CAR-independent mechanisms of adenovirus entry into cells are believed to exist as well.

As regards the cell type relevant in this thesis, CAR is reported to be expressed in human MSCs (Conget and Minguell, 2000). Upon induction of human MSCs to differentiate into osteoblasts or adipocytes, the cells lose their capability of being adenovirally transduced...
Establishment and optimization of methodology

(Hung et al., 2004). In contrast, MSCs that were transduced with adenovirus prior to the induction to differentiate preserved their differentiation potential, suggesting that for gene delivery purposes, adenoviral transduction of undifferentiated MSCs can solve difficulties of loss of adenoviral susceptibility that come along with MSC differentiation. Thereby, the level of CAR expression on the cell surface of human MSCs determined the transduction efficiency, and not the integrin expression.

3.1.1.2 Adenoviruses for gene transfer

Recombinant adenovirus vectors containing exogenous genes for transfer which are made replication-deficient have been used successfully for the delivery of foreign genes into cells, be it for gene therapy purposes or for transient reporter expression. Gene therapy is a technique whereby a gene is introduced into target cells for the purpose of correcting or treating a human disease. Recombinant adenoviruses deliver a multifaceted system for gene expression studies as well as therapeutic applications such as gene transfer in vitro and vaccination in vivo (Berkner, 1988; Miller, 1992; Morgan and Anderson, 1993). Several characteristics of adenovirus biology have led to their routine use and popularity in these applications. Advantageous characteristics of recombinant adenoviruses are their ability to infect both dividing and non-dividing cells, high levels of transgene expression, their simple production, high efficiency of transduction of most tissues and cell types, along with the capacity to accommodate large foreign DNA cassettes (Ghosh et al., 2006; Benihoud et al., 1999). Despite several advantages, there are disadvantages. There are safety concerns when using adenoviral vectors, especially for the treatment of non-lethal diseases. Furthermore, transduced cells are immunogenic and adenoviruses are cytotoxic when used at high doses. Last but not least, adenoviral vectors are non-insertional which leads to loss of the transgene after a certain defined time span (for review see (Russell, 2000)). This is often referred to as a disadvantage, however, for this thesis it is an advantage.

The most commonly used human adenovirus is serotype 5 (Ad5), which is replication defective due to deletion of E1 and E3 genes. E1 gene is required for the assembly of infectious virus particles, E3 gene is responsible for avoidance of host immunity. These deletions enable accommodation of up to 7.5 kb of foreign DNA (Benihoud et al., 1999), leading to a recombinant adenovirus. The vector is typically propagated in a cell line such
as human embryonic kidney cell line 293 (HEK-293) that complements E1 gene (Crystal, 1995).

The main limitation of first-generation adenoviral vectors which are deleted in either one or two viral early genes (E1 and E3) is the vector-mediated immunogenicity (for review see (Thomas et al., 2003)). Attempts to reduce the immunogenicity and toxicity of adenoviral vectors were addressed by introducing additional deletions in other early genes (E2 and/or E4). These second- and third-generation adenoviral vectors have been reported to show reduced toxicity in animal models indeed (O’Neal et al., 1998; Andrews et al., 2001), but the most important step towards decreased immunogenicity was achieved by the development of helper-dependent (also called gutless) adenoviral vectors that are deleted for all viral genes, leading to an increase in the safety (for review see (Morsy and Caskey, 1999)).

When adenoviruses are used for gene delivery purposes, the transient expression needs to be considered, which is due to their persistence as extrachromosomal episomes and the immune response against the vector. Although non-integrating adenoviruses can mediate persistent transgene expression under certain conditions, adenoviruses are most suitable for applications requiring transient expression. To date, 456 gene therapy clinical trials utilizing adenoviral vector, representing 23.1% of all vector-based gene therapy clinical trials, have been either ongoing, pending or completed, according to the Journal of Gene Medicine Clinical Trial Database, update July 2013 (www.abedia.com/wiley). Indications addressed by gene therapy clinical trials using adenoviral vectors include cancer, HIV infection, cystic fibrosis, coronary and peripheral artery diseases, glaucoma and age-related macular degeneration, hemophilia A, hepatitis C, as well as malaria, according to the Journal of Gene Medicine Clinical Trial Database, update July 2013 (www.abedia.com/wiley).

After the establishment of the above-mentioned methods, different conditions for the planned FACS experiment needed to determined and tailored to the experimental procedure. This circumstance originates from the fact that the expression level of a particular transgene is dependent on the activity of the promoter that drives its expression, which is unknown in the case of Ad.Runx2. Viral promoters, such as the very popular constitutively and ubiquitously active CMV promoter present in Ad.GFP, is able to drive high levels of transgene expression in a wide range of mammalian cell lines and
tissue types (for review see Papadakis et al., 2004). In contrast, eukaryotic promoters drive much lower levels of transgene expression. The promoter of Ad.Runx2 (Figure 3.4) which is composed of a 12x tandemly arranged 7 bp long Runx2 binding site (AACCACA), followed by a 136 bp minimal promoter sequence from the 5'-flanking region of the human MMP13 gene represents a tailor-made promoter of which the expression characteristics are unknown.

Figure 3.4: Schematic illustration of the promoter of Ad.Runx2. The promoter of Ad.Runx2 is composed of a 12x tandemly arranged 7 bp long Runx2 binding site (AACCACA), followed by a 136 bp minimal promoter sequence from the 5'-flanking region of the human MMP13 gene.

Due to the known difference that exists between the expression levels driven by viral and eukaryotic promoters, initial experiments were performed to determine the optimal MOI. The MOI describes the ratio of infectious viral particles to target cells which are transduced with the virus. Although for transduction experiments in cell culture, defined MOIs are applied to the target cells, the actual number of viral particles that enter a target cell is the result of a statistical process. Some cells become transduced with more, other cells with less viral particles than what the MOI predicts. Generally, both the probability that a cell becomes transduced with a particular number of viral particles and the total number of viral particles entered per target cells increases with increasing MOI applied. However, high titers of recombinant adenoviruses lead to cytotoxicity. This may be caused by different mechanisms such as direct toxicity of viral proteins and impairment of the synthesis of host proteins due to overexpression of the transgene (Durham et al., 1996), which becomes more pronounced the higher the viral titer applied to the cells.

To obtain optimal expression of the reporter gene, transduction of the adenoviral reporter into BMSCs needs to be performed with a suitable MOI. In fact, the MOI is an easy way to control the expression level of a transgene of an adenoviral vector. Transgene levels increase as the MOI increases, however increasing the MOI also increases the cytotoxic
potential of the viral titer applied. Therefore, the optimal MOI is determined by balancing the transgene expression level and the cytotoxicity. Once the optimal MOI had been determined, additional parameters such as the cell seeding density, the time point of sorting, and the cell preparation protocol for the FACS experiment were established. The use of primary human BMSCs makes these parameters more difficult to establish, a fact that is often overlooked.

Therefore, the content of this chapter encompasses different introductory experiments which can be classified into two parts. First, the central part of the whole thesis, the construction of the adenoviral reporter Ad.Runx2 is covered, followed by its validation of its specificity and responsiveness to RUNX2, along with its validation in MSCs. Secondly, the conditions for the planned FACS experiments to be determined and optimized are described. These experiments set the base for the subsequent FACS experiments detailed in the next chapter to identify and isolate osteoprogenitors from human BMSCs.

3.2 Results

3.2.1 Construction/cloning of Ad.Runx2

The Runx2-responsive adenoviral reporter Ad.Runx2 was generated as detailed in section 2.2.2. During the duration of the thesis, a total of seven batches of Ad.Runx2 were produced by amplifying aliquots in AD-293 cells. To determine the total and infectious viral titers, respectively, two different approaches were utilized. First, total viral particle concentration was determined by OD_{260} measurement. Viral particle concentration can be calculated according to the general rule that an OD_{260} of 1 corresponds to approximately 10^{12} viral particles/ml, resulting in the OD_{260}-based viral titer. Secondly, infectious viral particle concentration was determined based on plaque assay. The number of isolated plaques due to cytopathic effects of AD-293 cells was counted, and by multiplying this value with the corresponding dilution factor at which the virus was applied, the infectious viral titer was determined. Applying to the widely applied rule that approximately one in 100 viral particles is supposed to be infectious, the infectious viral titer was converted into the pfu-based viral titer, i.e. the corresponding total viral titer based on plaque assay, by multiplying the infectious viral titer by a factor of 100, which then is referred to as pfu-based viral titer.
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The pfu-based viral titer determination is considered to be more accurate since it takes the actual infectivity of the virus batch into account. Therefore, the pfu-based viral titer was used as the starting point to calculate the volume of virus to be applied to cells in the cell culture experiments at a given MOI.

Overall, the OD$_{260}$-based viral titers achieved were estimated to be between $0.61 \text{ - } 3.78 \times 10^{12}$ particles/ml, while the pfu-based viral titers were determined to be between $0.06 \text{ - } 1.19 \times 10^{12}$ particles/ml assuming that one out of 100 viral particles is supposed to be infectious (Table 3.1). As expected, plaque assays performed to substantiate the OD$_{260}$-based viral titer determination led to consistently lower viral titers than the OD$_{260}$ measurement, with differences ranging from a factor 1.7 to 18.3 (Table 3.1).

<table>
<thead>
<tr>
<th>Ad.Runx2 batch</th>
<th>OD$_{260}$-based viral titer</th>
<th>pfu-based viral titer</th>
<th>discrepancy OD$_{260}$ vs. pfu</th>
<th>OD$_{260}$ relative to the best batch</th>
<th>pfu relative to the best batch</th>
</tr>
</thead>
<tbody>
<tr>
<td>batch no. 1</td>
<td>0.86</td>
<td>0.50</td>
<td>1.7</td>
<td>23%</td>
<td>42%</td>
</tr>
<tr>
<td>batch no. 2</td>
<td>2.59</td>
<td>0.40</td>
<td>6.5</td>
<td>69%</td>
<td>34%</td>
</tr>
<tr>
<td>batch no. 3</td>
<td>1.71</td>
<td>0.70</td>
<td>2.4</td>
<td>45%</td>
<td>59%</td>
</tr>
<tr>
<td>batch no. 4</td>
<td>1.10</td>
<td>0.06</td>
<td>18.3</td>
<td>29%</td>
<td>5%</td>
</tr>
<tr>
<td>batch no. 5</td>
<td>0.81</td>
<td>0.06</td>
<td>10.2</td>
<td>16%</td>
<td>5%</td>
</tr>
<tr>
<td>batch no. 6</td>
<td>3.58</td>
<td>0.36</td>
<td>9.9</td>
<td>95%</td>
<td>30%</td>
</tr>
<tr>
<td>batch no. 7</td>
<td>3.78</td>
<td>1.19</td>
<td>3.2</td>
<td>100%</td>
<td>100%</td>
</tr>
</tbody>
</table>

Table 3.1: Overview of the Ad.Runx2 batches produced. For each of the seven batches, the OD$_{260}$-based and pfu-based viral titer, respectively is highlighted (viral titers were transformed by dividing the viral titer by a factor $10^{12}$). Overall, the pfu-based viral titer resulting from plaque assay led to lower viral titers than the OD$_{260}$-based viral titer resulting from OD$_{260}$ measurement, with differences ranging from a factor 1.7 to 18.3.

### 3.2.2 Responsiveness of Runx2 reporter to RUNX2 protein

To functionally analyze the 12x tandemly arranged Runx2 binding sites for its responsiveness to RUNX2 protein, cotransfection experiments using Runx2 expressing effector plasmid and Runx2-responsive luciferase reporter were performed. The Runx2-responsive luciferase reporter, pCBG68-12xRunx2-MinPro-control vector (section 2.4.1), as well as non-functional Runx2 and functional Runx2 expressing plasmids were generated (section 2.4.2). Cotransfection experiments were performed using HeLa cells which are reported to not endogenously express RUNX2, and luminescence was assessed 48 hours post-transfection by means of Chroma-Glo Luciferase Assay (Figure 3.5).
Upon cotransfecting HeLa cells with a Runx2 expressing effector plasmid and a luciferase reporter plasmid driven by a 12x tandemly arranged Runx2 binding site, a statistically significant higher transactivation could be seen compared to the Runx2 non-expressing cotransfection control (Figure 3.5). These findings indicate that the Runx2 binding sites are responsive to RUNX2 protein. However, leaky expression, meaning that initiation of transcription of the reporter gene takes place even in the absence of RUNX2 protein produced, could be observed in the Runx2 non-expressing cotransfection control, which reached half the luminescence from the Runx2 expressing cotransfection.

### 3.2.3 Cell seeding density – persistence of reporter expression

Once the responsiveness of Ad.Runx2 to functional RUNX2 protein was investigated, the Ad.Runx2 adenoviral reporter was utilized to identify and isolate osteoprogenitors from osteogenically induced BMSC populations. The overall experimental procedure for the isolation of osteoprogenitors is illustrated in Figure 3.6.
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**Figure 3.6:** Overall experimental procedure for FACS-mediated isolation of osteoprogenitors. Primary human BMSCs are obtained from bone marrow aspirates, plating onto tissue culture plastic, and allowing adherent cells to attach. BMSCs are expanded in culture in the presence of bFGF. Reseeded cells are transduced with Ad.Runx2 and thereafter stimulated towards the osteoblast phenotype. Cells induced to the osteogenic lineage will express RUNX2 and cause the expression of EGFP, thus the cell becomes green. The fluorescing cells can be identified and sorted by means of FACS. Reporter positive (i.e. green), negative, and unsorted cells are thereafter separately expanded in monolayer culture thereby losing the adenovirus. Once the required cell number is achieved, the different cell populations are subjected to in vitro osteogenic differentiation assay, and comparative investigation of osteogenic differentiation potential is performed.

There are three unknowns in the above-mentioned experimental procedure which need to be addressed prior to performing the FACS experiments, which are: (1) cell density at which the BMSCs are seeded for adenoviral transduction the next day, (2) optimal MOI for transduction using the adenoviral reporter Ad.Runx2, and (3) time point at which cell sorting is performed. These three parameters are addressed in the sections 3.2.3 and 3.2.4.
In this section, the seeding density at which cells are seeded for adenoviral transduction is defined. The optimal cell seeding density needs to take into account both the fact that osteogenic induction is modulated by cell-cell contact of the BMSCs and that the adenovirus persists in the transduced cells for the analyzed time period, as cell division leads to the target cell's loss of adenovirus, which is undesirable. It was decided for a seeding density of 60,000 cells/cm$^2$ for two reasons. First, this way, the number of cell culture flasks could be kept low, i.e. many cells can be processed per extent of experiment. Second, the high seeding density favors cellular persistence of adenovirus and rapid osteogenesis.

To examine the persistence of reporter expression in a cell seeding density setup suitable for future FACS experiments, i.e. 60,000 cells/cm$^2$, BMSCs expanded in monolayer culture in the presence of bFGF were reseeded at 60,000 cells/cm$^2$, and transduced with Ad.GFP the following day. Ad.GFP is a reporter adenovirus expressing EGFP driven by a constitutively active CMV promoter whose generation is described in section 2.2.1. The transduction was carried out by lanthofection at a MOI of 10 for 4 hours. Thereafter, cell culture medium was switched from expansion medium to control (unstimulated) and osteogenic (stimulated) medium, respectively. The following donor was used: donor Pat 53 (female, born 1992). This experiment was performed with only one donor as one run; since the issue of persistence of reporter expression is donor-independent, but solely dependent on the cell seeding density, one run was considered as enough. Assessment of GFP expression in the transduced cells was performed on day 2, 4, 5, and 6 post-transduction. On all time points investigated, GFP expression could be observed in both unstimulated and stimulated conditions (Figure 3.7). An increase in GFP expression could be observed until day 4 post-transduction, and then stayed sustained without significant reduction throughout the rest of the time frame analyzed. Overall, the GFP expression was more pronounced in the case of control medium. These results clearly demonstrate that GFP reporter expression mediated by the reporter adenovirus persists in human BMSCs seeded at 60,000 cells/cm$^2$ for up to 6 days post-transduction.
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Figure 3.7: Persistence of GFP expression in human BMSCs seeded at 60,000 cells/cm². BMSCs were seeded at 60,000 cells/cm², transduced with Ad.GFP at 10 MOI the next day, and GFP expression was assessed for up to day 6 post-transduction by means of fluorescence microscopy in control (upper row) and osteogenic (lower row) medium, respectively. The exposure times were kept constant at 833 ms for GFP.

To investigate what differences are seen at different cell seeding densities, especially as there is no consensus as to what cell seeding density should be used, the persistence of reporter expression was investigated in a low cell seeding density setup, i.e. 15,000 cells/cm². To examine the persistence of reporter expression in the lower cell seeding density setup, i.e. 15,000 cells/cm², BMSCs expanded in monolayer culture in the presence of bFGF were reseeded at 15,000 cells/cm², and transduced with Ad.GFP the following day. The transduction was carried out by lanthofection at a MOI of 10 for 4 hours. Thereafter, cell culture medium was switched from expansion medium to control (unstimulated) and osteogenic (stimulated) medium, respectively. The following three donors were used: first run: donor Pat 53 (female, born 1992); second run: donor Pat 55 (female, born 1994); third run: donor Pat 62 (female, born 1958).

Assessment of GFP expression in the transduced cells was performed every day from day 1 until day 8 post-transduction. At each time point investigated in each of the three donors, GFP expression could be observed in both unstimulated and stimulated conditions (Figure 3.8). An increase in GFP expression could be observed over time, which was more pronounced in the case of osteogenic medium. These results clearly demonstrate that GFP expression persists in BMSCs seeded at 15,000 cells/cm² for up to 8 days post-transduction.
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However, donor-dependent differences in the GFP positivities resulting from adenoviral transduction with Ad.GFP at 10 MOI were observed, whereby Pat 62 exhibited higher GFP positivity than Pat 53 and 55 which showed similar GFP positivities. Time course of GFP expression in both control and osteogenic medium, respectively as assessed by fluorescence microscopy of Pat 53 as representative donor is depicted in Figure 3.8.

![Fluorescence Microscopy Images](image)

**Figure 3.8:** Persistence of GFP expression in human BMSCs seeded at 15,000 cells/cm². BMSCs were seeded at 15,000 cells/cm², transduced with Ad.GFP at 10 MOI the next day, and GFP expression was assessed for up to day 8 post-transduction by means of fluorescence microscopy in control (upper row) and osteogenic (lower row) medium, respectively.
Representative images (donor Pat 53) are shown. The exposure times were kept constant at 833 ms for GFP, same like for the persistence of GFP expression experiment at 60,000 cells/cm², to ensure comparison of the GFP intensities amongst different independent experiments.

In conclusion, since viral persistence and osteogenesis are likely to be better in the higher cell seeding density, we started tending to the higher cell seeding density, i.e. 60,000 cells/cm² to perform FACS experiments.

### 3.2.4 Optimal MOI and time point of sorting based on cell viability and fluorescence expression

In this subchapter, the first question to be answered is: What is the optimal MOI for transduction using Ad.Runx2? Since the promoter of Ad.Runx2 represents a tailor-made promoter, the optimal MOI needs to be established to obtain an idea of the reporter expression level. The optimal MOI was chosen from 10, 100, and 1000 MOI based on high Ad.Runx2-mediated GFP positivity, low cytotoxicity, and cell monolayer intactness.

To determine the optimal MOI for Ad.Runx2, BMSCs expanded in monolayer culture in the presence of bFGF were reseeded at 60,000 cells/cm² in 6-well plates, and a range of MOI (10, 100, and 1,000) was tested for Ad.Runx2 transduction the following day. Non-transduced and mock transduced cells served as control cells.

The transduction was carried out by lanthofection for 4 hours at the above-mentioned MOIs. Thereafter, cell culture medium was switched from expansion medium to osteogenic (stimulated) medium. The following three donors were used: first run: donor Pat 53 (female, born 1992); second run: donor Pat 55 (female, born 1994); third run: donor Pat 62 (female, born 1958).

Cells were cultured for up to 8 days post-transduction whereby the temporal levels of GFP expression were assessed every day by means of fluorescence microscopy. In addition, on selected time points as illustrated in Figure 3.9, cell viability was assessed using Cell Titer Blue assay.
The results for each donor separately are as follows:

First run: donor Pat 53
Ad.Runx2 transduction at 10 MOI did not lead to marked GFP expression during the analyzed period. A few cells (up to 3 cells per well of a 6-well plate) expressed GFP from day 6 post-transduction on. Differentiation occurred but was undetectable at such a low level of virus. Cell monolayer stayed intact during the course of the experiment. Ad.Runx2 transduction at 100 MOI led to marked GFP expression, detectable from day 1 post-transduction on, which then increased in terms of number of green cells, and finally decreased again. Cell monolayers stayed intact and did not show signs of cytotoxicity in consequence of adenoviral transduction. Ad.Runx2 transduction at 1,000 MOI led to cell death from day 1 post-transduction on, and was prominent from day 2 post-transduction on, reflecting the severe cytotoxic effect of adenovirus applied to cells at such high adenovirus particle concentrations. Adenoviral GFP expression was observable during the time frame analyzed, and was slightly higher compared to an MOI of 100.

Second run: donor Pat 55
Ad.Runx2 transduction at 10 MOI did not lead to marked GFP expression during the analyzed period. A few cells (up to 4 cells per well of a 6-well plate) expressed GFP from day 6 post-transduction on. Differentiation occurred but was undetectable at such a low level of virus. Cell monolayer stayed intact during the course of the experiment. Ad.Runx2 transduction at 100 MOI led to marked GFP expression, detectable from day 2 post-transduction on, which then increased in terms of number of green cells, and finally decreased again. Cell monolayers stayed intact and did not show signs of cytotoxicity in consequence of adenoviral transduction. Ad.Runx2 transduction at 1,000 MOI led to cell death from day 2 post-transduction on, and was prominent from day 3 post-transduction on, reflecting the severe cytotoxic effect of adenovirus applied to cells at such high adenovirus particle concentrations. Adenoviral GFP expression was observable during the time frame analyzed, and was slightly higher compared to an MOI of 100.
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decreased again. Cell monolayers stayed intact and did not show signs of cytotoxicity. Ad.Runx2 transduction at 1,000 MOI led to cell death which was prominent from day 1 post-transduction on, reflecting the severe cytotoxic effect of adenovirus applied to cells at such high adenovirus particle concentrations. Adenoviral GFP expression was observable during the time frame analyzed, and was slightly higher compared to an MOI of 100.

Third run: donor Pat 62

Ad.Runx2 transduction at 10 MOI did not lead to marked GFP expression during the analyzed period. A few cells (up to 40 cells per well of a 6-well plate) expressed GFP from day 3 post-transduction on. Differentiation occurred but was undetectable at such a low level of virus. Cell monolayer stayed intact during the course of the experiment. Ad.Runx2 transduction at 100 MOI led to marked GFP expression, detectable from day 1 post-transduction on, which then increased in terms of number of green cells, and finally decreased again. Cell monolayers stayed intact and did not show signs of cytotoxicity. Ad.Runx2 transduction at 1,000 MOI, in contrast to the previous two donors, did not lead to adverse cytotoxic effects. Adenoviral GFP expression was observable during the course of the experiment. More precisely, the cells dose-dependently showed an increase in GFP expression compared with an MOI of 100, although the increase was much weaker compared with the one observed from an MOI of 10 to 100.

The results of the experiment for the determination of the optimal MOI for transduction of BMSCs with Ad.Runx2 are summarized in Table 3.2.

<table>
<thead>
<tr>
<th>MOI</th>
<th>10</th>
<th>100</th>
<th>1000</th>
</tr>
</thead>
<tbody>
<tr>
<td>Donor</td>
<td>GFP</td>
<td>Toxic</td>
<td>GFP</td>
</tr>
<tr>
<td>Pat 53</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Pat 55</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Pat 62</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

Table 3.2: Summary of the results of the experiment for the determination of the optimal MOI for transduction of BMSCs with Ad.Runx2. Three different BMSC donors have been investigated with regard to GFP expression (present (+); absent (-)) and cytotoxic effect (prominent cytotoxic effect (++); no cytotoxic effect (-)) upon adenoviral transduction with Ad.Runx2 at a range of MOI (10, 100, and 1,000) the day after cell seeding at 60,000 cells/cm².
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Time course of both light microscopy images to assess cell monolayer and GFP expression as assessed by fluorescence microscopy of Pat 53 as representative donor are depicted in Figures 3.10 until 3.12.

**Figure 3.10:** Determination of the optimal MOI for transduction of BMSCs with Ad.Runx2 based on assessment of GFP positivity and cell morphology – 10 MOI. BMSCs were seeded at 60,000 cells/cm², transduced with Ad.Runx2 at 10 MOI the next day. Both cell monolayer intactness, as assessed by light microscopy (upper panel), and GFP expression, as assessed by fluorescence microscopy (lower panel), were assessed every day for up to day 8 post-transduction in osteogenic medium. Representative images (donor Pat 53) are shown. The exposure times were kept constant at 833 ms for GFP.
Figure 3.11: Determination of the optimal MOI for transduction of BMSCs with Ad.Runx2 based on assessment of GFP positivity and cell morphology – 100 MOI. BMSCs were seeded at 60,000 cells/cm², transduced with Ad.Runx2 at 100 MOI the next day. Both cell monolayer intactness, as assessed by light microscopy (upper panel), and GFP expression, as assessed by fluorescence microscopy (lower panel), were assessed every day for up to day 8 post-transduction in osteogenic medium. Representative images (donor Pat 53) are shown. The exposure times were kept constant at 833 ms for GFP.
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Figure 3.12: Determination of the optimal MOI for transduction of BMSCs with Ad.Runx2 based on assessment of GFP positivity and cell morphology – 1,000 MOI. BMSCs were seeded at 60,000 cells/cm², transduced with Ad.Runx2 at 1,000 MOI the next day. Both cell monolayer intactness, as assessed by light microscopy (upper panel), and GFP expression, as assessed by fluorescence microscopy (lower panel), were assessed every day for up to day 8 post-transduction in osteogenic medium. Representative images (donor Pat 53) are shown. The exposure times were kept constant at 833 ms for GFP.

Donor-dependent differences in the GFP positivity resulting from adenoviral transduction with Ad.Runx2 at a particular MOI were observed. Irrespective of which of three analyzed
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MOIs was considered, Pat 62 exhibited higher GFP positivity than Pat 53 and 55 which showed similar GFP positivity.

The microscopically observed toxicity effects were confirmed using Cell Titer Blue assay. Cell viability was assessed on day 1, 3, and 7 post-transduction, and results are depicted in Figure 3.13. Increase of adenoviral dose from an MOI of 10 to 100 did not result in a marked increase in cell death. At an MOI of 1,000, however, cell viability was markedly reduced. However, donor-dependent differences in their susceptibility to cytopathic effects upon adenoviral transduction with Ad.Runx2 were observed. Whereas the cell viability of donor Pat 53 and 55 was more intensely impaired, donor Pat 62 exhibited considerably less cell death at all time points analyzed (Figure 3.13). Generally, cell viability was lowest on day 1 post-transduction, and then cells started to recover, which was most marked in the case of Pat 53. Recovery of the cells led to cell viability values which even exceeded 100% as the value set for mock transduced cells.

![Figure 3.13: Cell viability of BMSCs transduced with Ad.Runx2 at three different MOIs. BMSCs seeded at 60,000 cells/cm² were transduced with Ad.Runx2 at an MOI of 10, 100, or 1,000 the following day. Cells were cultured in osteogenic medium and cell viability was assessed using Cell Titer Blue assay on day 1, 3, and 7 post-transduction. Increase of the MOI of Ad.Runx2](image-url)
from an MOI of 10 to 100 did not result in a marked increase in cell death. At an MOI of 1,000, however, cell viability was markedly reduced and the extent of impairment of cell viability was dependent on the donor. Cell viability is depicted related to mock transduced control cells set to a 100%. Results are displayed as average of duplicates from three different donors (n=3).

To summarize, these results clearly support that an MOI of 100 is the optimal MOI (Figure 3.11). The argument against an MOI of 10 is too low GFP expression (Figure 3.10), the argument against an MOI of 1,000 is the prominent cytotoxic effect on the BMSCs (Figure 3.12 and 3.13).

In addition to the Cell Titer Blue assay to assess cell viability, propidium iodide (PI) staining was performed and the percentage of dead cells was determined by flow cytometry. Percentages of dead cells substantiated the findings from the Cell Titer Blue assay. PI staining on day 3 post-transduction of BMSCs transduced at the optimal MOI, i.e. 100, showed an only slight degree of cell death as a consequence of adenoviral transduction. Pat 53 had 94.4% PI\(^{-}\) cells, Pat 55 had 96.9% PI\(^{-}\) cells, and Pat 62 had 95.3% PI\(^{-}\) cells. Thereby, the percentage of PI\(^{-}\) indicates the percentage of live cells. The mentioned PI staining percentage values resulted from gating on single cells only. The single viable cells were subsequently analyzed for GFP expression.

The GFP positivity of the three donors were as follows: Pat 53: 15.0%, Pat 55: 12.9%, and Pat 62: 18.0%. The GFP positivity assessed on day 3 post-transduction reflected the observation made by fluorescence microscopy, whereby Pat 62 consistently showed higher GFP positivity than Pat 53 and 55.

In addition to Ad.Runx2, Ad.GFP as a control adenoviral reporter was applied in a range of MOI (5, 50, and 500) for transduction of the same BMSCs and investigated as described above. Irrespective of the donor, Ad.GFP transduction at 5 MOI did lead to prominent GFP expression during the analyzed time frame. Cells expressed GFP from day 1 post-transduction on. Cell monolayer stayed intact during the course of the experiment. Ad.GFP transduction at 50 MOI led to more intense GFP expression compared to an MOI of 5, detectable from day 1 post-transduction on. Cell monolayers stayed intact and did not show signs of cytotoxicity. Ad.GFP transduction at 500 MOI resulted in even more intense
GFP expression from day 1 post-transduction on. In conclusion, Ad.GFP consistently mediated high level GFP expression which stayed sustained without significant reduction throughout the time frame analyzed. However, donor-dependent differences in the GFP positivities resulting from adenoviral transduction with Ad.GFP at a particular MOI were observed and were found to be in accord with the tendencies found in the case of Ad.Runx2, whereby Pat 62 exhibited higher GFP positivity than Pat 53 and 55 which showed similar GFP positivities.

A marked dose response between adenovirus particle concentration applied and GFP positivity was found in BMSCs transduced with Ad.GFP. The percentage of GFP+ cells reached 100% at 50 MOI due to visual estimation, and the GFP intensity increased even more when an MOI of 500 was applied. Against expectation, signs of cytotoxicity in the case of Ad.GFP at MOI of 500 started to occur from around day 4 post-transduction on, which is relatively late compared to Ad.Runx2 applied at a comparable MOI of 1000. In addition, the present results indicate that adenovirus infection was very efficient, even at a low MOI of 5, at least 40% of the cells were GFP+ (based on visual estimation), and cell viability as assessed by microscopic observation was not reduced. Only when Ad.GFP was applied at an MOI of 500, impaired cell viability was found.

Time course of both light microscopy images to assess cell monolayer and GFP expression as assessed by fluorescence microscopy of Pat 53 as representative donor are depicted in Figures 3.14 until 3.16.
Figure 3.14: Assessment of transduction efficiency of BMSCs using control adenoviral reporter Ad.GFP – 5 MOI. BMSCs were seeded at 60,000 cells/cm², transduced with Ad.GFP at 5 MOI the next day. Both cell monolayer intactness, as assessed by light microscopy (upper panel), and GFP expression, as assessed by fluorescence microscopy (lower panel), were assessed every day for up to day 8 post-transduction in osteogenic medium. Representative images (donor Pat 53) are shown. The exposure times were kept constant at 833 ms for GFP, same like for the determination of the optimal MOI for transduction of BMSCs with Ad.Runx2 to ensure comparison of the GFP intensities amongst Ad.GFP and Ad.Runx2.
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Figure 3.15: Assessment of transduction efficiency of BMSCs using control adenoviral reporter Ad.GFP – 50 MOI. BMSCs were seeded at 60,000 cells/cm², transduced with Ad.GFP at 50 MOI the next day. Both cell monolayer intactness, as assessed by light microscopy (upper panel), and GFP expression, as assessed by fluorescence microscopy (lower panel), were assessed every day for up to day 8 post-transduction in osteogenic medium. Representative images (donor Pat 53) are shown. The exposure times were kept constant at 833 ms for GFP, same like for the determination of the optimal MOI for transduction of BMSCs with Ad.Runx2 to ensure comparison of the GFP intensities amongst Ad.GFP and Ad.Runx2.
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Figure 3.16: Assessment of transduction efficiency of BMSCs using control adenoviral reporter Ad.GFP – 500 MOI. BMSCs were seeded at 60,000 cells/cm², transduced with Ad.GFP at 500 MOI the next day. Both cell monolayer intactness, as assessed by light microscopy (upper panel), and GFP expression, as assessed by fluorescence microscopy (lower panel), were assessed every day for up to day 8 post-transduction in osteogenic medium. Representative images (donor Pat 53) are shown. The exposure times were kept constant at 833 ms for GFP, same like for the determination of the optimal MOI for transduction of BMSCs with Ad.Runx2 to ensure comparison of the GFP intensities amongst Ad.GFP and Ad.Runx2.

Cytotoxicity of the adenoviral vectors following transduction was analyzed by measurement of cell viability using Cell Titer Blue assay. Cell viability was assessed on day 1, 3, and 7 post-transduction, and results are depicted in Figure 3.17. No significant adverse effect on cell viability was apparent when compared with mock transduced control cells. Increase of adenoviral dose from an MOI of 5 to 50 did not result in a marked increase in cell death. At an MOI of 500, however, cell viability was reduced to 80% in donors Pat 53 and 55.
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Figure 3.17: Cell viability of BMSCs transduced with Ad.GFP at three different MOIs. BMSCs seeded at 60,000 cells/cm² were transduced with Ad.GFP at an MOI of 5, 50, or 500 the following day. Cells were cultured in osteogenic medium and cell viability was assessed using Cell Titer Blue assay on day 1, 3, and 7 post-transduction. Increase of the MOI of Ad.GFP from an MOI of 5 to 50 did not result in a marked increase in cell death. At an MOI of 500, however, cell viability was reduced to 80% in donors Pat 53 and 55. Cell viability is depicted related to mock transduced control cells set to a 100%. Results are displayed as average of duplicates from three different donors (n=3).

After the determination of the optimal MOI for the future FACS experiments, the second question to be answered is: What is the optimal time point to perform cell sorting of Ad.Runx2-transduced BMSCs for the purpose of functionally isolating osteoprogenitors?

To approach the determination of the optimal time point to perform cell sorting, BMSCs expanded in monolayer culture in the presence of bFGF were reseeded at 60,000 cells/cm², transduced with Ad.Runx2 at an MOI of 100 the following day. The transduction was carried out by lanthofection for 4 hours. Thereafter, cell culture medium was switched from expansion medium to control (unstimulated) and osteogenic (stimulated) medium, respectively. The following three donors were used: first run: donor Pat 53 (female, born
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Cells were cultured for up to 8 days post-transduction whereby the temporal levels of GFP expression in each condition (unstimulated as well as stimulated) were assessed every day by means of fluorescence microscopy. Thereby, different approaches were performed to quantify the extent of GFP expression.

In a first attempt, GFP expression level was assessed and quantified based on subjective impressions, whereby arbitrary fluorescence intensities were attributed to the different conditions and time points by comparative estimation amongst conditions, donors as well as time points.

Pat 53 showed a course of GFP expression level which was quite differently intense between the unstimulated and stimulated conditions. On day 1 post-transduction, stimulated condition showed GFP expression as opposed to the unstimulated condition which did not show any GFP expression. Throughout the rest of the time frame analyzed, GFP expression level at any particular time point was higher in the unstimulated condition than in the stimulated condition. A steady increase in the GFP expression level from day 1 post-transduction on changed over to the peak or rather a temporary plateau of GFP expression level on day 3 to 4 post-transduction. From day 4 post-transduction on, GFP expression level decreased whereby unstimulated condition showed higher (day 6 post-transduction) or same (day 5, 7, and 8 post-transduction) GFP expression than stimulated condition.

Pat 55 showed same GFP expression levels in the unstimulated and stimulated conditions from the onset of detectable GFP expression on day 2 up to day 3 post-transduction. Only on day 4 post-transduction, at which time point the peak occurred, stimulated condition showed higher GFP expression than unstimulated condition. After the peak was reached, GFP expression in both conditions steadily decreased throughout the rest of the time frame analyzed, whereby the GFP expression level at any particular time point after the peak was higher in the stimulated condition than in the unstimulated condition.

Pat 62 showed comparable GFP expression levels in the unstimulated and stimulated conditions from day 1 to 3 post-transduction at which point the peak occurred. Only on day 1 post-transduction, unstimulated condition showed higher GFP expression than stimulated condition. After the peak was reached, GFP expression in both conditions steadily decreased throughout the rest of the time frame analyzed, whereby the GFP
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expression level at any particular time point after the peak was higher in the stimulated condition than in the unstimulated condition.

In sum, GFP expression started to be observed from day 1 (Pat 53: stimulated condition; Pat 62: unstimulated and stimulated conditions) and day 2 (Pat 53: unstimulated condition; Pat 55: unstimulated and stimulated conditions) post-transduction on, respectively. Irrespective of the donor variation, GFP expression level steadily increased until reaching a peak between day 3 to 4 post-transduction, after which GFP expression level decreased and finally reached low expression levels. Overall, stimulated condition resulted in at least same, but predominantly higher GFP expression than unstimulated condition at any time point analyzed up to day 8 post-transduction (exception: Pat 53).

The subjective graphs of time course of GFP expression of all three donors analyzed are depicted in appendix 8.2 (Figure 8.1A, 8.2A, and 8.3A).

Then, in a second attempt, analysis of the time course of GFP expression was performed by image analysis of the fluorescence microscopic images taken of the different conditions, donors as well as time points.

For each condition of a particular donor and time point, fluorescence microscopic images were taken in a non-random way of spots with differential degrees of GFP positivity to record the whole range of GFP positivity observed in different fields of view with positive events present in a well of a 6-well plate.

The first level of image analysis was performed using representative images. Image analysis was performed as described in section 2.7.1, percentaged pixel area was determined using one image per condition each that was considered as representative image of the particular condition, that is an image that reflects an average green fluorescence within the range of positive green fluorescence expression levels.

Pat 53 showed a course of green percentaged pixel area which was quite differently intense between the unstimulated and stimulated conditions. On day 1 post-transduction, stimulated condition exhibited small green percentaged pixel area as opposed to the unstimulated condition which did not show any green percentaged pixel area. Throughout the rest of the time frame analyzed, green percentaged pixel area at any particular time point was higher in the unstimulated condition than in the stimulated condition. A steady increase in the green percentaged pixel area from day 1 post-transduction on changed over to the peak of green percentaged pixel area on day 3 post-transduction. From day 3 post-transduction on, green percentaged pixel area decreased whereby unstimulated condition
showed higher (day 6 post-transduction) or comparable (day 5, 7, and 8 post-transduction) green percentaged pixel area compared with stimulated condition.

Pat 55 showed comparable green percentaged pixel area in the unstimulated and stimulated conditions from the day 2 up to day 8 post-transduction. On day 3 and 5 post-transduction where two peaks occurred, the stimulated condition showed a higher green percentaged pixel area than unstimulated condition. After the second peak on day 5 post-transduction was reached, green percentaged pixel area in both conditions steadily decreased throughout the rest of the time frame analyzed, whereby the green percentaged pixel area on day 6 and 8 post-transduction was comparable between unstimulated and stimulated conditions, and was higher in the case of stimulated condition on day 7 post-transduction.

