Targeting the Cell Cycle
in Postmitotic Cardiomyocytes
Genetic Engineering of Terminally Differentiated Cells

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Zurich, 2002
To my parents

*Ruth and Ulrich*

for their love and for their continuous support and encouragement
they offered throughout my education
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## Abbreviations

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<tr>
<td>AAV</td>
<td>adeno-associated virus</td>
</tr>
<tr>
<td>AdV</td>
<td>adenovirus</td>
</tr>
<tr>
<td>APC</td>
<td>anaphase-promoting complex</td>
</tr>
<tr>
<td>APS</td>
<td>ammonium persulfate</td>
</tr>
<tr>
<td>araC</td>
<td>cytosine arabinoside</td>
</tr>
<tr>
<td>ARC</td>
<td>adult rat cardiomyocytes</td>
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<tr>
<td>AVET</td>
<td>adenovirus-enhanced transferrinfection</td>
</tr>
<tr>
<td>BHK</td>
<td>baby hamster kidney</td>
</tr>
<tr>
<td>BrdU</td>
<td>5’-Bromo-2’-deoxyuridine</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>C</td>
<td>cyclosome</td>
</tr>
<tr>
<td>Cak</td>
<td>Cdk activating kinase</td>
</tr>
<tr>
<td>CCD</td>
<td>charge-coupled devise</td>
</tr>
<tr>
<td>Cdc2</td>
<td>cell division control protein 2 (p34/Cdk1)</td>
</tr>
<tr>
<td>Cdk</td>
<td>cyclin-dependent kinase</td>
</tr>
<tr>
<td>CITE</td>
<td>cap-independent translation element</td>
</tr>
<tr>
<td>Cki</td>
<td>cyclin-dependent kinase inhibitor</td>
</tr>
<tr>
<td>CMV</td>
<td>cytomegalovirus</td>
</tr>
<tr>
<td>COS</td>
<td>African Green Monkey kidney</td>
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<tr>
<td>CRS</td>
<td>cytoplasmic retention sequence</td>
</tr>
<tr>
<td>Cy3</td>
<td>cyanine-3 (indocarbocyanine)</td>
</tr>
<tr>
<td>Cy5</td>
<td>cyanine-5 (indodicarbocyanine)</td>
</tr>
<tr>
<td>DAPI</td>
<td>4,5 diamino-2-phenylindoldihydrochlorid</td>
</tr>
<tr>
<td>DNA-Tf-PEI</td>
<td>DNA-transferrin-polyethylenimine</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylene diamine tetraacetic acid</td>
</tr>
<tr>
<td>EGFP</td>
<td>enhanced green fluorescent protein</td>
</tr>
<tr>
<td>EGTA</td>
<td>Ethylene glycol-bis[β-aminoethyl ether] N,N,N’,N’ tetraacetic acid</td>
</tr>
<tr>
<td>ES</td>
<td>embryonic stem</td>
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<tr>
<td>FCS</td>
<td>fetal calf serum</td>
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FITC fluorescein isothiocyanate
G0 Gap-zero (cell cycle phase)
G1 Gap 1 (cell cycle phase)
G2 Gap 2 (cell cycle phase)
GFP green fluorescent protein
H3 histone H3 protein
HA hemagglutinin protein (tag)
HEK human embryonic kidney
HeLa Henrietta Lacks
HRPO horseradish peroxidase
Ig immunoglobulin
IGF-1 insulin-like growth factor 1
IPTG isopropyl β-D-thiogalactopyranoside
IRES internal ribosomal entry site
IU/ml infectious units per milliliter
kb kilobases
LS low salt (buffer)
M mitosis (cell cycle phase)
MHC myosin heavy chain
MLC myosin light chain
mM monoclonal mouse
MOI multiplicity of infection
MP microtubule protective (buffer)
MPF M phase-promoting factor/mitosis-promoting factor
NF-Y nuclear factor Y
NRC neonatal rat cardiomyocytes
ns not significant
nsP(1-4) nonstructural proteins (1-4)
PAGE polyacrylamide gel electrophoresis
PBS phosphate buffered saline
PCNA proliferating cell nuclear antigen
PCR polymerase chain reaction
<table>
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<tr>
<td>PEI</td>
<td>polyethylenimine</td>
</tr>
<tr>
<td>PFA</td>
<td>paraformaldehyde</td>
</tr>
<tr>
<td>Plk1</td>
<td>polo-like kinase 1</td>
</tr>
<tr>
<td>pR</td>
<td>polyclonal rabbit</td>
</tr>
<tr>
<td>pRb</td>
<td>product of the retinoblastoma susceptibility gene</td>
</tr>
<tr>
<td>PTB</td>
<td>polypyrimidine tract binding protein</td>
</tr>
<tr>
<td>PTTG</td>
<td>pituitary tumor-transforming gene</td>
</tr>
<tr>
<td>RT</td>
<td>room temperature</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>reverse transcription polymerase chain reaction</td>
</tr>
<tr>
<td>S</td>
<td>Synthesis (cell cycle phase)</td>
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<tr>
<td>SDS</td>
<td>sodium dodecylsulfate</td>
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<tr>
<td>SEM</td>
<td>standard error of the mean</td>
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<tr>
<td>SG RNA</td>
<td>subgenomic RNA</td>
</tr>
<tr>
<td>SIN</td>
<td>Sindbis virus</td>
</tr>
<tr>
<td>STAV-pL</td>
<td>streptavidin-poly(L)lysine</td>
</tr>
<tr>
<td>SV</td>
<td>Simian virus</td>
</tr>
<tr>
<td>TAE</td>
<td>tris-acetate EDTA (buffer)</td>
</tr>
<tr>
<td>TdT</td>
<td>terminal deoxynucleotidyl transferase</td>
</tr>
<tr>
<td>Tf-PEI</td>
<td>transferrin-polyethylenimine</td>
</tr>
<tr>
<td>Tf-pL</td>
<td>transferrin-poly(L)lysine</td>
</tr>
<tr>
<td>TUNEL</td>
<td>TdT-mediated dUTP nick-end labeling</td>
</tr>
<tr>
<td>TxRed</td>
<td>Texas Red</td>
</tr>
<tr>
<td>wt</td>
<td>wild-type</td>
</tr>
<tr>
<td>X-Gal</td>
<td>5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside</td>
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SUMMARY