Pat 62 showed comparable green percentaged pixel area in the unstimulated and stimulated conditions from day 1 to 2 post-transduction. A steady increase in the green percentaged pixel area from day 1 post-transduction on changed over to a temporary plateau of green percentaged pixel area on day 3 to 4 post-transduction in the stimulated condition, whereas the green percentaged pixel area in the unstimulated condition exhibited a temporary minimum on day 3 post-transduction. From day 4 post-transduction on, green percentaged pixel area in both conditions steadily decreased throughout the rest of the time frame analyzed, whereby the green percentaged pixel area at any particular time point throughout the rest of the time frame analyzed was higher in the stimulated condition than in the unstimulated condition except for day 5 post-transduction, at which time point unstimulated condition showed larger green percentaged pixel area than stimulated condition.

The graphs of time course of GFP expression based on image analysis of representative images of all three donors analyzed are depicted in appendix 8.2 (Figure 8.1B, 8.2B, and 8.3B).

A second level of image analysis was performed in an extended manner and included all images taken. Image analysis was performed as described in section 2.7.1, cumulative pixel area was determined for each image taken. Per condition, all images taken reflect the whole range of positive green fluorescence expression levels.

Further data analysis of the cumulative pixel area of individual images encompassed the steps mentioned in the following. First, since different numbers of images were taken of the different conditions, donors as well as time points, the maximal number of images
amongst all conditions, donors as well as time points was determined to standardize the image analysis. The maximal image number was 17 which was achieved by only one donor analyzed in a particular condition at one particular time point; in all other cases, fewer images were taken as no more green cells could be found, and the remaining images up to 17 were considered as black, that is a cumulative pixel area of 0. This was based on the fact that where fewer images were taken it was due to no further field of view containing positive cells. Based on the cumulative pixel area determined for each individual image including those images considered as black to standardize the maximal number of images taken between all conditions, the sum of the cumulative pixel area of individual images per condition of a particular donor and time point was determined. To calculate the green percentaged pixel area per condition of a particular donor and time point, the corresponding sum of the cumulative pixel area was divided by the maximal number of images (i.e. 17) and further divided by the total 2040 x 1536 pixel image area.

Pat 53 showed a course of green percentaged pixel area which was comparable between the unstimulated and stimulated conditions up to day 4 post-transduction. A steady increase in the green percentaged pixel area from day 1 post-transduction on changed over to the peak of green percentaged pixel area on day 4 post-transduction. From day 4 post-transduction on, green percentaged pixel area decreased whereby stimulated condition showed higher green percentaged pixel area than stimulated condition throughout the rest of the time frame analyzed. From day 7 post-transduction on, green percentaged pixel area of stimulated condition started to increase again, hinting at a possible second peak.

Pat 55 showed same green percentaged pixel area in the unstimulated and stimulated conditions until day 2 post-transduction. From day 4 until 8 post-transduction, stimulated condition showed higher green percentaged pixel area than unstimulated condition. On day 4 and 6 post-transduction, two peaks occurred in the stimulated condition, while in the unstimulated condition, green percentaged pixel area steadily decreased from day 4 post-transduction on. After the second peak on day 6 post-transduction in the stimulated condition was reached, green percentaged pixel area steadily decreased throughout the rest of the time frame analyzed.

Pat 62 showed higher green percentaged pixel area from day 2 until 8 post-transduction in the stimulated than unstimulated condition, whereas on day 1 post-transduction, stimulated condition temporarily exhibited smaller green percentaged pixel area. A steady increase in the green percentaged pixel area from day 1 post-transduction on changed over to the peak of green percentaged pixel area on day 4 post-transduction. From day 4 post-
transduction on, green percentaged pixel area decreased. From day 7 post-transduction on, green percentaged pixel area of both unstimulated and stimulated conditions started to increase again, hinting at a possible second peak.

The graphs of time course of GFP expression based on image analysis of all images representing the most accurate approach to quantify the extent of GFP expression of all three donors analyzed are depicted in the left panel of Figure 3.18. For the sake of completeness, these graphs (Figure 8.1C, 8.2C, and 8.3C) are also depicted together with the graphs of the other two approaches to quantify the extent of GFP expression of the corresponding donors in the appendix 8.2 (Figure 8.1 until 8.3).

To investigate what differences are seen at different cell seeding densities, especially as there is no consensus as to what cell seeding density should be used, the determination of the optimal time point to perform cell sorting as described above was performed in addition in a lower cell seeding density setup, i.e. 15,000 cells/cm², as detailed in section 4.2.3.4. To ease comparison of the two different cell seeding density setups, part of the corresponding results of that experiment are added here as well and are shown in the right panel of Figure 3.18.
Figure 3.18: Graphical representation of the image analysis of the time course of GFP expression in BMSCs mediated by Ad.Runx2 transduction at an MOI of 100. BMSCs seeded at 60,000 and 15,000 cells/cm$^2$, respectively were transduced with Ad.Runx2 at an MOI of 100 the following day. Cells were cultured in control (unstimulated) and osteogenic (stimulated) medium, respectively. GFP expression was assessed for up to day 8 post-transduction by means of fluorescence microscopy. The exposure times were kept constant at 833 ms for GFP. From the three different approaches performed to quantify the extent of GFP expression, image analysis to determine green percentaged pixel area using all images taken of the different conditions, donors as well as time points, which represents the most accurate image analysis approach, is shown. Overall, the GFP expression reached higher levels in the higher cell seeding density, irrespective of the donor investigated. Furthermore, stimulated condition
resulted in at least same, but predominantly higher GFP expression than unstimulated condition at any time point analyzed up to day 8 post-transduction. The discrepancy in the GFP expression level between stimulated and unstimulated condition was larger in the lower cell seeding density setup, clearly seen in the case of donor Pat 62. Based on the image analysis approach by which the green percentaged pixel area is determined using all images taken, the peak of GFP expression was reached on day 4 post-transduction in the higher cell seeding density setup, and in the lower cell seeding density setup, depending on the donor, between day 3 and 7 post-transduction.

To conclude, the time course of Ad.Runx2-mediated GFP expression level in BMSCs seeded at 60,000 cells/cm² revealed the peak level of GFP expression to be on day 3 to 4 post-transduction in the case of the three donors analyzed. This time frame of the peak fluorescence could be determined and located to day 3 to 4 post-transduction by means of different methods of GFP expression analysis such as subjective comparative estimation of GFP expression levels as well as by image analysis of either representative or all images. The peak in the GFP expression level was taken as criterion to temporally localize the day of sorting for the upcoming FACS experiments to identify and isolate osteoprogenitors.

To adequately deal with the inherent donor variation that comes along with the FACS experiments performed with different donors, we decided to standardize the day of sorting by setting a defined time point of sorting, which is day 3 post-transduction in the case of a seeding density of 60,000 cells/cm².

First of all, comparison of the time course of Ad.Runx2-mediated GFP expression level in BMSCs seeded at 60,000 and 15,000 cells/cm² showed that the peak level of GFP expression in the case of a seeding density of 15,000 cells/cm² occurred later compared with a seeding density of 60,000 cells/cm². Furthermore, the discrepancy in the GFP expression level between stimulated and unstimulated condition was larger in the lower seeding density setup, clearly seen in the case of donor Pat 62. This observation can be explained by the fact that high density causes spontaneous differentiation and represents an additional trigger for osteogenic stimulation besides the osteogenic ingredients present in the osteogenic medium (dexamethasone). However, the density effect is expected to reduce the osteogenic effect of the dexamethasone. As our aim was to obtain Runx2 GFP+ cells, regardless of whether confluence (high density) or dexamethasone was the trigger, for the reasons previously mentioned, we chose to go forward with the cell seeding density of 60,000 cells/cm² to perform FACS-mediated isolation of osteoprogenitors.
3.2.5 Validation of Ad.Runx2 in rat MSCs

The previous experiments using human BMSCs clearly showed that adenoviral transduction by lanthofection was highly efficient in the first place, and that Ad.Runx2 was responsive to Runx2 protein, and when applied at an ideal MOI of 100, leads to GFP positivities ranging from 12.9-18.0% based on the three donors tested, whereby the percentages are related to the gated events of the preceding (parent) cell population representing viable single cells.

We sought to determine whether rat MSCs could be adenovirally transduced, and to test whether we could achieve Ad.Runx2-mediated GFP expression in rat MSCs similar to human cells. Rat MSCs expanded in monolayer culture were reseeded at either 15,000 or 60,000 cells/cm² in 6-well plates, and adenovirally transduced with Ad.GFP at an MOI of 10 and Ad.Runx2 at an MOI of 100, respectively the following day. The transduction was carried out by lanthofection for 4 hours at the above-mentioned MOIs. Thereafter, cell culture medium was switched from rat MSC culture medium to control (unstimulated) and osteogenic (stimulated) medium, respectively. Rat MSCs were used from the common stock at the AO Research Institute Davos, Davos, Switzerland. Assessment of GFP expression in the transduced cells was performed every day from day 1 up to day 6 post-transduction by means of fluorescence microscopy. On all time points investigated, GFP expression could be observed in both unstimulated and stimulated conditions in cells transduced with Ad.GFP (Figure 3.19). Whereas the GFP positivity was very low and estimated to be around 2% on day 1 post-transduction, the GFP positivity increased to around 10% and stayed at the same level for the rest of the time frame analyzed. In contrast, no GFP expression was observed in any of both cell seeding densities chosen and in any of both unstimulated and stimulated conditions in cells transduced with Ad.Runx2 during the whole time frame analyzed. First, these results demonstrate that rat MSCs can be adenovirally transduced, as assessed by Ad.GFP transduction, although less efficiently compared with human BMSCs. Secondly, the fact that rat MSCs transduced with Ad.Runx2 stayed negative up to day 6 post-transduction indicates that Ad.Runx2 indeed exhibits specificity with regard to its responsiveness and consequently its GFP expression mediated by Runx2 protein.
Figure 3.19: Validation of Ad.Runx2 in rat MSCs. Rat MSCs were seeded at either 15,000 or 60,000 cells/cm², transduced with Ad.GFP at 10 MOI and Ad.Runx2 at 100 MOI, respectively, the next day, and GFP expression was assessed for up to day 6 post-transduction by means of fluorescence microscopy in control (unstimulated) and osteogenic (stimulated) medium, respectively. Representative images of 15,000 cells/cm² condition in osteogenic medium on day 6 post-transduction are shown, which are representative for the other conditions (60,000 cells/cm² and control medium, respectively) as well. The exposure times were kept constant at 833 ms for GFP.

3.2.6 Establishment of cell preparation protocol for FACS and cell viability of sorted cells

To establish an optimal cell preparation protocol to get viable single cells for the FACS experiments, different parameters were addressed. The starting point of this subchapter was the observation that trypsinization alone is not suitable to generate single cell suspension of osteogenically induced BMSCs, mainly due to the fact that these cells, after 3 days of osteogenic induction, have produced an extensive
extracellular matrix composed of, amongst others, collagen type I. Therefore, the inclusion of a collagenase step was expected to be more effective than trypsin-EDTA alone.

Six concentrations of Collagenase type 2 (93.75, 187.5, 375, 750, 1500, and 3,000 units/ml, respectively) were tested with uninfected human BMSCs osteogenically induced for 3 days for the ability to generate single cell suspension. Only samples treated with 3,000 units/ml of Collagenase type 2 showed proper single cell suspension. Hence, this concentration was chosen as concentration of choice for subsequent experiments.

We decided for Collagenase type 2 and compared different combinations of treatment with Collagenase type 2 (3,000 units/ml) and Accutase for their ability to generate single cell suspension of BMSCs transduced with Ad.Runx2 at 100 MOI. The combinations were as follows:

- Accutase for 20 minutes, followed by Collagenase type 2 for 60 minutes
- Collagenase type 2 for 60 minutes, followed by Accutase for 20 minutes
- Accutase for 20 minutes, dilute with PBS, centrifuge at 300 g for 10 minutes, followed by Collagenase type 2 for 60 minutes (this way, the Collagenase type 2 is not added to the Accutase, but replaced, which leads to a higher effective concentration of Collagenase type 2)

Based on microscopic observations, Accutase treatment for 20 minutes, diluted with PBS, centrifuged at 300 g for 10 minutes, followed by Collagenase type 2 treatment for 60 minutes was the best combination to generate single cells suspensions. In the other combinations of Accutase and Collagenase type 2 which were also tested, cell aggregates remained after the treatment (Figure 3.20). On the one hand side, these findings indicate that Accutase needs to be removed prior to performing the Collagenase type 2 treatment, possibly due to mutual impairment of their enzymatic activities or mutual enzymatic degradation. On the other hand side, the centrifugation step followed by the aspiration of the Accutase and resuspension of the cell pellet in Collagenase type 2 enables exposure to a more concentrated enzyme solution which leads to a more efficient enzymatic degradation of the extracellular matrix without leaving cell aggregates behind.
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**Figure 3.20:** Combinations of Accutase and Collagenase type 2 treatment to generate single cells suspension of human BMSCs transduced with Ad.Runx2 at 100 MOI and osteogenically induced for 3 days. The upper left image represents the situation in an uninfected, osteogenically induced control, which was same independent of which of the three treatment combinations was applied.

After the Collagenase type 2 incubation for 60 minutes, we observed that a remarkable fraction of the single cells attached to the tissue culture plastic. Therefore, in order to detach those cells, we decided to include a trypsin-EDTA step for 5-10 minutes thereafter.

Altogether, these establishment steps led to the cell preparation protocol for FACS experiments of osteogenically induced BMSCs (section 2.1.7), achieving the generation of a single cell suspension to be subjected to FACS sorting.

Next, we determined any additive effect of adenoviral transduction and cell sorting with regard to cytotoxicity. Adenoviral transduction and cell sorting by means of FACS are considered as the two main stressors in the experimental procedure of the FACS experiments (Figure 3.6). For this purpose, BMSCs expanded in monolayer culture in the
presence of bFGF were reseeded at 60,000 cells/cm² and transduced with Ad.Runx2 at 100 MOI the following day. The following three donors were used: first run: donor Pat 53 (female, born 1992); second run: donor Pat 55 (female, born 1994); third run: donor Pat 62 (female, born 1958).

After 3 days of osteogenic induction, cells were sorted by means of FACS. Ungated PI-negative cells were sorted into collection tubes containing 20% FBS containing culture medium, counted using Trypan blue staining, and recultured into 6-well plates at a seeding density of 20,000 cells/cm². Unsorted cells (cells that did not see the cell sorter) serving as control cells were kept in FACS buffer during cell sorting of ungated cells. Thereafter, unsorted cells were subjected to the same reculture procedure: cell counting using Trypan blue staining and reculture into 6-well plates at a seeding density of 20,000 cells/cm². Both ungated and unsorted cells were cultured in expansion medium for up to 4 days, whereby cell viability was assessed on day 2 and 4 post-sort by means of Cell Titer Blue assay. Cell viability assay revealed that cell sorting as such did not cause cell death of the BMSCs transduced with Ad.Runx2 when compared with unsorted cells, neither on day 2 post-sort nor on day 4 post-sort. Average cell viabilities were 97% on day 2 post-sort and 94% on day 4 post-sort, referred to the cell viabilities of unsorted cells set to 100%. The experiments were performed with three donors of which each was analyzed in duplicate samples (n=6).

3.3 Discussion

Recombinant adenoviruses are considered as the vectors of choice for gene delivery and expression application. Features such as capability of infecting a broad range of cell types, adenoviral infection being independent of host cell division, but also obtaining of high viral titers constitute advantages favoring usage of adenoviruses. We obtained infectious viral titers of the adenoviral reporter Ad.Runx2 up to \(1.19 \times 10^{10}\) pfu/ml. Infectious viral titers of \(10^{11}\) pfu/ml can be achieved upon concentrating the virus by CsCl gradient centrifugation (Tollefson et al., 2007). Hence, the preparations of the adenoviral reporter Ad.Runx2 we performed were quite efficient. The comparison of OD\(_{260}\)-based and pfu-based viral titers exemplifies the potential discrepancy between the two methods which becomes recognizable in a twofold respect. First, the discrepancy between OD\(_{260}\)-based and pfu-based viral titer determination ranges from a factor 1.7 to 18.3. Secondly, while the lowest OD\(_{260}\)-based viral titer obtained reached 16% of the value of the batch with the best OD\(_{260}\)-based...
based viral titer, the lowest pfu-based viral titer obtained reached only 5% of the value of the batch with the best pfu-based viral titer. These findings indicate that the variation in the case of OD\textsubscript{260}^-based viral titers is smaller than in the case of pfu-based viral titers. This can be explained by the fact that while the OD\textsubscript{260}^- measurement is an objective measurement based on spectrophotometry, the plaque assay is more prone to mistakes. On the one hand, counting of the plaques can be incorrect by including plaques that did not arise due to cytopathic effects of an infectious viral particle, but rather due to cell death caused by either the warm agarose-medium overlay or the high cell seeding density. On the other hand, fusion of plaques can lead to underestimation of the actual infectious viral titer. Since calculations of the volume of virus to be applied to cells in the cell culture experiments at a given MOI are based on the viral titers, error propagation on the cell culture experiments is to be expected.

By means of cotransfection experiments using Runx2 expressing effector plasmid and Runx2-responsive luciferase reporter, we proved that the 12x tandemly arranged Runx2 binding sites which represent the Runx2-specific feature in the adenoviral reporter Ad.Runx2 are responsive to RUNX2 protein. These findings are in accordance with the publication based on which we designed the Runx2 binding site (Jimenez et al., 1999). By means of cotransfection experiments in HeLa cells, Jimenez and colleagues came to the following two conclusions. First, they showed that within several human MMP13 promoter deletion constructs, activity could still be induced if the Runx2 binding site was present. Secondly, the activation of the human MMP13 promoter by RUNX2 could be potentiated when an 8x tandemly arranged Runx2 binding site was used instead of a single Runx2 binding site placed upstream of the 83 bp minimal human MMP13 promoter. In this case, luciferase reporter activity was stimulated 25-fold upon cotransfection with a functional Runx2 expressing effector plasmid as opposed to basal level. However, we observed leaky expression in the Runx2 non-expressing cotransfection control which reached half the luciferase reporter expression level of the functional Runx2-expressing cotransfection. Such a pronounced leaky expression is in contrast to (Jimenez et al., 1999). They reported much stronger relative differences between functional and non-functional (basal) Runx2 expressing cotransfection (25-fold increase in the case of 8x tandemly arranged Runx2 binding site placed upstream of the 83 bp minimal human MMP13 promoter). The usage of a longer minimal promoter sequence in our cotransfection experiments might explain why we found higher basal luciferase reporter expression level.
In contrast to Jimenez and colleagues who made use of a 83 bp minimal human MMP13 promoter, we utilized a 136 bp minimal human MMP13 promoter reported by (Tardif et al., 1997). This promoter sequence was reported to have markedly increased transcriptional activity compared with a 1.6 kb promoter fragment when transfected into chondrocytes. Furthermore, a 39 bp minimal human MMP13 promoter exhibited no detectable promoter activity, indicating that already small changes in the fragment length of the promoter can lead to distinct changes in the basal promoter activity, which might be the case when a 83 bp instead of a 136 bp minimal promoter is utilized. Besides, it cannot be ruled out that tandemly arranging more Runx2 binding sites does not necessarily further potentiate the promoter activity.

Although Jimenez and colleagues performed cotransfection experiments in HeLa cells as well, leaky expression can also be explained by the fact that HeLa cells have a baseline Runx2 expression in the absence of the Runx2 expressing effector plasmid. According to the Human Protein Atlas (www.proteinatlas.org), a publicly available database of expression profiles of human proteins in tissues and cells, HeLa cells show weak RUNX2 protein expression. As the HeLa cell line has been subject to significant phenotypic drift, it is entirely possible our cell line has a higher Runx2 expression than that used in a paper 15 years ago. This would lead to a higher background, with a lower induced effect.

Despite the leaky expression in the Runx2 non-expressing cotransfection control, we can see an induction of reporter expression in response to Runx2 expression. As a more suitable control, HeLa cells that either do not express endogenous RUNX2 protein by means of Runx2 knockdown by RNA interference, or do express RUNX2 but which is rendered non-functional by impairing its phosphorylation state, should be used. In addition, a GFP construct will be used for the FACS experiments. A low level of leaky baseline GFP expression can be controlled for by gating out weak GFP expressing cells.

The first of the three unknowns in the experimental procedure of the planned FACS experiments which was addressed was the cell seeding density. High cell seeding density turned out to result in persistent GFP reporter expression over a period of 6 days of culture after transduction. Besides, low cell seeding density, i.e. 15,000 cells/cm², resulted in a persistent GFP reporter expression as well. Therefore, the cell density might not be a big variable. However, high cell seeding density was considered to be beneficial for osteogenic induction of the cells by representing a natural trigger for spontaneous osteogenic induction.
Next, the MOI as another factor to be determined was addressed. A dose response between adenovirus particle concentration applied and GFP positivity was found in BMSCs transduced with Ad.Runx2. Whereas a marked increase in the level of GFP expression came along with an increase of the MOI from 10 to 100, there was only a little if any increase in the overall GFP expression observed with an increase from 100 to 1,000 MOI, which was only observed in one of the three donors due to marked cell death in the other two donors investigated. The finding that only a weak increase in GFP positivity was observed with an increase in the MOI from 100 to 1,000 might indicate that the dose response reached an apparent plateau, which would not justify further increasing the MOI beyond 100 when aiming at increasing the GFP positivity due to the observed toxic effects.

In accordance with the experiments using Ad.Runx2, the same relative tendencies with regard to GFP positivity were found upon adenoviral transduction of the three donors with Ad.GFP. Irrespective of whether Ad.GFP or Ad.Runx2 was used, under the same conditions Pat 62 exhibited higher GFP positivity than Pat 53 and 55, respectively. Whereas GFP expression mediated by Ad.Runx2 reflects the expression of RUNX2 protein, and differences in Ad.Runx2-mediated GFP expression can be linked to differences in the osteogenic stage or responsiveness of a particular donor, the GFP expression mediated by Ad.GFP is dependent on the activity of the CMV promoter. These are two completely different mechanisms, and therefore these tendencies suggest variable donor-dependent viral susceptibility, with Pat 62 being more easily infected.

Furthermore, differential donor resistance to adverse cytotoxic effects to adenoviral transduction were observed. Whereas two of the donors (Pat 53 and 55) showed signs of cell death from day 1 post-transduction on, the third donor (Pat 62) appeared to withstand the cytopathic effects induced by transduction with Ad.Runx2 at an MOI of 1,000. These findings indicate that different donors are differentially susceptible to the cytopathic effects adenoviruses exert on cells. Differential susceptibility to cytopathic effects might represent an indicator for the health of a particular donor’s BMSCs.

By means of Cell Titer Blue assay to assess cell viability, the findings that adenoviral transduction impairs cell viability and that donors are differentially susceptible to adenoviral transduction’s cytotoxic effect assessed by microscopic observation could be substantiated.
As a consequence of the fact that adenoviral transduction impairs cell viability, higher adenoviral titers applied to cells tend to more strongly impact the cell viability. In accordance with this consideration, adenoviral transduction using Ad.Runx2 dose-dependently reduced cell viability, which was most prominently visible when comparing an MOI of 100 and 1,000. However, in contrast, adenoviral transduction using Ad.GFP did no markedly impair the cell viability. This finding was surprising considering that the high-level CMV-driven GFP expression is non-physiological and has the potential to impair cellular signaling systems and lead to cytotoxicity (Edholm et al., 2001).

After the optimal MOI had been determined, the peak of GFP expression level could be located to day 3 to 4 post-transduction in the case of the three donors analyzed. Not only was this temporal location consistently reported for all three donors analyzed, but also was it consistent irrespective of which of three methods was applied to determine the temporal course of GFP expression level. GFP expression analysis was performed either based on subjective comparative estimation of GFP expression levels or by image analysis of either representative or all images taken. To adequately deal with the inherent donor variation that comes along with the FACS experiments performed with different donors, the day of sorting was standardized by setting a defined time point of sorting, which is day 3 post-transduction in the case of a seeding density of 60,000 cells/cm$^2$. We decided to go forward with a seeding density of 60,000 cells/cm$^2$ after having compared the seeding densities of 60,000 and 15,000 cells/cm$^2$ with regard to persistence of reporter expression and time course of GFP expression level. While both seeding densities fulfilled the requirement of enabling persistent GFP expression, the time course and the intensity of GFP expression levels favored 60,000 cells/cm$^2$ as optimal cell seeding density. As expected, high seeding density, i.e. 60,000 cells/cm$^2$, led to spontaneous osteogenic differentiation where dexamethasone had a reduced effect. The seeding density of 15,000 cells/cm$^2$ had a more robust dexamethasone effect as the spontaneous differentiation was lower. Furthermore, the overall GFP expression level was higher in the case of the seeding density of 60,000 cells/cm$^2$. Since our aim was to obtain Runx2 GFP$^+$ cells, regardless of whether confluence (high density) or dexamethasone was the trigger, the seeding density of 60,000 cells/cm$^2$ was more beneficial to achieve this aim. The seeding density of 60,000 cells/cm$^2$ was chosen for these reasons, together with the fact that the time variation of the peak of GFP expression level amongst different donors was lower (day 3 to
4 post-transduction) at a seeding density of 60,000 cells/cm², while it was day 3 to 7 post-transduction in the case of 15,000 cells/cm².

Although the temporal localization of the peak of GFP expression level set to day 3 to 4 post-transduction was consistent amongst the methods used to determine GFP expression level, the different methods resulted in graphs with differing curve progression. Differences are in consequence of both the operator who performed the experiment as well as the mathematics behind the different image analysis methods. First of all, it needs to be mentioned that the images based on which the GFP expression level was determined were not taken randomly within the individual wells. Instead, spots with green cells were specifically looked for in order to record the whole range of GFP positivity observed in different fields of view present in individual conditions’ wells of 6-well plates. The non-random approach for image capturing represents a limitation of this study since it tends to assign higher GFP positivity to donors with lower GFP expression levels. Next, as regards the subjective comparative estimation of GFP expression, its weakness lies in the inherent error-proneness. Therefore, the subjective graphs of the time course of GFP expression level need to be interpreted with care. An attempt to more objectively determine the GFP expression levels was made by performing image analysis of the fluorescence microscopic images taken. First, the image analysis was performed using representative images. For this purpose, the total area of green fluorescent cells was quantified by measuring the cumulative pixel area that was considered as green. Although computerized determination of the total area of green fluorescent cells is an objective method, selecting images which then are considered as representative as a preparative step for the subsequent image analysis brings subjectivity. Keeping these arguments at the back of our minds, the graphs generated based on subjective estimation indeed look very different to the ones generated based on image analysis using representative images, at least in the case of donor Pat 55 and 62. Differences in the number of peaks, as well as inversed relative GFP expression levels between unstimulated and stimulated condition were observed between graphs generated based on subjective estimation and those generated based on image analysis using representative images. In contrast, donor Pat 53 exhibited a graph generated based on subjective estimation which was very comparable with the one generated based on image analysis using representative images. The only noticeable difference was that the peak in the graph generated based on subjective estimation was rather a plateau as opposed to a peak in the graph generated based on image analysis using representative images. However, what was unexpected was the observation that the unstimulated
condition exhibited higher GFP expression levels than the stimulated condition, which was the case in both the graphs generated based on subjective estimation and those generated based on image analysis using representative images. A very high seeding density (60,000 cells/cm\(^2\)) which was the case in this experiment is considered as a natural trigger for osteogenic differentiation indeed and thus can start the osteogenic differentiation of cells even in the absence of osteogenic medium. Nevertheless, considering that in the stimulated condition, high seeding density would act as an additional stimulus besides the osteogenic stimulus due to the osteogenic ingredients in the culture medium, unstimulated condition exceeding the GFP expression level of stimulated condition was unexpected. This finding would indicate that dexamethasone, the main osteogenic factor present in the osteogenic medium, rather acts inhibitory on Runx2 expression and/or function in high seeding density culture. In human osteoblast (HOB) cell lines, the synthetic glucocorticoid dexamethasone induced an increase in both protein level and DNA binding activity of RUNX2 (Prince et al., 2001). In contrast, rodent osteoblasts responded differently upon treatment with dexamethasone: rat osteoblasts showed decreased RUNX2 protein levels, while the RUNX2 protein level in mouse osteoblasts was not affected (Prince et al., 2001). The opposed effects dexamethasone had in the latter study and in our experiment might be explained by the different cell seeding densities applied. While we seeded the cells at high seeding density at 60,000 cells/cm\(^2\), Prince and colleagues seeded their cells in around 8,000 cells/cm\(^2\) and grew them until confluence (from four to six days).

However, what needs to be kept in mind is the fact that we observed the inversed relative GFP expression levels between unstimulated and stimulated condition in only one of the three donors analyzed, and therefore and even this was not consistent between the three methods applied, interpretations need to be made with care. An explanation for this discrepancy may be due to the reason why differing numbers of images were taken. In many cases every green cell was imaged as few were found. When then analyzing this condition the assumption that the image is a representative image is incorrect, as every other area in the same well would be black.

Therefore, the image analysis was extended and performed using all images taken, enabling the most objective way to analyze the course of GFP expression level. For this purpose, the sum of the cumulative pixel area of individual images per condition of a particular donor and time point was determined, and this value was further processed by relating it to the maximal number of images (i.e. 17) and to the total 2040 x 1536 pixel...
image area. The graphs generated based on image analysis using all images taken look very different to the ones generated using the representative images in all three donors analyzed. Differences that stand out encompass number and temporal location of peaks, inversed relative GFP expression levels between unstimulated and stimulated condition, along with the level of green percentaged pixel area at the end of the time frame analyzed. The individual differences in each donor’s graphs which comprise combinations of the above-mentioned characteristics of differences are explainable by either the discrepancy between the effective and maximal number of images or by the extent of how representative, i.e. average, the representative image actually is.

In conclusion, differences in a particular donor’s graphs generated by the three different methods to determine GFP expression level are a consequence of a combination of factors concerning the operator who performed the experiment, the number of available images (lower for poor donors), as well as the mathematics behind the different image analysis methods.

Besides human BMSCs which represent the standard cells used in this thesis, rat MSCs were also analyzed for their infectability by adenoviral vectors. Adenoviral transduction using Ad.GFP clearly showed that rat MSCs can be transduced by adenoviral vectors, although the transduction efficiency obtained was lower than with human BMSCs. Based on these results, we performed adenoviral transduction with Ad.Runx2. We could not observe any GFP expression mediated by Ad.Runx2 up to day 6 post-transduction. In principle, these negative results have several potential explanations. First, missing GFP expression might be merely due to the absence of RUNX2 protein. However, this explanation is very unlikely to be true since rat MSCs are reported not only to express Runx2, but also rat RUNX2 protein possesses transactivation potential indicating functionality of RUNX2 (Chang et al., 1998). Rather, it might indicate that rat RUNX2 protein does not bind to the human Runx2 binding sites, which then would explain the absent GFP expression. Alternatively, rat RUNX2 protein does bind human Runx2 binding sites indeed, but the general transcription factors such as TATA-binding protein and the transcription factor AP-1 which contribute to basal gene expression (Buttice et al., 1991), respectively of which binding sites are reported to be present in the 136 bp minimal promoter sequence from the 5'-flanking region of the human MMP13 gene (Tardif et al., 1997) only act species-specifically. This would lead to absent initiation of transcription of EGFP gene in the first place even in the presence of functional RUNX2 protein.
The suitable MOI used for adenoviral transduction is cell type dependent. We found that Ad.GFP transduction at 10 MOI of human BMSCs led to a markedly higher GFP expression level than in the case of rat MSCs. Therefore, the MOI in the case of rat MSCs would be needed to be increased to achieve comparable GFP expression levels. Accordingly, it would be necessary to increase the MOI of Ad.Runx2 in the case of rat MSCs to achieve a comparable situation with human BMSCs. Thus, it could potentially be the case that rat MSCs would have fluoresced green if transduced using an adjusted MOI which takes the smaller transduction efficiency based on Ad.GFP transduction into account.

In any case, based on the results obtained, we can conclude that these results substantiate the specificity of Ad.Runx2. Due to these findings, we can exclude the possibility that Ad.Runx2-mediated GFP expression exhibited unspecific background fluorescence in human BMSCs, as in this case, rat MSCs would also have been expected to exhibit some green fluorescence. This was not the case, but rather rat MSCs did not show any detectable GFP expression during a time frame at which GFP would have been expressed if conditions permitted it.
4 Runx2-responsive adenoviral reporter – in vitro validation

Part of the results presented in chapter 4 was introduced into the following manuscript which has been accepted for publication in Tissue Engineering.

In vitro osteogenic potential of human bone marrow derived MSCs is predicted by Runx2/Sox9 Ratio
Claudia Loebel, Ewa M. Czekanska, Marco Bruderer, Gian Salzmann, Mauro Alini, Martin J. Stoddart

Claudia Loebel, Ewa M. Czekanska, and Marco Bruderer: study conduct, data analysis and interpretation, manuscript writing. Specifically, Marco Bruderer provided mRNA data from time points day 2 and 7 and $^{45}$Ca incorporation data from day 28 of osteogenic and control hMSCs. Gian Salzmann: study conduct, data analysis and interpretation, final approval of manuscript. Mauro Alini and Martin J. Stoddart: conception and design, data analysis and interpretation, manuscript writing, final approval of manuscript. Martin J. Stoddart takes responsibility for the integrity of the data analysis.

Preliminary remark:
In this chapter, osteogenically induced human BMSCs were subdivided based on GFP reporter expression mediated by the Runx2-responsive adenoviral reporter Ad.Runx2 with which the BMSCs were transduced. The reporter-positive cell population exhibited in vitro characteristics of osteoprogenitors, as evidenced by the characterization experiments detailed in section 4.2.1. The initial milestone of different cell population characterization was achieved. The pattern of in vitro osteogenic differentiation capability amongst the cell populations isolated by FACS initiated follow-up experiments addressing a possible crosstalk between cell populations, addressed in section 4.2.2. From this moment, inconsistent results were obtained and we were no longer able to reliably predict the functionality of the sorted cell populations. Even a subsequent thorough re-evaluation phase, which constitutes section 4.2.3, addressing a variety of possible influencing factors could not concludingly answer which factor(s) resulted in the irreproducible experimental outcomes. Although a new hypothesis was proposed, based on exciting new insights in the
process of osteogenic commitment while the re-evaluation experiments were ongoing, the end of this chapter leaves the reader with some open questions. For example: why did the initial characterization experiments reproducibly work? With the completion of the present thesis, any concrete explanation remains speculation, although assumptions converge such that the explanation is linked to the quintessence of the novel hypothesis, according to which osteogenic commitment and differentiation is not triggered exclusively by Runx2, but by an interplay between Runx2, Sox9, and possibly additional transcriptional players.
4.1 Introduction

In the current chapter, we report a novel method for the FACS-mediated isolation of osteoprogenitors from osteogenically induced human BMSCs, conceptually illustrated in Figure 4.1. A subpopulation of osteogenically induced BMSCs was functionally identified and isolated by coupling the expression of the key osteogenic transcription factor RUNX2 to the expression of EGFP via the Ad.Runx2 adenoviral reporter. On that basis, fluorescing cells could be selected by means of FACS. Besides the fluorescing reporter-positive cells, the reporter-negative cells as well as the original unsorted cell population were used for comparative characterization.

Figure 4.1: Concept of the novel approach for the functional identification and isolation of a homogeneous osteogenic subpopulation of BMSCs.

The overall experimental procedure to practically approach the novel concept for the isolation of osteoprogenitors is illustrated in Figure 4.2.
Figure 4.2: Overall experimental procedure for FACS-mediated isolation of osteoprogenitors. Human BMSCs are obtained from bone marrow aspirates, plating onto tissue culture plastic, and allowing adherent cells to attach. BMSCs are expanded in culture in presence of bFGF. Reselected cells are transduced with Ad.Runx2 and thereafter stimulated towards the osteoblast phenotype. Cells induced to the osteogenic lineage will express RUNX2 and cause the expression of EGFP, thus the cell becomes green. The fluorescing cells can be identified and sorted by means of FACS. Reporter positive (i.e. green), negative, and unsorted cells are thereafter separately expanded in monolayer culture thereby losing the adenovirus. Once the required cell number is achieved, the different cell populations are subjected to in vitro osteogenic differentiation assay, and comparative investigation of osteogenic differentiation potential is performed.

The characterization of the resulting cell populations is expected to follow the tendencies depicted in Figure 4.3.
In chapter 3, the following three key parameters defining the experimental procedure had been determined and optimized, and were applied in this chapter:

- Cell seeding density for adenoviral transduction the next day: 60,000 cells/cm²
- Optimal MOI for adenoviral transduction using Ad.Runx2: 100 MOI
- Time point of cell sorting: day 3 post-transduction, i.e. day 3 of osteogenic induction

In the current chapter, the Ad.Runx2 adenoviral reporter was further validated in vitro, and the different expanded sorted cell populations resulting from FACS sorting were characterized.

The aim of this chapter was to develop a novel approach for the functional identification and isolation of osteoprogenitors from osteogenically induced human BMSCs. To familiarize with the cell population this chapter focuses on, an introduction into osteoprogenitors is given in the following.

Committed osteoprogenitors can be identified in BMSC populations by means of the CFU-O assay to assess the proliferation and differentiation potential in vitro. Bone nodules represent the in vitro end product of the proliferation and differentiation of individual osteoprogenitors, and there exists a linear relationship between the formed mineralized nodules and the number of seeded cells (Bellows et al., 1986). Independently performed limiting dilution studies have come to the common conclusion that osteoprogenitors are rarely present in primary cell sources. In 1989, Bellows and colleagues showed that under standard conditions, 0.3% of a mixed isolated rat calvaria cell population represent bone nodule-forming osteoprogenitors (Bellows and Aubin, 1989). Furthermore, this number was found to be increased by exogenous agents such as glucocorticoids (dexamethasone) (Bellows and Aubin, 1989). Estimation of the cell numbers present in a bone nodule revealed that osteoprogenitors are capable of undergoing six to seven PDs to form the...
bone nodule composed of functional osteoblasts. The bone nodule formation-based colony assay described in the foresaid publication to quantify osteoprogenitor numbers had been referred to as CFU-O assay due to the analogy to the termination in the field of hematopoietic stem cells. Time course studies of nodule formation revealed that there exist two different cell populations with osteoprogenitor characteristics, and that those osteoprogenitors requiring dexamethasone as a stimulus for osteogenic differentiation might be at an earlier stage in the differentiation pathway to osteoblasts (Bellows et al., 1987).

The fact that many additives have been reported to enhance osteogenesis supports the presence not only of committed, but also of inducible osteoprogenitors in bone marrow- and bone-derived populations. Turksen and colleagues subdivided osteoprogenitors from mixed fetal rat calvaria cell populations based on the expression of the osteoblast marker ALP into an ALP^+ and an initially ALP^- population (Turksen and Aubin, 1991). Thereby, almost all osteoprogenitors forming bone in the absence of glucocorticoids resided in the ALP^+ fraction, whereas those osteoprogenitors requiring dexamethasone for differentiation were enriched in the ALP^- fraction (Turksen and Aubin, 1991). Those requiring dexamethasone were considered as more primitive osteoprogenitors than those not requiring dexamethasone to differentiate (Turksen and Aubin, 1991). Treatment of the ALP^+ fraction with dexamethasone led to a 5-fold increase in the formation of bone nodules, while treating the ALP^- fraction led to a 30-fold increase. On the basis of the findings, mesenchymal stem cells pass through two osteoprogenitor stages in the osteoblast differentiation pathway, namely ALP^- early osteoprogenitors and ALP^+ late osteoprogenitors.

Besides usage of a single marker such as ALP for identification of osteoprogenitors, dual marker approaches have also been performed. For example, dual-color sorting using the stromal precursor cell marker STRO-1 and the bone/liver/kidney isoform of ALP as osteoblast marker, although not specific to the osteogenic lineage, to subdivide human bone marrow stromal cells and normal human bone cells obtained from trabecular bone, respectively resulted in an osteoblast lineage cell hierarchy. STRO-1^-/ALP^- osteoprogenitors progress to the intermediate preosteoblast stage characterized by a STRO-1^-/ALP^+ immunophenotype, and further advance into STRO-1^-/ALP^- osteoblasts, before the osteoblast lose the osteoblast marker ALP and become STRO-1^-/ALP^-, representing osteocytes and bone lining cells (Gronthos et al., 1999).
Here, we report a completely novel approach to identify and isolate osteoprogenitors, namely by basing the identification upon an intracellular transcription factor whose function is associated with osteogenic differentiation. Therefore, this approach is clearly set apart from identification methods based on single or multiple cell surface markers.

4.2 Results

4.2.1 Characterization of osteoprogenitors functionally isolated by a Runx2-responsive adenoviral reporter

As detailed in the experimental procedure in Figure 4.2, osteogenically induced BMSCs which had been transduced with Ad.Runx2 to cause the expression of EGFP mediated by the presence of functionally active RUNX2 protein were sorted based on the presence of green fluorescence. The FACS sorting procedure is explained in the following (Figure 4.4). Untransduced, osteogenically induced control cells served as negative control cells to determine the level of background green fluorescence. Based on the determined background fluorescence, in the transduced, osteogenically stimulated sample, after having taken a gap region into consideration to eliminate potential leaky expression of the vector, everything above the range of background green fluorescence was considered as GFP positivity (Figure 4.4).
Figure 4.4: Representative images of the FACS experiment procedure. A) Untransduced, stimulated control cells served as negative control to set the background fluorescence. B) Transduced, stimulated cells represented the actual sample. Actual GFP positivity was considered as a green fluorescence signal larger than the green background fluorescence considered as GFP negativity in the transduced, stimulated sample. Thereby, a gap region was taken into consideration to more clearly separate the ranges of GFP negativity and GFP positivity.

The FACS sorting procedure constitutes a part of a more extended gating procedure with which the data is systematically reduced starting from a record of all events, which besides the cells also included cellular debris (Figure 4.5). By successively selecting populations with specific characteristics of interest, the original population consisting of all events is reduced to single cells, which then are distinguished between GFP+, GFP-, and unsorted cells which reflected the ungated cell population, i.e. any event recorded at the first step of the gating procedure is considered as unsorted. In fact, we could not reveal any adverse effect of the cell sorting procedure on the viability of the sorted cells. Comparison of
unsorted (i.e. original transduced, osteogenically stimulated cell population which did not see the FACS sorter and thus did neither pass through the laser beams nor did they get deflected through high-voltage deflection plates into collection tubes) and ungated (i.e. original transduced, osteogenically stimulated cell population which passed through the FACS sorter, but were only unspecifically unsorted by collecting every event recorded at the first step of the gating procedure) revealed no impairment of the FACS sorting procedure on the cells and their viability. Therefore, the terms ‘unsorted’ and ‘ungated’ can be used interchangeably.
Figure 4.5: Illustration of the gating strategy. Proper cell sorting of one or several cell populations of interest significantly depends, amongst others, on an appropriate gating strategy. A correct gating strategy is crucial to identify and sort the true cell population. Essentially, gating aims at data reduction by selecting populations with specific characteristics. Depending on the cell population to be identified and the cells used, complex gating strategies are becoming more and more common. A) 1. Cells are distinguished from debris based on the forward scatter area (FSC-A) versus side scatter area (SSC-A) dot plot. FSC-
A is a measure of cell size, SSC-A is a measure of cell granularity. To make sure the detected signal arises from single cells, exclusion of doublets and cell aggregates is performed by gating them out based on their FSC width (FSC-W) versus FSC height (FSC-H) and SSC-W versus SSC-H dot plots. GFP+ and GFP- cells are gated starting from single cells as parent population based on the intensity of the green fluorescence signal. Where mentioned due to a specific interest in cell viability, an additional dead cell exclusion step was included. In this case, dead cells are excluded by means of propidium iodide (PI) staining due to its PI positivity. PI is a fluorescent dye that intercalates into double-stranded DNA. It is excluded from viable cells, but penetrates the cell membrane of dead cells, i.e. dead cells are positive for PI. In turn, GFP+ and GFP- cells are gated starting from single viable cells as parent population based on the intensity of the green fluorescence signal. B) The gating tree reflects the sequential procedure to finally sort the cells of interest, i.e. GFP+ and GFP- cells. Furthermore, at each hierarchical level, the gating tree shows the resulting percentage of each gating step. Thereby, "%Parent" and "%Total" are distinguished. "%Parent" refers to the percentage related to the gated events of the preceding cell population, whereas "%Total" refers to all events with which the gating procedure was started. Percentages in the context of FACS in this thesis mean "%Parent". Data acquisition and cell sorts were performed at 4°C using a FACS Aria cell sorter (BD Biosciences). Data were recorded and analyzed with FACSDiva software (BD Biosciences).

Upon cell sorting, reporter positive (Runx2 GFP+), reporter negative (Runx2 GFP-), and unsorted cells were separately expanded in monolayer culture to obtain the cell number required for the different experiments to characterize the different cell populations.

4.2.1.1 Runx2 GFP+ cells show slower proliferation rate in vitro

One way to approach the characterization of the different expanded sorted cell populations was the assessment of the proliferation potential. The three different cell populations were plated into 100 mm tissue culture plates in BMSC expansion medium in triplicate at a seeding density of 100 cells/plate. BMSC expansion medium was changed twice a week. After 14 days of culture, cultures were subjected to Crystal violet staining. To estimate proliferation, colonies with diameter larger than 5 mm were referred to as fast-growing CFU, distinguishing them from slow-growing CFU. Colonies were counted microscopically and distinction of fast-growing and slow-growing CFUs was conducted.
using AxioVision software. The experiments were performed with two donors of which each was analyzed in triplicate samples (n=6). The following two donors were used: first run: donor Pat 03 (female, born 1930); second run: donor Pat 04 (female, born 1970). CFU analysis at day 14 revealed that the total number of CFUs for all three cell populations were similar (Figure 4.6).
**Figure 4.6:** Representative images of tissue culture dishes seeded with expanded Runx2 GFP+, Runx2 GFP−, and unsorted cell populations, subjected to colony forming unit (CFU) assay. Each cell population was plated at 100 cells per 100 mm tissue culture plate and cultured in presence of bFGF. Crystal violet staining at day 14 identified the CFUs.

However, when distinguishing fast-growing from slow-growing colonies differing in the overall colony size, it arose that Runx2 GFP+ cells had the smallest fraction of fast-growing colonies at the expense of slow-growing ones (Table 4.1). Runx2 GFP− cell population showed a CFU behavior similar to the one of unsorted cell population. This suggests that Runx2 GFP+ cells show a more committed/differentiated phenotype than the other two cell populations, reflected as a decreased proliferation potential.

<table>
<thead>
<tr>
<th></th>
<th>Runx2 GFP+</th>
<th>Runx2 GFP−</th>
<th>unsorted</th>
</tr>
</thead>
<tbody>
<tr>
<td>fast-growing</td>
<td>5.5%</td>
<td>18.4%</td>
<td>19.8%</td>
</tr>
<tr>
<td>slow-growing</td>
<td>94.5%</td>
<td>81.6%</td>
<td>80.2%</td>
</tr>
</tbody>
</table>

**Table 4.1:** Self-renewal capability in vitro of expanded Runx2 GFP+, Runx2 GFP−, and unsorted cell populations. Each cell population was plated at 100 cells per 100 mm tissue culture plate and cultured in presence of bFGF. Crystal violet staining at day 14 identified the colony forming units (CFU). Frequencies of fast-growing and slow-growing CFUs for each of the three analyzed cell populations are presented (n=6).

### 4.2.1.2 Runx2 GFP+ cells show more osteoblast-like characteristics upon *in vitro* osteogenic differentiation

Next, to investigate the *in vitro* osteogenic differentiation potential of Runx2 GFP+, Runx2 GFP−, and unsorted cells, the three different expanded sorted cell populations were subjected to *in vitro* osteogenic differentiation for a total of 21 days.

To functionally test the *in vitro* osteogenic differentiation potential, ALP activity was determined at day 7, 14, and 21 of osteogenic differentiation (Figure 4.7). The experiments were performed with two donors of which each was analyzed in duplicate samples (pooled samples each from two wells) (n=4). The following two donors were used: first run: donor Pat 03 (female, born 1930); second run: donor Pat 25 (male, born 1970).

When comparing the ALP activities with the schematic representation of the exemplified course of ALP activity of MSCs subjected to osteogenic differentiation, which peaks at
around day 14, it arose that ALP activity of Runx2 GFP+ cell population was shifted towards earlier time points of osteogenic differentiation, showing highest ALP activity at day 7. Unexpectedly, Runx2 GFP+ cell population was the one with the next lower ALP activity, showing highest ALP activity at day 14. And unsorted cell population showed lowest ALP activity, not showing a distinct maximum at all until day 21 of osteogenic differentiation. These results indicate that Runx2 GFP+ cells are more osteogenic than the other two cell populations.

![Graph showing ALP activity](image)

**Figure 4.7:** Osteogenic differentiation in vitro of expanded Runx2 GFP+, Runx2 GFP-, and unsorted cell populations. Alkaline phosphatase (ALP) activity at day 7, 14, and 21. **: p≤0.05 vs. the other two cell populations of the same time point (d7); ##: p≤0.05 and ###: p≤0.01, respectively vs. the corresponding time point of the respective cell population. Results are presented as mean + SEM (n=4).

To further test the in vitro osteogenic differentiation potential by examining the matrix mineralization, ⁴⁵Ca incorporation of the different expanded sorted cell populations was determined at day 21 of osteogenic differentiation (Figure 4.8). The experiments were performed with five donors of which each was analyzed in quadruplicate samples (n=20). The following five donors were used: first run: donor Pat 03 (female, born 1930); second
run: donor Pat 04 (female, born 1970); third run: donor Pat 09 (female, born 1956); fourth run: donor Pat 25 (male, born 1970); fifth run: donor Pat 26 (male, born 1951).

$^{45}$Ca incorporation, related to the DNA amount, was significantly higher for Runx2 GFP$^+$ cell population treated with osteogenic medium than for Runx2 GFP$^-$ and unsorted cell populations treated with the same medium. This assay shows exactly the same tendencies than the ALP activity results, indicating that Runx2 GFP$^+$ cell population shows a higher capacity for in vitro osteogenic differentiation compared to Runx2 GFP$^-$ and unsorted cells.

**Figure 4.8:** Osteogenic differentiation in vitro of expanded Runx2 GFP$^+$, Runx2 GFP$^-$, and unsorted cell populations. $^{45}$Ca incorporation, related to the DNA amount, at day 21. ***: $p \leq 0.01$ vs. the other two cell populations treated with the same medium; ###: $p \leq 0.01$ vs. the same cell population treated with control medium. Results are presented as mean + SEM (n=20).

To conclude, we have utilized a Runx2-responsive reporter adenovirus to subdivide osteogenically induced human BMSCs and to isolate a BMSC subpopulation with high osteogenic differentiation potential. The novel isolation approach resulted in a cell population, namely Runx2 GFP$^+$ cell population, which displays characteristics appropriate for osteoprogenitors: (1) a slower proliferation rate, and (2) a more osteoblast-like phenotype upon in vitro osteogenic differentiation, as compared with Runx2 GFP$^-$ as well as original cell population.
ALP activity and $^{45}$Ca incorporation results are consistent and show the same trends: Runx2 GFP$^+$ cell population shows the highest in vitro osteogenic differentiation potential, Runx2 GFP$^-$ cell population takes a middle position, and unsorted cell population shows lowest osteogenic differentiation potential. While unexpected, both assays, which represent two completely different functional outcomes, show the same trends. Instead, one would have expected that Runx2 GFP$^+$ cell population would take one extreme in terms of in vitro osteogenic differentiation potential, Runx2 GFP$^-$ cell population would take the other extreme, and unsorted cell population as a mixture of both cell populations would take the middle position.

The observed tendencies suggest a crosstalk between the Runx2 GFP$^+$ and Runx2 GFP$^-$ cell populations. We expect the cell-cell interactions to be of inhibitory nature, namely that Runx2 GFP$^-$ cell population exhibits an inhibitory activity towards the Runx2 GFP$^+$ cell population. Once these two cell populations are separated from each other, Runx2 GFP$^+$ cell population is able to exert its actual high osteogenic potential we noticed in the in vitro osteogenic differentiation assays mentioned above.

### 4.2.2 Crosstalk experiments

To approach the cell-cell interactions between the Runx2 GFP$^+$ and Runx2 GFP$^-$ cells, we performed experiments whereby besides the original three analyzed expanded sorted cell populations, Runx2 GFP$^+$ and Runx2 GFP$^-$ cells were mixed after the post-sort expansion phase at different ratios to create three additional groups:

- group ‘50%+ & 50%-’: mixed cell population consisting of 50% Runx2 GFP$^+$ cells and 50% Runx2 GFP$^-$ cells
- group ‘85%+ & 15%-’: mixed cell population consisting of 85% Runx2 GFP$^+$ cells and 15% Runx2 GFP$^-$ cells
- group ‘95%+ & 5%-’: mixed cell population consisting of 95% Runx2 GFP$^+$ cells and 5% Runx2 GFP$^-$ cells

Crosstalk between Runx2 GFP$^+$ and Runx2 GFP$^-$ cell populations was investigated by determining the in vitro osteogenic differentiation potential by means of $^{45}$Ca incorporation of the six different cell populations at day 21 of osteogenic differentiation. The experiments were performed four times with a total of two donors of which each experiment was analyzed in quadruplicate samples. One of the two donors, namely Pat 03, was used for three out of four independent experiments. The following two donors were
used: first run: donor Pat 10 (female, born 1930); second, third, and fourth run: donor Pat 03 (female, born 1930).

For each individual crosstalk experiment, the results of $^{45}$Ca incorporation, related to the DNA amount, are shown in Figure 4.9.

**Figure 4.9:** Crosstalk experiment of Runx2 GFP$^+$ and Runx2 GFP$^-$ cell populations. Osteogenic differentiation in vitro of expanded Runx2 GFP$^+$, Runx2 GFP$^-$, and unsorted cell populations, along with three mixed populations consisting of different ratios between Runx2 GFP$^+$ and Runx2 GFP$^-$ cells was performed. $^{45}$Ca incorporation results, related to the DNA amount, at
day 21 of each individual experiment are shown. A) Results of donor Pat 10 (one experiment), B) Results of donor Pat 03 (three independent experiments). The bottom right graph represents a detail of the top right graph with stretched y-axis to accentuate the $^{45}$Ca incorporation rates and the comparison amongst the different cell populations.

Although the maximal $^{45}$Ca incorporation values obtained in the presence of osteogenic medium were very high while only resulting in basal values in the presence of control medium, Runx2 GFP$^+$ cell population was not necessarily the cell population with the highest $^{45}$Ca incorporation upon osteogenic differentiation. In that experiment where Runx2 GFP$^+$ cell population showed highest $^{45}$Ca incorporation (top right graph in Figure 4.9), the resulting $^{45}$Ca incorporation rates of the three additionally generated mixed cell populations were as expected (bottom right graph in Figure 4.9 which represents a detail of the top right graph in Figure 4.9). The $^{45}$Ca incorporation values increased as the fraction of Runx2 GFP$^+$ cells which originally contributed to the mixed cell population increased. In fact, the $^{45}$Ca incorporation values of the individual cell populations of this particular experiment correspond very well with the theoretical values calculated based on the ratios of Runx2 GFP$^+$ and Runx2 GFP$^-$ cells present in the cell populations. This would indicate that the observed $^{45}$Ca incorporation values of the different cell populations can be solely explained by the differential composition of a highly osteogenic and more weakly osteogenic cell population, and does not necessarily involve a crosstalk between the cell populations mutually affecting their osteogenic performance.

Returning to the indicated varying patterns of osteogenic performance of the different cell populations amongst the different experiments, what stands out even more considerably is the identification that inconsistent $^{45}$Ca incorporation patterns were observed even in experiments with the same donor (Figure 4.9B). This is in conflict with previous findings in the context of the experiments with only three analyzed groups (Runx2 GFP$^+$, Runx2 GFP$^-$, and unsorted cell populations) where the experiments repeatedly showed the same pattern with Runx2 GFP$^+$ as highly osteogenic cell population, or instead the experiment’s donor was a non-responder.

### 4.2.3 Troubleshooting experiments

The ambivalent results of the crosstalk experiments could be the consequence of a single or several possible influencing factors, which will be discussed and approached in the
following in the context of troubleshooting the crosstalk experiments. Due to the inconsistent results amongst experiments with the same donor, the influencing factor ‘donor’ could be excluded from further consideration in the first place.

### 4.2.3.1 Influencing factor ‘population doublings’

The first influencing factor we considered since it represents the most obvious deviation from the experimental procedure with the only three analyzed cell populations. Due to the larger numbers of cells required to perform the crosstalk experiments, the sorted cells needed to be expanded more extensively. Increase in the cumulative population doublings (PD) of cells affects the functional performance of cell populations, and the cell populations reach cellular senescence earlier.

The number of PDs the different cell populations underwent in the FACS experiments involving the three cell populations Runx2 GFP+, Runx2 GFP, and unsorted cell populations, referred to as ‘characterization experiments’, together with those from the crosstalk experiments involved the six different cell populations are summarized in Table 4.2. This compilation allows comparison between the two different types of experiments which are considered to differ in the overall PDs the cell populations underwent.

The compilation of the PDs shown in Table 4.2 reveals that at first sight, there is a trend towards higher PDs in the crosstalk experiments than in the characterization experiments in all three different types of PDs. To be more precise, the PDs of the characterization experiments spanned a range whose extremes are lower than in the case of the crosstalk experiments. However, the different PDs of both the number of initial PDs and number of post-sort PDs within the crosstalk experiments span a range of PDs which comprises the range set by the corresponding PDs from the characterization experiments. Therefore, this circumstance indicates that differences in the PDs amongst the characterization experiments and the crosstalk experiments are not strong enough to explain discrepant patterns of osteogenic performance of the different sorted expanded cell populations.

Nevertheless, within the crosstalk experiments, there is some tendency towards lower PDs in that particular experiment (third run) that showed the pattern of osteogenic performance that was repeatedly observed in the characterization experiments. However, the tendency is not convincing enough to consider the PDs as a predicting/influencing factor that affects the pattern of osteogenic performance of the different cell populations. The low PD numbers of Runx2 GFP+ cell population of the fourth run would predict a high
osteogenic performance; however, this particular experiment led to a conflictive pattern of osteogenic performance.

Even though we were not able to conclusively clarify the issue of PDs with regard to troubleshooting the crosstalk experiments and whether the increased PDs influenced the crosstalk experiments, we nevertheless went back to the experimental setup consisting of only the three originally analyzed cell populations Runx2 GFP+, Runx2 GFP−, and unsorted cell populations for the upcoming experiments, since overall, the PDs of the characterization experiments spanned a range whose extremes are lower than in the case of the crosstalk experiments (Table 4.2).

<table>
<thead>
<tr>
<th>Characterization Experiments</th>
<th>Number of Initial PDs (Pre-sort Expansion)</th>
<th>Number of Post-sort PDs</th>
<th>Number of Total PDs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Runx2 GFP+</td>
<td>Runx2 GFP-</td>
<td>Unsorted</td>
</tr>
<tr>
<td>First run: Pat 03</td>
<td>2.4</td>
<td>3.1</td>
<td>7.1</td>
</tr>
<tr>
<td>Second run: Pat 04</td>
<td>2.9</td>
<td>4.9</td>
<td>4.4</td>
</tr>
<tr>
<td>Third run: Pat 09</td>
<td>2.9</td>
<td>5.3</td>
<td>5.6</td>
</tr>
<tr>
<td>Fourth run: Pat 25</td>
<td>3.3</td>
<td>3.9</td>
<td>4.1</td>
</tr>
<tr>
<td>Fifth run: Pat 26</td>
<td>2.8</td>
<td>2.1</td>
<td>4.6</td>
</tr>
<tr>
<td>Mean ± STD</td>
<td>2.8 ± 0.3</td>
<td>3.9 ± 1.3</td>
<td>5.1 ± 1.2</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Crosstalk Experiments</th>
<th>Number of Initial PDs (Pre-sort Expansion)</th>
<th>Number of Post-sort PDs</th>
<th>Number of Total PDs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Runx2 GFP+</td>
<td>Runx2 GFP-</td>
<td>Unsorted</td>
</tr>
<tr>
<td>First run: Pat 10</td>
<td>3.1</td>
<td>6.6</td>
<td>7.1</td>
</tr>
<tr>
<td>Second run: Pat 03</td>
<td>4.5</td>
<td>5.0</td>
<td>5.3</td>
</tr>
<tr>
<td>Third run: Pat 03</td>
<td>2.3</td>
<td>3.2</td>
<td>6.8</td>
</tr>
<tr>
<td>Fourth run: Pat 03</td>
<td>1.8</td>
<td>2.7</td>
<td>13.9</td>
</tr>
<tr>
<td>Mean ± STD</td>
<td>3.0 ± 1.1</td>
<td>4.4 ± 1.8</td>
<td>8.2 ± 3.8</td>
</tr>
</tbody>
</table>

**Table 4.2:** Number of population doublings (PD) of the different initial BMSC populations and sorted cell populations, respectively of the characterization experiments and the crosstalk experiments. Number of initial PDs refers to the number of PDs the cells underwent from thawing the BMSCs until the end of the pre-sort expansion phase at which the cells were reseeded for the adenoviral transduction the next day. Number of post-sort PDs refers to the number of PDs the cells underwent from reculturing the cells after cell sorting until the end of the post-sort expansion phase after which the different cell populations were subjected to in vitro osteogenic differentiation assay. The number of total PDs refers to the sum of the number of initial PDs and the number of post-sort PDs.

4.2.3.2 Influencing factor ‘Ad.Runx2 batch – function-impairing mutation’

The next influencing factor we considered was the Ad.Runx2 batch used for transduction prior to cell sorting. The origin of this influencing factor to be included in the troubleshooting experiments is the fact that we produced the Ad.Runx2 batches in house ourselves, along with the general observation that in the course of the hitherto performed
FACS experiments, there was a shift towards higher GFP positivities assessed at day 3 post-transduction when the cells were sorted, although this might be due to mere donor variability. In the course of the previously performed cell culture experiments, several batches which were successively produced were utilized. Production of a fresh batch of the reporter adenovirus was performed by amplifying aliquots of the batch that is running low. This circumstance, in theory, harbors the risk that a potentially function-impairing mutation could occur. The consequence thereof can be meaningful, as exemplified by a single point mutation (S65T) of GFP which amongst others resulted in increased brightness (Heim et al., 1995). A single point mutation could potentially impair the stability, the sensitivity, or even the functionality of Ad.Runx2, which in turn would ruin the FACS experiment.

To eliminate this influencing factor for future FACS experiments, we freshly produced Ad.Runx2 starting from the pAdEasy vector stage. pAdEasy-1-pShuttle-Runx2-MinPro-EGFP-polyA_clone 8.1 that had been stored at -20°C was first verified by sequencing of the appropriate regions using pShuttle forward and pShuttle-CMV reverse primers in two different reactions. Positive sequencing results allowed us to proceed with the production of fresh Ad.Runx2. Production of primary adenoviral stock and its subsequent amplification in AD-293 cells were performed as described in the section 2.2. Viral titer was estimated according to optical density (OD\textsubscript{260}), whereas the infectious titer was subsequently determined by performing a standard plaque assay on confluent AD-293 cultures.

Furthermore, the functionality of the freshly produced Ad.Runx2 was tested by its ability to lead to GFP expression levels similar to those of previous Ad.Runx2 batches, assessed by means of flow cytometry analysis of osteogenically induced human BMSCs.

In conclusion, we freshly produced a new batch of Ad.Runx2 starting from the corresponding AdEasy vector that was sequenced and confirmed to exhibit the predicted nucleotide sequence. Therefore, we can safely eliminate the influencing factor of a possible function-impairing mutation to influence future FACS experiments.

### 4.2.3.3 Influencing factor ‘serum used for cell culture’

Another putative influencing factor and causer of the ambivalent crosstalk experiment results was that a new batch of heat-inactivated FBS was used for the four crosstalk experiments. The change in the batch of heat-inactivated FBS as influencing factor was approached based on the assumption that a new batch can potentially lead to a shift in
the timing of the osteogenic induction of the BMSCs. This would have drastic consequences for the whole experiments because a defined timing of osteogenic induction for the identification and isolation of the osteoprogenitor population is a key component which had been established and optimized prior to the actual experiments. The consequences might even be that pronounced that the cells of interest are missed due to inappropriate timing of the FACS sort.

The planned experimental procedure to approach the influencing factor ‘serum used for cell culture’ is highlighted in Figure 4.10. To simulate the effects of the change in batch of heat-inactivated FBS on the sorted cells, two additional different FBS types were included in this troubleshooting experiment. The freshly produced Ad.Runx2 from now on was used as standard.

![Diagram of three conditions with serum for cell expansion and sorted cell populations: Runx2 GFP+, Runx2 GFP-, unsorted, reseeding 60,000 cells/cm², Ad.Runx2, 100 MOI, osteogenic induction, FACS, re-culture, monolayer expansion, osteogenic differentiation, and GFP positivity at sort.]

**Figure 4.10:** Experimental procedure in the context of the troubleshooting of the crosstalk experiments that takes the issue ‘serum used for cell culture’ into account. From the stage of thawing of the BMSCs on, the cells were split into three portions, and each cell fraction was exposed to any of the following three different FBS types: heat-inactivated FBS, referred to as ‘G-FBS’; hMSC-FBS, referred to as ‘M-FBS’, and Biochrom FBS Superior ‘B-FBS’. Use of two additional different FBS types and their effects on the sorted cells was considered as a replacement for the effects the change in the batch of heat-inactivated FBS had on the sorted cells. Thereby, uniform FBS was used for both the expansion of the cells prior to sorting and...
the post-sort expansion and in vitro osteogenic differentiation. For each of the three FBS types, in vitro osteogenic differentiation of expanded Runx2 GFP⁺, Runx2 GFP⁻, and unsorted cell populations was performed. ⁴⁵Ca incorporation rates were determined at day 21. Furthermore, the freshly produced Ad.Runx2 (section 4.2.3.2) was used for adenoviral transduction.

The in vitro osteogenic differentiation potential of the three cell populations (Runx2 GFP⁺, Runx2 GFP⁻, and unsorted) of each FBS condition was determined by means of ⁴⁵Ca incorporation at day 21 of osteogenic differentiation. The experiment was performed with one donor and was analyzed in quadruplicate samples. The following donor was used: donor Pat 53 (female, born 1992).

For each individual FBS type used, the results of ⁴⁵Ca incorporation are shown in Figure 4.11. Overall, in contrast to only basal ⁴⁵Ca incorporation values in presence of control medium, osteogenic medium led to increased ⁴⁵Ca incorporation values.

M-FBS led to highest ⁴⁵Ca incorporation rates in the presence of osteogenic medium in all three analyzed cell populations amongst the three different FBS tested. Furthermore, using M-FBS for the cell culture of the BMSCs and sorted cells, respectively, Runx2 GFP⁺ cell population showed the highest ⁴⁵Ca incorporation rates, followed by Runx2 GFP⁻ cell population which took an intermediate position, and unsorted cell population showed lowest ⁴⁵Ca incorporation rates. In this sub-experiment, the pattern of the osteogenic performance amongst the three different analyzed cell populations was as repeatedly found in previous FACS experiments to characterize the different cell populations. When G-FBS was used for the cell culture of the BMSCs and sorted cells, only weak ⁴⁵Ca incorporation rates were reported in osteogenic medium, which were comparable between the three analyzed cell populations. In the case of B-FBS, only weak ⁴⁵Ca incorporation rates were measured in osteogenic medium, which were lowest and comparable to the value in control medium in the case of Runx2 GFP⁺ cell population. In the case of Runx2 GFP⁻ and unsorted cell populations, ⁴⁵Ca incorporation rates in the presence of osteogenic medium were higher than the one of Runx2 GFP⁺ cell population. Nevertheless, the overall ⁴⁵Ca incorporation values of the cell populations in the G-FBS and B-FBS conditions, respectively were considered as low, when related to the values obtained of previous FACS experiments exceeding a value of 100,000 CPM which is generally considered as a meaningful ⁴⁵Ca incorporation rate in our FACS experiments.
The findings of this troubleshooting experiment which addressed the issue ‘serum used for cell culture’ revealed varying \(^{45}\text{Ca}\) incorporation intensities depending on the FBS used to uniformly supplement cell culture medium for both the expansion and osteogenic stimulation of BMSCs and sorted cell populations, respectively. Depending on which FBS was used, the pattern of osteogenic performance amongst the three cell populations was different, ranging from one case where the repeatedly observed pattern could be reproduced, one case where all cell populations had an even osteogenic performance, over to a case where the pattern was opposite to the one that could be repeatedly observed in previous characterization experiments.

**Figure 4.11:** Troubleshooting experiment considering the issue ‘serum used for cell culture’. For each of the three FBS types ‘G-FBS’, ‘M-FBS’, and ‘B-FBS’ investigated, osteogenic differentiation in vitro of expanded Runx2 GFP\(^{+}\), Runx2 GFP\(^{-}\), and unsorted cell populations was performed. \(^{45}\text{Ca}\) incorporation rates not related to the DNA amount at day 21 for each individual FBS type are shown.
4.2.3.4 Cell sorting at early and late time point

In the previous troubleshooting experiment, the FBS type used for the cell culture was reported to be an influencing factor that is able to lead to the observed changes in the pattern of osteogenic performance amongst the three different cell populations. One explanation for this observation might be that a particular FBS type differentially intensely favors the cellular performance and viability, whereby this can happen on the level of the total BMSC fraction prior to sorting and/or on the level of the different sorted cell populations, whereby in this case this can potentially happen at different extents amongst the different sorted cell populations.

Another possible explanation for the observation regarding the impact of the FBS type on the pattern of osteogenic performance amongst the different cell populations might be that the exposure of the cells to a particular FBS type rather changes the timing of the osteogenic stimulation, either beneficially or adversely by speeding up or slowing down the progression of osteogenic stimulation, respectively. To exemplify, amongst the three FBS types investigated in the troubleshooting experiment, the M-FBS is specifically tested for the ability to support the expansion and function of MSCs while maintaining them at a more primitive stage. In turn, the M-FBS was the one whose supplementation of the cell culture medium resulted in the best osteogenic performance in all three analyzed cell populations amongst the three different FBS tested. Hence, this example suggests a link between the stage of primitiveness or differentiation and the osteogenic performance of the expanded cell populations which were sorted at a standardized day independent of whether the time point was longer matched with the progression of osteogenic differentiation in response to the corresponding FBS type used to cultivate the cells.

Taking into account that day at which we perform cell sorting was standardized by setting a defined time point of sorting, this experimental setup does not incorporate changes in the timing of the progression of osteogenic stimulation, e.g. due to the choice of a particular FBS used for the cell culture. The possible consequences of a change in the progression of osteogenic stimulation on the composition and characteristics of the cell populations sorted on a standardized day which no longer represents the optimal time point are developed in the following, and leads us to the ‘early vs. late responder’ concept.

Early vs. late responder concept

The bone marrow contains, amongst others, osteoprogenitors. It has to be noted that there exist several subpopulations of osteoprogenitors, all finally reaching the similar endpoint.
to make and mineralize a bone matrix. Osteoprogenitors are characterized by their limited self-renewal capability and their extensive proliferation potential. At least some progenitor cell pools appear to undergo osteogenic differentiation as a default pathway in vitro, whereas others require a stimulus beyond the presence of serum. Therefore, the following two subsets of osteoprogenitors can be classified:

- **Immature (also referred to as uncommitted, primitive, inducible, glucocorticoid-requiring) osteoprogenitors**
  - They require a stimulus such as dexamethasone for osteogenic differentiation
  - They are early in the differentiation pathway from committed cells to osteoblast

- **Mature (also referred to as committed) osteoprogenitors**
  - These are progenitor cells restricted to the osteoblast development and bone formation under default differentiation in vitro
  - They are relatively late in the differentiation pathway from committed cells to osteoblast

Translated to the Runx2-responsive reporter expression system, this leads to the following circumstances:

First, there are the committed osteoprogenitors undergoing osteogenic differentiation in the absence of glucocorticoids which are relatively late in the differentiation. Independent of whether the cells are osteogenically induced or not, this osteoprogenitor population accounts for a particular Runx2-mediated reporter expression in a particular time frame. These progenitors are early responders, since they are already committed and do not need to have responded to dexamethasone to undergo osteogenic differentiation and to start expressing Runx2. In addition, a second class of osteoprogenitors is present in the BMSC population, which form bone nodules in culture only upon activation, such as the presence of dexamethasone or upon reaching high density. Any additional reporter expression in the presence of dexamethasone resulted from dexamethasone acting upon this second osteoprogenitor population which requires glucocorticoids to proliferate and/or differentiate along the osteogenic pathway. These progenitors are late responders since the onset of Runx2 expression depends on dexamethasone having affected the progenitor cells, which causes delayed reporter expression.

Due to inconsistent cell culture conditions (such as different FBS type used or differences in the number of PDs) to expand BMSCs (containing the osteoprogenitors), kinetics of the
osteoprogenitor populations can vary. Conditions that favor faster transition from a primitive to a more mature state of osteoprogenitors (such as extended expansion time or ‘lower quality’ FBS used to supplement the cell culture medium) shift the immature osteoprogenitors forward in their differentiation pathway towards osteoblast. Thus, osteoprogenitors require a shorter osteogenic stimulation period following the initial expansion phase until reaching a particular stage of induction. Or in contrast: Conditions that support the primitive state of immature progenitors (such as FBS type used to supplement the cell culture medium) require an extended osteogenic stimulation period following the initial expansion phase until a particular stage of induction is reached.

By means of Ad.Runx2, we aimed at identifying and isolating osteoprogenitors from osteogenically stimulated BMSCs. Knowing that there exist at least two subsets of osteoprogenitors in the bone marrow, the question of which subset of osteoprogenitors we isolated by means of the FACS experiment remained unaddressed. The current hypothesis was that the Runx2 GFP$^+$ cell population consisted of both uncommitted and committed osteoprogenitors, at least at the time when the cells were sorted. Furthermore, the GFP expression on day 3 of osteogenic stimulation, when the cells were sorted, was a combined GFP expression caused by both the uncommitted and committed osteoprogenitors (Figure 4.12A). Upon post-sort expansion of the sorted cell populations, the composition of the different cell population underwent changes due to enrichment of fast-growing progenitors and depletion of slow-growing progenitors. Hence, the more immature/primitive uncommitted osteoprogenitors are considered to more and more get the upper hand over the overall composition of the Runx2 GFP$^+$ cell population during the post-sort expansion period, and finally accounting for the major portion of the osteogenic performance as assessed by $^{45}$Ca incorporation. Instead, the committed osteoprogenitors which are more mature are believed to become senescent by the time the osteogenic differentiation assay is initiated. In sum, we hypothesize that the uncommitted osteoprogenitors are mainly responsible for the high in vitro osteogenic differentiation potential we repeatedly observed in the characterization experiments. The consequences of the insight that the uncommitted osteoprogenitors mainly account for the in vitro osteogenic differentiation potential with regard to a change in the kinetics in the osteoprogenitor populations due to unlike cell culture conditions are considerable. Under cell culture conditions that support the primitive state of immature progenitors leading to extended period of osteogenic stimulation required to achieve a particular stage
of induction, a standardized day of sorting we established to be at day 3 of osteogenic stimulation in the case of a seeding density of 60,000 cells/cm² becomes imprecise and tends to miss the uncommitted osteoprogenitors whose fluorescence shifts towards later time points of osteogenic stimulation.

Identification and characterization of uncommitted, dexamethasone-responsive osteoprogenitors requires separating them from the committed osteoprogenitors in the first place. A clear separation of uncommitted osteoprogenitors from committed osteoprogenitors can be approached by a longer period of osteogenic induction (which includes dexamethasone). Relating to the experimental procedure, osteogenic induction for 7 days instead of only 3 days leads to a clearer separation of the two classes of osteoprogenitors, whereby the inducible osteoprogenitors get more time to advance in the progression towards osteoblasts and can finally be clearly distinguished from the committed osteoprogenitors upon longer osteogenic induction (Figure 4.12B). However, to isolate the inducible osteoprogenitors, the day of sorting needs to be adjusted towards a later time point.
Figure 4.12: Idealized schematic representation of the course of GFP expression caused by uncommitted and committed osteoprogenitors present in BMSCs which were transduced with Ad.Runx2 at an MOI of 100 upon osteogenic stimulation. A) After 3 days of osteogenic stimulation, the overall GFP expression observed at this time point is potentially a combination of the GFP expression caused by both the uncommitted and committed osteoprogenitors. B) After 7 days of osteogenic stimulation, a more clear separation between the uncommitted and committed osteoprogenitors can be obtained, whereby the uncommitted osteoprogenitors require more time to advance in the progression of osteoblast differentiation and hereby can be clearly distinguished from the committed osteoprogenitors. Due to these temporally distinguishable responses of the two subsets of osteoprogenitors, the
committed osteoprogenitors are referred to as ‘early responders’ and the uncommitted osteoprogenitors as ‘late responders’.

In addition to the early vs. late responder concept developed above, the idea arose to reduce the cell seeding density for transduction with Ad.Runx2, which should serve the purpose of reducing the required number of cells to perform a particular FACS experiment, which in turn beneficially influences the issue of PDs.

Reduced cell seeding density – persistence of reporter expression

In order to reduce both the number of PDs the BMSCs undergo in the pre-sort expansion phase of the FACS experiments and the required number of cells to perform the FACS experiment at a particular cell culture flask extent, we aimed at reducing the cell seeding density for Ad.Runx2 transduction from 60,000 cells/cm\(^2\) to 15,000 cells/cm\(^2\). We already examined the persistence of reporter expression in a lower cell seeding density setup, i.e. 15,000 cells/cm\(^2\) in section 3.2.3. We have previously shown that GFP expression persists in BMSCs seeded at 15,000 cells/cm\(^2\) for up to 8 days post-transduction (Figure 3.8). Besides the persistence of reporter expression, changing the cell seeding density requires checking and redefining another parameter, namely the time point of sorting based on the peak of fluorescence.

Reduced cell seeding density – redefinition of time point of sorting based on fluorescence expression

To approach the redetermination of the optimal time point to perform cell sorting, BMSCs expanded in monolayer culture in the presence of bFGF were reseeded at 15,000 cells/cm\(^2\), transduced with Ad.Runx2 at an MOI of 100 (the optimal MOI is independent of the cell seeding density used; therefore, the optimal MOI does not need to be redetermined) the following day. The transduction was carried out by lanthofection for 4 hours. Thereafter, cell culture medium was switched from expansion medium to control (unstimulated) and osteogenic (stimulated) medium, respectively. The following three donors were used: first run: donor Pat 53 (female, born 1992); second run: donor Pat 55 (female, born 1994); third run: donor Pat 62 (female, born 1958).
Cells were cultured for up to 8 days post-transduction whereby the temporal levels of GFP expression in each condition (unstimulated as well as stimulated) were assessed every day by means of fluorescence microscopy. Thereby, different approaches were performed to quantify the extent of GFP expression.

In a first attempt, GFP expression level was assessed and quantified based on subjective impressions, whereby arbitrary fluorescence intensities were attributed to the different conditions and time points by comparative estimation amongst conditions, donors as well as time points.

GFP expression started to be observed from day 2 (Pat 62) and day 3 (Pat 53 and Pat 55) post-transduction on, respectively. Irrespective of the donor variation, GFP expression level steadily increased until reaching a peak between day 4 to 6 post-transduction, after which GFP expression level decreased and finally reached low expression levels.

Pat 53 showed a course of GFP expression level which started to become quite differently intense between the unstimulated and stimulated conditions from day 4 post-transduction on. GFP expression level from day 4 was higher in the stimulated condition than in the unstimulated condition. In the stimulated condition, a steady and marked increase in the GFP expression level from day 3 up to 5 post-transduction took place at which point the peak occurred. In contrast, in the unstimulated condition, an only slight increase in the GFP expression level from day 3 to 4 post-transduction changed over to the peak or rather a temporary plateau of GFP expression level on day 4 to 5 post-transduction.