Cardiomyocytes cease to divide shortly after birth and an irreversible cell cycle arrest becomes evident. Proliferation of differentiated cardiomyocytes and an eventual tissue repair are blocked. The here presented work focused on the question how the control of the cell cycle of this specific cell type is organized. Particular attention was given to the G2/M transition and the molecules involved in the mentioned checkpoint. Primary cultures of ventricular adult rat cardiomyocytes (ARC) were established and served as model for the postmitotic phenotype in the adult mammalian heart. They provided the platform for the presented investigations. The functional recovery of the mitosis-promoting factor (MPF) by genetic means was of special interest; it consists of cyclin B1 and the cyclin-dependent kinase Cdc2. This implicated the evaluation of appropriate gene delivery systems for ARC, which are known for their resistance to transfection. Sindbis virus (SIN)-based vectors proved to be a powerful gene transfer vehicle for cultured ARC. Coming along with an infection efficiency of more than 80%, SIN exerted a remarkably lower cytopathogenicity on cultured ARC than on other cell types investigated in parallel. SIN achieved high transgene expression levels within a short period of time and allowed the expression of genetically engineered contractile proteins in cardiomyocytes. Localization of fluorescence protein-tagged myofibrillar components could thus be readily accomplished, revealing SIN as a straightforward means to study sarcomeric protein interactions.

The implementation of the adenovirus-enhanced transferrinfection (AVET) system allowed the reliable expression of cell cycle-dependent genes in cultured ARC. The AVET system permitted plasmid transfection of cultured ARC, reaching under optimized conditions and depending on the plasmid used an efficiency of more than 20% without affecting cell morphology and behavior. The AVET system represented a novelty, able of plasmid transfection of cultured ARC without the need of active viral particles. This made AVET not only very useful for the present work but will turn out to be attractive for a wide range of applications in cardiobiology research.

The influence of the in vitro environment on the cell cycle in cardiomyocytes was assessed. Whereas ARC proved to be resistant to cell cycle re-entry, neonatal rat
cardiomyocytes (NRC) in culture displayed eventually mitotic figures that allowed to propose ongoing cytokinesis in a few instances.

Reactivation of MPF in cultured cardiomyocytes was accomplished by applying elaborated gene engineering techniques. Functional characterization of exogenous cyclin B1 and Cdc2 in cultured ARC became feasible using AVET-mediated gene transfer. Proteolysis of mitotic cyclins, being in cells present in late M and G1 phase, was not active enough in ARC to degrade exogenous cyclin B1. Simultaneous ectopic expression of wild-type versions of cyclin B1 and Cdc2 was sufficient to induce MPF activity, which hold also true for cultured NRC. Re-established MPF resulted in an arrest of the cells with a mitotic phenotype, characterized by abnormal condensation of the nuclei, histone H3 phosphorylation and a variable degree of decay of the myofibrillar apparatus. Although cell division was not observed, the results provided first evidence that cell cycle-related events could be triggered by genetic intervention at the G2/M boundary in postmitotic cardiomyocytes.

Taken together, the findings offer not only novel insights about the G2/M checkpoint and its regulatory factors in postnatal cardiomyocytes, but will also be of importance to formulate novel strategies to overcome the proliferation arrest in adult cardiomyocytes.
ZUSAMMENFASSUNG


Eine weitere Transfektionsmethode zeigte sich als hervorragenden Weg, um ARC zu transduzieren. Der Vektor besteht aus einem Polykation-DNA-Protein Komplex, der mit inaktivierten Adenoviren gekoppelt ist. Diese sogenannte AVET (engl. “adenovirus-enhanced transferrinfection”) Methode zeichnete sich durch eine relativ hohe Effizienz in kultivierten ARC aus, welche in gewissen Fällen mehr als 20% erreichte. Es zeigte sich auch, dass zytotoxische Effekte auf die Zellen kaum nachzuweisen waren und damit das grosse Potenzial dieser Methode für weitere Anwendungen in der kardiobiologischen Grundlagenforschung.


Die hier gemachten Befunde liefern nicht nur neue Erkenntnisse über die Regulation am G2/M Kontrollpunkt in postmitotischen Herzmuskelzellen, sondern geben wichtige Hinweise für die Ausarbeitung von neuen experimentellen Ansätzen, um die Zellteilung wieder in Gang zu bringen.
1 INTRODUCTION

1.1 REGULATION OF THE MAMMALIAN CELL CYCLE

According to Webster’s New Collegiate Dictionary, the word ‘cycle’ is defined as a course or series of events or operations that recur regularly and usually lead back to the starting point. Nature is full of processes matching this definition precisely. Whether it is the ‘solar cycle’, in which the sun undergoes a period of activity with a peak in sunspots and massive coronal mass ejections every eleven years, or the ‘cardiac cycle’, in which the heart exerts its vital role by cyclic action spanning from systole through diastole, we are influenced by or rely on these highly orchestrated mechanisms. The beauty and functioning of such processes but also their importance for life on the molecular level are nowhere better manifested than in the cell cycle, being the fundamental means by which all living things are propagated.

The intention of the following chapter is to present the main concept of the mammalian cell cycle and its control factors by travelling through the different stages. On this short journey, only a glimpse of the diversity of the molecular players and their interaction can be given. But the brief touch of this fascinating network should assist to appreciate the second part of this introduction, where the mentioned cell cycle-regulatory factors are discussed with their relevance and their putative role in cell cycle control of cardiomyocytes.

1.1.1 THE CYCLIN-DEPENDENT KINASES AND THEIR ROLE IN CELL CYCLE REGULATION

The cell cycle represents a collection of highly ordered processes that results in the duplication of a cell (Elledge, 1996). The two most important events during the passage through the cell cycle are the S phase (S = synthesis) and the M phase (M = mitosis), when chromosomes are replicated and segregated into the dividing cells (Nurse, 1994). The interval between the completion of M phase and the beginning of S phase, called the G1 phase (G = gap), and the interval between the end of S phase and the beginning of M phase, called the G2 phase, form the links necessary to fulfill the image of the cell cycle as a circle.
As the cells progress through the cell cycle, they undergo several checkpoints, which ensure that each newly dividing cell receives a full complement of the hereditary material. Several of these checkpoints are regulated by a family of protein kinases, the cyclin-dependent kinases (Cdk), and their obligate activating partners, the cyclins (Figure 1-1) (Hunter and Pines, 1994). In complex cell cycles as the mammalian cell cycle, the CdkS are not only the engines that drive the cell through the different stages but act also as information processors that integrate extracellular and intracellular signals for smooth coordination of cell cycle events in the face of environmental changes (Morgan, 1997).