In both unstimulated and stimulated conditions, from day 5 post-transduction on, GFP expression level decreased again down to low GFP expression levels.

Pat 55 showed a course of GFP expression level which started to become quite differently intense between the unstimulated and stimulated conditions from day 4 post-transduction on. In the stimulated condition, a steady and marked increase in the GFP expression level from day 3 up to 5 post-transduction took place at which point the course changed over to the peak or rather a temporary plateau of GFP expression level on day 5 to 6 post-transduction. From day 6 post-transduction on, GFP expression level decreased again down to low GFP expression levels. In contrast, in the unstimulated condition, an only slight increase in the GFP expression level from day 3 to 4 post-transduction changed over to the peak or rather a temporary plateau of GFP expression level on day 4 to 6 post-transduction. From day 6 post-transduction on, GFP expression level decreased again down to low GFP expression levels.
Pat 62 showed a course of GFP expression level which started to become differently intense between the unstimulated and stimulated conditions from the onset of observable GFP expression on day 2 post-transduction. In both unstimulated and stimulated conditions, GFP expression level steadily increased up to day 4 post-transduction at which point the peak occurred. After the peak was reached, GFP expression in both conditions steadily decreased throughout the rest of the time frame analyzed, whereby the GFP expression level at every time point after the peak was higher in the stimulated condition than in the unstimulated condition.

In sum, GFP expression started to be observed from day 2 (Pat 62) and day 3 (Pat 53 and Pat 55) post-transduction on, respectively. Irrespective of the donor variation, GFP expression level steadily increased until reaching a peak between day 4 to 6 post-transduction, after which GFP expression level decreased and finally reached low expression levels. Overall, stimulated condition resulted in higher GFP expression than unstimulated condition at every time point analyzed from day 4 to day 8 post-transduction. Further to this, also irrespective of the donor variation, the difference between the GFP expression levels of stimulated compared with unstimulated condition throughout most of the time frame analyzed was much more pronounced than in the case of a cell seeding density of 60,000 cells/cm², namely the stimulation led to higher GFP expression levels compared with the unstimulated condition. This general finding can be explained by the fact that in cell seeding density condition with the lower seeding density of 15,000 cells, the only present trigger for osteogenic stimulation are the osteogenic ingredients present in the osteogenic medium, which sets the stimulated condition more clearly apart from the unstimulated condition, as opposed to the high cell seeding density situation where both unstimulated and stimulated conditions have the high density as a natural trigger for osteogenic stimulation.

The subjective graphs of time course of GFP expression of all three donors analyzed are depicted in appendix 8.3 (Figure 8.4A, 8.5A, and 8.6A).

Secondly, analysis of the time course of GFP expression was performed by image analysis of the fluorescence microscopic images taken of the different conditions, donors as well as time points.

For each condition of a particular donor and time point, fluorescence microscopic images were taken in a non-random way of spots with differential degrees of GFP positivities to
record the whole range of GFP positivities observed in different fields of view with positive events present in a well of a 6-well plate.

The first level of image analysis was performed using representative images. Image analysis was performed as described in section 2.7.1, percentaged pixel area was determined using one image per condition each that was considered as representative image of the particular condition, that is an image that reflects an average green fluorescence within the range of positive green fluorescence expression levels.

Pat 53 showed comparable green percentaged pixel area in the unstimulated and stimulated conditions from the day 3 up to day 8 post-transduction. On day 4 and 5 post-transduction, stimulated condition showed higher green percentaged pixel area than unstimulated condition, whereby the peak occurred on day 4 post-transduction in the stimulated condition. After the peak on day 4 post-transduction was reached, green percentaged pixel area steadily decreased back to the level of unstimulated condition by day 6 post-transduction, and the green percentaged pixel area was comparable for unstimulated and stimulated conditions throughout the rest of the time frame analyzed.

Pat 55 showed comparable green percentaged pixel area in the unstimulated and stimulated conditions from the day 3 to day 4 post-transduction. On day 4 post-transduction, at which time point the peak occurred in the unstimulated condition, the unstimulated condition showed slightly higher green percentaged pixel area than stimulated condition. Instead, green percentaged pixel area further increased in the stimulated condition and changed over to the peak of green percentaged pixel area on day 5 post-transduction. After second peak on day 5 post-transduction was reached in the stimulated condition, green percentaged pixel area in the stimulated condition steadily decreased throughout the rest of the time frame analyzed, whereby the green percentaged pixel area on day 7 and 8 post-transduction was comparable again between unstimulated and stimulated conditions.

Pat 62 showed comparable green percentaged pixel area in the unstimulated and stimulated conditions from day 1 to 2 post-transduction. A steady increase in the green percentaged pixel area from day 1 post-transduction on changed over to the peak of green percentaged pixel area on day 3 post-transduction in the stimulated condition, whereas the green percentaged pixel area in the unstimulated condition exhibited a temporary minimum on day 4 post-transduction. From day 3 post-transduction on, green percentaged pixel area in the stimulated condition steadily decreased throughout the rest of the time frame analyzed, whereby the green percentaged pixel area in the stimulated condition was
comparable again with the unstimulated condition from day 5 post-transduction on throughout the rest of the time frame analyzed.

The graphs of time course of GFP expression based on image analysis of representative images of all three donors analyzed are depicted in appendix 8.3 (Figure 8.4B, 8.5B, and 8.6B).

The second level of image analysis was performed in an extended manner and included all images taken. Image analysis was performed as described in section 2.7.1, cumulative pixel area was determined for each image taken. Per condition, all images taken reflect the whole range of positive green fluorescence expression levels. Further data analysis of the cumulative pixel area of individual images encompassed the steps mentioned in the following. First, since different numbers of images were taken of the different conditions, donors as well as time points, the maximal number of images amongst all conditions, donors as well as time points was determined to standardize the image analysis. The maximal image number was 11; if fewer images were taken it was because no more green cells could be found in a particular condition, donor and time point, respectively, the remaining images up to 11 were considered as black, that is a cumulative pixel area of 0. Based on the cumulative pixel area determined for each individual image including those images considered as black to standardize the maximal number of images taken between all conditions, the sum of the cumulative pixel area of individual images per condition of a particular donor and time point was determined. To calculate the green percentaged pixel area per condition of a particular donor and time point, the corresponding sum of the cumulative pixel area was divided by the maximal number of images (i.e. 11) and further divided by the total 2040 x 1536 pixel image area.

Pat 53 showed slightly higher green percentaged pixel area in the unstimulated than in the stimulated condition on day 3 post-transduction. From day 4 until 8 post-transduction, stimulated condition showed higher green percentaged pixel area than unstimulated condition. On day 4 and 7 post-transduction, two peaks occurred each in the stimulated and unstimulated conditions. In the stimulated condition, the peak on day 4 post-transduction represented the major peak, while both peaks in the unstimulated condition reached same green percentaged pixel area.

Pat 55 showed same green percentaged pixel area in the unstimulated and stimulated conditions on day 3 post-transduction. From day 4 until 8 post-transduction, stimulated condition showed higher green percentaged pixel area than unstimulated condition. On
day 5 and 7 post-transduction, two peaks occurred in the stimulated condition, while in the unstimulated condition, the peak in green percentaged pixel area occurred on day 5 post-transduction, and steadily decreased from day 5 post-transduction throughout the rest of the time frame analyzed.

Pat 62 showed same green percentaged pixel area in the unstimulated and stimulated conditions on day 2 post-transduction. From day 3 until 8 post-transduction, stimulated condition showed much higher green percentaged pixel area than unstimulated condition. From day 4 post-transduction, green percentaged pixel area in the stimulated condition stayed on a high level and did not show a distinct peak. In the unstimulated condition, green percentaged pixel area exhibited a temporary minimum on day 4 post-transduction.

The graphs of time course of GFP expression based on image analysis of all images representing the most accurate approach to quantify the extent of GFP expression of all three donors analyzed are depicted in the right panel of Figure 3.18. For the sake of completeness, these graphs (Figure 8.4C, 8.5C, and 8.6C) are also depicted together with the graphs of the other two approaches to quantify the extent of GFP expression of the corresponding donors in the appendix 8.3 (Figure 8.4 until 8.6).

The determination of the optimal time point to perform cell sorting as described above was also performed in a high cell seeding density setup, i.e. 60,000 cells/cm$^2$, as detailed in section 3.2.4. To ease comparison of the two different cell seeding density setups, the sets of graphs of both seeding density setups have been compiled and are shown in Figure 3.18.

To conclude, the time course of Ad.Runx2-mediated GFP expression level in BMSCs seeded at 15,000 cells/cm$^2$ revealed the peak level of GFP expression to be on day 3 to 7 post-transduction in the case of the three donors analyzed, depending on the approach used to quantify the extent of GFP expression. This time frame of the peak fluorescence could be determined and located to day 3 to 7 post-transduction by means of different methods of GFP expression analysis such as subjective comparative estimation of GFP expression levels as well as by image analysis of either representative or all images. The peak in the GFP expression level was taken as criterion to temporally localize the day of sorting for the upcoming FACS experiments to identify and isolate osteoprogenitors.

These findings show that the peak level of GFP expression in the case of a seeding density of 15,000 cells/cm$^2$ which could be located to day 3 to 7 post-transduction occurred later compared with a seeding density of 60,000 cells/cm$^2$, in which case the peak level of GFP expression occurred on day 3 to 4 post-transduction.
To adequately deal with the inherent donor variation observed more pronouncedly in chapter 3, it was decided to standardize the day of sorting by setting a defined time point of sorting, which is day 7 post-transduction in the case of a seeding density of 15,000 cells/cm².

**Early vs. late responder experiment**

To experimentally investigate the early vs. late responder concept mentioned above, we performed an experiment whereby Runx2 GFP⁺, Runx2 GFP⁻, and unsorted cell populations were identified and sorted on day 3 as well as day 7 post-transduction from osteogenically induced BMSCs seeded at a seeding density of 15,000 cells/cm² which had been transduced with Ad.Runx2 at 100 MOI the following day.

To investigate the *in vitro* osteogenic differentiation potential of Runx2 GFP⁺, Runx2 GFP⁻, and unsorted cells, each sorted on day 3 and day 7 post-transduction, the six different expanded sorted cell populations were subjected to *in vitro* osteogenic differentiation whereby ⁴⁵Ca incorporation rates were determined at day 21 of osteogenic differentiation. The experiment was performed once with one donor. The experiment was subdivided into two subexperiments differing in the FBS type used. From the stage of thawing of the BMSCs on, the cells were split into two portions, and each cell fraction was exposed to either ‘M-FBS’ or ‘B-FBS’ containing cell culture medium for the whole period of the experiment. The experiment was analyzed in quadruplicate samples. The following donor was used: donor Pat 53 (female, born 1992).

For each sorting day and each of the FBS type used, the results of ⁴⁵Ca incorporation, related to the DNA amount, are shown in Figure 4.13.
Figure 4.13: Troubleshooting experiment addressing the ‘early vs. late concept’ along with the ‘serum used for cell culture’ and reduced cell seeding density (15,000 cells/cm²). For each of the two FBS types ‘M-FBS’ and ‘B-FBS’ investigated, osteogenic differentiation in vitro of expanded Runx2 GFP⁺, Runx2 GFP⁻, and unsorted cell populations, each sorted on day 3 and 7 post-transduction, was performed. ⁴⁵Ca incorporation, related to the DNA amount, at day 21 is shown.

The ⁴⁵Ca incorporation rates of the expanded different cell populations which had been sorted on day 7 post-transduction showed a pattern which was comparable to the one observed in the troubleshooting experiment which addressed the issue ‘serum used for cell culture’ (Figure 4.11), with the only difference being that Runx2 GFP⁺ cell population showed high ⁴⁵Ca incorporation in the presence of control medium. Thereby, M-FBS turned out to be most beneficial with regard to ⁴⁵Ca incorporation rates and led to the highest ⁴⁵Ca incorporation rates in the presence of osteogenic medium in all three analyzed cell populations, while B-FBS only led to weak ⁴⁵Ca incorporation in the presence of osteogenic medium. This could be reproduced in the present experiment in the cell populations sorted on day 3 post-transduction. However, in the M-FBS condition on day 3 post-transduction, the unsorted cell population behaved differently. First, the ⁴⁵Ca incorporation rate in
osteogenic medium was higher than the one from Runx2 GFP cell population. Furthermore, control medium led to even higher $^{45}$Ca incorporation than osteogenic medium, although this difference was not statistically significant. As regards the $^{45}$Ca incorporation rates of the expanded different cell populations which had been sorted on day 7 post-transduction, the B-FBS condition showed a pattern which was comparable to the one observed in the cell populations sorted on day 3 post-transduction in the B-FBS condition. Instead, the cell populations sorted on day 7 post-transduction in the M-FBS condition behaved differently to the corresponding cell populations sorted on day 3 post-transduction. While Runx2 GFP cell populations were comparable amongst sorting on day 3 and 7, the $^{45}$Ca incorporation rates of Runx2 GFP$^+$ and unsorted cell populations in the presence of osteogenic medium were half as high as the corresponding ones from the cell populations sorted on day 3 post-transduction. However, while the $^{45}$Ca incorporation rate of the unsorted cell population in the presence of control medium was basal as opposed to the comparable cell population sorted on day 3 post-transduction, the $^{45}$Ca incorporation rate of the Runx2 GFP$^+$ cell population in the presence of control medium was as high as the one in osteogenic medium. In conclusion, while the B-FBS condition led to consistently low osteogenic performance, cell culture medium supplemented with M-FBS led to higher osteogenic performance, which was more marked in the expanded cell populations sorted on day 3 post-transduction indeed, but did not reach a value of 100,000 CPM/µg DNA obtained in previous FACS experiments which is generally considered as a meaningful $^{45}$Ca incorporation rate in our FACS experiments. Furthermore, like previously mentioned, the pattern of $^{45}$Ca incorporation rates of the expanded different cell populations which had been seeded at 15,000 cells/cm$^2$ and sorted on day 7 post-transduction (Figure 4.13, lower panel) resembled the pattern in the troubleshooting experiment which addressed the issue ‘serum used for cell culture’, i.e. cells seeded at 60,000 cells/cm$^2$ and sorted on day 3 post-transduction (Figure 4.11). This observation raises the following question: as high density is an osteogenic stimulus, could the cells seeded at 15,000 cells/cm$^2$ have proliferated more over the 7 days of culture and then behaved more like the cells seeded at 60,000 cells/cm$^2$ after 3 days of culture? It might be the case that the 4 more days of proliferation makes the cells behave more like the cells in the high seeding density setup.
4.2.3.5 Influencing factor ‘viral titer determination method’

Another putative influencing factor and causer of the ambivalent crosstalk experiment results was considered the increasingly discrepant results in the viral titers obtained by any of the two methods to determine the viral titer.

The amplification of a new batch of adenovirus is completed by the determination of viral titer required for consistency amongst downstream cell culture experiments in which the adenovirus is applied in a clearly defined amount. The viral particle concentration was estimated according to optical density (OD$_{260}$) as a physical assay, while the infectious viral titer was determined by means of plaque assay as a biological assay (Mittereder et al., 1996). In general, the infectious viral titer obtained by plaque assay was considered as more accurate and therefore its results served as basis for the MOI calculations.

The viral titer as determined by OD$_{260}$ measurement along with the infectious viral titer as assessed by plaque assay of all the Ad.Runx2 batches which were successively produced in the course of the thesis are summarized in Table 3.1. As highlighted in section 3.2.1 in the context of the construction of Ad.Runx2, plaque assay resulting in the pfu-based viral titer to substantiate the OD$_{260}$-based viral titer determination led to consistently lower viral titers than the OD$_{260}$ measurement, with differences ranging from a factor 1.7 to 18.3. Since the OD$_{260}$-based viral titer determined by a spectrophotometer represents a more objectively determined viral titer compared with the pfu-based viral titer which is prone to mistakes by the operator, the discrepant differences in the two viral titers are an expression of the more or less accurately performed counting of the plaques in the context of the plaque assay. Considering that in fact the more error-prone pfu-based viral titer served as basis for the MOI calculations, large variation in the actual MOI applied to different FACS experiments is considered to have arisen. In essence, this raises the potential that one or more of the later FACS experiments, where the discrepancy was higher (Table 3.1, batches no. 4, 5, and 6) and its results might have been impaired by too large amounts of Ad.Runx2 applied to the cells, which in turn is considered to affect the cell populations' behavior in a negative manner. The discrepancy between the OD$_{260}$-based and the pfu-based viral titer determination increased in the course of the different Ad.Runx2 batches produced, which indicates that the repeatedly observed in vitro osteogenic performance pattern of the characterization experiments performed with earlier Ad.Runx2 batches were obtained owing to accurate determination of the pfu-based viral titer.
To experimentally investigate the discrepancy between OD\textsubscript{260} -based and pfu-based viral titer determination and its consequences on the osteogenic performance of expanded sorted cell populations, an experiment was performed whereby Runx2 GFP\textsuperscript{+}, Runx2 GFP\textsuperscript{-}, and unsorted cell populations were identified and sorted on day 3 as well as day 7 post-transduction from osteogenically induced BMSCs seeded at a seeding density of 15,000 cells/cm\textsuperscript{2} which had been transduced with Ad.Runx2 at 100 MOI the following day. Thereby, the MOI was calculated either due to OD\textsubscript{260} -based or pfu-based viral titer, respectively. According to the Ad.Runx2 batch which was in use at that time, there was a difference of a factor 3.2 between OD\textsubscript{260} -based and pfu-based viral titer.

To investigate the \textit{in vitro} osteogenic differentiation potential of Runx2 GFP\textsuperscript{+}, Runx2 GFP\textsuperscript{-}, and unsorted cells, each sorted on day 3 and day 7 post-transduction, the six different expanded sorted cell populations were subjected to \textit{in vitro} osteogenic differentiation whereby \textsuperscript{45}Ca incorporation rates were determined at day 21 of osteogenic differentiation. The experiment was subdivided into two subexperiments differing in whether the OD\textsubscript{260} -based or the pfu-based viral titer was used to calculate the MOI for adenoviral transduction. From the stage of thawing of the BMSCs on, the cells were uniformly cultured in ‘M-FBS’ containing cell culture medium for the whole period of the experiment as ‘M-FBS’ turned out to be the most beneficial FBS type amongst the three tested ones with regard to osteogenic performance of the different cell populations. The experiment was analyzed in quadruplicate samples using donor Pat 55 (female, born 1994).

For each of sorting day and each of the two viral titers used to calculate the MOI, the results of \textsuperscript{45}Ca incorporation, related to the DNA amount, are shown in Figure 4.14.
Figure 4.14: Troubleshooting experiment addressing the 'viral titer determination method' along with the reduced cell seeding density (15,000 cells/cm²). For each of the two viral titers used (OD₂₆₀-based and pfu-based, respectively (difference: factor 3.2)) to calculate the MOI, osteogenic differentiation in vitro of expanded Runx2 GFP⁺, Runx2 GFP⁻, and unsorted cell populations, each sorted on day 3 and 7 post-transduction, was performed. ⁴⁵Ca incorporation, related to the DNA amount, at day 21 is shown.

Overall, the ⁴⁵Ca incorporation rates were very high and the threshold of 100,000 CPM/µg DNA considered as meaningful ⁴⁵Ca incorporation rate was exceeded in nearly all conditions of nearly all different cell populations in the presence of osteogenic medium, while control medium resulted in only basal ⁴⁵Ca incorporation rates. Both day 3 and 7 post-transduction sorted cell populations of the OD₂₆₀-based viral titer condition and the day 3 post-transduction sorted cell populations of the pfu-based viral titer condition showed a comparable osteogenic performance pattern, whereby Runx2 GFP⁺ cell population was inferior to Runx2 GFP⁻ and unsorted cell populations in the presence of osteogenic medium. In the day 7 post-transduction condition of the pfu-based viral titer condition, ⁴⁵Ca incorporation rates of the three different cell populations were similar and did not exceed the threshold of 100,000 CPM/µg DNA considered as meaningful ⁴⁵Ca
incorporation. These findings indicate that depending on whether the cell populations were sorted on day 3 or 7 post-transduction, adenoviral transduction at MOIs of 100 and 320 resulted in a change in both the absolute $^{45}$Ca incorporation rates and the $^{45}$Ca incorporation pattern amongst Runx2 GFP$^+$, Runx2 GFP$^-$, and unsorted cell populations. In the cell populations sorted on day 3 post-transduction, the osteogenic performance was comparable irrespective of whether OD$_{260}$-based or pfu-based viral titer was used to calculate the MOI of 100 for Ad.Runx2 transduction. However, using pfu-based viral titer to calculate the MOI resulted in a reduction in the $^{45}$Ca incorporation rates of all three cell populations sorted on day 7 post-transduction. In conclusion, adenoviral transduction at the two different MOIs of 100 and 320 does not lead to impairment of the osteogenic performance of the sorted cell populations when sorted at day 3 post-transduction, but an effect is noticeable when cells are exposed to the larger amount of virus for an extended period of time such as 7 days.

The different expanded sorted cell populations from day 7 post-transduction condition of the pfu-based viral titer condition were further characterized by means of CFU-ALP assay to quantify the number of ALP$^+$ CFUs representing osteoprogenitor cells (Nishida et al., 1999). The three different cell populations were plated into 100 mm tissue culture plates in BMSC expansion medium in triplicate at a seeding density of 100 cells/plate. BMSC expansion medium was changed twice a week. After 7 days of culture, medium was switched to osteogenic medium, and this medium was kept for up to 14 days while changing the medium twice a week. At different time points (day 7, 11, and 14) of osteogenic stimulation, cultures were subjected to ALP staining to assess number of CFU-ALP. To assess the number of CFU-ALP, colonies stained blue were counted. The experiment was analyzed in triplicate samples.

The highest number of ALP$^+$ CFUs at all three time points analyzed were observed in Runx2 GFP$^+$ cell population, Runx2 GFP$^-$ cell population exhibited lower number of ALP$^+$ CFUs, and unsorted cell population had lowest number of ALP$^+$ CFUs (Table 4.3). These results suggest that Runx2 GFP$^+$ cell population exhibits the highest number of osteoprogenitors. Furthermore, these tendencies in the CFU-ALP behavior corresponded to the pattern of osteogenic performance amongst three cell populations of day 7 post-transduction condition of the pfu-based viral titer condition, as assessed by $^{45}$Ca incorporation, thereby substantiating each assay’s results (Figure 4.14).
4.2.4 Derivation of new hypothesis – isolation of osteoprogenitors based on the ratio of Runx2/Sox9 instead of mere Runx2

As detailed in section 3.1, in human systems, there exists a discrepancy between Runx2 mRNA and protein level (Shui et al., 2003). Osteoblast differentiation in human BMSCs has been demonstrated to be primarily associated with increases in RUNX2 protein activity, and not with increased Runx2 mRNA or protein levels. These findings render even Runx2 mRNA expression levels useless as criterion for identification of osteoprogenitors. This fact constitutes the central basis which motivated the generation of the adenoviral reporter responsive to functionally active human RUNX2 protein.

At the same while the troubleshooting experiments from section 4.2.3 were running, the issue of the Runx2/Sox9 ratio in the context of gene expression analysis was raised. That is, osteogenic differentiation of BMSCs was found to be reflected in a combined change of Runx2 and Sox9 mRNA levels. While changes in Runx2 mRNA levels did not serve as robust indicator for osteogenic differentiation, the ratio of Runx2 to Sox9 however consistently correlated with whether cells were cultured in osteogenic medium, or control medium instead.

These findings were obtained due to experiments which addressed the timing of stimulation mentioned in the following.

<table>
<thead>
<tr>
<th></th>
<th>Runx2 GFP+</th>
<th>Runx2 GFP-</th>
<th>unsorted</th>
</tr>
</thead>
<tbody>
<tr>
<td>d7</td>
<td>12</td>
<td>8</td>
<td>6</td>
</tr>
<tr>
<td>d11</td>
<td>13</td>
<td>10</td>
<td>7</td>
</tr>
<tr>
<td>d14</td>
<td>14</td>
<td>14</td>
<td>11</td>
</tr>
</tbody>
</table>

**Table 4.3:** Number of CFU-ALP of expanded Runx2 GFP+, Runx2 GFP-, and unsorted cell populations sorted on day 7 post-transduction from BMSCs which had been seeded at reduced cell seeding density (15,000 cells/cm²) and transduced with Ad.Runx2 at 100 MOI, as calculated by pfu-based viral titer. Each cell population was plated at 100 cells per 100 mm tissue culture plate and cultured in presence of bFGF for 7 days. Thereafter, medium was switch to osteogenic medium and cells were further cultured for up to 14 days. ALP staining at day 7, 11, and 14 identified the CFU-ALP. Average number of ALP+ CFUs of triplicate samples are presented.
4.2.4.1 Gene expression timing of *in vitro* osteogenic stimulation

To investigate the gene expression timing of *in vitro* osteogenic stimulation of BMSCs, BMSCs expanded in monolayer culture in the presence of bFGF were reseeded at 15,000 and 60,000 cells/cm\(^2\), respectively. The following day, for each cell seeding density, cell culture medium was switched from expansion medium to control (unstimulated) and osteogenic (stimulated) medium, respectively. Cells were subjected to *in vitro* osteogenic differentiation (or not) for up to 8 days. At different time points (day 2, 4, 6, and 8) of osteogenic stimulation, cultures were subjected to RNA isolation, followed by gene expression analysis by means of real time PCR. Gene expression analysis was performed for the following genes: human Runx2, human Runx2 osteo, human Sox9, while Rpl13a served as housekeeping gene (Figure 4.15). Untreated cells at day 0 were chosen as calibrator sample. The experiment was performed once with one donor. From the stage of thawing of the BMSCs on, the cells were uniformly cultured in ‘M-FBS’ containing cell culture medium for the whole period of the experiment. The experiment was analyzed in duplicate samples using donor Pat 51 (female, born 1963).

The Runx2 osteo and Runx2 genes showed the same general pattern in their expression profile. Irrespective of the seeding density condition, the time point analyzed and whether cells were unstimulated or stimulated, mRNA upregulation relative to day 0 was in the range of 1 to 2. As regards the gene Sox9, gene expression was considerably different between the unstimulated and stimulated condition in the 15,000 cells/cm\(^2\) seeding density condition, irrespective of the time point analyzed. Sox9 upregulation relative to day 0 was consistently higher in the unstimulated condition than in the stimulated condition. In the 60,000 cells/cm\(^2\) seeding density condition, Sox9 gene expression was higher in the unstimulated condition as well. However, the difference in Sox9 gene expression between unstimulated and stimulated condition, which at day 2 was similar to the 15,000 cells/cm\(^2\) condition, became less pronounced in the course of the experiment. The ratio of Runx2 mRNA to Sox9 mRNA was considerably larger in the stimulated condition, in both 15,000 and 60,000 cells/cm\(^2\) conditions, at all time points analyzed. On the one hand, these findings support the elsewhere reported insight that Runx2 mRNA expression levels do not correlate with the extent or progression of osteogenic differentiation. On the other hand, the results indicate that the Runx2/Sox9 ratio in terms of mRNA expression levels consistently differs depending on whether cells are stimulated in osteogenic medium or are unstimulated in control medium, and this change is very rapid. Thereby, a Runx2/Sox9
ratio above 3.5 is characteristic for stimulated condition, while a Runx2/Sox9 ratio below 3.5 is characteristic for unstimulated condition.
Figure 4.15: Gene expression analysis of BMSCs during in vitro osteogenic stimulation. BMSCs seeded at 15,000 and 60,000 cells/cm², respectively were subjected to osteogenic stimulation for up to 8 days. Unstimulated condition in control medium served as control. Gene expression analysis for the genes Runx2 osteo, Runx2, and Sox9 was performed. In addition, the ratio of Runx2 to Sox9 was calculated. Rph3a was used as housekeeping gene, and untreated cells at day 0 were chosen as calibrator sample.

4.3 Discussion

The present chapter describes a new method to identify and isolate osteoprogenitors from human BMSCs. The actual novelty consists of not approaching the isolation based on CD markers, but utilizing an adenoviral reporter system specific for the functionally active key osteogenic transcription factor RUNX2, which is linked to the cell phenotype of interest. The resulting Runx2 GFP⁺ cell population isolated by means of FACS due to the expression of the reporter gene EGFP, when cultured in osteogenic medium, is superior to Runx2 GFP⁻ subpopulation as well as the original cell population as regards in vitro osteogenic differentiation capability. This was evidenced using two different functional assays of osteogenic differentiation, namely ALP activity and radioactive ⁴⁵Ca incorporation to assess formation of mineralized matrix. Thereby, both assays which independently addressed different osteogenic properties, were in accordance with each other. In other words, a cell population with (1) similar number of CFUs, but more slow-growing CFUs compared to MSCs, (2) higher in vitro osteogenic differentiation capability than BMSCs is referred to as osteoprogenitors of the human bone marrow.

However, against the expected behaviors of the three different cell populations analyzed as highlighted in Figure 4.3, Runx2 GFP⁻ cell population does not behave contrariwise to Runx2 GFP⁺ cell population, but rather like the unsorted cell population itself. A possible explanation might be that withdrawal of Runx2 GFP⁺ cell population (which only constitutes a minor fraction of the unsorted cell population) from unsorted cell population, leading to Runx2 GFP⁻ cell population, only results in minor changes in the cellular behavior of the resulting cell population.

The unexpected tendencies of the three different analyzed cell populations in their osteogenic performance we observed suggested a crosstalk between the Runx2 GFP⁺ and Runx2 GFP⁻ cell populations. To approach the cell-cell interactions between the Runx2 GFP⁺
and Runx2 GFP+ cells, we performed experiments whereby besides the original three analyzed expanded sorted cell populations, three additional groups were created by mixing Runx2 GFP+ and Runx2 GFP− cells at different ratios, leading to ‘50%+ & 50%−’, ‘85%+ & 15%−’, and ‘95%+ & 5%−’ mixed cell populations. After a couple of experiments performed, we were faced with the fact that varying patterns of osteogenic performance of the different cell populations amongst the different experiments were observed even in experiments with the same donor. This was in conflict with the findings of previous characterization experiments with only three analyzed groups (Runx2 GFP+, Runx2 GFP−, and unsorted cell populations), where the experiments repeatedly showed the same pattern with Runx2 GFP+ as highly osteogenic cell population, or instead the experiment’s donor was a non-responder. Therefore, we were prompted to troubleshoot the findings of the crosstalk experiments and considered several influencing factors as candidates that ruined the crosstalk experiments.

First, we addressed the issue of PDs that we considered as the most obvious influencing factor owing to the fact that for the crosstalk experiments with additional groups, more cells were required, leading to a more extensive expansion of both the BMSCs at the beginning of the experiment as well as the sorted cells prior to seeding the cells for the in vitro osteogenic differentiation assays. However, based on the performed experiments, we were not able conclusively clarify the issue of PDs and their effect on a possible failure of the crosstalk experiments. Nevertheless, we went back to the experimental setup consisting of only the three originally analyzed cell populations Runx2 GFP+, Runx2 GFP−, and unsorted cell populations for the upcoming experiments, since overall, the PDs of the characterization experiments spanned a range whose extremes are lower than in the case of the crosstalk experiments. Next, a new batch of Ad.Runx2, which was sequenced and confirmed to exhibit the predicted nucleotide sequence, was freshly produced. Therefore, we could safely eliminate the influencing factor of a possible function-impairing mutation to, at least in future, ruin FACS experiments.

The troubleshooting experiment which addressed the issue ‘serum used for cell culture’ revealed varying 45Ca incorporation intensities depending on the FBS used for both the expansion and osteogenic stimulation of BMSCs and sorted cell populations. Depending on which FBS was used, the pattern of osteogenic performance amongst the three cell populations markedly differed. These findings indicate that there is a link
between the pattern of osteogenic performance observed amongst the three cell populations and the FBS used for the cell culture, and that depending on the FBS type or batch used, the osteogenic performance results of a particular FACS experiments do not represent the actual potential of the corresponding cell population.

Furthermore, to be precise, the cultivation of the cells in the presence of different FBS types brought along differences in the PDs. The PDs in the troubleshooting experiment which addressed the issue ‘serum used for cell culture’ were as indicated in Table 4.4.

<table>
<thead>
<tr>
<th>FBS Type</th>
<th>Number of Initial PDs (pre-sort expansion)</th>
<th>Number of Post-sort PDs</th>
<th>Number of Total PDs</th>
</tr>
</thead>
<tbody>
<tr>
<td>G-FBS</td>
<td>3.9</td>
<td>4.2</td>
<td>9.4</td>
</tr>
<tr>
<td>M-FBS</td>
<td>4.7</td>
<td>3.0</td>
<td>9.4</td>
</tr>
<tr>
<td>B-FBS</td>
<td>4.2</td>
<td>6.5</td>
<td>9.4</td>
</tr>
</tbody>
</table>

Table 4.4: Number of population doublings (PD) of the different initial BMSC populations and sorted cell populations, respectively of the troubleshooting experiment which addressed the issue ‘serum used for cell culture’. Number of initial PDs refers to the number of PDs the cells underwent from thawing the BMSCs until the end of the pre-sort expansion phase at which the cells were reseeded for the adenoviral transduction the next day. Number of post-sort PDs refers to the number of PDs the cells underwent from reculturing the cells after cell sorting until the end of the post-sort expansion phase after which the different cell populations were subjected to in vitro osteogenic differentiation assay. The number of total PDs refers to the sum of the number of initial PDs and the number of post-sort PDs.

The compilation of the PDs shown in Table 4.4 reveals that on the one hand, the number of initial PDs, along with the number of post-sort PDs of Runx2 GFP+ and unsorted cells amongst the three different FBS were comparable. Instead, the number of post-sort PDs of Runx2 GFP+ cells amongst the three different FBS showed distinctive differences. Cell culture in M-FBS resulted in much lower number of post-sort PDs than in the other two conditions. And this lowest number of post-sort PDs in the case of M-FBS came along with the best osteogenic performance of the Runx2 GFP+ cell population, which could be explained by the presence of the best cellular vitality due to lowest number of PDs they underwent after cell sorting. Further, this would indicate that the post-sort culture period is particularly critical as well as susceptible to stressors that might impair the cellular vitality of progenitor cell populations. Although the number of post-sort PDs of Runx2 GFP+ and unsorted cells amongst the three different FBS were comparable, the $^{45}$Ca incorporation rates were markedly different within a particular cell population amongst
the three different FBS used. This would suggest that the number of post-sort PDs is a less critical factor that determines the osteogenic performance, at least with regard to Runx2 GFP and unsorted cells.

In sum, the FBS used to cultivate the cells and/or the PDs the different cell populations underwent were revealed as parameters which might determine the osteogenic performance of the expanded sorted cell populations in the context of the troubleshooting experiment which addressed the issue ‘serum used for cell culture’.

Experiments were performed using BMSCs seeded at a lower density (15,000 cells/cm²) to determine the peak level of fluorescence upon a change in the cell seeding density from 60,000 to 15,000 cells/cm². The results revealed that the peak level of GFP expression in the case of a seeding density of 15,000 cells/cm² which could be located to day 3 to 6 post-transduction occurred later compared with a seeding density of 60,000 cells/cm², in which case the peak level of GFP expression occurred on day 3 to 4 post-transduction (section 3.2.4). This observation can be explained by the fact that high density represents an additional trigger for osteogenic stimulation besides the osteogenic ingredients present in the osteogenic medium. The stronger total osteogenic stimulus leads to a faster progression of both the osteogenic stimulation and also of the induction of Runx2 gene expression. This leads to a shift of the GFP fluorescence and its peak towards earlier time points.

A reduction of the seeding density from 60,000 to 15,000 cells/cm² was addressed mainly to reduce the total number of cells required for a particular FACS experiment, which in turn reduces the risk of expanding the BMSCs too heavily at the beginning of the FACS experiment. Persistence of the GFP reporter expression at a seeding density of 15,000 cells/cm² for up to 8 days post-transduction could be shown. To determine the optimal time point of sorting in the reduced seeding density condition, peak level of GFP expression was determined and could be located to day 3 to 6 post-transduction. In conclusion, upon the reduction in the seeding density, not only was the peak level of GFP expression shifted towards later time points, but also was the window broader in which the peak was located amongst three donors.

The ‘early vs. late responder’ concept, which proposes that an osteogenic stimulation period lasting longer than the originally defined 3 days leads to a clearer separation
between two subsets of osteoprogenitors, the uncommitted and committed osteoprogenitors, was investigated. While a mixture of both osteoprogenitor subpopulations as a whole is able to show high in vitro osteogenic performance according to this concept, a clearer separation of both osteoprogenitor subpopulations, which at the end serves to isolate the different osteoprogenitor subpopulations, can give insight into whether one of two osteoprogenitor subpopulations is mainly responsible for the high in vitro osteogenic performance observed in the characterization experiments. Whereas the $^{45}\text{Ca}$ incorporation rates of cell populations exposed to B-FBS containing cell culture medium were comparable irrespective of whether they had been sorted on day 3 or 7 post-transduction, M-FBS containing cell culture revealed differential osteogenic performance in cell populations sorted on day 3 vs. 7 post-transduction. In vitro osteogenic performance overall was more marked in the expanded cell populations sorted on day 3 post-transduction. In particular, Runx2 GFP$^+$ cell population showed double the $^{45}\text{Ca}$ incorporation rate compared with when sorted on day 7 post-transduction. Furthermore, the difference in $^{45}\text{Ca}$ incorporation rate in osteogenic medium compared with control medium was much higher in the Runx2 GFP$^+$ cell population sorted on day 3 post-transduction, whereas the $^{45}\text{Ca}$ incorporation rates amongst the two different media were comparable in the Runx2 GFP$^+$ cell populations sorted on day 7 post-transduction. This was against our expectations. By isolating Runx2 GFP$^+$ cell population on day 7 post-transduction as a late time point, we expected to isolate uncommitted osteoprogenitors which required a stimulus, such as dexamethasone, for osteogenic stimulation. Therefore, we expected a much more marked difference in the $^{45}\text{Ca}$ incorporation rates in response to osteogenic medium (which includes dexamethasone) compared with control medium when Runx2 GFP$^+$ cell population was sorted on day 7 post-transduction. However, this was not the case. One explanation might be the fact that in this experiment, we additionally addressed the reduced cell seeding density. Redefinition of the peak level of GFP expression upon reduction of the seeding density from 60,000 to 15,000 cells/cm$^2$ could locate the peak to day 3 to 6 post-transduction, which represents a shift towards a later time point compared with the one established for a seeding density of 60,000 cells/cm$^2$. In turn, the change in seeding density not only changes the peak level of GFP expression, but potentially also changes the kinetics of the two subsets of osteoprogenitors, which brings along a shift towards later in the time points referred to as early and late time points. A possible mismatch between the standardized time points and the donor specific peak
having changed in response to a reduction in the seeding density could lead to a change in the populations obtained.

The discrepancy between OD$_{260}$-based and pfu-based viral titer determination and its consequences on the osteogenic performance of expanded sorted cell populations was investigated.

In fact, there is no widely accepted method used for the determination of viral titers. There are several methods in use such as OD$_{260}$ measurement which is a physical method based on optical density, and plaque assay as a biological assay. The OD$_{260}$ measurement as a physical measurement is independent of biological functionality and stands out due to reproducibility, and for the determination of the physically present viral titer, it represents a more precise method. The plaque assay as a biological assay relies on the infection of AD-293 cells in culture followed by further events which depend on biological aspects of the adenovirus. Given the complexity of the plaque assay, correct assessment of viral titers is more difficult to achieve and misestimation of the functionality of the adenovirus is a known consequence (Mittereder et al., 1996).

The experiment revealed that depending on whether the cell populations were sorted on day 3 or 7 post-transduction, upon adenoviral transduction at MOIs of 100 and 320, a change in both the absolute $^{45}$Ca incorporation rates and the $^{45}$Ca incorporation pattern amongst Runx2 GFP$^+$, Runx2 GFP$^-$, and unsorted cell populations could be observed. In the cell populations sorted on day 3 post-transduction, the osteogenic performance was comparable irrespective of whether OD$_{260}$-based or pfu-based viral titer was used to calculate the MOI of 100 for Ad.Runx2 transduction. However, when the pfu-based viral titer was used to calculate the MOI, a reduction in the $^{45}$Ca incorporation rates of all three cell populations sorted on day 7 post-transduction was observed. In addition, the $^{45}$Ca incorporation pattern amongst the three different cell populations of day 7 post-transduction condition of the pfu-based viral titer condition was substantiated by CFU-ALP assay. Therefore, the adenoviral transduction at the two different MOIs of 100 and 320 does not lead to impairment of the osteogenic performance of the sorted cell populations when sorted at day 3 post-transduction, but a difference can be observed when cells are exposed to a larger titer of virus for an extended period of time such as 7 days. One explanation for the rather weak impact of a higher than planned MOI applied might be that the difference between the OD$_{260}$-based and pfu-based viral titer used in this experiment was 3.2 and thus rather small, compared with differences of a factor of 18.3 in...
Ad.Runx2 batch no. 4 (Table 3.1). Thus, the Ad.Runx2 batch with its rather low discrepancy between the OD$_{260}$-based and the pfu-based viral titer used in this particular experiment did not reproduce the most extreme condition that had been present in Ad.Runx2 batch no. 4 according to Table 3.1 with a discrepancy of 18.3 between the OD$_{260}$-based and the pfu-based viral titer. Therefore, it cannot be excluded that MOIs which were that far beyond 100 like it could potentially have been the case in Ad.Runx2 batches with a discrepancy of nearly 20 between OD$_{260}$-based and pfu-based viral titer did indeed lead to impairment of the final performance of the different cell populations. Thereby, the different cell populations could potentially be differentially intensively affected.

In sum, the characterization experiments reproducibly showed that Runx2 GFP$^+$ cell population displayed characteristics appropriate for osteoprogenitors. Experiments became inconsistent when using an extended assay extent to approach the crosstalk between Runx2 GFP$^+$ and Runx2 GFP$^-$ cell populations. This circumstance prompted us to perform a phase of thorough troubleshooting. Different influencing factors were addressed which were considered as candidates that affected the crosstalk experiments. Factors such as the number of PDs, the possibility of function-impairing mutation in the Ad.Runx2 batch used at the time, the serum used for cell culture, along with the viral titer determination method were investigated. In the context of these influencing factors being experimentally addressed, we also focused on the ‘early vs. late responder concept’ which arose based on findings we made during the troubleshooting phase.

Despite the many influencing factors that were addressed and the fact that even smaller experiments did not result in a return to the expected pattern, a pattern was emerging. The behavior of the cell population in respect to fluorescence intensity was always as expected; suggesting the reporter virus works, yet the later behavior of the isolated cells was functionally inconsistent. This raised questions regarding the original hypothesis which aimed at identifying and isolating osteoprogenitors by means of Ad.Runx2 using RUNX2 protein activity as a unique criterion that is sufficient to judge whether a particular cell is an osteoprogenitor or not.

At the same while the troubleshooting experiments were running, the issue of the Runx2/Sox9 ratio in the context of gene expression analysis was raised as a consequence of the findings made by our group, as well as by the findings of experiments addressing gene expression timing of in vitro osteogenic stimulation. Accordingly, osteogenic differentiation of BMSCs was found to be reflected in a combined change of Runx2 and
Sox9 mRNA levels. While changes in Runx2 mRNA levels did not serve as robust indicator for osteogenic differentiation, the ratio of Runx2 to Sox9 however consistently correlated with whether cells were cultured in osteogenic medium, or control medium instead. Since the Runx2/Sox9 ratio has turned out to be a reliable and consistent criterion and indicator for osteogenic differentiation, we believe that the original hypothesis needs to be adapted. While the original hypothesis claims that isolation of osteoprogenitors can be approached with sufficient specificity based on the presence of functionally active and phosphorylated RUNX2 protein, the novel hypothesis claims that isolation of osteoprogenitors is required to be based on the Runx2/Sox9 ratio, either on the mRNA or rather protein level (Figure 4.16A).

Once preliminary data will have been generated which support the newly derived hypothesis, one issue that needs clarification is whether the Runx2/Sox9 ratio is required to be assessed rather on the protein level than on the mRNA level. In this case, dual adenoviral reporters for Runx2 and Sox9 will be generated in the near future to answer this question. According to the novel hypothesis, mere selection based on Runx2 is not sufficient to identify osteoprogenitors. Instead, discrimination using the two different factors Runx2 and Sox9 is required. According to the novel hypothesis, osteogenically induced BMSCs are distinguished into the following four cell populations, differing in whether any of the two factors is expressed at low or high level (Figure 4.16B):

- \( \text{Runx2}^{\text{low}}/\text{Sox9}^{\text{high}} \) (population 1), representing the chondrogenic progenitors
- \( \text{Runx2}^{\text{high}}/\text{Sox9}^{\text{high}} \) (population 2), representing progenitors with an unknown phenotype
- \( \text{Runx2}^{\text{low}}/\text{Sox9}^{\text{low}} \) (population 3), representing uncommitted progenitors, or committed progenitors from other lineages
- \( \text{Runx2}^{\text{high}}/\text{Sox9}^{\text{low}} \) (population 4), representing the osteogenic progenitors
Figure 4.16: Adaptation of hypothesis for the identification and isolation of osteoprogenitors. A) Schematic illustration of the adaptation of the hypothesis. While the original hypothesis claims that isolation of osteoprogenitors can be approached with sufficient specificity based on the presence of functionally active and phosphorylated RUNX2 protein, the novel hypothesis claims that isolation of osteoprogenitors is required to be based on the Runx2/Sox9 ratio, either on the mRNA or protein level. B) Distinction of osteogenically...
induced BMSCs according to the novel hypothesis is based on the two different factors Runx2 and Sox9, which results in four cell populations differing in whether any of the two factors is expressed at low or high level.

Despite all the uncertainty about the original hypothesis the inconsistent results of the crosstalk experiments and the troubleshooting experiments detailed in the current chapter have evoked, it needs to be clearly stated that the functioning of Ad.Runx2 is unquestionable. Across all experiments performed, Ad.Runx2 has always behaved in the way it was expected, be it the responsiveness of Ad.Runx2 to RUNX2 protein detailed in chapter 3, but also the time course of GFP expression level mediated by Ad.Runx2 transduction of BMSCs detailed in the current chapter. In contrast, it is the functioning of the sorted cell populations which has become inconsistent. It is this insight that prompted us to question the original hypothesis. From the perspective of the novel hypothesis, mere selection of Runx2 GFP$^+$ cells only results in a more or less intensely mixed cell population, whereof the true osteoprogenitors are only a fraction. This circumstance then explains the inconsistent results in the crosstalk experiments and troubleshooting experiments.

Cells are inherently complex. The expression of a given gene is controlled by several transcription factors, which in turn are regulated by multiple signal transduction pathways. Therefore, the data obtained from a single reporter may be insufficient for achieving reliable results. Likewise, Dennis and colleagues reported that although candidate master transcription factors for osteogenesis, chondrogenesis, as well as adipogenesis have been reported, no individual master gene for a particular mesenchymal lineage has been identified that can alone account for the differentiation into the corresponding lineage (Dennis and Charbord, 2002). Rather, there are several activators and repressors that together lead to the differentiation along a particular pathway and induce the expression of cell type-specific marker proteins (Dennis and Charbord, 2002). For example, PPARγ2 which is a repressor of osteogenesis needs to be low for osteogenesis to proceed, besides that RUNX2 needs to be present since it acts as an activator of osteogenesis (Dennis and Charbord, 2002). This substantiates that single reporter approaches which focus on only one factor are not sufficient to identify MSC progenitors.

Another illustration concerns collagen type II which is a chondrogenic marker. Using a collagen type II reporter, the assumption would be that it would identify a chondrogenic phenotype. If collagen type I was also active, it would be fibrocartilage, which would behave differently to cells where the collagen type II was active, but the collagen type I was
inactive. If collagen type X was active and the collagen type II was active, it would be hypertrophic chondrocytes, a third phenotype whereby the collagen type II expression alone may be similar for all three phenotypes.

In sum, reporter systems relying on detection of a single transcription factor can be strongly influenced by many different factors, and each of these interact to produce a large number of variables. Combinatorial reporters may be required to more accurately identify a particular phenotype.
5 Runx2-responsive lentiviral reporters – construction, in vitro validation and usage for drug testing

5.1 Introduction

Osteoporosis is a progressive skeletal disorder which is characterized by a decrease in bone mass and density, leading to an increased risk of bone fracture. In all different types of osteoporosis, the underlying mechanism is an imbalance between bone resorption by osteoclasts and bone formation by osteoblasts, whereby bone resorption exceeds bone formation (Rodan and Martin, 2000). Drugs that inhibit the formation or activity of osteoclasts are commonly used for the treatment of osteoporosis. Worth mentioning is the class of drugs referred to as bisphosphonates, which prevent the loss of bone by inhibition of osteoclasts. Instead, factors that promote bone formation are far less considered (Rodan and Martin, 2000). Therefore, application of drugs that enhance the osteogenic differentiation of osteogenic cells such as MSCs might potentially be beneficial in boosting bone formation in osteoporosis (Rodan and Martin, 2000). Such bone forming agents might comprise growth factors such as BMP-2, or hormones such as parathyroid hormone (PTH) (Kha et al., 2004).

There are a few known osteoinductive factors that can drive the lineage-specific differentiation of MSCs into fully differentiated mature osteoblasts. BMPs are the best known osteoinductive factors that induce osteoblastic differentiation and bone formation in vitro and in vivo. However, despite their potent osteoinductive properties, the use of BMPs is currently hindered by the large concentrations required to induce adequate bone formation (Kha et al., 2004). PTH has been reported to stimulate bone growth and slow down the rate of bone loss. Teriparatide is a human PTH analog which has been approved by the U.S. Food and Drug Administration (FDA) (for review see (Das and Crockett, 2013)). However, due to its unknown long-term effects and high costs, treatment is reserved for patients with severe osteoporosis.

Only a few other candidate sites of action have been reported so far as targets for bone formation. Sclerostin is expressed by osteocytes and represents a negative regulator of bone formation. Different humanized monoclonal antibodies against sclerostin are currently in clinical development, aiming at neutralizing the anti-anabolic effects on bone formation of sclerostin (for review see (Das and Crockett, 2013)). Furthermore, amongst drugs that inhibit bone resorption, denosumab prevents RANKL from interacting with
RANK and in this way inhibits both differentiation of osteoclasts and the function of mature osteoclasts (for review see (Das and Crockett, 2013)). Denosumab was approved by the FDA for the indication osteoporosis (for review see (Das and Crockett, 2013)). In sum, the lack of potential candidate molecules that could stimulate bone formation and induce the lineage-specific differentiation of precursor cells into osteoblasts, respectively makes the investigation of new candidates an interesting area of research.

In the previous chapter, we made use of an Ad.Runx2 adenoviral reporter to identify and isolate osteoprogenitors. This novel approach to identify and isolate osteoprogenitors stands out due to the fact that the reporter responds to an active, representative, lineage-relevant protein, and is not based on CD markers or mRNA. In addition to the fact of being a functional assay, the approach is also characterized by being a live cell approach. This property was only partially exploited in the previous chapter and harbors the potential for being utilized for additional purposes.

Generally speaking, a live cell approach enables less time-consuming experiments as opposed to destructive assays involving laborious sample taking and preparations steps, which in addition are based on sample taking time points which requires preceding establishment and optimization. Furthermore, live cell approaches enable high-throughput analysis and screening, which in the case of destructive assays would lead to an analysis no longer being within the bounds of possibility.

In the current chapter, we hypothesize that biomaterial- or drug-mediated differences in the protein activity levels of functional RUNX2 proteins between conditions can be linked to the osteoinductive capability of the corresponding biomaterial or drug. Hence, this approach might represent a potent approach for the identification of new bone forming drug candidates or biomaterials, and enables a high-throughput approach.

Based on this hypothesis, the Runx2-responsive reporter which senses differences in the levels of functional RUNX2 protein allows utilizing the reporter for different possible applications, which comprise:

- Testing of (bio)materials for their osteoinductive capability
- Comparison/screening of various osteogenic induction protocols
- Chemical library screening to identify new bone forming drug candidates
- Early time point donor screening of osteogenic performance
Besides the different possible applications that can be explored due to the novelty of the approach, the Runx2-responsive reporter system can also be used for rather basic osteoblast biology purposes. As RUNX2 controls osteoblast lineage commitment and differentiation, as well as expression of bone-related genes, characterization of mechanisms that modulate RUNX2 protein activity harbor the potential to identify biological factors critical for bone formation (Drissi et al., 2000).

With regard to the usage of the Runx2-responsive reporter system as a screening tool, the adenoviral Runx2-responsive GFP reporter involves some limitations. First, the adenoviral Runx2-responsive GFP reporter is a transient reporter. In principle, there is no reason that screening assays cannot be performed with transient reporters. However, the reality of logistics would make it difficult. For example, in a large screen, reproducibility across all of the plates and wells in a large screen, and across multiple screens is critical, and this is harder to achieve with the inherent variability of transient reporter systems. Adenoviral transduction would be required to be repeated for every screen, for every plate, and even for every well. In addition, before screening thousands of wells, one would want to have some assurance that the particular adenoviral transduction used was of reproducible high efficiency. Secondly, a GFP reporter brings about the constraint being low signal sensitivity. The transientness and lack of sensitivity as limitations motivate the idea to generate a stable lentiviral Runx2 luciferase reporter.

The content of this chapter follows three main milestones mentioned in the following. First, we designed and ordered a custom-made lentiviral-based luciferase reporter construct whose expression is activated by Runx2. Based on the lentiviral plasmid backbone construct, the lentivirus was produced. Secondly, this construct was planned to then be used to generate stable immortalized reporter hTERT-MSCs. Finally, from the above-mentioned applications, we focused on the aspect of bone forming drugs. The aim was to utilize a candidate drug approach to validate the stable immortalized reporter hTERT-MSCs.

Lentiviruses are enveloped, single stranded RNA viruses 80-100 nm in diameter and form a genus within the family of retroviruses. The most famous representative of lentiviruses is the human immunodeficiency virus (HIV).
Like all retroviruses, lentiviruses possess the three main genes of retroviruses *gag*, *pol* and *env* which encode for viral proteins. In addition, they have several additional genes referred to as accessory genes, such as *tat*, *tas*, *rev*, *vif*, *vpr*, *vpu*, *vpx*, and *nef*. These accessory genes encode for additional regulatory proteins (for review see (Tang et al., 1999)).

As regards the lentiviral infectious cycle, once the lentivirus has entered the target cell, in the cytoplasm, the viral single stranded RNA genome is reverse-transcribed into double stranded DNA by the viral reverse transcriptase (for review see (Stone et al., 2000)). After the viral genome has been actively imported into the nucleus, the viral integrase randomly and stably integrates the viral genome into the host genome (for review see (Stone et al., 2000)).

Concerning the features of lentiviruses for their application in gene transfer applications, advantages of lentiviral vector encompass their ability to transduce dividing and non-dividing cells, their stable gene expression, and their large insert capacity (up to 10 kb) (for review see (Walther and Stein, 2000)). One drawback of the usage of lentiviral vectors is the potential for insertional mutagenesis (for review see (Walther and Stein, 2000)).

The use of human telomerase reverse transcriptase (hTERT)-immortalized BMSCs for tissue engineering applications is summarized in the following (Kassem et al., 2004). From the currently present clinical data, it has become obvious that large numbers of *ex vivo* expanded primary BMSCs are required for bone tissue engineering applications (Kon et al., 2000). The efficiency of bone tissue engineering is further complicated given that human primary BMSCs exhibit limited life span and progressively lose their stem cell characteristics upon *in vitro* expansion (Banfi et al., 2000). Primary BMSCs have been reported to gradually lose their bone-forming capability upon *in vitro* expansion (Banfi et al., 2000).

In analogy to studies with fibroblasts and epithelial cells which demonstrated that ectopic expression of hTERT elongates their telomere lengths as well as extends their replicative life span (Bodnar et al., 1998), the idea arose that ectopic expression of hTERT might be able to extend the replicative life span of BMSCs *in vitro*. Ectopic expression of hTERT in human BMSCs has been reported to not only extend the replicative life span, but also to enhance their osteogenic differentiation potential *in vitro* and their bone-forming capability *in vivo* (Shi et al., 2002; Simonsen et al., 2002). However, the potential genomic instability of hTERT-overexpressing human BMSCs and the risk for cell transformation represent safety concerns (Kassem et al., 2004). In sum, genetically modified immortalized...
BMSCs represent a potential alternative cell source for tissue engineering applications, although safety concerns need to be solved prior to their introduction in clinical trials.

5.2 Results

5.2.1 Generation of recombinant Runx2-specific lentiviruses – Lv.1xRunx2 and Lv.12xRunx2

Two different recombinant lentiviruses were generated, starting from custom-made lentiviral Runx2-specific Metridia luciferase (MetLuc) reporter constructs. They are referred to as Lv.1xRunx2 and Lv.12xRunx2, as detailed in section 2.5. The properties of each recombinant lentivirus are as follows:

- Lv.1xRunx2: lentiviral reporter driving MetLuc expression controlled by the full rodent Runx2 promoter, i.e. the reporter reflects Runx2 promoter activity
- Lv.12xRunx2: lentiviral reporter driving MetLuc expression controlled by the 12x tandemly arranged 7 bp sequence, AACCACA, of the Runx2 binding site in the MMP13 promoter (Jimenez et al., 1999), followed by a 136 bp minimal promoter sequence from the 5'-flanking region of the human MMP13 gene reported to be essential for basic transcription (Tardif et al., 1997), i.e. the reporter reflects RUNX2 protein activity

MetLuc is secreted into the medium, allowing for non-destructive analysis at every medium change.

5.2.2 Introductory experiments with hTERT-MSCs – in vitro osteogenic differentiation

After we had successfully acquired an immortalized hTERT-MSC cell line (Dr. Dario Campana, St. Jude Children’s Research Hospital, Memphis, TN), we first demonstrated the in vitro osteogenic differentiation potential of the hTERT-MSCs. For this purpose, hTERT-MSCs were subjected to in vitro osteogenic differentiation as detailed in section 2.1.5 for up to 35 days. The experiments were analyzed in quadruplicate samples.

hTERT-MSCs readily differentiated into the osteogenic lineage, with the osteogenic phenotype confirmed by ALP staining on day 7, 14, 17, and 21, and Alizarin Red S staining on
day 28 and 35 of osteogenic differentiation (Figure 5.1). As shown in Figure 5.1A, under stimulation in osteogenic medium for up to 21 days, ALP staining could be revealed from day 14 as opposed to control medium where no ALP staining could be observed up to day 21 of osteogenic differentiation. Furthermore, calcification as assessed by Alizarin Red S staining was present on day 28 and even more intensely on day 35 in contrast to its absence in control medium on both mentioned time points (Figure 5.1B). These results clearly reveal that hTERT-MSCs express ALP and exhibit calcification upon osteogenic stimulation. These results are especially important for future testing of drugs and biomaterials which will be performed with the hTERT-MSCs stably transduced with Runx2-specific lentiviruses, as the results have defined reference time points at which the readout of the testing experiments could be performed.

Figure 5.1: Preliminary testing experiment of the immortalized hTERT-MSCs. hTERT-MSCs were subjected to in vitro osteogenic differentiation for up to 35 days. A) ALP staining on day 7, 14, 17, and 21, as well as B) Alizarin Red S staining on day 28 and 35 were performed to assess the osteogenic differentiation capability of the hTERT-MSCs.
5.2.3 Generation of stable Runx2 reporter hTERT-MSCs

Two different stably transduced Runx2 reporter hTERT-MSC cell lines were generated. They are referred to as Lv.1xRunx2-hTERT-MSCs and Lv.12xRunx2-hTERT-MSCs, as detailed in section 2.6.

To generate a fully transduced population of cells, it is important to determine the minimum amount of a selection antibiotic required to eliminate untransduced cells. Cell lines vary in the level of resistance to antibiotics, so the level of resistance to a particular cell line must be tested before attempting stable selection of the cells. A killing curve analysis will determine the minimum concentration of the antibiotic needed to kill non-transduced cells. The antibiotic concentration for selection will vary depending on the cell type and the growth rate. Once the effective concentration of antibiotic has been determined, transduced cells can be selected for resistance.

The effective selection concentration of Puromycin was determined to be 2 µg/ml according to killing curve analysis investigating 2 µg/ml, 6 µg/ml, and 10 µg/ml of Puromycin.

Based on these results, transduced cells were selected by culture in the presence of 2 µg/ml of Puromycin over a period of 12 days.

5.2.4 In vitro validation of stable Runx2 reporter hTERT-MSCs

After the generation of recombinant Runx2-specific lentiviruses and their utilization to stably transduce hTERT-MSCs, we sought to validate the stable reporter hTERT-MSCs in vitro. In vitro validation encompassed finding answers to the following questions:

- Does in vitro osteogenic differentiation, which leads to the expression of Runx2, cause MetLuc expression?
- At which extent does in vitro osteogenic differentiation cause MetLuc expression mediated by the rodent Runx2 promoter and the promoter consisting of tandemly arranged Runx2 binding sites and a minimal promoter, respectively?
- How large are the differences in the reporter expression in osteogenic medium compared with control medium?
- How large are the differences in the reporter expression in high seeding density and in low seeding density conditions?
For this purpose, Lv.1xRunx2-hTERT-MSCs and Lv.12xRunx2-hTERT-MSCs expanded in monolayer culture were reseeded at either 60,000 (high seeding density) or 15,000 (low seeding density) cells/cm² in 48-well plates. The next day, cell culture medium was switched from hTERT-MSC culture medium to hTERT-MSC control (unstimulated) and osteogenic (stimulated) medium, respectively. Cells were subjected to in vitro osteogenic differentiation (or not) for a period of 8 days, whereby assessment of MetLuc expression was performed on day 0 (to assess basal level of MetLuc expression), and then every second day from day 2 up to day 8 of osteogenic differentiation. High density and low density experiment each was performed once and was analyzed in triplicate samples each. MetLuc expression was determined by means of Renilla Luciferase Assay System, as detailed in section 2.7.2.2. Analysis of MetLuc expression was based on both ‘MetLuc expression per day’ as well as ‘cumulative MetLuc expression’.

In the high seeding density condition (Figure 5.2), irrespective of the time point investigated, measured counts per second (CPS) values were considerably higher in the case of Lv.1xRunx2-hTERT-MSCs compared to Lv.12xRunx2-hTERT-MSCs. In the Lv.1xRunx2-hTERT-MSCs, osteogenic medium resulted in higher CPS values per day on day 2 and 4 of osteogenic differentiation. On day 6 and 8 of osteogenic differentiation, the pattern changed and control medium resulted in higher CPS values per day. Cumulative CPS values revealed comparable MetLuc expression during the whole course of the experiment between control and osteogenic medium. In the Lv.12xRunx2-hTERT-MSCs, osteogenic medium resulted in higher CPS values per day on all time points analyzed from day 2 of osteogenic differentiation. Accordingly, cumulative MetLuc expression was higher in the presence of osteogenic medium at all time points analyzed. These results indicate that in high seeding density condition, MetLuc expression in Lv.1xRunx2-hTERT-MSCs controlled by the full rodent Runx2 promoter did not reveal differences between control and osteogenic medium, respectively. In contrast, MetLuc expression in Lv.12xRunx2-hTERT-MSCs reflecting RUNX2 protein activity in osteogenic medium was superior to control medium.
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**Figure 5.2:** Metridia luciferase (MetLuc) expression analysis of Lv.1xRunx2-hTERT-MSCs and Lv.12xRunx2-hTERT-MSCs subjected to in vitro osteogenic differentiation in high seeding density condition. Expanded cells were seeded at 60,000 cells/cm$^2$ (high seeding density), and cell culture medium was switched to either hTERT-MSC control (unstimulated) or osteogenic (stimulated) medium the next day. Cells were subjected to osteogenic differentiation (or not) for a period of 8 days. MetLuc expression was determined on day 0 (basal level), 2, 4, 6, and 8 of osteogenic differentiation. Both 'MetLuc expression per day' and 'cumulative MetLuc expression' were determined and are shown in a separate graph.

In the low seeding density condition (Figure 5.3), irrespective of the time point investigated, measured CPS values were considerably higher in the case of Lv.1xRunx2-hTERT-MSCs compared to Lv.12xRunx2-hTERT-MSCs. In the Lv.1xRunx2-hTERT-MSCs, osteogenic medium resulted in smaller CPS values per day on all time points analyzed from day 2 of osteogenic differentiation except for day 6. Cumulative CPS values revealed comparable MetLuc expression during the whole course of the experiment between control and osteogenic medium. In the Lv.12xRunx2-hTERT-MSCs, osteogenic medium resulted in smaller CPS values per day on all time points analyzed from day 2 of osteogenic differentiation except for day 6. Cumulative MetLuc expression was higher in the presence of control medium at
all time points analyzed. These results indicate that in low seeding density condition, MetLuc expression in Lv.1xRunx2-hTERT-MSC controlled by the full rodent Runx2 promoter did not reveal differences between control and osteogenic medium, respectively. In contrast, MetLuc expression in Lv.12xRunx2-hTERT-MSCs reflecting RUNX2 protein activity in control medium was superior to osteogenic medium.

**Figure 5.3:** Metridia luciferase (MetLuc) expression analysis of Lv.1xRunx2-hTERT-MSCs and Lv.12xRunx2-hTERT-MSCs subjected to in vitro osteogenic differentiation in low seeding density condition. Expanded cells were seeded at 15,000 cells/cm² (low seeding density), and cell culture medium was switched to either hTERT-MSC control (unstimulated) or osteogenic (stimulated) medium the next day. Cells were subjected to osteogenic differentiation (or not) for a period of 8 days. MetLuc expression was determined on day 0 (basal level), 2, 4, 6, and 8 of osteogenic differentiation. Both ‘MetLuc expression per day’ and ‘cumulative MetLuc expression’ were determined and are shown in a separate graph.
5.2.5 Candidate drug testing using stable Runx2 reporter hTERT-MSCs

In this section, we wanted to address the question whether the stable Runx2 reporter hTERT-MSCs can detect a drug-mediated increase in the Runx2 expression. Herewith, we wanted to perform preparatory work towards the final aim being chemical library screening for the identification of bone forming drug candidates. Therefore, it was desirable that the system is applicable in a 96-well plate format for screening approaches. The stable Runx2 reporter hTERT-MSCs were applied to test whether we could detect an increase in Runx2 expression mediated by the following drug candidate: resveratrol.

Resveratrol (3,4',5-trihydroxystilbene) is a naturally occurring phytoalexin produced in a number of plants such as grapes (Kupisiewicz et al., 2010). Resveratrol and its role in osteogenesis are detailed in the following.

As regards the bone metabolism, resveratrol is reported to have the potential to antagonize osteoclasts and to promote osteoblasts (Kupisiewicz et al., 2010). However, these effects on osteoclasts and osteoblasts can only be seen when resveratrol is used at concentrations between 50 and 100 µM, whereas lower concentrations seem to show no effect (Kupisiewicz et al., 2010).

The first osteotrophic effect of resveratrol was shown by Mizutani and colleagues who demonstrated that resveratrol directly stimulated cell proliferation and differentiation of osteoblasts (Mizutani et al., 1998). These effects could be blocked by tamoxifen, an anti-estrogen, so that they concluded that resveratrol acts via estrogen receptors. This assumption could be confirmed by experimental in vivo data showing that resveratrol prevented femoral bone loss in ovariectomized rats (Mizutani et al., 2000).

Further insight into the bone-protective effect of resveratrol came from a study that demonstrated that resveratrol stimulated BMP-2 production by osteoblasts through Src kinase-dependent estrogen receptor activation and elevated serum concentrations of BMP-2 in ovariectomized rats (Su et al., 2007). Thereby, the forkhead protein FOXA1 was shown to be required for the resveratrol-induced BMP-2 expression.

Besides the BMP-2 pathway, data of experiments with MSCs revealed a second pathway, involving Sirt1 (Backesjo et al., 2006): MSCs can differentiate into osteoblasts, adipocytes, chondrocytes, as well as cell types of other connective tissue. It has been suggested that a reciprocal relationship exists between differentiation into osteoblasts and adipocytes. PPARγ2 is a key regulator for adipogenic differentiation. Activation of Sirt1 has recently...
been shown to decrease adipogenic differentiation via inhibition of PPARγ2. Here, resveratrol comes into play: resveratrol is an activator of Sirt1. Correspondingly, it could be shown that treatment of the mesenchymal cell line C3H10T1/2 as well as of rat primary bone marrow stromal cells with resveratrol increased osteoblast development at the expense of adipogenic differentiation (Backesjo et al., 2006).

Insight into resveratrol-mediated promotion of osteogenesis via Sirt1 in human MSCs came from Tseng and colleagues who found that besides Sirt1, FOXO3A is involved in the mentioned mechanism (Tseng et al., 2011). In this way, resveratrol upregulated the expression of the Runx2 gene and hence promoted osteogenesis (Tseng et al., 2011).

Summarizing the biological effect of soluble resveratrol, it has been reported that resveratrol upregulates Runx2 gene expression (Tseng et al., 2011).

In the following, we wanted to make use of these properties of resveratrol and involve them in the further *in vitro* validation of the stable Runx2 reporter hTERT-MSCs, but also use this drug for a candidate drug testing approach with the stable Runx2 reporter hTERT-MSCs.

For this purpose, Lv.1xRunx2-hTERT-MSCs and Lv.12xRunx2-hTERT-MSCs expanded in monolayer culture were reseeded at either 60,000 (high seeding density) or 15,000 (low seeding density) cells/cm² in 96-well plates. The next day, cell culture medium was switched from hTERT-MSC culture medium to any of the following six different hTERT-MSC medium conditions:

- control medium
- osteogenic medium
- osteogenic medium + 1:100 96% EtOH (carrier control)
- osteogenic medium + 1 µM resveratrol
- osteogenic medium + 10 µM resveratrol
- osteogenic medium + 50 µM resveratrol

Cells were subjected to *in vitro* osteogenic differentiation (or not) for a period of 8 days, whereby assessment of MetLuc expression was performed on day 0 (to assess basal level of MetLuc expression), and then every second day from day 2 up to day 8 of osteogenic differentiation. The resveratrol study with both high and low seeding density condition each was performed once and was analyzed in triplicate samples each. MetLuc expression was determined by means of Renilla Luciferase Assay System, as detailed in section 2.7.2.2.
Analysis of MetLuc expression was based on both ‘MetLuc expression per day’ as well as ‘cumulative MetLuc expression’. The resulting values were related to the cell numbers as assessed by Cell Titer Blue assay in the identical cultures which was measured directly after the MetLuc expression measurement, finally resulting in ‘MetLuc expression/Cell Titer Blue per day’ as well as ‘cumulative MetLuc expression/Cell Titer Blue’.

Prior to performing the MetLuc expression analysis, Cell Titer Blue assay was analyzed in order to assess relative cell numbers and to reveal possible cytotoxic effects of any of the resveratrol concentrations. Resveratrol at the highest concentration tested (50 µM) markedly impaired cell viability (Figure 5.4). The cytotoxic effect of resveratrol was more prominent in the low seeding density condition. Furthermore, the second highest resveratrol concentration (10 µM) started to exert a cytotoxic effect in the low seeding density condition during the time frame analyzed, but became noticeable only from day 8 of osteogenic differentiation.

![Figure 5.4: Cell Titer Blue assay analysis of Lv.1xRunx2-hTERT-MSCs and Lv.12xRunx2-hTERT-MSCs subjected to the resveratrol study in either low or high seeding density condition. Expanded cells were seeded at either 15,000 cells/cm² (low seeding density) or 60,000](image-url)
cells/cm² (high seeding density). The next day, cell culture medium was switched to either hTERT-MSC control medium (unstimulated), osteogenic medium (stimulated), or any of four additional media containing either carrier only or resveratrol at either 1, 10, or 50 µM. Cells were subjected to osteogenic differentiation (or not) for a period of 8 days. Cell Titer Blue assay was performed on day 0 (basal level), 2, 4, 6, and 8 of osteogenic differentiation.

Due to the previous findings, the highest resveratrol concentration tested (50 µM) was excluded from the subsequent MetLuc expression analysis.

Analyzing the resveratrol study overall resulted in the following observations:
In the Lv.1xRunx2-hTERT-MSCs (RUNX2 mRNA synthesis) low seeding density condition (Figure 5.5, left panels), osteogenic medium + 10 µM resveratrol resulted in markedly higher MetLuc expression/Cell Titer Blue per day on day 4 of osteogenic differentiation, while due to the large standard deviations at day 2 and 6 of osteogenic differentiation, osteogenic medium + 10 µM resveratrol only hinted at higher MetLuc expression/Cell Titer Blue per day. The cumulative MetLuc expression/Cell Titer Blue was highest in osteogenic medium + 10 µM resveratrol for the whole course of the experiment.
In the Lv.1xRunx2-hTERT-MSCs high seeding density condition (Figure 5.5, right panels), no tendencies and differences, respectively across all different medium conditions could be identified. The cumulative MetLuc expression/Cell Titer Blue was comparable amongst all different medium conditions investigated.
**Low seeding density**  
**Lv.1xRunx2**  
**High seeding density**

**Figure 5.5:** Metridia luciferase (MetLuc) expression analysis of Lv.1xRunx2-hTERT-MSCs subjected to the resveratrol study in either low or high seeding density condition. Expanded cells were seeded at either 15,000 cells/cm² (low seeding density) or 60,000 cells/cm² (high seeding density). The next day, cell culture medium was switched to either hTERT-MSC control medium (unstimulated), osteogenic medium (stimulated), or any of three additional media containing either carrier only or resveratrol at either 1 or 10 µM. Cells were subjected to osteogenic differentiation (or not) for a period of 8 days. Both ‘MetLuc expression/Cell Titer Blue per day’ and ‘cumulative MetLuc expression/Cell Titer Blue’ were determined by involving normalization of the MetLuc expression with Cell Titer Blue assay data, and are shown in a separate graph.

In the Lv.12xRunx2-hTERT-MSCs (RUNX2 protein activity) low seeding density condition (Figure 5.6, left panels), control medium demonstrated markedly higher MetLuc expression/Cell Titer Blue per day on day 4 of osteogenic differentiation. The cumulative MetLuc expression/Cell Titer Blue was highest in control medium for the whole course of the experiment.

In the Lv.12xRunx2-hTERT-MSCs high seeding density condition (Figure 5.6, right panels), osteogenic medium + 10 µM resveratrol showed markedly lower MetLuc expression/Cell Titer Blue per day on day 2 and 4 of osteogenic differentiation than all the other medium
conditions analyzed. The cumulative MetLuc expression/Cell Titer Blue was comparable between control medium and osteogenic medium thereby exhibiting the highest cumulative MetLuc expression/Cell Titer Blue amongst all different medium conditions investigated.

As an additional finding from the cumulative MetLuc expression analysis, it arose that the patterns of cumulative MetLuc expression (control vs. osteogenic medium in both low vs. high seeding density condition) observed in the in vitro validation experiments of Lv.1xRunx2-hTERT-MSCs and Lv.12xRunx2-hTERT-MSCs from section 5.2.4 could be
reproduced in the present resveratrol study when MetLuc expression was not related to the Cell Titer Blue assay data (data not shown).

When the MetLuc expression was related to the Cell Titer Blue assay data as a measure of the cell number, in the case of Lv.1xRunx2-hTERT-MSCs low seeding density condition, osteogenic medium resulted in higher cumulative MetLuc expression/Cell Titer Blue than control medium (Figure 5.5, bottom left panel), while the pattern of cumulative MetLuc expression/Cell Titer Blue stayed comparable between control medium and osteogenic medium in the high seeding density condition (Figure 5.5, bottom right panel). In the case of Lv.12xRunx2-hTERT-MSCs low seeding density condition, control medium resulted in higher cumulative MetLuc expression/Cell Titer Blue than osteogenic medium (Figure 5.6, bottom left panel), while the pattern of cumulative MetLuc expression/Cell Titer Blue stayed comparable between control medium and osteogenic medium in the high seeding density condition (Figure 5.6, bottom right panel). These findings indicate that upon normalization of the cumulative MetLuc expression of control medium and osteogenic medium conditions with the Cell Titer Blue assay data as a measure of the cell number, relatively speaking, the trends changed towards better performance of osteogenic medium in the Lv.1xRunx2-hTERT-MSCs low seeding density condition and in the Lv.12xRunx2-hTERT-MSCs high seeding density condition, while the trends stayed the same in the remaining two conditions. One explanation that might partially explain the observed change in pattern upon normalization with the Cell Titer Blue assay data are the differential cell numbers arising in control medium compared with osteogenic medium. Upon normalization of the MetLuc expression with the Cell Titer Blue data, measured MetLuc expression (which is not normalized with the cell number) is no longer prone to artifacts caused by differential cell numbers. This artifact would be greater at low density where there is a greater potential for proliferation, and this is what we observed.

5.3 Discussion

The present chapter describes the generation of two different recombinant lentiviruses, one of which reflects Runx2 promoter activity, referred to as Lv.1xRunx2, while the other one reflects RUNX2 protein activity, referred to as Lv.12xRunx2. Human immortalized hTERT-MSCs stably transduced with either Lv.1xRunx2 or Lv.12xRunx2 were utilized to perform in vitro validation and candidate drug testing.
First, *in vitro* osteogenic differentiation experiments of hTERT-MSCs proved that the cells indeed undergo osteogenic differentiation in the presence of osteogenic medium. However, compared with primary human BMSCs, *in vitro* osteogenic differentiation progressed more slowly. Whereas in primary human BMSCs, ALP activity on day 7 and 14 was comparably high and started to decrease from day 14 till 21 of osteogenic differentiation, ALP staining assessed up to day 21 was most intense on day 21 of osteogenic differentiation.

*In vitro* validation of stable Runx2 reporter hTERT-MSCs was performed to assess whether Lv.1xRunx2-hTERT-MSCs and Lv.12xRunx2-hTERT-MSCs, respectively indeed responded to changes in the Runx2 expression with an increase in MetLuc expression. Runx2 expression was planned to be induced by means of *in vitro* osteogenic differentiation, whereas exposure to control medium was considered to lead to a weaker MetLuc response.

When comparing the measured MetLuc CPS values between Lv.1xRunx2-hTERT-MSCs and Lv.12xRunx2-hTERT-MSCs, overall, it arose that the Lv.1xRunx2-hTERT-MSCs showed massively higher CPS values compared with Lv.12xRunx2-hTERT-MSCs: a difference in a factor 24 to 66 in the high seeding density condition (Figure 5.2), and a difference in a factor 19 to 40 in the low seeding density condition (Figure 5.3). These differential scales can be explained by differences in the promoter properties of Lv.1xRunx2 and Lv.12xRunx2. The promoter of Lv.1xRunx2 is a naturally occurring full rodent Runx2 promoter containing binding sites for many more transcription factors, co-activators and other regulatory proteins, whereas the promoter of Lv.12xRunx2 is a short tailor-made promoter composed of only few regulatory elements, which leads to lower expression levels compared with the long naturally occurring full rodent Runx2 promoter. In brief, the promoter of Lv.1xRunx2 is more powerful due to the more cooperative binding sites for transcription factors and other regulatory proteins. Additionally, the high CPS values in Lv.1xRunx2-hTERT-MSCs clearly demonstrated that the rodent Runx2 promoter worked and was activated in a human cell model.