Key regulators in the major cell cycle checkpoint in late G1, known as the restriction point in mammalian cells, include the Cdk4 and Cdk6, which preferentially assemble into holoenzymes with either cyclin D1, cyclin D2 or cyclin D3 (Sherr, 1994). The D-type cyclins are generally highly inducible by growth factors. This characteristic led to the idea these cyclins act as growth factor sensors mediating extracellular cues directing the cell to another division or withdrawing it from the cell cycle into a resting state called G0 (Sherr, 1993). Cyclin D-dependent kinase activities are first manifested by mid-G1, increase to a maximum near the G1/S transition, and persists through the first and subsequent cycles as long as mitogenic stimulation continues (see Figure 1-2 on page 3).
The activity of these kinases can directly be blocked by members of the first cyclin-dependent kinase inhibitor (Cki) family, or INK4 proteins, consisting of p16 (INK4a), p15 (INK4b), p18 (INK4c) and p19 (INK4d) (see Figure 1-1 on page 2) (Sherr and Roberts, 1995). These proteins selectively inhibit the activity of cyclin D-dependent kinase by competitively binding to these kinases and thus preventing cyclin D interaction.

One primary target of cyclin D-dependent kinases is the product of the Retinoblastoma susceptibility gene (pRb) (Ewen et al., 1993; Kato et al., 1993). This protein comprises, together with p107 and p130, the known members of the pRb family of proteins, often also called pocket proteins (Classon and Dyson, 2001). The proteins of this class control gene expression mediated by a family of transcriptional regulators, collectively termed the E2Fs (Nevins, 1992). The activity of E2F depends on the heterodimers composed of one of five different E2F subunits (E2F-1 to E2F-5) and one of three so-called DP family members (DP-1, DP-2, and DP-3). Each pocket protein sequesters different E2F-DP complexes in a characteristic manner throughout the G0-G1 to the S phase interval (Sherr, 1996). In their hypophosphorylated form, pRb proteins bind to a subset of E2F complexes, rendering them to repressors that constrain expression of E2F targets (Weintraub et al., 1995). Phosphorylation of pRb and its relatives initiated by the cyclin D-dependent kinases (Ewen et al., 1993; Kato et al., 1993) results into the release of the E2F complexes, enabling them to trigger the expression of genes required for DNA
synthesis and further cell cycle-regulating molecules as cyclin E, cyclin A, the mitotic kinase Cdc2 (p34/Cdk1), and E2F-1 itself (Sherr, 1996).

The expression of cyclin E is normally periodic and maximal at the G1-S transition where it enters into active complexes with its catalytic partner Cdk2 to accelerate the phosphorylation of the pRb proteins (Hinds et al., 1992). Because the cyclin E gene is itself E2F-responsive, cyclin E-Cdk2 acts through a positive feedback to facilitate progressive rounds of pRb phosphorylation and E2F release (Weinberg, 1995). This positive crossregulation of E2F and cyclin E leads to an irreversible commitment to enter S phase, whereby pRb inactivation shifts from being mitogen-dependent to mitogen-independent (Sherr, 1996).

The onset of cyclin A synthesis in late G1 is an additional important event in the G1/S transition, because inhibition of cyclin A function in cultured cells can also inhibit S phase entry (Girard et al., 1991; Pagano et al., 1992). Cyclin A forms with Cdk2 active complexes and contributes to the maintenance of pRb in its hyperphosphorylated state as the cycle moves ahead (Sherr, 1996). Further substrates for cyclin A-Cdk2 and cyclin E-Cdk2 complexes potentially include proteins at replication origins whose phosphorylation might promote DNA synthesis (Stillman, 1996). Once the cell enters the S phase, the timely inactivation of cyclin E and E2F activities seems to be equally crucial for cell cycle progression. Cyclin E is rapidly degraded by the ubiquitin-dependent proteolysis pathway (Won and Reed, 1996) and E2F-1 transactivation activity is decreased by accumulating cyclin A-Cdk2 complexes that phosphorylate the E2F complexes and thus preclude their DNA binding ability (Krek et al., 1994; Dynlacht et al., 1994).

Activity of cyclin E-, and cyclin A-dependent kinases are negatively regulated by the second Cki family, termed the KIP/CIP family, which is composed of p21 (CIP1/WAF1/SDI1) (Xiong et al., 1993; Harper et al., 1993; el-Deiry et al., 1993; Dulic et al., 1993), p27 (KIP1) (Polyak et al., 1994; Toyoshima and Hunter, 1994) and p57 (KIP2) (Lee et al., 1995; Matsuoka et al., 1995). Members of this family are also potent inhibitors of cyclin D-dependent kinases and thus characterized by a broader substrate specificity than the INK4 family, which is limited in their inhibitory function to cyclin D-dependent kinases (Pines, 1997). Besides its function as a Cki, p21 is linked to the control of DNA synthesis since it binds and inactivates components of the DNA replication machinery, in
particular to the so-called proliferating cell nuclear antigen (PCNA), which is necessary for the elongation of primed DNA templates by DNA polymerase δ (Waga et al., 1994). Transcription of the p21 gene is induced by wild-type p53, a transcription factor which also functions as a tumor suppressor (el-Deiry et al., 1993). However, it was demonstrated that p21 is also induced by a p53-independent pathway (Michieli et al., 1994). One of the roles of p53 is to act as a central integrator of signals indicating DNA damage, genotoxic stress and senescence (for a review: Ko and Prives, 1996). In some cell types, p53 induces apoptosis when overexpressed and is required to induce apoptosis in case of severe DNA damage, chemotherapeutic drugs, or overexpression of the adenoviral oncoprotein E1A (Lowe et al., 1993a; Lowe et al., 1993b; Lowe and Ruley, 1993). However, the p53-dependent onset of apoptosis does not rely on p21 (Deng et al., 1995), and p53 may directly activate transcription of death genes, such as bax, or down-regulate survival genes, such as bcl-2 (Miyashita et al., 1994; Miyashita and Reed, 1995).

P27 of the Cip/Kip family may be the Cki most directly involved in restriction point control (Sherr, 1996). Expression of p27 is high in cells in the G0 state, but falls immediately once the cells enter the cycle (Kato et al., 1994) (see Figure 1-2 on page 3). Mice nullizygous for the gene encoding p27 grow faster than control animals and display tissues with increased numbers of smaller cells demonstrating the importance of this protein in regulating both cell size and cell number (Nakayama et al., 1996). In contrast to p27, most of the p57-deficient mice died already in the perinatal period, with multiple developmental defects (Yan et al., 1997).