Further, the *in vitro* validation experiments of stable Runx2 reporter hTERT-MSCs revealed that in both high and low seeding densities, Lv.1xRunx2-hTERT-MSCs did not reveal any difference between control and osteogenic medium, indicating comparable rodent Runx2 promoter activity, irrespective of the seeding density condition (high vs. low) and the culture medium. This finding is in line with the fact that osteoblast differentiation in human BMSCs is primarily associated with increases in RUNX2 protein activity, and not
Runx2-responsive lentiviral reporters – construction, in vitro validation and usage for drug testing

with increased Runx2 mRNA or protein levels. Runx2 promoter activity which is a measure of the Runx2 mRNA level was expected to stay the same during the course of the validation experiment both in high and low seeding density conditions. As regards the Lv.12xRunx2-hTERT-MSCs which reflects RUNX2 protein activity, differential MetLuc expression was expected to be measured between control and osteogenic medium on the one hand side, and between high and low seeding density on the other hand side, analogous to the previous experiments using Ad.Runx2 adenoviral reporter. In practice, we expected higher overall basal reporter expression in high seeding density condition as opposed to low seeding density condition as high seeding density serves as a natural trigger for osteogenesis. A more distinct difference in reporter expression between control and osteogenic medium was expected in the low seeding density condition since the dexamethasone present in osteogenic medium while absent in control medium serving as the only osteogenic trigger has the potential to diverge the MetLuc expression between control and osteogenic medium. Against our expectations, the CPS values per day, at least from day 4 of osteogenic differentiation, were higher in the low seeding density condition compared with the high seeding density condition, irrespective of the cell culture medium used. This observation is even more noticeable given that the CPS values are not normalized with the cell number, although it has to be mentioned that by day 4 of osteogenic differentiation, the difference in the cell numbers between the high and low seeding density condition is considered to no longer be that considerable because the low seeding density will likely have caught up with the confluence of the high seeding density due to ongoing cell proliferation at subconfluent stages. Next, although osteogenic medium revealed higher MetLuc expression in the case of high seeding density condition on all time points analyzed from day 2 of osteogenic differentiation, osteogenic medium was inferior to control medium in Lv.12xRunx2-hTERT-MSCs in low seeding density condition not only on all time points analyzed from day 2 of osteogenic differentiation except for day 6, but also in the cumulative expression. One explanation of the unanticipated response to the two cell culture media investigated in the case of Lv.12xRunx2-hTERT-MSCs in low seeding density condition might the differential cell proliferation. Seeding cells at 15,000 cells/cm$^2$ (low seeding density) allows the cells to proliferate until confluence is reached. Control medium favors proliferation at higher extent than osteogenic medium which triggers the cells to differentiate rather than proliferate. This circumstance can lead to discrepant cell numbers present in a well depending on whether the cells are cultured in control or osteogenic medium. Even if
MetLuc expression per cell stays the same, higher cell number lead to higher CPS values measured (which are not normalized with the cell number). Since only the low seeding density condition which leads to subconfluence allows further proliferation of the cells, the argument of the cell numbers has a major strength in the low seeding density condition. In contrast, in the high seeding density condition, due to the confluence already achieved upon seeding the cells, there is no space for potential differential cell numbers to arise due to different cell culture media used, which then would cause differences in the MetLuc expression due to mere differences in the cell numbers. Accordingly, the superiority of control medium with regard to MetLuc expression was noticed only in the low seeding density condition, and further to this, it was more pronounced at earlier time points prior to confluence. This is in line with the fact that the cell numbers became more similar to each other upon reaching confluence, and thus the total MetLuc expression is more correlated to the MetLuc expression per cell, and is no longer prone to artifacts caused by differential cell numbers. This would indicate that a more reliable, online method to monitor cell number over time would need to be implemented.

The resveratrol study aimed at answering the question whether stable Runx2 reporter hTERT-MSCs can detect a drug-mediated increase in the Runx2 expression. Thereby, resveratrol served as candidate drug given the reported capability of resveratrol to upregulate Runx2 gene expression (Tseng et al., 2011). First, from the three concentrations of resveratrol investigated, the highest concentration (50 µM) had cytotoxic effects on stable Runx2 reporter hTERT-MSCs, as assessed by Cell Titer Blue assay. The cytotoxic effect of 50 µM resveratrol on hTERT-MSCs was especially marked in the low seeding density condition. This can be explained by the higher 'drug per cell’ exposure in the low seeding density condition, which was mainly the case at earlier time points of the assay when the low seeding density condition was at subconfluent stages.

As part of the experience obtained in the previous in vitro validation experiments, the MetLuc expression was normalized with the Cell Titer Blue data in the resveratrol study in order to determine the MetLuc expression related to the cell number. We assessed the MetLuc expression both as 'MetLuc expression/Cell Titer Blue per day' as well as 'cumulative MetLuc expression/Cell Titer Blue’. Overall, the 'MetLuc expression/Cell Titer Blue per day' analysis only resulted in selective marked differences of an individual condition of a particular time point, and was not strong enough to reveal overall
characteristic trends of an individual subexperiment. Only resveratrol at a concentration of 10 µM resulted in selective marked differences, which were as follows: in the Lv.1xRunx2-hTERT-MSCs low seeding density condition, resveratrol resulted in markedly higher MetLuc expression/Cell Titer Blue per day on day 4 of osteogenic differentiation, while in the Lv.12xRunx2-hTERT-MSCs high seeding density condition, resveratrol showed markedly lower MetLuc expression/Cell Titer Blue per day on day 2 and 4 of osteogenic differentiation. In all other conditions and at all other time points, resveratrol at most only hinted at markedly different MetLuc expression/Cell Titer Blue per day compared to the other medium conditions analyzed. Besides, the ‘cumulative MetLuc expression/Cell Titer Blue’ analysis only in one subexperiment resulted in an overall characteristic trend of resveratrol, namely in Lv.1xRunx2-hTERT-MSCs low seeding density condition where resveratrol at a concentration of 10 µM resulted in the highest cumulative MetLuc expression/Cell Titer Blue for the whole course of the experiment. In all other conditions and at all other time points, resveratrol did not result in any differences. Given that the resveratrol study was analyzed in a refined way which took the cell numbers into account and was not based on mere MetLuc expression resulting from a whole well, it would suggest that the stable Runx2 reporter hTERT-MSC system might not be sensitive enough to detect differences in the Runx2 gene expression mediated by a drug which has been reported to upregulate Runx2 gene expression. Or rather that the changes in Runx2 expression upon treatment with resveratrol are not marked enough to be recordable with the stable Runx2 reporter hTERT-MSCs.

Tseng and colleagues reported an upregulation of Runx2 and osteocalcin expression upon resveratrol treatment in human embryonic stem cell-derived mesenchymal progenitors (Tseng et al., 2011). The regulation of resveratrol on Runx2 was assessed by real time PCR. By means of real time PCR, they showed that after treatment with 5 µM resveratrol for 9 days, expression of Runx2 was upregulated 2.3 fold. By means of luciferase reporter assay, resveratrol dose-dependently (0-10 µM resveratrol) stimulated the promoter activity of the human Runx2 promoter-luciferase reporter plasmid (hRUNX2-luc) approximately three-fold, as assessed 48 hours post-transfection. However, it needs to be mentioned that they determined the effect of resveratrol by comparison of control (basal) medium with control medium + resveratrol. This is in contrast to our experiment where osteogenic medium + resveratrol was compared with either osteogenic medium or control medium. In other words, they assessed the effect of resveratrol in the presence of unstimulated medium background, while we investigated resveratrol in the presence of osteogenic ingredients.
However, this issue can be ruled out by another study comparing osteogenic medium and osteogenic medium + resveratrol with regard to its effect on osteogenic differentiation in human BMSCs (Dai et al., 2007). In a range of resveratrol concentrations that contained the resveratrol concentrations we investigated, they found that resveratrol stimulated osteoblastic differentiation as determined by real time PCR analysis for Runx2 expression, ALP activity, and calcium deposition into the extracellular matrix, compared with osteogenic medium. Resveratrol at a concentration of 1 µM increased Runx2 mRNA message 2.9 fold compared with osteogenic medium (Dai et al., 2007).

Based on the results of the two publications that addressed the effect of resveratrol on Runx2 expression, resveratrol treatment at concentrations we used led to marked changes in Runx2 expression, as assessed on the mRNA message level. In fact, these findings emphasize the assumption that the stable Runx2 reporter hTERT-MSC system might not be sensitive enough to detect differences in the Runx2 gene expression mediated by resveratrol at the concentrations analyzed. Alternatively, cumulative assessment might not be the optimal analysis for genes whose expression fluctuates over time.

Although insufficient sensitivity of the stable Runx2 reporter hTERT-MSC system might be a plausible explanation for the overall insignificant and only selective differences observed in the present resveratrol study, another issue should not be disregarded. More specifically, it is the discrepancy between promoter activity, mRNA levels, and protein levels. Strictly speaking, Lv.1xRunx2-hTERT-MSCs drive MetLuc expression controlled by the full rodent Runx2 promoter, while Lv.12xRunx2-hTERT-MSCs reflect RUNX2 protein activity. One also has to consider post-transcriptional regulation including mRNA stability that might change without differences in gene promoter activity. Therefore, gene expression should have been confirmed by both promoter activity and mRNA level. To determine the mRNA level, real time PCR should have been performed to safely demonstrate whether resveratrol indeed upregulated Runx2 gene expression on the mRNA level. Change in mRNA and protein level, respectively in some conditions does not necessarily require an increase in the transcription rate, i.e. promoter activity. Thus, increased mRNA levels cannot automatically be correlated to increased promoter activity. And changes in the protein level or activity, in turn, cannot automatically be correlated to increased mRNA levels, as Shui and colleagues showed using the example of RUNX2 protein in the context of osteogenic differentiation (Shui et al., 2003).
In sum, promoter activity, mRNA levels, and protein levels are not necessarily tightly connected with each other, which renders directly correlated responses of Runx2 gene expression to resveratrol rather optional than mandatory.
6 HoxA9- and Vezf1-responsive adenoviral reporters – construction and in vitro validation

Part of chapter 6 has been published as a review article in Journal of Vascular Research.

Role of HOXA9 and VEZF1 in endothelial biology
Marco Bruderer, Mauro Alini, Martin J. Stoddart

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Marco Bruderer: manuscript writing. Mauro Alini and Martin J. Stoddart: manuscript writing, final approval of manuscript.
6.1 Introduction

There is a general consensus that endothelial progenitor cells (EPC) play a crucial role in postnatal neovascularization and thus have a promising potential in cell therapy for cardiovascular diseases and ischemic diseases, in tissue engineering of blood vessels as well as in engineering vascularized tissue such as bone or skin (Rouwkema et al., 2009; Hendrickx et al., 2011). One prerequisite for EPCs to become clinically more relevant is the establishment of safer and more reproducible isolation protocols, leading to more homogeneous endothelial cell populations, in order to guarantee consistent outcome of clinical studies.

A detailed knowledge of the molecular mechanisms, signaling pathways, and transcriptional regulation governing EPC differentiation is still unknown. This is partly due to the absence of a specific transcription factor defining EPCs.

Inspired by other research fields where master transcription factors of specific differentiation pathways are known, such as osteogenesis and adipogenesis, we aimed at searching for putative EPC-specific transcription factors based on literature search. A large body of publications has shown that HOXA9 and VEZF1 are two transcription factors involved in endothelial biology. In particular, these two transcription factors have been linked with involvement in endothelial commitment of progenitor cells as well as expression restricted to endothelial cells and their precursors (Rossig et al., 2005; Xiong et al., 1999).

Therefore, in the current chapter, these two transcription factors were considered as candidate endothelial-/EPC-specific transcription factors. Besides potentially providing clarity into the field of endothelial biology and facilitating the elucidation of mechanisms that regulate EPC differentiation, an endothelial-/EPC-specific transcription factor could be used for specific endothelial cell monitoring and the study of endothelial cell functions. The same system could also be applied for a more precise and novel isolation procedure for EPCs, which in turn would provide facilitation in research of EPCs as well as their clinical application.

Since to our knowledge, there was no comprehensive overview of HOXA9 and VEZF1 available at that time, the then present knowledge of human HOXA9 and VEZF1 was published as a review article.

The current chapter is based on the decision we made to consider both HOXA9 and VEZF1 respectively as EPC-specific transcription factors. Given this assumption, the hypothesis
aimed to be tested was whether EPCs could be functionally identified and isolated from mononuclear cells by means of adenoviral reporters responsive for HOXA9 and VEZF1.

### 6.2 Results

### 6.2.1 Construction/cloning of Ad.HoxA9 and Ad.Vezf1

The HOXA9- and VEZF1-responsive adenoviral reporters Ad.HoxA9 and Ad.Vezf1 were generated as detailed in sections 2.2.3 and 2.2.4. For cell culture experiments during the whole duration of the thesis, one batch each of Ad.HoxA9 clone 7 and clone 10, and one batch each of Ad.Vezf1 clone 8 and clone 11 were utilized. To determine the total and infectious viral titers, two different approaches were utilized: OD\textsubscript{260} measurement and plaque assay, resulting in OD\textsubscript{260}-based and pfu-based viral titers, as detailed in section 3.2.1. Since the pfu-based viral titer determination is considered to be more accurate since it takes the infectivity of the virus into account, the pfu-based viral titer was used as the starting point to calculate the volume of virus to be applied to cells in the cell culture experiments at a given MOI.

Overall, the OD\textsubscript{260}-based viral titers achieved were estimated to be between $1.44-2.58 \times 10^{12}$ particles/ml, while the pfu-based viral titers were determined to be between $0.25-0.70 \times 10^{12}$ particles/ml assuming that one out of 100 viral particles is supposed to be infectious (Table 6.1). Plaque assay performed thereafter to substantiate the OD\textsubscript{260}-based viral titer determination led to consistently lower viral titers than the OD\textsubscript{260} measurement, with differences ranging from a factor 2.3 to 5.8 (Table 6.1).

<table>
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<th>adenoviral reporter</th>
<th>OD\textsubscript{260}-based viral titer</th>
<th>pfu-based viral titer</th>
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</tr>
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</table>

*Table 6.1: Overview of the Ad.HoxA9 and Ad.Vezf1 clones produced. For each clone, the OD\textsubscript{260}-based and pfu-based viral titer, respectively is highlighted (viral titers were transformed by dividing the viral titer by a factor $10^{12}$). Overall, the pfu-based viral titer resulting from plaque assay led to lower viral titers than the OD\textsubscript{260}-based viral titer resulting from OD\textsubscript{260} measurement, with differences ranging from a factor 2.3 to 5.8.*
6.2.2 In vitro validation of Ad.HoxA9 and Ad.Vezf1

We sought to determine whether we could achieve Ad.HoxA9- and Ad.Vezf1-mediated GFP expression in HUVEC cells as a positive control cell line, while HeLa cells were taken as a negative control cell line (Patel et al., 1999). HUVEC cells and HeLa cells expanded in monolayer culture were reseeded in 6-well plates at the following seeding densities: HUVEC cells at 33,000 cells/cm²; HeLa cells at 10,000 cells/cm². The following day, the cells were adenovirally transduced with Ad.GFP at an MOI of 10 and Ad.HoxA9 as well as Ad.Vezf1, two viral clones each, at an MOI of 100 (in the case of HUVEC cells which had been seeded at 33,000 cells/cm²) and 50 (in the case of HeLa cells which had been seeded at 10,000 cells/cm²), respectively. The transduction was carried out by lanthofection for 4 hours at the above-mentioned MOIs. Thereafter, cell culture medium was replenished with fresh HUVEC and HeLa culture medium, respectively. Assessment of GFP expression in the transduced cells was performed every day from day 4 up to day 7 post-transduction by means of fluorescence microscopy. On all time points investigated, GFP expression could be observed in both HUVEC cells (Figure 6.1) and HeLa cells (Figure 6.2) transduced with Ad.GFP, Ad.HoxA9 clone 7 and 10, and Ad.Vezf1 clone 8 and 11, whereby day 6 post-transduction was chosen as representative time point to show the images. First of all, these observations confirm that Ad.HoxA9 and Ad.Vezf1 are generally functional based on their ability to induce GFP reporter expression in human cells. However, against our expectations, HeLa cells also expressed GFP. Although the GFP expression intensities between HUVEC cells and HeLa cells are not properly comparable due to different seeding densities and different MOIs of Ad.HoxA9 and Ad.Vezf1, the findings give reason to claim that, assuming that the responsiveness of Ad.HoxA9 and Ad.Vezf1 is specific to the respective transcription factor, HeLa cells express HOXA9 and VEZF1 protein at levels comparable with HUVEC cells. This is evidenced by the fact that the GFP positivities of Ad.HoxA9 and Ad.Vezf1 relative to those of Ad.GFP in the respective cell line are similar between HUVEC cells and HeLa cells. Herewith, possible differential transduction efficiencies were taken into account.

In sum, the results suggest that either Ad.HoxA9 and Ad.Vezf1, respectively are not specific enough in terms of their responsiveness, or instead that the cell lines analyzed express HOXA9 and VEZF1 protein, as opposed to what we have referenced (Patel et al., 1999).
**Figure 6.1:** Validation of Ad.HoxA9 and Ad.Vezf1 in HUVEC cells. HUVEC cells seeded at 33,000 cells/cm² were transduced with Ad.GFP at 10 MOI and Ad.HoxA9 and Ad.Vezf1, both clones for each, at 100 MOI, respectively the next day. GFP expression was assessed from day 4 up to day 7 post-transduction by means of fluorescence microscopy. Representative images taken on day 6 post-transduction are shown.
Figure 6.2: Validation of Ad.HoxA9 and Ad.Vezf1 in HeLa cells. HeLa cells seeded at 10,000 cells/cm² were transduced with Ad.GFP at 10 MOI and Ad.HoxA9 and Ad.Vezf1, both clones for each, at 50 MOI, respectively the next day. GFP expression was assessed from day 4 up to day 7 post-transduction by means of fluorescence microscopy. Representative images taken on day 6 post-transduction are shown.

As a follow-up experiment, the extent of the previous validation experiment was extended such that human BMSCs as additional cell model and Ad.Runx2 as additional adenoviral reporter was also analyzed.
For this purpose, HeLa cells and human BMSCs expanded in monolayer culture were reseeded at either 15,000 or 60,000 cells/cm² in 6-well plates, and adenovirally transduced as follows the following day: HeLa cells with Ad.HoxA9 clone 7 and clone 10, Ad.Vezf1 clone 8 and clone 11, Ad.Runx2, at an MOI of 100 each, and with Ad.GFP at an MOI of 10; human BMSCs with Ad.HoxA9 clone 7 and clone 10, Ad.Vezf1 clone 8 and clone 11, at an MOI of 100 each. The transduction was carried out by lantofection for 4 hours at the above-mentioned MOIs. Thereafter, cell culture medium was replenished with fresh HeLa culture medium, or instead switched from BMSC expansion medium to control (unstimulated) and osteogenic (stimulated) medium, respectively. Assessment of GFP expression in the transduced cells was performed every day from day 1 up to day 7 post-transduction by means of fluorescence microscopy. For a particular condition (defined by the cell type analyzed, cell seeding density chosen, cell culture medium used, adenoviral reporter used), GFP expression could be observed in both HeLa cells (Figure 6.3) and human BMSCs (Figure 6.4) with comparable GFP positivities and expression intensities across all time points investigated. Representative images are shown for day 2 post-transduction, at the seeding density of 60,000 cells/cm², and in the case of human BMSCs in osteogenic medium. These experiments generated two different insights:

First, HeLa cells showed weak levels of GFP expression mediated by Ad.Runx2. This finding is substantiated by the Human Protein Atlas (www.proteinatlas.org), a publicly available database of expression profiles of human proteins in tissues and cells, according to which HeLa cells show weak RUNX2 protein expression. However, this finding is in contrast to many publications stating that HeLa do not endogenously express RUNX2 protein (Inman and Shore, 2003; Javed et al., 2005; Pratap et al., 2005). This indicates a level of phenotypic shift over time by HeLa cells.

Secondly, human BMSCs express HOXA9 and VEZF1 protein assuming that the responsiveness of Ad.HoxA9 and Ad.Vezf1 is specific to the respective transcription factor. In contrast to the first validation experiment, Ad.GFP-mediated GFP expression was stronger in intensity compared to the ones mediated by Ad.HoxA9, Ad.Vezf1, and Ad.Runx2. Ad.HoxA9 and Ad.Vezf1 GFP expression intensities were weaker compared with the previous validation experiment. As an additional difference to the first validation experiment, overall, Ad.Vezf1 revealed stronger GFP expression compared with Ad.HoxA9, although the discrepancy was more marked in human BMSCs, whereas in HeLa cells, GFP expression intensities were rather comparable between Ad.HoxA9 and Ad.Vezf1.
Validation of Ad.HoxA9 and Ad.Vezf1, along with Ad.Runx2, in HeLa cells. HeLa cells seeded at either 15,000 or 60,000 cells/cm² were transduced with Ad.HoxA9 and Ad.Vezf1, both clones for each, and Ad.Runx2 at 100 MOI, and with Ad.GFP at 10 MOI the next day. GFP expression was assessed from day 1 up to day 7 post-transduction by means of fluorescence microscopy. Representative images taken on day 2 post-transduction, at the seeding density of 60,000 cells/cm² are shown.
In the last section, we revealed GFP reporter expression in response to HOXA9 and VEZF1 protein, respectively. These findings prompted us to search for available data on protein expression of these two proteins. According to the Human Protein Atlas (www.proteinatlas.org), a publicly available database of expression profiles of human proteins in tissues and cells, HeLa cells show weak VEZF1 protein expression, illustrating that VEZF1 indeed is not specific. At that time, data for HOXA9 was not present. Based on
the validation experiments and the search for protein expression data, it became apparent that neither transcription factor is endothelial-/EPC-specific.

To concludingly answer the question whether HOXa9 and VEZF1, respectively are indeed expressed in HUVEC and HeLa cells at the protein level, Western Blot analysis of HUVEC and HeLa cell extracts was performed. For this purpose, HUVEC cells and HeLa cells were expanded in monolayer culture in 100 mm tissue culture plates. Upon confluence, cytosolic and nuclear extracts were prepared from cell monolayers, whereby protein concentration was determined by means of Bradford assay. SDS-PAGE was performed by loading 6 µg total protein of cytosolic and nuclear fractions per lane on a polyacrylamide gel, followed by Western Blot analysis using specific antibodies as detailed in section 2.12.4.

We confirmed expression of both human HOXa9 and VEZF1 in both HeLa and HUVEC cell nuclear extracts, while cytosolic fractions were devoid of the presence of any of the two transcription factors analyzed (Figure 6.5). HOXa9 expression was revealed to be present at weak levels, while VEZF1 was expressed at comparatively high levels.

![Western Blot Image](image)

**Figure 6.5**: HOXa9 and VEZF1 protein levels in HeLa and HUVEC cells. Western Blot detection of human HOXa9 and VEZF1 in cytosolic and nuclear extracts of HUVEC and HeLa cells, respectively. 6 µg total protein of cytosolic and nuclear extracts, respectively was used for each sample and Lamin B1 served as control for equal loading.
6.3 Discussion

The present chapter addressed two different adeno viral reporters which were generated with the aim to identify and isolate EPCs from mononuclear cells. We decided for the transcription factors HOXA9 and VEZF1 to account for the specificity of the adeno viral reporters, referred to as Ad.HoxA9 and Ad.Vezf1, supported by several publications summarized in our review article (Bruderer et al., 2013). HOXA9 is mainly characterized by the endothelial specificity of its target genes, whereas VEZF1 is mainly distinguished by the endothelial specificity of its own expression (Bruderer et al., 2013).

In the course of the in vitro validation experiments, it was observed that HeLa cells, which we considered as a negative control cell line, expressed GFP mediated by Ad.HoxA9 and Ad.Vezf1. Assuming that the responsiveness of Ad.HoxA9 and Ad.Vezf1 is specific to the respective transcription factor, this finding gave first evidence that non-endothelial cells express HOXA9 and VEZF1 protein as well, in fact at levels comparable with the endothelial HUVEC cell line.

The expression of HOXA9 and VEZF1 protein not being specific to endothelial cells could be substantiated by Western Blot analysis, which showed that HOXA9 (at weak levels) and VEZF1 (at comparatively high levels) protein are present in HeLa and HUVEC nuclear extracts.

Collectively, the results of the validation experiments of Ad.HoxA9 and Ad.Vezf1 and the assessment of HOXA9 and VEZF1 protein levels in HeLa and HUVEC cells confirmed that both transcription factors are not specifically expressed in endothelial cells.

Technically speaking, to prove that a particular protein can be referred to as specific for a particular cell type or lineage, examination of tissue distribution in situ and protein expression in all possible cell types needs to be performed. However, protein expression in most cases is studied and reported only in a series of tissues and cell types, leading to an incomplete overall picture of the expression pattern. As a consequence, a possible statement about tissue-specific or cell type-specific expression is made prematurely. Whereas in the case of HOXA9, endothelial-specific distribution has not been reported, VEZF1 has been termed ‘a zinc finger transcription factor restricted to endothelial cells and their precursors’ (Xiong et al., 1999). This statement was based on the spatial and temporal expression during murine embryogenesis, along with the examination of adult organs and established endothelial and hematopoietic cell lines, all of which were assessed on the RNA transcript level only (Xiong et al., 1999). In reality, the expression of VEZF1 appeared to be higher in HeLa cells and MSCs than that of HOXA9. Due to the expression of VEZF1,
HOXA9 and RUNX2 in HeLa cells, an alternative conclusion may be that this cell line has a massively dysregulated phenotype and may not be the ideal choice of negative control in such studies. It may be prudent to attempt further work with more primary cells. To conclude, the two transcription factors presented in this chapter are associated with the endothelial cell phenotype as highlighted by our review article (Bruderer et al., 2013); however, they are not specific solely for endothelial cells/EPCs. Rather, they are more selective for endothelial cells or their progenitors, assuming cell populations not associated with endothelial phenotype have been previously excluded using other means. This circumstance is not favorable for being applied as a developmental biology tool in order to specifically label/track EPCs in vivo due to co-labelling of other cell populations. However, it does not hinder their use in in vitro cell cultures from which cell populations not associated with endothelial phenotype had been excluded. In this pre-selected setting allowing only the desired starting cell populations to be present, expression of HOXA9 and VEZF1, respectively could be a useful tool for monitoring the development for endothelial cells and their progenitors. This way, occurrence of endothelial cells and their progenitors can be reliably monitored using reporter-based live cell approaches coupling expression of HOXA9 and VEZF1, respectively to the expression of a reporter gene. Therefore, although the experiments performed in the context of this chapter suggested that HOXA9 and VEZF1 are not specifically expressed in endothelial cells/EPCs, the two adenoviral reporters are not automatically rendered useless for the anticipated isolation of EPCs, but could be used for monitoring and cell isolation approaches of starting cell populations consisting of a limited spectrum of cell types. In fact, RUNX2 protein expression and usage of Ad.Runx2 exemplifies that a particular transcription factor must not necessarily be cell type-specific in order to still be used as tool for monitoring of differentiation processes or isolation of cell populations. The fact that RUNX2 protein is (weakly) expressed in HeLa cells does not interfere with using Ad.Runx2 for osteoprogenitor isolation from bone marrow-derived mesenchymal stem cells devoid of cervical epithelial cancer cells such as HeLa cells.
The present thesis aimed at establishing a novel concept for the identification, and in particular for the isolation of osteoprogenitor and endothelial progenitor cell (EPC) populations from human BMSCs and mononuclear cells, respectively. The rationale for establishing a novel approach for the isolation of osteoprogenitors and EPCs is based on the fact that no specific, unique, and robust cell surface marker is known for osteoprogenitors and EPCs, which would enable a more homogeneous progenitor cell population to be obtained. This would lead to more consistent experimental results. To approach this aim, we made use of several self-generated adenoviral GFP reporter constructs, each of which was responsive to a particular relevant transcription factor considered to be selective for the cell type of interest. With this thesis, we have contributed a number of important pieces of insight to the field of adult stem and progenitor cell isolation, while at the same time having raised new questions.

The first part of this thesis describes a new method to identify and isolate osteoprogenitors from human MSCs. The actual novelty consists of not approaching the isolation based on CD markers as currently performed, but utilizing an adenoviral reporter system specific for the key osteogenic transcription factor Runx2, which is linked to the cell phenotype of interest. The resulting reporter positive, Runx2 GFP+ cell population isolated by means of FACS was repeatedly revealed to be superior to Runx2 GFP- subpopulation, as well as the original cell population, as regards the in vitro osteogenic differentiation capability. This was evidenced using two different functional assays of osteogenic differentiation, namely ALP activity and radioactive 45Ca incorporation to assess formation of mineralized matrix. Additionally, cells were investigated regarding their colony formation, while taking size of colony into account. The Runx2 GFP+ cell population resulted in a cell population with a) similar number of CFUs, but more slow-growing, smaller CFUs compared to MSCs, b) higher in vitro osteogenic differentiation capability than unsorted MSCs. The percentage of Runx2 GFP+ cells corresponds to estimates of osteoprogenitor frequency in adherent human stromal cell layers, which are in the range of 1-10% of adherent cells (Bianco et al., 2006).

Despite the clear nomenclature developed beforehand, we should emphasize that it cannot be excluded that the isolated Runx2 GFP+ cell population are cells with a more mature osteoblastic phenotype, i.e. preosteoblasts or even immature osteoblasts. This issue highlights both the strength, but also the weakness of the here presented isolation
method. The novel isolation method is completely based on the Runx2 reporter and thus on the expression and presence of functional RUNX2 protein. Runx2 expression starts early in osteogenic differentiation and its expression levels stay high during the course of osteogenic differentiation, thus also enabling isolation of osteogenic cells that are not exactly at the stage of osteoprogenitor, when considering that MSCs constitute a heterogeneous cell population including early- as well as late-committed progenitors. In contrast, CD marker expression varies depending on the differentiation stage of the cell. Therefore, CD marker-based isolation approaches have less probability of simultaneous osteoprogenitor/pre-osteoblast/osteoblast co-isolation, whereas isolation based on RUNX2 makes co-isolation likely. This fact represents a clear advantage over the cell surface marker based isolation methods. However, when it comes to the definition of the isolated cell population, the principle of the isolation method causes difficulties, since the resulting Runx2 GFP\(^+\) cell population cannot be stated as homogeneous osteoprogenitor population due to the presence of contaminating, more mature osteogenic cells which still express RUNX2. Although, these contaminating cells might be diluted away during the post-sorting cell expansion process. This issue represents an unavoidable limitation because the experiments are performed with primary human cells whose timing of osteogenic differentiation varies from patient to patient, thus it is impossible to determine a reproducible point in time when all cells are at an optimal stage for cell sorting. However, even if the nomenclature of the isolated Runx2 GFP\(^+\) cell population is not finalized at this stage of investigation, it is fact that this cell population represents a precursor stage of osteoblasts. Another issue that has not even been started to be addressed is the multidifferentiation potential of the different cell populations. Our data do not indicate whether the Runx2 GFP\(^+\) cells are progenitors restricted to the osteogenic lineage, or rather represent progenitors with osteo-chondrogenic or even osteo-chondro-adipogenic potential (Muraglia et al., 2000).

The results of this isolation method brought along striking questions regarding the cellular behavior of the three cell populations. Osteogenic differentiation capability, as assessed by ALP activity and \(^{45}\)Ca incorporation assays, was as follows: Runx2 GFP\(^+\) was superior to Runx2 GFP\(^-\), which in turn was superior to unsorted cell population. This was against the expectations which were that Runx2 GFP\(^+\) cell population would perform best in terms of osteogenic differentiation capability, Runx2 GFP\(^-\) cell population being the least efficient, and the unsorted cell population capturing the intermediate position. A potential
explanation included a crosstalk taking place between the cell populations in the way that Runx2 GFP cell population might act as a feeder layer, playing a pro-proliferative, but anti-osteogenic role for the Runx2 GFP+ cell population. This issue was addressed, but could not be conclusively answered due to the ambivalent results of the crosstalk experiments that arose.

The isolation method presented here stands out due to the concept of getting access to the cell populations of interest by means of a key transcription factor-responsive reporter, representing the major novelty compared to isolation approaches reported so far. Furthermore, three novelties of the approach are worth being mentioned. First, the present approach represents a functional assay. The adenoviral reporter responds to an active, representative, lineage-relevant protein, and is not based on CD markers or mRNA that might not even be able to characterize BMSC-derived osteoprogenitors. In fact, CD marker profiles and gene expression patterns of MSCs are considered to be unrobust characteristics which can be significantly affected by cell culture conditions. Supplementation of in vitro MSC culture with growth factors such as FGF-2 was shown to significantly change the MSC surface marker profile (Hagmann et al., 2013). Lee and colleagues revealed that gene expression patterns of MSCs vary with different seeding densities and culture times (Lee et al., 2013). Hence, CD markers and gene expression patterns due to their unrobustness are non-ideal properties to be chosen as isolation criteria. Second, the approach stands out due to its potential versatility. By replacing the specific transcription factor binding site with one for another transcription factor, this approach is not limited to MSC-derived osteoprogenitors. It can be adopted to other progenitor populations present in human MSCs such as chondro- or adipoprogenitors, but also opens usage in other stem cell fields, e.g. cancer stem cells, where there is a need for identification of specific markers that might distinguish cancer stem cells from the bulk of the tumor (Al Hajj et al., 2003). Third, the adenoviral reporter-based isolation method represents a live cell approach, which enables less time-consuming experimental procedures such as comparative screening of various osteogenic induction protocols or testing of (bio)materials for their osteoinductive capability, to name only two examples. In contrast to the second and third mentioned novelties which mainly concern applications of the novel reporter-based isolation method, the first novelty addressing the basic difference to previously reported isolation approaches of MSCs leads us back to the issue which is prominent in all discussions about isolation of MSCs, namely CD markers.
As already mentioned several times, MSCs from bone marrow represent a heterogeneous population of cells, and are not a true population of stem or progenitor cells. As a consequence, researchers have tried to isolate subpopulations of MSCs using different approaches. The most often reported attempt is by using specific antibodies against various CD markers, such as CD105 (Majumdar et al., 2000), CD106, Stro-1 (Simmons and Torok-Storb, 1991b), Stro-1 in combination with CD106 (Gronthos et al., 2003), and CD49a (Deschaseaux and Charbord, 2000; Stewart et al., 2003). All these antibodies have been reported to facilitate the isolation of CFU-F from adult human bone marrow. Stewart and colleagues, comparing the efficiency of CFU-F capability following selection using Stro-1, CD63, CD49a, and CD166, found that CD49a was the most efficient (Stewart et al., 2003). However, it is important to keep in mind that despite the marked CFU-F enrichment obtained to date by using CD marker-based isolation methods, the selected cell fractions still contain other, contaminating cell types of both stromal and hematopoietic origin (Simmons and Torok-Storb, 1991b; Gronthos et al., 1994).

Many different methods to isolate MSCs exist, but fewer methods are described for the isolation or purification of osteoprogenitors from bone marrow. Gronthos and colleagues used a positive selection procedure using the monoclonal antibody Stro-1 to isolate all clonogenic stromal precursors in human bone marrow, including all osteoprogenitors (Gronthos et al., 1994). Others have reported that in mice, osteoprogenitors could be sorted from bone marrow based on their binding to wheat germ agglutinin (WGA) and Sca-1 expression (Van Vlasselaer et al., 1994). Falla and colleagues purified/enriched osteoprogenitors from murine bone marrow based on in vivo treatment with 5-fluorouracil. This nucleotide analogue massively diminishes the number of cycling cells but preserves the quiescent ones of the marrow (Falla et al., 1993). Therefore, they concluded that osteoprogenitors represent a quiescent cell population in the murine bone marrow. Furthermore, against our expectations, the osteoprogenitors they have isolated were characterized by nonadherence to tissue culture plastic (Falla et al., 1993). In addition, several other groups reported nonadherent, nonhematogeneous cell populations present in human, rat, and murine bone marrow which express bone proteins and respond to osteogenic growth factors (Falla et al., 1993; Long et al., 1990; Clarke and McCann, 1991; Van Vlasselaer et al., 1994; Long et al., 1995). Besides CD marker-based isolation methods, selection of MSC (sub)populations making use of size properties are reported (Colter et al., 2000; Colter et al., 2001).
Selection of subpopulations of MSCs based on cell surface marker expression is potentially a fast and clinically efficient way to purify a heterogeneous populations of MSCs. Results presented so far suggest that this approach is a realistic possibility, but generation of antibodies recognizing novel antigens is still necessary. The more reports are being published, the more likely is becomes that the use of several antibodies rather than a single antibody will be necessary to establish the most efficient means to select MSCs and subpopulations thereof. The future identification of novel unique and specific cell surface markers for MSCs and subpopulations thereof might be enabled by making use of the novel isolation approach by means of proteomic analyses of the Runx2 GFP⁺, Runx2 GFP⁻, and unsorted cell populations. In the end, from a clinical application perspective, it is easier to go into clinics with a CD marker-based isolation method as opposed to procedures involving adenoviral vectors due to their associated ethical issues.

In conclusion, although the usage of adenoviral reporters for the isolation of progenitor populations represents a proof of principle approach, it might potentially facilitate the path into clinics by elucidation of potential novel cell surface markers specific for MSCs or subpopulations thereof by means of proteomics approaches. The adenoviral reporters might also be used for purity checking of isolation procedures using currently used cell surface markers. Moreover, usage of the adenoviral reporters might have even more far-reaching applications such as in vivo bioluminescence imaging (BLI). BLI allows for noninvasive imaging of ongoing biological processes in small laboratory animals (for review see (Keyaerts et al., 2012)). While common applications of BLI encompass cancer progression using a bioluminescent cancer cell line (Rehemtulla et al., 2000) and in vivo infection studies using bioluminescent pathogens (Xiong et al., 2005), RUNX2-responsive luciferase reporters might be used in the context of BLI to evaluate bone forming processes in vivo. While fluorescence imaging (FLI) has also been developed, BLI which is based on the activity of luciferases is more sensitive, does not require excitation and does not bring along issues with background signals from naturally occurring fluorescent substances present in the animal (for review see (Keyaerts et al., 2012)).

After having performed the initial characterization experiments of the three different sorted cell populations yielding reproducible in vitro osteogenic performance patterns amongst the cell populations, crosstalk experiments of the different cell populations were performed. Thereby, in addition to the original three expanded sorted cell populations analyzed, Runx2 GFP⁺ and Runx2 GFP⁻ cells were mixed after the post-sort expansion phase
at different ratios to create three additional groups. In the course of the subsequent crosstalk experiments, conflicting results as regards the \textit{in vitro} osteogenic performance of the cell populations arose. The inconsistency also concerned the original three analyzed cell populations. The incidence of inconsistent patterns of \textit{in vitro} osteogenic differentiation capability prompted us to perform a set of troubleshooting experiments, addressing the following influencing factors: population doublings, function-impairing mutation of Ad.Runx2, serum used for cell culture medium, change in cell seeding density, and viral titer determination method. After having performed the different troubleshooting experiments, the conclusion was that none of the influencing factors turned out to be the causer of the inconsistent results.

At this point, we started to take stock of the current body of results of all the previously performed experiments. All present data evidenced that there exists a discrepancy between the functionality of the Ad.Runx2 adenoviral reporter and the functionality of the cell populations resulting from the isolation by means of Ad.Runx2.

The functionality of the Ad.Runx2 adenoviral reporter was proven by several lines of evidence. First, the 12x tandemly arranged Runx2 binding sites as part of the promoter of the Ad.Runx2 adenoviral reporter was proven to be responsive to RUNX2 protein, and together with the minimal promoter to cause luciferase reporter expression (section 3.2.2). Second, the course of Ad.Runx2-mediated GFP reporter expression is in accordance with what is expected: the GFP reporter expression steadily increases in a high seeding density condition, whereby the GFP expression is increased in osteogenic medium as opposed to control medium, then reaches a peak on day 3 to 4 post-transduction, and thereafter decreases again to basal levels. However, when interpreting time courses of reporter expression such as the Ad.Runx2-mediated GFP reporter expression, the GFP reporter protein level needs to be clearly separated from the actual level of functional RUNX2 protein. The GFP protein level at a particular time point does not simply reflect the RUNX2 protein level at that particular moment, but rather the cumulative result of protein synthesis, degradation, and dilution (upon population doublings) up to that particular moment (Brown and Lostroh, 2008). The stability of GFP protein directly impacts the extent of the history of gene expression at each time point. Hence, the time course of RUNX2 protein level may not parallel the time course of GFP reporter expression obtained by visual inspection. It is not the amount of GFP protein that has accumulated by a particular time point, but rather the rate of synthesis of GFP protein at that particular moment which reflects the RUNX2 protein level present at that particular moment, and
that is what is actually of note. Further to this reasoning, promoter activity and thus GFP reporter gene expression can be influenced in an unpredictable manner by other factors such as genetic and epigenetic features associated with the artificial environment of the Ad.Runx2 promoter. That is, the endogenous locus of the RUNX2 binding sites does not reflect the artificial environment of the tailor-made promoter of Ad.Runx2, which can lead to situationally different genetic and epigenetic regulation of the promoter and RUNX2 binding sites, respectively.