After progressing through the S phase, cyclin A is getting implicated in the G2/M transition of the cell cycle where it can form active complexes with the mitotic cyclin-dependent kinase Cdc2 (Nigg, 1995). Preventing cyclin A function or its expression at the G2/M boundary results in cell cycle arrest, suggesting, besides its essential function in the G1/S transition, a role in the control of M phase entry (Pagano et al., 1992). However, the main regulator of M phase entry is known as MPF, the mitosis-promoting factor (King et al., 1994).
1.1.2 MPF: Regulating the Onset of Mitosis

The last phase of the cell cycle before cell division is the M phase comprising mitosis and cytokinesis, which at its end leads back to the starting point thereby initiating an new round of the cyclic molecular interplay. The main purpose of mitosis is to segregate sister chromatids into two nascent cells, such that each daughter cells inherits one complete set of chromosomes. Mitosis is usually separated into five distinct stages: prophase, prometaphase, metaphase, anaphase and telophase. At the end of mitosis, the process of cell cleavage occurs, termed cytokinesis, whose regulation is linked intimately to mitotic progression (for a review: Nigg, 2001).

The initiation of mitosis in eukaryotic cells is governed by a spatially and temporally complex phosphorylation cascade, which culminates in the activation of the mitosis-promoting factor (MPF) (Ohi and Gould, 1999). MPF is composed of the cyclin-dependent kinase Cdc2 and its B-type cyclin regulatory subunit. Only two B-type cyclins, cyclin B1 (Pines and Hunter, 1989) and cyclin B2 (Chapman and Wolgemuth, 1992), have been identified in mammals, although chickens, frogs, flies and nematode worms possess a third, more distant relative, termed cyclin B3 (Gallant and Nigg, 1994; Kreutzer et al., 1995). The genes of cyclin B1 and cyclin B2 display very little similarity in the first 100 residues, and about 57% identity in the remaining 300 residues (Chapman and Wolgemuth, 1993). Both cyclins assemble with Cdc2 and are active only in mitosis (Jackman et al., 1995), but only the activity of cyclin B1 is essential since mice lacking cyclin B1 die in utero (Brandeis et al., 1998). Mice nullizygous for cyclin B2 survive, but are smaller in size and have a reduced litter sizes, indicating that this B-type cyclin confers a growth advantage (Brandeis et al., 1998).

Concerning cyclin B, mitosis can be divided into two main events: In the first, cyclin B activates MPF and initiates prophase. In the second, MPF activates a ubiquitin-dependent proteolytic system, causing both cyclin B destruction and the initiation of anaphase (King et al., 1994) (see Figure 1-3 on page 7). The ball is set rolling towards M phase by an accumulation of cyclin B above a threshold level in the late G2 phase (see Figure 1-2 on page 3) (Solomon et al., 1990) as a consequence of cyclin B mRNA synthesis starting at the end of S phase (Pines and Hunter, 1989). After association of cyclin B with Cdc2, the activity of the MPF complex is regulated by a number of phosphorylation events, resulting either in its activation or inhibition (Smits and Medema, 2001).
Phosphorylation of the conserved threonine residue 161 (in human Cdc2) in the T-loop of Cdc2 was demonstrated to be required for MPF activation (Desai et al., 1992). The phosphorylation activity has been attributed to a complex composed of Cdk7 and cyclin H, exerting its role as a Cdk activating kinase (Cak) (Fisher and Morgan, 1994). However, the importance of this complex in vivo as a Cak in the mammalian cell cycle has not been clearly demonstrated (Harper and Elledge, 1998).

**Figure 1-3:** Regulation of M phase viewed from the perspective of the mitosis-promoting factor MPF, consisting of Cdc2 and cyclin B. Entry into mitosis is the consequence of MPF activation which depends in mammalian cells primarily on the dephosphorylation of Cdc2. This occurs when the activity of the phosphatase Cdc25C exceeds the inhibitory function of the kinases Wee1 and Myt1. Exit from mitosis depends on the inactivation of MPF, which itself depends on cyclin destruction by the APC ubiquitin ligase (adapted from Nigg, 2001).

Most emphasis has been placed on the two negative regulatory sites on Cdc2, which are threonine 14 (T14) and tyrosine 15 (Y15). These residues located in the ATP-binding site of Cdc2 are phosphorylated by the Wee1/Myt1 protein kinases thus maintaining the pool of cyclin B/Cdc2 complexes in an inactive state. Phosphorylation of Y15 by the Wee1 protein kinase has been proposed to interfere with the transfer of phosphate from Cdc2 to a bound substrate (Heald et al., 1993; McGowan and Russell, 1995; Watanabe et al., 1995). Additionally, T14 together with Y15 are phosphorylated by the Myt1 protein kinase (Mueller et al., 1995; Liu et al., 1997). Whereas Wee1 is a nuclear protein, Myt1 is a cytoplasmic membrane-bound protein, mainly associated with the endoplasmic reticulum and the Golgi apparatus (Kornbluth et al., 1994; Mueller et al., 1995; Liu et
al., 1997). Phosphorylation on the two negative residues by these two kinases have been both implicated in preventing mitosis in the presence of damaged DNA (Jin et al., 1996; see Figure 1-3 on page 7).

The inhibition imposed on Cdc2 by the Wee1 protein kinase family is released by the Cdc25 phosphatases, which dephosphorylate Thr14 and Tyr15 on Cdc2. In mammals, three known Cdc25 protein phosphatases exist, namely Cdc25A, Cdc25B and Cdc25C (Nilsson and Hoffmann, 2000). Both Cdc25B and Cdc25C have been implicated in the regulation of M phase entry. The Cdc25C phosphatase is found in the cytoplasm in interphase cells where it is bound to the 14-3-3 protein and thus rendered inactive (Takizawa and Morgan, 2000). Activation of Cdc25C requires phosphorylation by MPF itself (Hoffmann et al., 1993) as well as by a further kinase, the Polo-like kinase 1 (Plk1) (Takizawa and Morgan, 2000). The ability of MPF to activate its own activator provides the potential for a positive feedback loop in Cdc2 kinase activity (Takizawa and Morgan, 2000). This positive feedback loop may be additionally enhanced by the ability of MPF to phosphorylate and inactivate Wee1 (Patra et al., 1999).

During interphase, cyclin B1/Cdc2 complexes are entirely found in the cytoplasm, either in soluble form or associated with centrosomes and the microtubule network (Pines and Hunter, 1991; Bailly et al., 1992; Jackman et al., 1995). The cytoplasmic localization of cyclin B1 during interphase is in fact the result of continuous export from the nucleus (Hagting et al., 1998). During prophase, MPF is suddenly translocated into the nucleus as a result of changes in both export and import rates. Detailed analysis of the kinetics in live human cells suggested that phosphorylation of the so-called cytoplasmic retention sequence (CRS) within cyclin B1 lead to an increase in the rate of cyclin B1 import (Hagting et al., 1998). Recently, Plk1 has been identified as a protein kinase that phosphorylates cyclin B1 and targets it to the nucleus during prophase playing an important role in cyclin B1 translocation (Toyoshima-Morimoto et al., 2001). Furthermore, it was demonstrated that MPF itself can phosphorylate two residues in the CRS region in vitro, proposing that Cdc2 help to promote its own translocation to the nucleus (Izumi and Maller, 1991).

As the cells enter mitosis, MPF phosphorylates key components of the subcellular structures thereby causing the complete reorganization of the cell architecture (Smits and Medema, 2001) (see Figure 1-3 on page 7). Besides regulating the activity of itself by
phosphorylating Cdc25 and potentially Wee1, MPF also induces changes in the microtubule network, the Golgi apparatus and the nucleus. MPF-induced phosphorylation of Eg5, a kinesin-related motor protein required for establishing a biopolar spindle, regulates its association with the spindle apparatus (Blangy et al., 1995). MPF triggers also the induction of Golgi fragmentation by phosphorylating the cis-Golgi matrix protein GM130 (Lowe et al., 1998). Furthermore, MPF phosphorylates lamins leading to the disassembly of the karyoskeletal structure and finally to nuclear envelope breakdown (Peter et al., 1990).

Another early mitotic event is chromosome condensation, which is accompanied by extensive phosphorylation of both histone and non-histone proteins. Histone modifications, including phosphorylation, acetylation and methylation, have long been correlated with changes in chromatin condensation states (Nigg, 2001). The linker histone H1 has been demonstrated to be an excellent substrate of the MPF complex, but despite extensive investigations, the significance of this phosphorylation still remains unknown (Nigg, 2001). Several studies demonstrated that histone H1 phosphorylation is not a prerequisite for chromatin condensation. The less dramatic phosphorylation of histone H3 may play a role in this event (Guo et al., 1995; Hendzel et al., 1997; Sauve et al., 1999). Phosphorylation of histone H3 has been attributed to NIMA (De Souza et al., 2000), a protein kinase identified in the filamentous fungus Aspergillus nidulans and a possible MPF substrate (Ye et al., 1995). The mammalian homologue of this protein kinase is Nek2, which is implicated in the control of centrosome structure (Fry et al., 1998). No role of phosphorylation of histone H3 in animal cells has been assigned to Nek2, but to B-type aurora kinase, a member of the aurora kinase family (Adams et al., 2001; Murnion et al., 2001). Evidences that this kinase is in turn also involved in H3 phosphorylation in the mammalian cell cycle has not been reported up to now.

1.1.3 INACTIVATION OF MPF: ANAPHASE ONSET AND MITOTIC EXIT

The activation of MPF induces the cell to divide by altering cell structure and function through mitotic phosphorylation. After the cytoskeleton has been reorganized for division and the condensed chromosomes are aligned on the metaphase plate, MPF activates the cyclin degradation system, thereby inducing anaphase and its own inactivation (King et al., 1994).
Mitotic cyclins are stable throughout S-, G2- and early M phase until the onset of anaphase, in which they are destroyed, preventing further activity of their associated kinases. The destruction of A- and B-type cyclins was demonstrated to be dependent on a conserved motif of nine residues known as the destruction box and located 40-50 residues from the amino-terminus (Brandeis and Hunt, 1996). Using real time microscopy, it has been shown that mammalian cyclin B1 begins to be proteolytically degraded just at the end of prometaphase when the chromosomes align (Clute and Pines, 1999). This cyclin proteolysis is mediated by the ubiquitin pathway, which involves the assembly of chains of the small polypeptide ubiquitin on substrate proteins and thus targets them for degradation by the 26S proteasome (Peters, 1999). All physiological ubiquitination reactions characterized to date require three types of enzymes: the ubiquitin-activating enzyme E1, the ubiquitin-carrier protein E2 and the ubiquitin-protein ligase E3. In the system responsible for cyclin B degradation, the E3-like function is carried out by a large complex called cyclosome (C) or anaphase-promoting complex (APC) (Morgan, 1999; Hershko, 1999).

The activity of MPF and Plk1 have been implicated in the activation of APC (Kotani et al., 1998; Kramer et al., 2000), and protein kinase A has been described as a negative regulator (Kotani et al., 1998). Which of these kinases acts directly on APC in vivo remains to be established (Nigg, 2001). One main characteristic of APC is that this complex acts on different substrates at different times during M phase. The substrate and time specificity is controlled besides phosphorylation events by binding of adaptor proteins as for instance the p55Cdc (hCdc20), which is the mammalian form of the yeast cell cycle protein Cdc20 (Weinstein, 1997; Kallio et al., 1998). The second adaptor protein hCdh1, the mammalian form of yeast Cdh1/Hct1 (Fang et al., 1998), binds APC in late M phase and regulates among other things the degradation of mitotic cyclins during G1 phase (Fang et al., 1998; Nasmyth, 1999).

The APC owes its name to the observation that its activity is essential for the initiation of sister chromatid separation in anaphase. To be segregated properly, each chromosome must be aligned in the centre of a biopolar spindle such that its two sister chromatids are attached by microtubules to opposite poles. The so-called spindle assembly checkpoint (see Figure 1-3 on page 7) controls the correct alignment, and in the case of an
inconsistency, Mad2 possibly sequesters p55Cdc and prevents thus APC activation (Nasmyth, 1999; Morgan, 1999).