While we can safely consider the functionality of Ad.Runx2 to be in the way it is to be expected, the functionality of cell populations resulting from the isolation by means of Ad.Runx2 is inconsistent, as shown by the different validation and crosstalk experiments. The \textit{in vitro} osteogenic differentiation potential of a particular cell population, even in experiments with the same donor, generated different patterns of results. One factor which brings inaccuracy and even randomness along and as a consequence contributes to the unpredictable behavior of the cell populations is adenoviral transduction itself. Although usage of a particular MOI for adenoviral transduction implies an exact ratio of virus to target cells, the probability of a cell’s infection underlies the statistical Poisson distribution: some cells may take up a multiple of the number of viral particles as foretold by the MOI, while other cells are not hit by any viral particle at all. In theory, the consequences thereof can be so far-reaching that a cell with a high RUNX2 protein level that was transduced with an actual low number of viral particles exhibits lower GFP expression compared with a cell with a low RUNX2 protein level that is transduced with a much higher actual number of viral particles. In this example, the GFP expression level would indirectly correlate with the RUNX2 protein level of a particular cell, which in turn would lead to more or less intensely mixed cell populations. Although this factor accounts for a certain randomness of the functionality of the sorted cell populations, this should statistically average out over the large number of cells being used. Thus, the functionality of the sorted cell populations can be considered as generally controllable as regards the general concept of the novel isolation approach.

Collectively, the only residual way the discrepancy between the functionality of Ad.Runx2 and the functionality of the sorted cell populations could be explained was that the hypothesis was wrong. Our hypothesis was that a single transcription factor-responsive reporter is sufficient to specifically distinguish osteoprogenitors from the original BMSC population.
While the troubleshooting experiments were performed, the issue of the Runx2/Sox9 ratio in the context of gene expression analysis was raised. Interest in Runx2/Sox9 arose as a result of the findings from *in vitro* differentiation experiments with BMSCs within our group. As opposed to Runx2 and Sox9 mRNA levels, which could not be robustly linked to whether the corresponding sample was subjected to osteogenic differentiation or not, Runx2/Sox9 ratio in terms of mRNA expression levels consistently differed depending on whether cells were stimulated in osteogenic medium or were unstimulated in control medium. In fact, the greatest change was observed in the Sox9 mRNA and not in the Runx2.

In embryology, a link between SOX9 and RUNX2 has been well documented. SOX9 is described as the master transcriptional regulator of chondrogenic differentiation and is expressed in all osteochondroprogenitors and chondrocytes except hypertrophic chondrocytes (Wright et al., 1995; Zhao et al., 1997; Akiyama et al., 2002; Akiyama et al., 2005). It is required for all sequential steps of the chondrocyte differentiation pathway during endochondral bone formation, ranging from commitment of undifferentiated mesenchymal cells to osteochondroprogenitors, mesenchymal condensation, chondrocyte differentiation to chondrocyte proliferation (Akiyama et al., 2002). It has also been proposed that osteochondroprogenitors acquire a chondrogenic fate when Sox9 expression remains high compared with Runx2, and that the cells undergo osteogenesis when Sox9 expression is lower than Runx2 expression (Akiyama et al., 2004; Akiyama et al., 2005). Furthermore, ectopic Sox9 overexpression in Runx2-expressing osteoblasts resulted in abnormal bone formation, which indicates that SOX9 is capable of inhibiting the osteoblast regulator RUNX2 (Zhou et al., 2006).

RUNX2 and SOX9 are expressed in osteochondroprogenitors during embryonic development. It has been reported that SOX9 directly interacts with RUNX2 and represses its activity *in vivo* (Zhou et al., 2006). The mechanism involved in this inhibition was identified to be an inhibition of the transactivation capability of RUNX2 by SOX9-mediated degradation of RUNX2 in a proteasome-independent, but phosphorylation-dependent manner (Cheng and Genever, 2010).

In sum, these findings make it plausible that Runx2 expression in itself is not an indicator of osteogenic potential, but rather Runx2 in conjunction with Sox9 which is indicative of the osteogenic potential of human BMSCs. Only taking Runx2 into account is not predictable enough to estimate the osteogenic potential of human BMSCs.
The original hypothesis was maintained as a mainstay of this project for a substantial proportion of the time of the project as Runx2 and its key role in osteogenic differentiation is such a confirmed concept. As witnessed by the chapter dedicated to trying to determine where the error in the protocol lay, a significant number of experiments were performed in order to locate the source of the discrepancy.

We experienced that reporter experiments regarding only one single factor in isolation were not robust. Consideration of only one factor inherently brings about an incomplete picture, unlike gene expression analysis by real time PCR which is widely regarded as “gold standard” measurement, where several genes are verified to give a much broader picture of gene expression behavior. A broader approach that considers multiple factors as opposed to only a single factor to predict the osteogenic potential was addressed by Platt and colleagues (Platt et al., 2009). They demonstrated that multipathway kinase signatures of human MSCs comprising eight intracellular phosphoproteins are predictive for osteogenic differentiation (Platt et al., 2009).

The lack of a reliable outcome from single reporter constructs is a common occurrence and it is likely that the conclusions made from the work presented in this thesis go some way to accounting for the results of a number of other groups.

The future usage of a dual reporter such as a combination of Runx2-responsive GFP reporter (Ad.Runx2) and Sox9-responsive red fluorescent protein (RFP) reporter (Ad.Sox9) is likely to provide sorted cell populations which are more homogeneous and which possess more consistent characteristics as regards their in vitro osteogenic performance. Once the Sox9-responsive RFP reporter Ad.Sox9 will be available, dual reporter experiments using Ad.Runx2 and Ad.Sox9 will enable osteogenically induced BMSCs to be subdivided into four cell populations. Cells can be sorted based on the relative expression of the two factors RUNX2 and SOX9 rather than whether either of the two factors is expressed at a low or high level. The Runx2 GFP+/Sox9 RFP or rather Runx2^{high}/Sox9^{low} cell population, which will be considered to represent the osteoprogenitors, will then be subjected to in vitro investigation of differentiation and its in vitro osteogenic performance will be compared with the other three cell populations resulting from the subdivision of osteogenically induced BMSCs. Once this will have been achieved and repeatedly confirmed, it will be even more interesting to examine how the different cell populations behave in vivo with regard to their in vivo bone forming capability. In future, it will also be exciting to re-
address the issue of crosstalk of the different cell populations, which will have newly been sorted by means of the dual reporter approach.

Even if the Runx2/Sox9 ratio instead of the mere functional RUNX2 protein level represents a more reliable indicator of the osteogenic potential, RUNX2 is nonetheless required to induce osteoblastogenesis. However, it would appear that the SOX9 level needs to decrease in order to allow this to occur.

Stem cell differentiation and maintenance of stemness are complex processes involving the coordinated expression of several genes. Waddington introduced the ‘epigenetic landscape’ as a conceptual picture for how gene regulation modulates cell lineage specification (for review see (Macarthur et al., 2009)). Similarly to a marble that rolls down a sloping landscape containing hills and valleys, cells take different paths down this landscape and in this way adopt different fates. Thereby, the location of a particular marble represents a gene expression profile, while a phenotypic cell fate is represented by a valley state. Let us consider that osteogenic and chondrogenic specification in MSCs or rather osteochondroprogenitors is controlled by the transcription factors Runx2 and Sox9, as described earlier (Akiyama et al., 2004; Akiyama et al., 2005). In this two-gene relationship, the state of indeterminacy is characterized by a balanced expression pattern of Runx2 and Sox9. While small perturbations of the state are buffered by the rim and the marble rolls back into the valley, larger perturbations above a distinct threshold will kick a marble out of the basin. In gene expression terms, a perturbation of the state in a two-dimensional (two-gene: Runx2, Sox9) system into any of two directions corresponds to priming of the cell towards osteogenic (Runx2 > Sox9) and chondrogenic (Runx2 < Sox9) phenotype, respectively. In case the perturbation is large enough and the marble crosses the hill, the cell is specified towards osteogenic (Runx2 >> Sox9) and chondrogenic (Runx2 << Sox9) lineage, respectively. While this concept highlights that transcription factor expression levels need to be viewed more broadly to serve as reliable indicator for cell lineage specification, it also plausibly substantiates that an osteochondroprogenitor requires a certain intensity of a trigger in order to be induced towards osteogenic phenotype. It would be inadequate indeed if this cell would already start to differentiate into the osteogenic lineage upon only a weak trigger.

In a second approach towards the linkage of the level of functional RUNX2 protein and the osteogenic potential, we generated two stable lentiviral Runx2 luciferase reporter cell lines,
of which Lv.1xRunx2-hTERT-MSCs served to address the mRNA expression, while Lv.12xRunx2-hTERT-MSCs served to address the protein activity. The aim of the generation of stably transduced cell lines was to achieve a two-fold improvement of the adenoviral Runx2-responsive GFP reporter. First, a stable reporter cell line leads to more robust, reproducible and consistent results compared with a transient reporter. Second, a luciferase reporter represents a more sensitive reporter compared with GFP. Not only did it represent a more sensitive system by using a luciferase reporter instead of a GFP reporter, but also did a naturally secreted luciferase, MetLuc, allow for a non-destructive and much less laborious way to readout the assay. From a high-throughput analysis and screening point of view, this circumstance represents a massive advantage.

After the in vitro validation of the stable Runx2 reporter hTERT-MSCs had been performed, we aimed at determining whether our stable Runx2 reporter cell model could be used to detect increases in Runx2 expression mediated by resveratrol. Resveratrol is a natural compound that, in the context of its ability to modulate osteogenesis of human BMSCs, has been reported to upregulate Runx2 gene expression (Tseng et al., 2011). Due to this capability, we became interested in testing resveratrol using the stable Runx2 reporter hTERT-MSCs. We decided for three different resveratrol concentrations based on the publication by Tseng and colleagues (Tseng et al., 2011). We did not initially test and optimize the concentrations of resveratrol for the stable Runx2 reporter hTERT-MSCs. In the drug testing experiment, resveratrol at the highest concentration tested (50 µM) revealed a marked cytotoxic effect, hence setting an upper limit of a putatively optimal resveratrol concentration to upregulate Runx2 gene expression. This way, we could indirectly exclude the possibility that the dose was insufficient to lead to a sufficient upregulation of Runx2 gene expression.

Despite both the fact that we have tested sufficiently high concentrations of resveratrol and the improvement in sensitivity we had hoped for by utilizing stable lentiviral Runx2 luciferase reporter cell lines, resveratrol did not lead to marked increases in the MetLuc reporter expression normalized to the cell number compared with medium lacking in resveratrol. Therefore, insufficient sensitivity of the stable Runx2 reporter hTERT-MSC system was considered as a plausible explanation for the overall insignificant and only selective differences in the candidate drug testing experiment using resveratrol. It will be exciting to see whether the use of clonal populations of stable Runx2 reporter hTERT-MSCs could lead to a more robust response compared with stable Runx2 reporter hTERT-MSC used as an inherently mixed/pooled population.
The second part of this thesis aimed at establishing a novel approach to functionally isolate EPCs from mononuclear cells. No specific transcription factor defining EPCs was known at the start of the PhD project, which prompted a search for putative EPC-specific transcription factors in the literature. A large body of publications hinted at HOXA9 and VEZF1 as relevant transcriptional regulators of endothelial biology. Both were investigated in order to maximize the potential for a successful outcome. However, it was demonstrated that neither are endothelial/EPC-specific based on the in vitro validation experiments. Even so, they still might represent a useful tool for monitoring the development of endothelial cells and their progenitors in an in vitro setting, from which cell populations possessing the transcription factors, but not associated with the endothelial phenotype, had been excluded.

Importantly, the fact that the adenoviral reporters Ad.HoxA9 and Ad.Vezf1 led us to the knowledge that VEZF1 and HOXA9 are not specific as first thought indirectly fortifies the specificity of Ad.Runx2. Compared with the Ad.HoxA9- and Ad.Vezf1-mediated GFP expression level, overall, Ad.Runx2-mediated GFP expression level in BMSCs was much less intense. Since the only difference between the different adenoviral reporters Ad.Runx2, Ad.HoxA9, and Ad.Vezf1 was the tandemly arranged transcription factor binding site, differential GFP expression levels across the different adenoviral reporters due to features of the adenoviral reporters themselves can only be due to the difference in the transcription factor and its binding sites. In contrast, other features of the adenoviral reporters such as the minimal promoter do not account for a possible unspecificity of the adenoviral reporters, because otherwise the minimal promoter-mediated effect would have been observed across all different adenoviral reporters. From a proof of principle point of view, this fact adds further confirmation that the Runx2-responsive adenoviral reporter Ad.Runx2 is indeed specific.

The development of a novel approach for the functional identification and isolation of osteoprogenitors from human BMSCs aimed at identifying a subpopulation of BMSCs that is more homogeneous than the initial BMSC population. Attempts to isolate more homogeneous human BMSC populations have been made, which were based on size, cell surface markers, but also defined culture conditions (Table 1.1). Our novel approach fills a niche in the spectrum of possibilities to isolate more homogeneous human BMSC subpopulations. In addition, our approach is also different in the way that we intentionally
commit the BMSCs towards the osteogenic phenotype in order to be able to identify the osteogenic subpopulation of cells. Hence, the isolated subpopulation of interest cannot be considered as a naïve progenitor population.

The central element of the transcription factor-responsive reporter system which accounts for the specificity of the reporter is the transcription factor itself. Choice of an appropriate and ideal transcription factor is essential and lays the cornerstone for successful and reliable downstream experiments.

In the case of the approach to isolate osteoprogenitors, Runx2 as the major transcriptional regulator of osteoblast differentiation was considered a robust and central transcription factor, which in addition had the potential to account for sufficient osteogenic specificity. However, although being necessary, Runx2 is not sufficient for proper osteogenic differentiation (for review see (Satija et al., 2007)). The zinc finger-containing transcription factor Osterix (Osx) acting downstream of Runx2 is required for complete osteogenic differentiation. This circumstance raises the question whether Osx instead could be used as an alternative transcription factor to account for the specificity of the transcription factor-responsive reporter system to functionally isolate osteoprogenitors. Similar to Runx2, Osx seems not to be sufficient to induce osteoblast differentiation (Kim et al., 2006b). Kim and colleagues reported the inability of Osx to induce osteogenic lineage commitment in NIH-3T3 fibroblasts (Kim et al., 2006b). Instead, it appears likely that a combination of Runx2 and Osx serves as an ideal candidate pair of transcription factors for dual reporter-mediated isolation of osteoprogenitors.

Taken together, transcription factor-specific reporters can serve both as basis to functionally subdivide and isolate committed subpopulations of stem cell populations and as tool for applied approaches such as drug screening and biomaterials testing. However, the addressed transcription factors need to be considered as players within a regulatory network, situationally necessitating usage of multiple reporters to serve as a satisfying indicator of cell lineage specification potential. Furthermore, transcription factor-specific reporters are not necessarily dependent on cell type- and tissue-specific expression of the corresponding transcription factors, but need to meet the criterion for specificity only in the range of cell fates the starting cell population can give rise to. The absence of specific MSC surface markers and the heterogeneity of MSCs represent two significant stumbling blocks in the field of MSC biology as well as in the field of therapeutic applications of MSCs.

In the present thesis, we addressed these two aspects. The original hypothesis of the thesis
was to establish a new method to isolate subpopulations of MSCs based on criteria other than cell surface markers, and hoped for obtaining more homogeneous cell populations. However, what should not be forgotten is the fact that the two mentioned issues are consequences of the traditional method to isolate MSCs. The traditional isolation method of MSCs comprising adherence of cells to tissue culture plastic and in vitro culture of cells involves steps which are unpredictable and cannot be properly controlled, rendering it a black box procedure. In the experiments in the present thesis, we applied the novel isolation method to MSCs which in turn had been isolated by means of traditional adherence to tissue culture plastic. Recent developments have started to focus on the identification of novel surface markers for the in vivo localization and prospective isolation of MSCs representing a pure population of multipotent, naïve MSCs devoid of contaminating cells arising due to in vitro culture (for review see (Mabuchi et al., 2013)).

Although the present thesis encompasses basic research experiments, the two progenitor cell populations which had been addressed in this thesis are clinically meaningful. The osteogenic potential of seeded cells is crucial for successful bone tissue engineering. Using a more homogeneously osteogenic cell population than MSCs harbors the potential for improvements in the outcome of bone tissue engineering approaches. Besides, sufficient vascularization which could be stimulated by co-seeding of cells with endothelial potential is another key prerequisite for successful cell-based bone tissue engineering. Keeping these two important prerequisites in mind, the two mentioned cell populations we aimed at isolating, as well as the novel isolation approach as such, have the right to enjoy adequate importance in the debate of clinical applications of stem and progenitor cell populations.
Appendix

8 Appendix
8.1

Nucleotide sequences

8.1.1 Nucleotides used for cloning
Nucleotide name
HoxA9F1 rev
HoxA9F2 rev
M13for(-20)
M13rev

Sequence
5'-AAGCTTATGCAATTT-3'
5'-CCGCGGAGGCCTATC-3'
5'-GTAAAACGACGGCCAG-3'
5'-CAGGAAACAGCTATGAC-3'
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HoxA9F1
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pShuttle forward
5'-GAAGTGAAATCTGAATAATTTTGTG-3'
pShuttle-CMV reverse 5'-GTGGTATGGCTGATTATGATCAG-3'
MinPro for ClaI
5'-GGGATCGATTAGGAAGTGGAAACCTATC-3'
EGFP rev XhoI
5'-GCACTCGAGTTACTTGTACAGCTCGTCCA-3'
5'-GGTACCATATTACCCCCACTCCATATATTACCCCCACTCCATATATTACCCCCACTCCAT
Vezf1F1
ATATTACCCCCACTCCATATATTACCCCCACTCCATATATTACCCCCACTCCATAAGCTT-3'
5'-AAGCTTATTACCCCCACTCCATATATTACCCCCACTCCATATATTACCCCCACTCCATA
Vezf1F2
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Vezf1F1 rev
5'-AAGCTTATGGAGTGGGGGT-3'
Vezf1F2 rev
5'-CTATCGATGGAGTGGGGGT-3'
pShuttle for_2
5'-TGTGGCAAAAGTGACGTTTTTGGT--3'
12xVezf1-MinPro rev
5'-ACTTCCTAATCGATGGAGTGGGGGTAA-3'
12xRunx2 for
5'-AAACTCGAGCATGCCATGGACCA-3'
MinPro rev HindIII
5'-GGGGAAGCTTGGTTTCTAGATTGAATGG-3'
pCMV-IE for
5'-CCATAGTAACGCCAATAG-3'

Table 8.1: Oligonucleotides and PCR primer sequences used for the cloning.

8.1.2 Sequence of DNA constructs
8.1.2.1

pShuttle-CMV-EGFP-polyA

Sequence of pShuttle-CMV-EGFP-polyA (8,203 bp). The map is shown in Figure 2.3.
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AAAAGGATCT  CAAGAAGATC  CTTTGATCTT  TTCTACGGGG  TCTGACGCTC  6150
AGTGGAACGA  AAACCTACGCT  TAAAGGATT  TGGTGATGAG  ATTATCAAA  6200
AGGATCTCCA  CCTAGATCCT  TTTAAATTTAA  AATGAAAGTT  TTTAAAATCT  6250
CTAAAGTATA  TATGAGTAAA  CTGTGCTGTA  CAGTTAAGGA  TGCTTAATGA  6300
GTAGGCGACC  TATCTACAGG  ATCTGTCTAT  TTGTTCTACG  CATAGTTGCC  6350
TGACTCCCCG  TCGTGTAAGAT  AACCTACGATA  CGGGAGGGCT  TACCATCTGG  6400
Appendix

CCCATGCTT GCAATGATAC CGCGAGACC CCAGCTCACCG GCTCCAGATT 6450
TATCAGCAAT AAACCAGCCA GCCGGAAGGG CCGAGGCGAG AAGTGCTCTT 6500
GCAACTTTAT CGCCCTCAT CATCGTCTATT AATTGTGGCC GGAAGCTAG 6550
AGTAAGTGTG TCAGGCTGTGA ATAGTCTGCC CACGTTGTT GCATTGGCTG 6600
CAGCCATGAG ATTATCAAAA AGGATCTTCA CTCAGTCTCT TTTCACTAGT 6650
AAAGCCAGCT CGCAGAAACG GTGCTGACCC CGGATGAATG TCAGCTACTG 6700
GGCTATCTGG ACAAGGGAAAA AGCGAAGCAG AAAGAGAAAAG CAGGCTGCTT 6750
GCAGTGGGCT TACATGGCGA TAGCTAGACT GGGCGGTGTT ATGGACAGCA 6800
AGCGAACGGG AAATTGCGAC TGGGCCGCC TCTGTTAAGG TTGGGAAGCC 6850
CTGCAAAAGTA AACTGGAGTG CTTCTCTGCC GCAAGGATTC TGATGGCGCA 6900
GGGATCAAG CTCTGATCAGA GAGACAGGAT GAGATGCTTT TGCGAATGAT 6950
GAACAAGATG GATGTCAGCG AGTTTCTCCG GCCGCTGGTG TGGAGAAGCT 7000
ATTCCGCTAT GACTGGGCAC AACAGACAAT CGGCTGTCTT GATGCGCGCG 7050
TGGTCCCGCT GTCAAGCGCG GGGCGGCCGG TTCTTTTAT GAAGACGCCA 7100
CTGTCCGCTG CCCTGAATGA ACTGCAAGAC GAGGACACGC GGCTATCGTG 7150
GCTGGCCAGG ACGGGCGTTC CTGGCGCAGC TGTCGCGAG TGTCGCTACTG 7200
AAAGCGGAGG GAGCTTGGCT CTTATTGGCGG AAGTGCGGCG GCAGGATCTC 7250
CTGTCATCTC A CCTTGCCTCC TGGCGAGAAA GTATCCATCA TGGCTAGTG 7300
AAATGGGGCC CTGGATACCG TGGAAGCGGC TACCTGCACA TGGAGGCACC 7350
AAACGAAAAC TCCCATCGAC CCAGACAGTA CTCCGATCCA AGCCGCTCCT 7400
CTGCAATCAG ATGATCTGGA CGAAGGACAT CACGGGGCTCC GGCGCAGC 7450
ACTGTCTGCC AGGCTCAAGG CGAACGATGCC CGACGCGGAG GATCTCTGCG 7500
TGACCCTATG CGATGCCTGC TTGCGCAGATA TCTATGGTGA AAATGGCAGC 7550
TPTTCTGGAT TCACTGGACTG TGAGCGGCCTG GGTGTTGCAG ACCGCTATCA 7600
GGACATAGCC TTGGCTACCC GTGATATTGC TGAAGAGCCT GCCGCGCAAT 7650
GGGCTGACC CGCTGTCTTG CTTTACGTTA TGGCCGCTCC GATGCGCAG 7700
CGCATCGGT CTCATGCGCT TCTTGACGAG TTCTTCTGAA TTTCGTTAAA 7750
ATTTTTGTTA ATATCGCTCA TTTTTTAAAC AAATGGCAGA AATCGCGGACC 7800
ATCCCCATATA AATCAAAGAG ATAGACGCGT ATAGGTTGGA GTGTTGTTCC 7850
AGTTTTGGAAC AAGATCCTAC TATTTAAGAA CGTGAGCTCC AAGCTCAAAG 7900
GGGAAAGAAA CGTCTATCGA GCGATGGGCG CACTACGTGA ACCATCACC 7950
TAATCAAGTT TTGTGTTGGTC GAGGTGCGGT AAAGATCTAA ATCGGAACCC 8000
Appendix

8.1.2.2 pShuttle-Runx2-MinPro-EGFP-polyA

Sequence of pShuttle-Runx2-MinPro-EGFP-polyA (8,004 bp). The map is shown in Figure 2.4.


```
TAAGGAGGAC CCCCGATTTTA GAGCTTGACG GGGAAAGCGG GCGAACGTTGG 8050
CGAGAAAGGA AGGGAAGAAA GCGAAAGGAG CGGGCGCTAG GCCGCTGGCA 8100
AGTGTAGCGG TCACGTGCAG CTGATACCAC ACCCCCGCGC GCTTAATGCG 8150
CCGCTACAGG GCGGTCCCAT TGCACCCATCA GGAATGAATT AATTCTTAAT 8200
TAA 8250
```

```
CATCATCAAT AATATACCTT ATTTTGACAT GAAGCGAAAT TGATATTGAG 50
GGGTTGGAGT TTGTGACGTT GCCTGACGGG TGGGAACGGG CCGGGTGACG 100
TAGTATGTGT GCGGAAGTGT GATGTGACCA GTGTGCGGGA ACACACGTA 150
CCGACCGGATG TGGGAAGAGT GACGTGGTTT GTGTGCACGG GTGTACAGC 200
GAAATGACAA TTTTACGGGCG ATTTTAGCGG GATGTTGTAG TAAATTTGGG 250
CGTAAACGAG TAAGATTTGG CCATTTTCGC GGGAAACTG AATAAGAGGA 300
AGATGAAATCT GAAATAATTT GTGTACTTCA TAGCCTGTAAT TACTGTACCC 350
CCGGCCGCCT CGAGTTCTAGA GATATCGCAAT TCCTGCAGCC CGGGGATCC 400
GCCCTTGCAG CATGCCATGG ACCCAAACC ACCACTCGGGA CCACAAACCA 450
CACCTCGGAC CAACAAACCAC AACTCGGGACC ACAAACACA CTCGGGTCAG 500
TGCCGACATG CCATGGACCA CAACACCAAC TCCGGGACAC AAACACACT 550
CGGACCACA AACCACACTC GGGACCACA ACCACACTCG GGACCACAAA 600
CCACACTCGG GACCACAAAC CACACTCGGG ACCAAACAAA AACTCGGGA 650
CCACAAACCA CACTCGGTC GCCTGGGAGT GACCTAGGAAG TGGAAACCTA 700
TCCATAAGTG ATTTTCTACC ATTGAAACC TATAAGATAG AAGGTAATCT 750
CTGCGGAAGG ACAACAGTCC CAGCGCATCA CATTCAATG TAGAAACCAT 800
GGTGAGCAG GCCGAGGAGC TGTCACCCGG GGTGGTGCCC ATCTGCTGTCG 850
AGCTGGACGG CGACGTACAA GCACCAAGGT TCGCGTGTCC CGGGAAGGSC 900
GAGGGCGATG CCACCTACGG CAAAGCTGACC CTGAAGTCCT CACTGCACCAC 950
CGGCAAGCTG CCCGTGCCTT GCCCACCCTT CGTGACCACC CTGACCTACG 1000
```
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CCGTGCAGTG  CTTCAGCGCC  TACCCGGACC  ACGTGAAGCA  GCACGACTTC  1050
TTCAAGTCGG  CCATGGCCGA  AGGGCTACGC  CAGGACCGCA  CCATCTCCTT  1100
CAAGGAGCGC  GGCACACTAC  AGACCCGCCG  CGAGGTGAAG  TTGAGGGGCG  1150
ACACCCTGTG  GAACCGGACTC  GAGCTGAAGG  GCATCGACTT  CAAGGAGGAC  1200
GGCAACATCC  TGGGGCACA  GCTGGAGTAC  AACTACAACA  GCCACAAACGT  1250
CTATATCATT  GCCGACAAGGC  AGAAGAAGGG  CATCAAGGTG  AACTCTCAAG  1300
TCGGCCACAA  CATCGAGGAG  GCGACCGGTG  AGGCTGCGGA  CCACATCAGG  1350
CAGAACACCC  CATCGGCAGA  CCGCCCGCGT  CAGCTGAGCCG  ACAACACTA  1400
CCTGAGACCC  CAGTCCGCCC  TGACGAAGAA  CCCCCACGAG  AAGCAGGATG  1450
ACATGCTGCT  GCTGGAGTTC  GTGACCAGCC  CGGGATACAC  TCTCGGCATG  1500
GAGGACGTGT  ACAAGTAACT  GATATCAGAA  CACCCGATCC  TAGATAACTG  1550
ATCATATCAA  GCCATACCA  ATTTTGAGAG  GTTTTACTTG  CTTTAAAAA  1600
CCCTCCACAC  CTCCCCCTGA  ACGTGAAACA  TAAAAATGAT  GAATTTGTGA  1650
TTGTTAAACTT  GTTTATGGCA  GCTTATAATG  GTTACAATAA  AAGCAATAGC  1700
ATCACAATATT  TCAAAATAAA  AGCAATTTTT  TCACCTGCAAT  CTAGTTGTGG  1750
TTTGTCAAAA  CTCACTCAATG  TATCTAAGCG  GGATCTGGG  CGTGGTAAGA  1800
GGGTTGGAAAG  AATATATAAG  GTTGGGGCTT  TATGTAGTTT  GTATCTCTGT  1850
TTGAGACGAC  CGCCGCGGCC  ATGAGACCAA  ACGTGTTCGA  TAGGAGCATT  1900
GTGAGCCTCAT  ATTTGACAAC  GCGCATGCCC  CCATGGGCGG  GGGTGCGCTA  1950
GAATGTGATG  GGCTCCAGCA  TTGATGGCTG  CCCGGTCCTG  CCCGCAAACGT  2000
CTACTACCTT  GACCTACGAG  ACCGTGCTG  GAAGCCGTTT  GGAGACTGCA  2050
GCCTCCGCGG  CGCGTACGCG  CCGTCGAGCC  ACGCCCGCGC  GGATTGTGAC  2100
TGACTTTTCT  TTTCCTGAGCC  CCCTTGCAAG  CAGTGCGACT  TCCGGTTAC  2150
CCGCCCGCGA  TGCAAGGTTT  ACGGTCTTTT  TGGCACATT  GGATTTTTGG  2200
ACCCGGGAAC  TTAATGTCGT  TTTCCTGAGA  CTGTTGGATC  TGCGCCAGCA  2250
GGTTTTCTCC  CTGAGGGCTT  CCTCCCTCTC  CAATGCGTTT  TAAAACATAA  2300
ATAAAAAACC  AGACTCTGTT  TGGATTGGGA  TCAAGCAAGT  GTCTTGTCTG  2350
TTTATTTTAG  GGGTGGTCG  CGCCGCGTAG  CCCCGGGACC  AGCGGTCTCG  2400
GTCTGTGAGG  GTCTCTCTGA  TTTTTTCCAG  GACGTTGGAA  AGGTGACTCT  2450
GGATGTTCAG  ATACATGGGC  ATAGCCCGGT  CTCTGGGGTG  GAGGTAGCAG  2500
CACTGCAGAG  CTTCATGCTG  CGGGTGCTTG  TTGTAGATGA  TCCAGCTGTA  2550
GCAGGAGGCG  TGAGCGTGTTG  GCCCTAAAAT  GTCTTTGAGT  AGCAAGCTGA  2600
Appendix

TTGCCAGGGG CAGGCCCTTG GTGTAAGTGT TTGACAAACG GTTAAAGCTGG 2650
GATGGGTGCA TACGCTGGGGA TATGAGATGC ATCTTTGGACCT GTATTTTTAG 2700
GTTGGCTATG TTCCCAGCCA TATCCTCTCG GGGATTCATG TTGTCGAA 2750
CCACCAGCAC AGTGTATCCG GTGCACTTGG GAAATTGTTC ATGTAGCTTA 2800
GAAGGAAATG CGTGGAAGAA CTTGGAGACG CCCTTTGTGAC CTCCAAGATT 2850
TTCCATGCAT TCGTCCCATAA TGATGCAAT GGGCCACGG GCCGGGCGCT 2900
GGGCGAAGAT ATTTCTGGGA TCACTAAGCT CATAGTTGTG TTCCAGGAAG 2950
AGATCGTCAT AGGCCATTTT TACAAGACGC GGGGGAGAGG TGCCGAGACT 3000
CGGTATAATG GTCCTTATCG GCCCAAGGGA GTAGTTAACC TCACAGATTT 3050
GaATTTCCCA CGTTTGTAGT TCAGATGTGC TACCTCGGCG 3100
GCCATGAAAG AAACGTTTTC CGGGGTAGG GAGATCAGCT GGAAGGAAG 3150
CAGCTTCTCG AGGACCTCCC ACTTACCACA CCGGTGCGGC CGTAAATCA 3200
CACCTTATTAC CGGGTCAAC TGGTAGTTAA GAGAGCTGCA GCTGCGCTCA 3250
TCCCTGAGCA GGGGGGCCAC TTCGGAAAGC ATGTCTCCTGA CTGGCATGTT 3300
TTCCCTGACC AAATCCGCCA GAAGGCGCTC GCCGCGCAAG GATAGCAGTT 3350
TTGCGAAGGA AGCAAATTCT TTCAACGGTT TGAGACCGTC CGCGGTAGGC 3400
ATGCTTTTGA GCCTTGTGCC AACGAGTTCC AGGCGTCTCC AACGCTCGGT 3450
CACCTGCTCT AGGCATCTCT GATCCAGCAT ATCTCTCTCTG TTGGCGGCTT 3500
CGGGCGCATT TGCCTGTACG GCAGTAGTGC GTGCTCGTCC AGACGGGCCA 3550
GGGTCACTGC TTTCRCCGCG GCCAGGCTTT TGTCAGCGCT AGTCCTGGGC 3600
ACGCTGAAAG GGTGCCGCTC GCCGCTGCGG CTGGCAGGGG TGCGCTTGA 3650
GCTGGCTCTG TGTTGTGCTGA AGCGCTGCCG GTCTTGCGCC TGCGGTGGG 3700
CCAGGTAGCA TTTGACCATTG GTGTCATAGT CCAGCCCTTC CGCGCGCTGG 3750
CCCTTGCCGC GCACCTGCCG TTGGCAGGGC CCCTGGCCACC AGGGCGACTG 3800
CAGACTTTTG AGGCCGCTAGA GCTTGCGGCG GAGAAAACTCC GATTCGCCG 3850
AGTAGCATTG GCAGCCCGAG GCCCGCGAGA CGGTCTCGCA TTCCACGACC 3900
CAGGTTGACT CTGGCCGGTTG GGGGATAAAA ACCAGGTTCG CCCCATGCTT 3950
TTTGGATGCGT TCTTATCCCTC TGGTTTCCAT GAGCCGCTGT CCACGCTCGG 4000
TGACGAAAAG GTGTCGCGTG TCCCCGTATA CAGACTTGGG AGGGAGTTTA 4050
AACGAAATTC ATAGCTTTGT GCATGGCGCG CATATAAAAA TGCAAGGTGC 4100
TGCTCAAAAA ATCAAGGCAA GCCTCGCGCA AAAAGAAAAG CATACGTAGA 4150
TCATGCTCAT GCAGATAAAG GCAGGTGACG TCCGGAACCA CCACAGAAAA 4200
AGACACCATT TTCTCTCAAA ACATGTCTGC GGGTTTCTGC ATAAACACAA 4250
AAATAAAATAA CAAAAACAATT TTTAACATTG AGAGCACTGT CTTCACACAG 4300
AAAAACACAC CCTTATAAACG ATAAACACGGA CTACGGCAGTT GCCGCCGCTGA 4350
CGTAAAAA AACTGTCACC GTGAIATAAAA AGCACCACCG ACAGCTCCTC 4400
GGTCACTGGG GGAGTCTAAA GTGAAGACTC GGTAAACACA TCAGGTTGAT 4450
TCATCGGCTCA GTGCATAAAAA GCGACCGAAA TAGCCCAGGG GATAACATAC 4500
CCGCAGGCCGTT AGAGACAAACA TTTACTAGCCCA CATAGGAGGT ATAAACCAAAT 4550
TAATAGGAGAG TAAACACACTGA AAAACCCTTC TTGCTTACGGCG 4600
AAAAATAGCACC CTTCCCCGCTC CAGAACAAACA TACACGCGTT CACAGCAGCA 4650
GCCTAACAGCT GAGCCTTACC ATGAAAAAAG AAAACCTATT AAAAACAAAC 4700
CACTCGACAC GCCACCACGCT CAATCTACCTA GATGTTAAAC AAAGCCGCAAG 4750
TGAGAGACGA GTATATATAG GAACAATAGAA TGACTCTAGACA GTTAAAGTCCC 4800
ACAAAAACAAC CCAAGAAACGC CGACCGGAAA CCTACGCCCA GAAACGAAAG 4850
CCAAAAAAC ACCAACTTCC TCAATACGTCA ACTTTCTGGGT TCCCCAGTTA 4900
CGTAACCTCC CATTCTTAAGA AAACCTAACAT TCCCAACACA TACAGTCTAC 4950
TCCCGCCTCAA AACCTCTAGTCA ACCGGCCCCG TTTCCAGCGC CGGCCACACG 5000
TAACAAAACTC CACCCCCCTCAA TTATCATATT GGCTTCAATCC AAAATATAAGG 5050
TATATTTATAG ATGATGTTAAA TTAACATGCA TGGATCCATA TGGGTTGTGA 5100
AATACGCAGC AGATCGGTAA GGAAGAATAA CCACACAGGG CGCTCTCCCG 5150
CTTCCTCGCT CACTGACTCG CTGGCGTCCGG TGTTGCGCTGT GCCGCAGCCG 5200
GTATCGAGTCA ACTCAAAAGGC GTGAAATCAGG TTATCCACAG AATCAGGGGA 5250
TAAGCCAGGA AAGAACATTGAT GAGCAAAGGG CCCAAGAAAG GCCAGGAACC 5300
GTAAAAAGGC CGGTGGTGCT CGGGATTTCAC ATAGGCTCAG CCCGCCGTGAC 5350
GACCATCAAA AAAATCGAGG CTCAGTCAAG AGTGGCGGAA ACCCGACAGG 5400
ACTATAAAGA TACCAGCGGT TTCCCTCGTGG AAGCCTACCT GTGGCTCTCTC 5450
CTTGTTCGAC CCTGCCGTTT ACCCGATACC TGGGCCGCTTT TCTCCCTCTG 5500
GAAACGGTGG CGTGTCTTCA TAGCTACGCG TGGATGTATC TCAGTTGCTGT 5550
GTAGGTCGTT CCGTCAGAAC TGSGGCTGTGT GCAGAAACCC CCCGTCCAGC 5600
CCGACGCTGG CGCTCGTATCC GGTAACCTAC GTCTTGAGCT CAAAGCGTGTA 5650
AGACAGCGACT TATTGCGCACT GCCAGCGCGC ACTGGTAAACA GGATAGCAGG 5700
AGCGAGGATG TAGGGCGGATG CTACAGAGTT CCTGAACTGG TGGCCTAATC 5750
ACGGTACACT TAGAAGGACA GTATTGGTAA TCTCGGCTCT GCTGAAGCCA 5800
GTTACCTTCG GAAAAAGAGT TGGTAGCTCT TGATCCGGCA AACAACCAC 5850
CGCTGGTAGG GGTGGTTTTT TTTTTGTGCA GCACGAGATT ACGGCGAGAA 5900
AAAAAGGATC TCAAGAGAAT CTTTGTACTT TTTCTACGGG GTCTGACGCT 5950
CAGTGAAAGC AAAAACTCAGC TTAGGGGATT TGAGGGCTGA ATATTACA 6000
AGGATCTTC ACCTGATCC TTTTAAATTA AAAATGGAAT TTAAATGCA 6050
CTTAAAGATG ATAGCAATGA ACTTGGTGTCG AGAATTAACG ATGCTTTAAT 6100
ATGGAGGGAC CTATCTCAGC GATCTGCTAA TTTGTTTGTG CCAATGTTGG 6150
CTGACTCCCG GTGTTGTAAG TAACTACAGT ACAGGGAGGC TTACCATCTG 6200
GCCCAGTGG TCGAATGATA CGCGAGACC CAGCCTGACC GCCTCCGATT 6250
TTATCGACAA TAAACCAGCC AGCCGAGGGG GCCGAGCGCA AGATGGGTCC 6300
TGAAACTTTA TGCGCGCTTCA TCCAGTCTTTA TAAATTGTGG CGGAGAAGTA 6350
GGATTAATAG TGCCGCCATT AAATAGTTTG CCAAGGCTG TGGGATTCTG 6400
GCAGCCATGA GATTATCAAA AAGGATCTTC ACCTAGATCC TTTTCAGCTA 6450
GAAGGCCAGT CCGCAAGAAG GGTGTCTGACC CCGGATGAAT GTCAAGCTCT 6500
GGGCTATCGG GACAAGGAAA AACGCAAGCG CAAAGAGAAA GCAGGTAGCT 6550
TGAGTGGGCT TTACATGGCG ATAGCTAGAC TGGCCGGTGT TATGGACAGC 6600
AGGCGAACCC GAATGGCGAG CTGGGGCGGC CTCTGTGGAAG GTGGGAAAGC 6650
CTGCAAAAGT AAACCTGATG GCTTTCTTGC CGCCAAGGAT CTGATGGCCG 6700
AGGGGATCAA GCTCTGATCA AGAGCAGAGA TGAGGATGTT TCAGCATGTA 6750
TGAACAAGAT GGAATGCACG CAGGTCTTCC GGCAGCTTGG GTGGAGAGGC 6800
TATTGGCTAT TGACTGGCCA CAAACAGACAA TGAGCTGCTC TGATGCCGCC 6850
GTGTTCGGGC TGATCGAGCA GCCCGGGCGC TTCTTCTTAC TGAAAGACGA 6900
CCTGCTCGGT GGCTCTGATG AACTGCAAGA CGAGGCAGG CGCTATGCTG 6950
GGCTGGCCAC GACGGGCATT CCTTGCAGCA CTGTGCTGCA CTGTGCTGCA 7000
GAGCGGGAAA GGGACTGGCT GCTATGGGCC GAAGTGCGGG GCCAGGATGCT 7050
CCTGTCACTT CACCTTGCTC TGCCGAGGAA AGTATCACA ATGGCTGATG 7100
CAATGCCGGC GCTGGATGAG CCATTGCTCG CTAACCTGCCC ATTCGACCAC 7150
CAAGCGAACCC ATCGCATCGA GCGAGCAGGT ACTGGATGGA AAGCCGGTCT 7200
TGCCATCACG CATGATCTGG ACACAGAGCA TACGGGGCTC CGCCAGCCCG 7250
AAGGGTTCGC CAGGCTCAAG GCGAGCATGC CGAGGGGCAG GGATCTGCTG 7300
GTGACCCCATG GCGATGCTTG CTTGCGGAATT ATCATGTTGG AAAATGGCCG 7350
CTTTTCTGGA TTCATGCCGCT GTGGCCGGCT GGTGTGCGGC GACCCTATAC 7400
8.1.2.3 pShuttle-HoxA9-MinPro-EGFP-polyA

Sequence of pShuttle-HoxA9-MinPro-EGFP-polyA (7,952 bp). The map is shown in Figure 2.5.