It was demonstrated that inhibition of the cyclin degradation machinery by microinjecting APC antibodies arrested mammalian cells at metaphase (Tugendreich et al., 1995). However, a nondestructible mutant of cyclin B was shown to prevent MPF inactivation, but sister chromatid separation occurred normally under these circumstances, indicating that MPF inactivation is not required for anaphase initiation. In contrast, overexpression of the amino-terminal fragment of cyclin B acting as a specific competitor for cyclin degradation, delayed sister chromatid separation, indicating that APC activity is required for anaphase triggering (Holloway et al., 1993). These findings made in lower vertebrates have led to the identification of another group of APC substrates, namely the anaphase inhibitors (Smits and Medema, 2001). Studies in yeast have revealed that sister-chromatid separation depends on the APC-mediated degradation of these anaphase inhibitors, termed securins (Funabiki et al., 1996). The human securin has been found to be identical to the pituitary tumor-transforming gene (PTTG), which is overexpressed in some tumors and exhibits transforming activity (Zou et al., 1999). Securin prevents the destruction of a multiprotein complex known as cohesin, which is responsible for sister chromatid cohesion (Nigg, 2001). The first wave of cohesin removal from chromosome arms already takes place during prophase to permit extensive chromosome condensation. This does not dependent on APC but requires phosphorylation of cohesin, which potentially involves MPF (Kimura et al., 1998).

1.1.4 The Final Stage of the Cell Cycle

After the separated sister chromatids arrive at the poles and a new nuclear envelope reforms around each group of them in telophase, cytokinesis occurs, representing the final stage of the M phase and the cell cycle. In this last step, actin, myosin and other cytoplasmic proteins are redistributed into the cleavage furrow, which forms midway between the segregated chromosomes in early anaphase (Fishkind and Wang, 1995). The resulting actin ring contracts and accomplished thus the separation of two daughter cells and the completion of cell division.

With regard to the molecular coordination of cytokinesis with mitotic progression in the mammalian cell cycle, it remains unclear whether mechanisms exist similar to them
known in yeast nowadays (Nigg, 2001). A study suggested a model for the timing of
 cytokinesis based on \textit{in vitro} findings that MPF can phosphorylate myosin regulatory
 light chain on inhibitory sites, thereby delaying cytokinesis until MPF activity falls at
 anaphase (Satterwhite \textit{et al.}, 1992). These results are in contrast to a more recent
 published paper, in which the authors could demonstrate that myosin inactivation does
 not influence the timing of cytokinesis (Shuster and Burgess, 1999). A role of Plk1 in the
 regulation of cytokinesis in mammalian cells has been additionally proposed (Nigg,
 2001), but experimental data are still missing. At least for the B-type aurora kinase, it
 was shown that overexpression of an inactive version of this kinase in cultured
 mammalian cells could disrupt cleavage furrow formation without affecting nuclear
 division, implying an involvement in cytokinesis regulation (Terada \textit{et al.}, 1998).

Thus far, an overview of the mechanisms that control the passage of mammalian cells
 through the specific cell cycle phases has been given with special focus on the protein
 phosphorylation pathways involving cyclins and their associated kinases. It should be
 emphasized that the view of the presented cell cycle molecules interactions has been
 confined to continuously dividing cells without giving special attention to the molecular
 causes leading to pathologic hyperplasia as it is encountered in cancer. Indeed, most
 mature tissues consist of some combination of continuously dividing cells, quiescent
 cells that occasionally enter the cell cycle, and nondividing cells (Cotran \textit{et al.}, 1999).
 Regarding to the latter, the elucidation of surveillance mechanism controlling the cell
 cycle arrest in this kind of cells has become of major interest due to the implications in
tissue regeneration.

\textbf{1.2 THE CELL CYCLE AND ITS ROLE IN CARDIOMYOCYTES}

The correct function of cell cycle control mechanisms is a prerequisite for the generation
 and structural modelling of all tissues and consequently of organs as the heart. The heart
 is of course absolutely crucial for the function of the organism by pumping blood
 through the body, thereby providing oxygen and nutrients, removing waste products, and
 refreshing the blood with oxygen and removing carbon dioxide in the lungs. The
fundamental importance of this organ has been known since the earliest days in history. The ancient Greeks regarded the heart as the seat of life and emotions (see Figure 1-4). Basis of the heart function represents the near-inexhaustible cardiac muscle, the myocardium, composed primarily of a collection of striated muscle cells, the cardiomyocytes (Schoen, 1999). These cells are the most physically active cells in the body, contracting constantly about 3 billion times or more in an average human lifespan by pumping 7000 liters of blood per day along 100000 miles of blood vessels (Severs, 2000).

The cardiomyocytes are not only outstanding in terms of their physiologic performance but exhibit extraordinary features regarding their cell cycle regulation. The main purpose of this chapter is to elucidate the cardiac cell cycle and the importance of its regulation factors in heart development and disease. Particular attention is also paid to experimental approaches and investigations for a better understanding of the cell cycle in cardiomyocytes.

1.2.1 THE CELL CYCLE IN HEART DEVELOPMENT

Mammalian cardiomyocytes show generally two developmentally distinguishable forms of growth. During fetal life, cardiac mass is increased by hyperplastic growth, in which the cardiomyocytes are actively proliferating. During neonatal life, there is a transition from hyperplastic to hypertrophic growth such that further increases in myocardial mass are typically not accompanied by cardiomyocyte proliferation (Rumyantsev, 1977; Parker and Schneider, 1991; Claycomb, 1992; Soonpaa and Field, 1998). Cardiomyocyte growth has been mainly investigated for ventricular cardiac muscle (MacLellan and Schneider, 2000). Most of the data available considering cell cycle-regulating factors
during mammalian heart development is derived from the mouse and rat model system. Analysis of cardiomyocyte growth during murine development indicates that DNA synthesis in these cells takes place primarily \textit{in utero}, reaching a labeling index of 33% in mid-gestation at day 12 \textit{post coitum} and decreasing progressively to 2% at birth (Soonpaa \textit{et al.}, 1996). At day 4 - 6 postnatal, the labeling index increases transiently to 10% and falls afterwards back to baseline levels. During this phase, karyokinesis occurs in the absence of cytokinesis, resulting in binucleation of ventricular cardiomyocytes (Clubb and Bishop, 1984; Li \textit{et al.}, 1996a). In the adult rat heart, approximately 89% of all ventricular cardiomyocytes exhibit binucleation, 10% of the cells are mononucleated and the rest shows tri- or even tetranucleation, whereby the nuclear DNA content is diploid in 93% of the cells with the remainder being tetraploid or even more (Kellerman \textit{et al.}, 1992). In humans, the withdrawal of ventricular cardiomyocytes from the cell cycle occurs within the first few weeks of life (Zak, 1974) and polyploidization has been reported to be associated with aging and increased heart weight (Adler and Friedburg, 1986; van der Laarse \textit{et al.}, 1989).

The withdrawal of postnatal cardiomyocytes from the cell cycle is linked with a change in the expression pattern of cell cycle regulatory molecules. During the embryonic stage, high levels of cyclins D1, D2, D3, A, B1 and E at both mRNA and protein levels in rat cardiomyocytes are expressed (Yoshizumi \textit{et al.}, 1995; Brooks \textit{et al.}, 1997; Kang \textit{et al.}, 1997; Flink \textit{et al.}, 1998). Additionally, PCNA and the cyclin-dependent kinases Cdc2, Cdk2, Cdk4, and Cdk6 are highly expressed and their associated kinase activity are also present (Yoshizumi \textit{et al.}, 1995; Brooks \textit{et al.}, 1997; Kang \textit{et al.}, 1997; Flink \textit{et al.}, 1998). The protein expression profiles of cyclins D1, D2, D3, A, B1 and E and their associated kinases become progressively and significantly downregulated in 2-day-old rat cardiomyocytes compared to the levels observed in the embryonic stage. Moreover, the protein levels of cyclin A, B, D1, E and Cdc2 become even undetectable by immunoblotting in adult cardiomyocytes (Yoshizumi \textit{et al.}, 1995; Brooks \textit{et al.}, 1997; Kang \textit{et al.}, 1997; Flink \textit{et al.}, 1998). Protein kinases implicated in Cdc2 kinase activity regulation, such as Cdk7 and Wee1, are either slightly increased in expression or do not change significantly (Kim \textit{et al.}, 1998). The kinase Plk1, involved in M phase regulation, was demonstrated to be downregulated on both the transcriptional and translational level during heart development (Georgescu \textit{et al.}, 1997).
The downregulation in the expression of cyclins and cyclin-dependent kinases during normal development of rat cardiomyocytes have been shown to be concomitant with the specific upregulation of the cyclin-dependent kinase inhibitor molecules p21 and p27 (Li and Brooks, 1997; Burton et al., 1999). Expression at the transcriptional level of the third member of the Cip/Kip family, p57, has been reported (Matsuoka et al., 1995), but the protein is detectable only at early stages in rat, however, it persist throughout life in man (Burton et al., 1999). Interestingly, cyclin-dependent kinase inhibitors of the INK4 family are hardly expressed, at least, demonstration of their protein expression in any developmental stages of the rat heart has not been achieved so far (Koh et al., 1998; Li and Brooks, 1999).

Regarding the tumor suppressor p53, transcript levels decrease markedly during the process of cardiomyocyte terminal differentiation and are very low or undetectable in adult animals (Kim et al., 1994). In contrast, the protein level of p53 seems to be upregulated in the adult compared to neonates (Magyar and Eppenberger, 1999).

The expression pattern for proteins of the pRb family in cardiomyocytes is controversial; studies concerning the expression of the retinoblastoma gene product pRb in the myocardium had shown conflicting results (MacLellan and Schneider, 1998). One group had suggested that the pRb mRNA and the protein be expressed in the developing ventricle, with pRb mRNA remaining constant throughout the development but with subsequent downregulating protein levels (Kim et al., 1994). Another report had failed to confirm the presence of pRb in the fetal heart (Zacksenhaus et al., 1996). Most reports confirm the presence of pRb in the neonatal and adult cardiomyocytes, albeit at low levels (Chen et al., 1996; Flink et al., 1998). The related protein p107 is mainly expressed in the embryonic heart and then downregulated after birth (Kim et al., 1995; Flink et al., 1998). The least studied pRb-like protein, p130, is expressed at low levels and remains present in the adult heart (Chen et al., 1996; Flink et al., 1998).

1.2.2 **THE CELL CYCLE IN THE PATHOLOGIC HEART**

Cardiac dysfunction may have devastating physiologic consequences for the organism. Heart disease is the predominant cause of disability and death in industrial nations accounting for about 40% of all postnatal deaths (Schoen, 1999). Cardiac mortality occurs in 80 to 90% of the cases as a result of myocardial ischemia. This pathologic
condition is defined as an imbalance between the supply, or perfusion, and the demand of the heart for oxygenated blood together with a reduced availability of nutrient substrates and inadequate removal of metabolites (Ganz and Braunwald, 1997). The most important form of ischemic heart diseases is the myocardial infarction, which is the consequence of coronary artery obstruction caused for instance by severe atherosclerosis. The insufficient supply of blood to the myocardium leads to cellular necrosis, and thus to the loss of cardiomyocytes resulting in a reduced pump function. The outcome is often heart failure, also called congestive heart failure, which is the inability to pump blood at a rate commensurate with the requirements of the metabolizing tissues or only from an elevated filling pressure (Schoen, 1999). In many pathologic states, the onset of heart failure is preceded by cardiac hypertrophy, the compensatory response of the myocardium to increased load and mechanical work (Schoen, 1999). This event is reflected on the cardiomyocyte level by an increased rate of protein synthesis, the amount of protein in each cell, the size of cardiomyocytes, and the up- and downregulation of specific genes including cell cycle-regulatory molecules.

In the rat model, a transient downregulation of p21 and p27 mRNA and protein levels, accompanied by a concomitant upregulation in the expression of cyclin D2 and cyclin D3, and in the activities of Cdk4 and Cdk6 complexes were detected in ventricular cardiomyocytes during the first two weeks following aortic constriction (Li and Brooks, 1997; Li et al., 1998). Also a reproducible upregulation of Cdk2 protein expression and activity was observed in the second week after imposition of the aortic constriction, although this was not significant compared to control animals (Li et al., 1998). In another study, induced myocardial infarction in the rat heart has been characterized by an increase in protein levels of cyclins A, B1, E and Cdc2 together with an enhanced activity of the Cdc2 kinase (Reiss et al., 1996). In human end-stage heart failure, p21 and p27 are reduced, whereas p57, which is found in the normal human heart throughout life, seems to be upregulated (Burton et al., 1999). These observations are consistent with the fact that injury to the myocardium may lead to a reversion to a fetal expression pattern (Petkova et al., 2000).

Considering DNA synthesis, cardiomyocyte labeling indexes have been reported in rats with either myocardial infarcts or with coronary artery narrowing (Nag et al., 1983; Capasso et al., 1992; Olivetti et al., 1992; Kajstura et al., 1994; Reiss et al., 1996).
the other hand, investigations by other groups have failed to confirm a significant increase in DNA synthesis in the suffering heart (Kellerman et al., 1992; Soonpaa and Field, 1994; Soonpaa and Field, 1997). It has been suggested that these controversial results be due to differences in animal species as well as methodology. Recently, the group of Anversa has even challenged the dogma that adult cardiomyocytes are postmitotic cells by showing some evidences of cell division after myocardial infarction in human patients (Beltrami et al., 2001). However, this report is highly controversial in its nature and does not elucidate potential molecular factors that are responsible to trigger proliferation in this cell type.