The sequence of pShuttle-HoxA9-MinPro-EGFP-polyA is identical to the one from pShuttle-Runx2-MinPro-EGFP-polyA (appendix 8.1.2.2), except for the 12xRunx2 binding site that was replaced by the 12xHoxA9 binding site (length: 214), which is shown in the following.

TTAATAAAAA TGGCATATATT AATAAAAATTG CATATATTTAA TAAAATTTGCA
TATATATAA AAATGCATA TATATATTTAA ATTCATATA TTAATAAAAA TGGCATATATT
AATATAAAAATG CATATATTTAA TAAAATTTGCA TATATATTTAA AAATGCATA
TATATATTTAA ATTG

8.1.2.4 pShuttle-Vezf1-MinPro-EGFP-polyA

Sequence of pShuttle-Vezf1-MinPro-EGFP-polyA (7,905 bp). The map is shown in Figure 2.6.

The sequence of pShuttle-Vezf1-MinPro-EGFP-polyA is identical to the one from pShuttle-Runx2-MinPro-EGFP-polyA (appendix 8.1.2.2), except for the 12xRunx2 binding site that was replaced by the 12xVezf1 binding site (length: 214), which is shown in the following.

AGGACATAGC GTTGGCTACC CGTCGATATGC CTTGAAGACCT TGCGGCACGA 7450
TTGGCTGACC GCTTGGTGCT CTGGTTACGTG ATCGGCGTTC CCGATCGGCA 7500
GCCGATCGCC TTCTATGCCCT TTCTTTCAAGA GTTCTTTCTGA ATTGTGTTAA 7550
AAATTTTGTG AAATCGAGTC ATTTTTTAAAC CAATAGGCGG AAATCGGCAAA 7600
CATCCCTTTAT AAATCTAAAG AATAGACCCG GATAGGTTTG AGTGTGTGTC 7650
CAGTTTGGA CAAGAGTCCA CTATTTAAGA AGCTGGACTC CAACGTCAAA 7700
GGGCGAAAAG CGCTCTATCA GGGCGAGTGC CCACTACGTC ACCACATACC 7750
CAAATCAAGT TTTTGGCCTCC CAGGTCCCG TAAAGCTCTA AATCGGACCC 7800
CTAAAGGGAG CCCCGACATTG AGAGCTTGAC GGGGAACGGG GGGCAAGTG 7850
GCCGAGAAGGG AAGGGAGAAGGA AGCGGAAGGA GGGCGCTGCTC GGGCGCTGCG 7900
AAGTGTAGCG GTCAACGCTGC GCGTAACCCAA CACACCCCGG CGCTTAATGC 7950
GCCGCTACAG GGGCGCTCCA TTCGCCATTTCA AGGATCGAAT TAACTTTTAA 8000
TTAA

8050
Appendix

8.1.2.5  pCBG68-12xRunx2-MinPro-control vector

Sequence of pCBG68-12xRunx2-MinPro-control vector (5,429 bp). The map is shown in Figure 2.7.

12xRunx2 binding site: 54 – 289, length: 236; Minimal Promoter: 290 – 425, length: 136;
CBG68luc: 461 – 2089, length: 1629; SV40 polyA: 2137 – 2358, length: 222

TTACCCCCAC TCCATATATT ACCCCCCACTC CATATATTAC CCCACTCCA
TATATTTCCC CCACTCCATA TATTACCCCA ACTCCATATA TTACCCCCAC
TCCATAAGCT TATTACCCCA ACTCCATATA TTACCCCCAC TCCATATATT
ACCCTACTAC CATATATTAC CCCACTCCCA TATATTACCC CCACTCCATA
TATTACCCCA ACTC

GGTACCGAGC TCTTACGCGT GCTAGCCCGG GCTGAGCAT GCCATGGACC 50
ACAAACACCAT CTCGGACAC CAAACACACAC TCCGGACACAC AAAAAACAACT 100
CGGSAACACA AACACACTTC GGGTCCGTTG GACATGACCA TGGACACAAA 150
AACCACACTCC CGACACAAAC CAACACCTCGG GACACAAAC AAAAAACAACT 200
AACCACAAACC AACTCGGAGG CCACACACAA CACACGGGAC ACAAACACAA 250
ACACCGGACC AAAAAACACCA CTCGGACCA CAAACACACAC TCGGCTCGGG 300
GGGGATGAC TAGAAATGGG AAACCTATCC ATAAGTGAAT TCTCACCATT 350
GCAGGCTAT ATAAAGTGAAAG TGAATCTCTG CGGAAGACA ACATCCCCCA 400
GGCATCACC CTTCAATCTAG AAACCAAGCT TGGCATTCCGG GTACTGTGGG 450
TAAAGCCACC ATGGTGAACA CCAGAAAGAA CGTGAATCTAC GGCCAGAAA 500
CAGGCATCC ACCGGGAGAAG CACACGCTGC GTGAGATGCT CTCACGACGA 550
CCTGTAAACC ATAGTCGACT CCTCAGACGA CTCGTTAGAC TGCTGGAGAA 600
CGAGGCTTC TACTCAGCGG AATTTTTCGA AGTACTGTG CTTGTTGGCCS 650
AAAGCCTCCA TAAATGCTGGG TACAAATGA ACGATGTGTT GAGCATTTGT 700
Appendix

GCTGAGAATA ACACCGGCTT CTCTTATTCCT GAAATGCTGT CTTGGTACAT 750
CGGCAATGATT GTGGCCCTCTG TGAATGAATC TTACATCCTCA GATGAGCTGT 800
GTAAGGTATT GGTATTAGAC AAACCTCAAA TGCTCTTTTAC TACCAAAAC 850
ATCTTGAAATA AGGCTTTTGGAA GTGCCAGTCTT CGTACTAACT TACATCAACG 900
CATCATTATT CTGGTACAGT TCAGAAACAT CCACGGCTGT GAGGCCCTCC 950
CTAACCTTCT CTCGCGTTTAC AGGCGAGTTA ATATCGTAAA TTTCAAGCCC 1000
TTGCATTTTG ATCCAGTTCGA GCAAGTGAGCT GTCTATTCTT GTCTCCTCCG 1050
CACACCTGCT TTGCTCAAAAG TGTCTCATGCA GACTCACCAG AATATCTGTG 1100
TGCGTTTGAT CCACCTCTCT CACCCTCTGT TGGGTACTCA ATPTGATCCCT 1150
GCGGTGACTG TGCCGTTGAT TATGGCTCCTT TATCCACGCTT TGTGTTCCTC 1200
TATTACCTGT GGCTATTTCAG TGGTCGCGTT GCGGTGCATC ATGTTTCGTC 1250
GCTTCGACCA AGAACCCCTCC TTGAAGGCTTA TTCAAGACTA CAGGTTGCTG 1300
TCGGTGATCA ACGTCCCTTC AGTCATTGTTG TCTCTGAGCA AATCTCCTTT 1350
GGTTGACAAG TATGACTCTGA GCAGCTTGGC TGAGCTGATG TGGGCGCTG 1400
CTCCTTTGCGCAAAGAAGTGG CCGGAGGTGCG CTGCTAAAGCG TCTGAAACCTC 1450
CCTGCGATCC GCTCCGGGTTTT GATTCTGACT GAGAGCCTTT CTGCTAACAT 1500
CCATACGCTTC CAGACGAGT AATGCTCTGG TAGCTCGGCTG CGCGTGCATC 1550
CTCTTATGCC TCGAAGAGTC GCAGAGCGTG AGACCGCCAA AGCATCGGCG 1600
CACAATCGAG TCGGTGAATT GTGTATTAAAG GGGCTATGGG GCTCTAAAGG 1650
CTACGGACG AATGTTGAGG CGACTAAAGA AGCCATTGAT GATGATGGCT 1700
GGCTCCATAG CGCGCAACTTC GGTTACTATG ATGAGGAGCA ACATCTCTAT 1750
GTGGTGCGATC COTACAAGAGA TPTGATTAAG TACAAGGGCT CTCAAGTTCGC 1800
ACCAGCCGAA CTGGAGAGAA TTGTCCTGAA GACCTTTTGT ATCCGCGACG 1850
TGGCCGGTGTG GGGTAACCCA GACTTGGAAG GTGGCGAGTT GCTAGCCGC 1900
TTTGTGGTGA AACAACCGGG CAAGGAGATC ACTGCTAAGG AGGCTCTAGA 1950
CTATTGCGGC GAGGGCCTGT CTGACACCAA ATATCTGCGT GCGGGCGTCC 2000
GCTGCCGCTG ATTCTTTCAG CGCAACGTTA CGGTAAGAT CACTCTTAAA 2050
GAGTTGCTGA AGCAGCCTCT CGAAAAAGCT GCGGCTAGT AAAGCTCTCA 2100
TGATATCTCT GAGTCGGGGGC GGCGGGCGGC TGGACGGAAA CATGATAAGA 2150
TACATTGATG AGTGGTAGGAC AACCACAATG AGAATGCAGT GAAAAAAATG 2200
CTTTATTTGTG GAATTTTTGAT ATGCTATTGGA TTTATTTAGA ACCATATAA 2250
GCTGCAATAA ACAAGTTAAC AACAACAAATG GCATTCAATT TATGTTTCAG 2300
Appendix

GTTTACGGGGG AGGTGTTGAGC GGGTTTTTAA AGCAGGAAGT ACTCTTACCA 2350
ATGTGGTAAC ATCGTAAAGG ATCTTGAACCA TCGAGCGAGG AATGCCGGGA 2400
ACTGGCGGCA GTAGGGGGCG GGATGGGCGG AGTGTAGGAA GGGACTATGG 2450
TTGCTGACTA ATTGAGATGC ATGCTTTGCA TACTTCTGCC TGCTGGGAGG 2500
CCTGGGGACT TTCCACACCT GGTGGCTGAC TAGATGAGAT GCATGCTTTG 2550
CATACCTTCTG CTTCTGCGGG AGCCCTGGGGA CTTCCACACAC CTAACCTGAC 2600
ACACATATTCA CAGCGGAGTCC GTGCAGGATTC GCCCTTGAGA GCTTCTACCC 2650
CAGTCAGCTC CTTCCGGTGG GCGCGGGGCA TGACTATGCTT GCAGCGACCTT 2700
ATGACTGTCAT CTCTTTTCTAT GCAACTCGTA GGACAGTGGC CGCGACCGCT 2750
CTTCCCGCTTC CTCTCGCACT GACTCGCTGC GCTCGGCTGT TCGGTGCGGG 2800
CGAGGGTAT CAGCTCTACTC AAAACCGGTA ATAGCTTATAT CCAGACAAATC 2850
AGGGGATAAC CGAGGAAAGA ACACTGACGC AAAGCCCAAGG CAAAACGGCA 2900
GGAACCGTAA AAAGGCGGCAG TGGCTGCGGT TTATCTCATAG CACTCCGGCC 2950
CCATGCAGGC ATCAAAAAAA TCGACGGCTA ATCGAGGTTT GCGGAACCCC 3000
GACAGGACTA TAAAGATACCC AGGCGTTTCC CCGTGGAAAT CTCCTCATGC 3050
GCTCTCCTGT TCGCACCCTGG CGCCTTACCG GATACCTGTCC GCCTTTTCTC 3100
CTTCCGGGAA GCCGCTGCGCT TCTCTCATAGC TCAGCAGCTAT GGTATCTCAG 3150
TTGGGTGTAG GTGCTTTCGCT CCAACGCCTGG CTGGTGGCAGC GAACCCCGGC 3200
TTCAGCCGGCA CCGCTCGGCCC TTATCCGGTA ACTATCGTCT TGAGTCAAAC 3250
CCGCTAACAG AGCAGTTATAC GCCACTGGCGA GCACCCACTG TGAACAGGAT 3300
TAGCAGAGCG AGGTATGTAG CGCGGTGCTAC AGATTCTTGG AAATGCGGAGG 3350
CTAACCTAGG CTTACATAGA AGAAACAGTAT TTGGTATCTGC CGCTCTGCTG 3400
AAGCCGTTTA CTTCCGAAAA AGAGCTTTGG AGCTTTGTAT CGCGCAAAAC 3450
AACCAACCGCT GTAGGGGGTG GTTTTTTTTT TTGCAAGCAG CAGATTACGG 3500
GCAGAAAAAA AGGTATCTCAA GAAAGCTCCTT TGATCTTTCT TTCGGGCTCT 3550
GACGCTCAGT GGAACGAAAA CTCAAGTTAA GGATTTTTGG TCTGAGATT 3600
ATCAAAAAAG ATCTTCACCT ATGATCCCTT AAATAAAAAA TGAAGTTTTA 3650
AATCAATCTA AGATATATAT AGTGAAGCTT GGGCTGACAG TTACACATGC 3700
TTACATCATG AGCGACCATAG CTCAGCGACG TGCTATTTTT TTCGATCCAT 3750
AGTTGCCCTG CTCCCCGCTCG TGTAGATAAC TAGATACGGAG GAGGGCTTAC 3800
CATCTGGCAG CAGTGCTCAGA ACGTAAACGG GAGGCCCAGC CTCACCAGGCT 3850
CCAGATTTAT CAGCAATATAA CCAGCCAGCC GGAAGGGGCG AGCGCAGAGA 3900
Appendix

TGCTCCTGCA ACTTTATCCG CCTCCATCCA GTCTATTAAT TGTGGCCGGG 3950
AAGCTAGAGT AAGTAGTTCCG CCGTTAAATA GTTTGCCGAA CTGTTGTTCC 4000
ATTGCTACAG GCATCGTGGT GTCACGCTCC TGGTTTGGA TGGCTCATT 4050
CAGCTCCGGT TCCCAACGAT CAAGCGGAGT TACATGATCC CCCATGTTGT 4100
GCAAATAGAC GATTAGCTCC TTCCGGTCTTC CGATCGTGTTG CAGAAGTAAG 4150
TGGGCCGCAG TGTATACACT CATGTTTATG GCAGCAGTGC ATAATTCTCT 4200
TACTGTCATG CCAATCCGTTA GATGTCTTTTC TGGACTGTTG GAGTACTCAA 4250
CCAAAGTCATT CTGAGAATAG TGTTAGCGGC GACCAGAATG CTCTTTGCCG 4300
CCCTCAATAAC GGGATAATAC CCCGCCACAT ACGGAACCTT TAAACGTGCT 4350
CATCATGGGA AAACCTTTCT CGGCGGGAAA ACTCTGAAAG ATCTTACCGC 4400
TGTGGAGATC CAGGTCGATT TAAACCACTC GTGCACCGAA CTGACTTCGA 4450
GCATCTTTTA TTTTCACCAG CGTTTCTGGG TGAGCAAAAA CAGGAAGGCA 4500
AAATGCGGCA AAAAGGGGAA TAAGGCAGAC ACGGAAATGT TGAATACCTA 4550
TACTCTTCTCT TTTTAAATAT TATTTGAAGCA TTTATCAAGG TTTATTTCTC 4600
ATGAGCGGAT ACATATTGGA ATGTATTAG AAAAAAAAC AAATAGGGGT 4650
TCCCGGCACA CTTCCCCGGA AAGTGCCACC TGACGCGCCC TGATAGGGCG 4700
CATTAAGGCG GCCGCGGTGTG GTGGTTACGC GCACGGTGAG GCCTACACTT 4750
GCCAGCGCCC TAGCGCCCGC TCTCTTGCGT TTCTTACCCT CTTTTCTGCC 4800
CACGTTCCGCC GGCATTCCCC GTCAAAGCTCT AAATCGGGGG CTCCCCCTAG 4850
GGTTCCGATT TAGTGCTTTA CGGCACCTCG ACCCAAATAA ACTTGATTTG 4900
GGTGATGGTT CACGTAGTGG GCCRTCAGCC TGAAGAGGCT TTTTTCGGCC 4950
TTTGAGCTTG CAGTCCACGT TCTTTAATAG TGGACTCTTG TTCCAAACTG 5000
GAACAACACT CAAACCCTATC TGGGTCTATT CTTTTGATT TATAAGGGATT 5050
TGCCGATTTC CGGCCTATAG GTAAAAAAT GAGCTGATTG AAAAAAAAT 5100
TAACGCCGAT TTTAAACAAA TATTAACGCT TACAATTTGC CATTACCGAT 5150
TCAGGGTGGG CAAGTTGTTGC GAAGCCCGAT CCGTGCGGCC CTCTTCGGTA 5200
TTACGCCAGC CCAAGCTACC ATGATAGTTA AGTAAATATT AGGTAACCGG 5250
GGTACTTGGGA GCGGCCGCAA TAAAATATCT TTAATTTTAC TACATCTCTG 5300
TTTGAGTTTTT TTTGTGGAAT CGATAAGTAC ACGATAAGCT CTCCACCCAA 5350
ACAAAAACGAA ACAACAAACA CTTGCACCAA AGGCTGCTCC CAGTCCAGTG 5400
GCAGGGTGCCA GAACATTTCT CTATCGGATA 5450
8.1.2.6  pCMV-Myc

Sequence of pCMV-Myc (3,282 bp). The map is shown in Figure 2.8.

CMV promoter: 27 – 549, length: 523

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GAGTTTCGAGC TTGCGATGCCT GCAGGCAGTT TCATATTAA CCGTCGATGG 50
CCCAGCCCTTG TGACAGCGCA ACGACCCCCG CCCATTCAGC TCAATAATGA 100
CTGATGTTG CATTATACG CCAATAGGGA CTTTTCAATG AGCTCAATGGA 150
GGGTAGTTT TACACCAAGC TGCCCATTGG GCAGTACATG AAGTGATATCA 200
TTTCTCAGCT ACACCCCGTCA TGAGCTCAGA TTGGCGCATG 250
GCTGCTACCG TGGGGATGCT ATATAAGCAG AGCTGATTTA GTGACGATGC 300
AGATCGCCTG AGAACGCGCAT CCACGCTTTT TGACCTCATC TAGAAGACAC 350
CGGGACCGAT CCAGCGCTTCG GACTCTAGAG TCGATCTGCA GGCATGCTAG 400
CTTTGCTAA TCAAGCTCTCG AGCTGTTTTA TGGTGAAAT TGTTATCCGC 450
TGACAAATTTC ACACCGATAT CCACCGCCGA GCATATACGT TAAGCTGTCG 500
GATGACCGAT TGGGGATTCT ATATAAGCAG AGCTGATTTA GTGACGATGC 550
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CTTATCCCA CTCGCCAGCAG CCACTGCTAA CAGGATTAGCC AGAGCCGAGGT 1450
ATGTAAGGCGG TGCTACAGGAG TTCTTGAGTGG GTGCGCTAAA CTACGCGTAC 1500
ACTAGAAGGA CAGTATTTTG TATCTGCGCT CTGCTGAAAGC CAGTTACCTT 1550
CGGAAAAAGA GTTGGTAGCT CTGGATCCCGG AAAACAAACC ACAGCCTGGTA 1600
GGGTTGATTT TTTTGGTTCGC AAGCAGCAGA TTACGCCGAG AAAAAGAGGA 1650
TCTCAAGAAG ATCTCTTGTAT CTTTTCTACTG GGCTGCTGAGC CTCACTGGA 1700
CGAAACTCTCA CTTTAAGGGGA TTTGCTGCAT GAGATTATCA AAAAGGATCT 1750
TCACCTAGAT CTTTTTAAAT TAAAATGAA GTTTTTAAAAT CACTAAAGGT 1800
ATATATAGGT AAAACTTTGTC TGACAGTTAC CAAATGCTAA TCAGTGAGGC 1850
ACCTATCTCA GCAGATCTGT TATTTCGTTTC ATCCAGATTG CTGAGACTCC 1900
CCGTCGTGTA GATAACTACG ATACGGGAGC GCTTACCCAT TGCCGCTAGT 1950
GCTGCAATGA TACCCGGAGA CCCACGCCTCA CGGCTCCGAG ATTTAAGCAG 2000
AATAAACCAG CCAGCCGGAAG GGGCCAGAGC CAGAAGTGGT CCTGCAACTT 2050
TATCCGCTCT CATCCAGTCT ATTAATTTGT GCCGGGAAAGC TAGATTAAGT 2100
AGTTCGCCAG TTTAAATTGT GCGCAACGTT GTGCCCATAG CTACAGGGCAT 2150
CGTGGTGTCA CGTCCGCTGT TTTGGTAGGTC TTCATTCGAG TCCGGTCCTC 2200
AACGATCCAAG GCGATTTACA TGATCCCCCA TGTTTGCGAA AAAACCGGTT 2250
AGCTCTCCTCG GTCTCCGAT CGTGCTCAGA AGTAAAGTGG CCGCAGTGTG 2300
ATCACTCATG GTTATGGCAG CACTGCAATA TTCTCTTACT GTCATGCCAT 2350
CCGTAAGATGT TTTTTCTGTG ACTGCTGAGT ACTCAACCAA GTCACTCCTG 2400
GAATAGTGTA TGCCGCGGACG GATGCGCTCT TGCCGCCTCG CCATACGGGA 2450
TAATACGGCG CCACATAGCA AACAATTAAA AGTGCTCATC ATTTGAAAAA 2500
GTCTTCGGGG CGGAAAATCTC TCAAGGATCT TACCCTGGTT GAGATCCAGT 2550
TCGATGTAAC CCACTGCTGC ACCCAACTGA TCTTCGCACT CTTTTACTTT 2600
CACCGCCGTT TCTGTTGGAG CAAAAACAGG AAGGAAAAT GCCGCAAAA 2650
AGGGAATAAG GCAGCACGCG AAATGGTTAA TACTCATACT CTTCCTTTTT 2700
CAATATTATT GAACCATTTA TCAGGGTTAT TGTCTCATGA GCGGATACAT 2750
ATTGGAATGT ATTTAGAAAA ATAAACAATT AGGGTTCCCG CCACATTTAC 2800
CCGAAAAGT GCCACCGTAG GTCTAAGAAA CCATTATTAT CATGACATTG 2850
ACCTATAAAA ATAGGCGGTAT CAGGAGGCCAC TTTGTTCTCG CCGTTTCTCG 2900
TGATGACCGGT GAAAAACCTCT GACACATGCA GCTCCGGGAG AGGGTCACAG 2950
CTTGCTCTGTA AGCGGATGCCC GGAGACAGAC AAGCCGGTCA GGGCCGCTCA 3000
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8.1.2.7 pLV.Ex.Si.P/Puro-Runx2-MetLuc

Partial sequence of pLV.Ex.Si.P/Puro-Runx2-MetLuc (10,490 bp). The full sequence map is shown in Figure 2.10.

The sequence of the \textit{Runx2} promoter (length: 2,514) is shown in the following.

\begin{verbatim}
GCGGGTTTGG CCGGGTGGCTG GGGCTGGCTT AACTATGCAG CATCAGAGCA 3050
GATTTGACTG AGAGTCACC ATATGCCTGT TGAATAACCG CACAGATCGC 3100
TAAAGGAGAA ATACCGCATC AGGCGCCATT CGCCATCCAG GCTGCCGAC 3150
TGTTGGAAG GCGATCTCGT GCGGCGCTCT TGCGTATTAC GCCGGCTGCC 3200
GAAAGGGGGA TGGTGTGCAA GGCATTAAG GTTGGTAAAG CCAGGGTTT 3250
CCCAGTCACG AGCTTGTAAG ACGACGGCCA GT 3300
\end{verbatim}

AACAATACATA AAGGCCACGT TCAGCAACCT CTACTAAAAC TTTGTATCAT
GAGATACAGA CCAATACTCA CAGACACAGG AAATAGGTGT AGGTACCTG
CAAAAACAGAC CATGTGACTA CTCACTCTGAT AATGAGATG GTGGTAATAAT
TTATTCAAAT TCATTTTGA TCAATTTGAA TACCAATATT TATATACCTT
TTTTTTAAAAA AATGTAAGGG AAAATTATT GCAAAAAAAA TTAGGACATA
AAAGACCTGG GCACCTCTAA AGAAAAGCAT TTGCTTACTA TCCTATAGCA
ACTACGCAAA CATCTTCAAC TGCCAGTGC TGCCACTTCT TGTACATATG
GAACATAAGTT CAGAAACTCC ACAAAATTATA TAGACAAAAAC CTTTTTTTTA
TTTACTTTGA ATAATAGAGA TAAAGATCAG ACTGCGACAC TTTATTTATG
AAAGAGGATA ATAGAGTAAC TTTTTCCTCC TCTGATGAA TAAATGCCCT
AAATGAAAAC TTCGTATAAA ATATCTGGTT TACAGTAATA CATGATCTA
GCCTCAAAAA TCAACAAAAA GAATGTATTT CTGTGGTTTT GTCATTAAAA
CTTTATTCTG AAAAATTAAA TAAATAAAC TATGCTCTTG AAAATAAGG
GGTTAAAGGC ATTACCAGTC TTTCCAGTA TATAGAAGAT AATGTCTAA
AGAATCTTAT GACATGATT TCATAGATAA CTTTAACCTA GAGGAAAAAC
AAACAGACAA TGAGTTATTT TGGGTGTAC AGACACAAGA ATATTTTACT
TCTGTCACC TCTAAGTCAC TCTCCTTCTA CGACTGCGT CACCCCAAAT
AATTTCTTGT ATCTCTGTGC CCCCACCCAC CATCAGAGTC ATCCGTGCAA
TGCCACTCTT GGTTACCATC ACAAATACAA AAGATCTAAG ATGCAAAATTC
\end{verbatim}
CAGGAAGCTC TAATCATAAA TGTTGAATTC ACTGCCATGG ATATAATAAC
AGGGATAGT TTCCCCAGAG TGGTTTCATG TTTCTTCTGA ATCAGAATTA
GCAAATCAAG ACGACTAACA TACTCTGTTG GTGTGCATTA TTTCTTCTTA
CACCAAGCAT TTTGTTATTT ATTCCAAGGC TTTCAATTAA AACAACAAAA
ACCTACAGTT TCTCTTAACC CCCCCTATTT GCAGCTATGG AAATTACTGC
ATATTTCATT ATATATGCCG AACTGCACCC AAAGTCTCTG TACAGTCACT
GTCACGCTG ATGAGGAAAT TATACAAAAA ATTTCTTGAG AAGATAAAAA
CATAATCAAC AGAAAACTAA CATTAGCTCA AAAAAATGTC CACCAAAATT
CTGCACATTT GTTTTTIAAG ATCCTCAGAG TAAACATGGA ATGATGGCAA
AAATAATGTA AACGATACTA ATTACATTTA ATCTTTATAG TAAAGAGGCC
CAGTAATAAA AAAAAAAA ATCAACTACA CAGCCATGGT TTAATATTTG
TAAAGGAAATC CCAAGCTCAA CACTTTTGTG ACAGCCAAAT ACGTGATTC
CGATCCCCGG CAAGGAGTGT GCAAGCAGAG CTCTGGAAAG GTAAACTCCT
TTTTACATCT AGTTACAGAT CCCCAGCTTT AGAAAGCACA GCAAAAGGCA
ACAGAAGGGA AGCAAGCCACC CTGGGAAATC CGAAAGCAGCC TTGCAAGTGA
TACAATCCCA AGATGCCAAT TACTGCAAAG CAGCACTTGT GTCGAAACG
CCACACACTC AGTTGAGACA ATTTTGCTCA TTTTTCCATA GACATAATAA
TGAAAGGAAAG AGGAGGGGGA TGGAGAGAG AGATGAAAAA GCAGAGGGAG
GAAGGGGGAG TAGGGGAGTG GCAGAAGAGG AAAGCTTACT CTACAGAGTT
CTGGTCTCAA GAGGGCTTAAC TTACAGGAGG TGTGGGCTCC TTTACGCAAT
GTTTCTAGGC AAAATCCTCA TGAGTCAAAA AAATTTAAAAA GCTATAACCT
TCTGAATGCC AGGAAGGCCT TACCAAAAAG CTTTTGTCAG AGGGAGAAGG
GAAGAGAGAG AGGGAGAGAG AGAGGGAGAG AGAGGGGAGG AGAGGAGAGG
AAGGAGAGA AGAGGACAC CCATAAGTAA AGAGACAGAA GGAAGGAAAG
GGAGAGGACA AGAGGAAGAG AGGGAGGGAG GGGAGGGGAG AAGAAAGAAG
ATGGAGAAGG AGGGAGGGGA GAGAGCAAGG GGGAGGCCAG AGTGTTAGGC
AGTCCACTTT TACTTTGAGT ACTGTGAGGT CACAAACCAC ATGATTTCTG
CTCTCCAGTA ATAGTGCTTG AAAAAATAG GAGTTTAAA GCTTTTGCTT
TTTGGATTG TGTG

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The sequence of MetLuc (length: 660) is shown in the following.

ATGGACATCA AGGTGGTGT T CACCTGTTT TTCACGCCC T GGTGCAGGC
CAAGAGGCC GAGTTCGACC CCAACATCGA CATCGTTGCC CTGGAAGGCA
AGTTCCGAT CACCAACCCTG GAAACCGACC TGTTCACCAT CTTGGAGACC
ATGGGAAAGTA TGATCAAGGC CGACATCGCC GACACCGACC GGGCAGCAA
CTTCGCTGCC ACCGAGACCG ACGCAAACCG GGGCAAGATG CCCGGCAAGA
AGCTGCCCTT GGGCGTCATC ATGAAATGG AAGCCAACGC TTCCAAGGCC
GGCTGCACC GGCTGCTGCT GATCTGCTTG AGCAAATCA AGTGGACCAGC
CAAGATGAGG GTGTACATCC CCGCAAGGTC CCAGCATACGG GGCAGCGACA
AGAAAAACCG CCAGGCGGCG ATCGTGGGCC CCGATCGTGA CATCCCCGAG
ATCAGCGGCT TCAAGAAAT GGGCCCATG GAACAGTCTA TCGCCAGGT
GGACAGATGC GCCAGCTGCA CACCCGGCTG CTTGAGGCG CTGCGCAAGG
TGAAGTGACG CGAGCTGCTG AAGAAGTGGC TGCCCACGG GTGCGCCAGC
TTCCCGCGCA AGATCCAGAA AGGTTGACG AACATCAAGG GCATGGCCGG
CGACAGGGTA

8.1.2.8 pLV.Ex.Si.P/Puro-Runx2_X12-MetLuc

Partial sequence of pLV.Ex.Si.P/Puro-Runx2_X12-MetLuc (8,348 bp). The full sequence map is shown in Figure 2.10.

The sequences of both the 12xRunx2 binding site (length: 236) and the Minimal Promoter (length: 136) are identical to the one from pShuttle-Runx2-MinPro-EGFP-polyA (appendix 8.1.2.2), the sequence of MetLuc (length: 660) is identical to the one from pLV.Ex.Si.P/Puro-Runx2-MetLuc (appendix 8.1.2.7).

8.2 Graphical representation of GFP expression level time course mediated by Ad.Runx2 transduction of BMSCs seeded at 60,000 cells/cm² – donors Pat 53, 55, and 62
Appendix

Figure 8.1: Graphical representation of time course of GFP expression in BMSCs mediated by Ad.Runx2 transduction at an MOI of 100 of donor Pat 53. BMSCs seeded at 60,000 cells/cm² were transduced with Ad.Runx2 at an MOI of 100 the following day. Cells were cultured in control (unstimulated) and osteogenic (stimulated) medium, respectively. GFP expression was assessed for up to day 8 post-transduction by means of fluorescence microscopy. The exposure times were kept constant at 833 ms for GFP. Three different approaches were performed to quantify the extent of GFP expression: A) Subjective impressions, whereby arbitrary fluorescence intensities were attributed to the different conditions and time points by comparative estimation amongst conditions, donors as well as time point. B) Image analysis to determine green percentaged pixel area using representative images of the different conditions, donors as well as time points. C) Image analysis to determine green percentaged pixel area using all images taken of the different conditions, donors as well as time points.
Appendix

Figure 8.2: Graphical representation of time course of GFP expression in BMSCs mediated by Ad.Runx2 transduction at an MOI of 100 of donor Pat 55. BMSCs seeded at 60,000 cells/cm² were transduced with Ad.Runx2 at an MOI of 100 the following day. Cells were cultured in control (unstimulated) and osteogenic (stimulated) medium, respectively. GFP expression was assessed for up to day 8 post-transduction by means of fluorescence microscopy. The exposure times were kept constant at 833 ms for GFP. Three different approaches were performed to quantify the extent of GFP expression: A) Subjective impressions, whereby arbitrary fluorescence intensities were attributed to the different conditions and time points by comparative estimation amongst conditions, donors as well as time point. B) Image analysis to determine green percentaged pixel area using representative images of the different conditions, donors as well as time points. C) Image analysis to determine green percentaged pixel area using all images taken of the different conditions, donors as well as time points.
Appendix

**Figure 8.3:** Graphical representation of time course of GFP expression in BMSCs mediated by Ad.Runx2 transduction at an MOI of 100 of donor Pat 62. BMSCs seeded at 60,000 cells/cm² were transduced with Ad.Runx2 at an MOI of 100 the following day. Cells were cultured in control (unstimulated) and osteogenic (stimulated) medium, respectively. GFP expression was assessed for up to day 8 post-transduction by means of fluorescence microscopy. The exposure times were kept constant at 833 ms for GFP. Three different approaches were performed to quantify the extent of GFP expression: A) Subjective impressions, whereby arbitrary fluorescence intensities were attributed to the different conditions and time points by comparative estimation amongst conditions, donors as well as time point. B) Image analysis to determine green percentaged pixel area using representative images of the different conditions, donors as well as time points. C) Image analysis to determine green percentaged pixel area using all images taken of the different conditions, donors as well as time points.
8.3 Graphical representation of GFP expression level time course mediated by Ad.Runx2 transduction of BMSCs seeded at 15,000 cells/cm² – donors Pat 53, 55, and 62

Figure 8.4: Graphical representation of time course of GFP expression in BMSCs mediated by Ad.Runx2 transduction at an MOI of 100 of donor Pat 53. BMSCs seeded at 15,000 cells/cm² were transduced with Ad.Runx2 at an MOI of 100 the following day. Cells were cultured in control (unstimulated) and osteogenic (stimulated) medium, respectively. GFP expression was assessed for up to day 8 post-transduction by means of fluorescence microscopy. The exposure times were kept constant at 833 ms for GFP. Three different approaches were performed to quantify the extent of GFP expression: A) Subjective impressions, whereby arbitrary fluorescence intensities were attributed to the different conditions and time points.
by comparative estimation amongst conditions, donors as well as time point. B) Image analysis to determine green percentaged pixel area using representative images of the different conditions, donors as well as time points. C) Image analysis to determine green percentaged pixel area using all images taken of the different conditions, donors as well as time points.

**Figure 8.5:** Graphical representation of time course of GFP expression in BMSCs mediated by Ad.Runx2 transduction at an MOI of 100 of donor Pat 55. BMSCs seeded at 15,000 cells/cm² were transduced with Ad.Runx2 at an MOI of 100 the following day. Cells were cultured in control (unstimulated) and osteogenic (stimulated) medium, respectively. GFP expression was assessed for up to day 8 post-transduction by means of fluorescence microscopy. The exposure times were kept constant at 833 ms for GFP. Three different approaches were performed to quantify the extent of GFP expression: A) Subjective impressions, whereby...
arbitrary fluorescence intensities were attributed to the different conditions and time points by comparative estimation amongst conditions, donors as well as time points. B) Image analysis to determine green percentaged pixel area using representative images of the different conditions, donors as well as time points. C) Image analysis to determine green percentaged pixel area using all images taken of the different conditions, donors as well as time points.

**Figure 8.6:** Graphical representation of time course of GFP expression in BMSCs mediated by Ad.Runx2 transduction at an MOI of 100 of donor Pat 62. BMSCs seeded at 15,000 cells/cm² were transduced with Ad.Runx2 at an MOI of 100 the following day. Cells were cultured in control (unstimulated) and osteogenic (stimulated) medium, respectively. GFP expression was assessed for up to day 8 post-transduction by means of fluorescence microscopy. The exposure times were kept constant at 833 ms for GFP. Three different approaches were
performed to quantify the extent of GFP expression: A) Subjective impressions, whereby arbitrary fluorescence intensities were attributed to the different conditions and time points by comparative estimation amongst conditions, donors as well as time point. B) Image analysis to determine green percentaged pixel area using representative images of the different conditions, donors as well as time points. C) Image analysis to determine green percentaged pixel area using all images taken of the different conditions, donors as well as time points.
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