1.2.3 Reactivation of the Cardiac Cell Cycle

The topic that mammalian adult cardiomyocytes irreversibly exit from the cell cycle is heavily discussed and remains controversial. While Anversa and colleagues have suggested that such cells divide (Anversa and Kajstura, 1998; Beltrami et al., 2001), Soonpaa and Field and many others have questioned this proposal (for a review: Soonpaa and Field, 1998). In the newt, it has been generally accepted that amphibian adult ventricular cardiomyocytes can divide (Oberpriller et al., 1995). Regarding mammalian ventricular cardiomyocytes, most investigators nowadays are convinced that these cells are not capable of re-entering the cell cycle to undergo cell division after birth (Schneider and MacLellan, 2001). This fact has begun to spur investigators to examine the function of cell cycle proteins in postmitotic cardiomyocytes and overcome the cell cycle block by gene delivery and transgenic animals.

Functional proof for the importance of diminished cyclin D1 expression during heart development had been demonstrated using transgenic mice, which overexpress this cyclin under the control of the cardiospecific α-myosin heavy chain (MHC) promoter (Soonpaa et al., 1997). Cyclin D1 overexpression resulted in an increase in Cdk4 levels in the adult myocardium together with modest increases in PCNA and Cdk2 levels. Further analyses revealed a marked increase in the number of cardiomyocyte nuclei and a persistent DNA synthesis in 0.05% of the cells in adult cardiomyocytes from the ventricle.

Transgenic mice expressing the cyclin-dependent kinase Cdk2 under the α-MHC promoter displayed an increase in the expression level of Cdk4, cyclin A, D3 and E
besides the forced Cdk2 expression (Liao et al., 2001). Also an increase in both DNA synthesis and PCNA levels in the adult transgenic hearts has been shown. An interesting feature of adult animals was that the preponderance of binucleated cardiomyocytes shifted toward mononucleated cells, which were also smaller in size.

Knock-out mice of cyclin-dependent kinase inhibitors p16 exhibited a normal cardiac phenotype (Serrano et al., 1996) while p18-deficient mice demonstrated an increase in heart weight, which was proportional to increased body size (Franklin et al., 1998), both pointing to the negligible role of Ink4 family protein members in cardiomyocytes. At least, overexpression of p16 by recombinant adenovirus has been demonstrated to lead to a decreased rat cardiomyocyte size in vitro and in vivo, which may represent a novel strategy for gene therapy in the treatment of cardiac hypertrophy (Nozato et al., 2001).

Although p21, a member of the Cip/Kip family, is strongly regulated in cardiomyocytes during life (Li and Brooks, 1997), null-mice of this cyclin-dependent kinase inhibitor did not show any alterations specific to the cardiac phenotype (Deng et al., 1995). On the other hand, p27 knockout mice displayed a significant upregulation of cyclin A, E, Cdk2 and Cdc2 shortly after birth, resulting in additional rounds of cardiomyocyte proliferation and, according to the authors, in delayed terminal differentiation of these cells seen by ongoing fetal gene expression (Poolman et al., 1999).

Other investigations have concentrated directly on the transcription factor family E2F and members of the pRb protein family, which are the substrates for cyclin D-associated kinases. The role of E2F complexes in the cardiac cell cycle has become of even more interest since E2F is complexed with p107 in proliferating fetal cardiomyocytes, whereas E2F is principally associated with p130 and a lower level of pRb in neonatal cardiomyocytes implying a direct involvement in the withdrawal from the cell cycle (Flink et al., 1998). Considering the role of pRb in postmitotic cardiomyocytes, a cardiac-restricted deletion of this protein using the Cre/lox system in the adult stage has resulted in cardiac enlargement and sustained DNA synthesis similar to what is observed in transgenic mice overexpressing cyclin D1 (MacLellan et al., 1998).
The function of pRb and its related proteins in mediating cardiac cell cycle control was further investigated by exploiting the ability of viral oncoproteins to bind and thus inactivate this class of proteins (Figure 1-5). The first of these viral proteins to be studied in cardiomyocytes had been SV40 large T antigen. Expression of this protein in cultured neonatal rat cardiomyocytes led to proliferation with reportedly normal expression of cardiac-specific proteins (Sen et al., 1988). Later studies had revealed that sarcomeric MHC and other indicators of cardiac differentiation were expressed only conditionally in such large T antigen-transformed cells (Jahn et al., 1996). The transformation of adult ventricular cardiomyocytes in culture by large T antigen had been additionally reported (Miller et al., 1994), but without stringent analysis of cardiomyocyte properties. Transgenic mice expressing the large T antigen under a variety of muscle-specific promoters resulted in hyperplasia of atrial and ventricular cardiomyocytes (Field, 1988; Katz et al., 1992), but cells derived from the tumors exhibited only a few of the ultrastructural characteristics typical for the cardiac phenotype.

Overexpression of the adenoviral protein E1A by adenoviral gene delivery in cultured embryonic and neonatal rat cardiomyocytes had resulted in the induction of DNA synthesis (Kirshenbaum and Schneider, 1995; Liu and Kitsis, 1996; Bishopric et al., 1997). The entry into S phase was accompanied by widespread apoptosis, which could be prevented by simultaneous expression of the adenoviral E1B protein (Kirshenbaum and Schneider, 1995), a known inhibitor of apoptosis (White et al., 1992). Further studies revealed that, like E1A, forced expression of E2F-1 reactivated the cell cycle in cultured neonatal rat cardiomyocytes leading to DNA synthesis and the suppression of cardiac gene transcription, underlying the effects of E1A and its mitogenic signaling (Kirshenbaum et al., 1996; von Harsdorf et al., 1999). In term of the E1A pathway, it has
been demonstrated in cultured neonatal cardiomyocytes that this adenoviral protein is able to induce S phase entry independent of Cdk2, which is activated by E2F-1 overexpression, suggesting the potential involvement of other endogenous pRb-binding proteins or alternative E1A targets (Akli et al., 1999). Even induced DNA synthesis has been observed in adult cardiomyocytes in vitro and in vivo by ectopic expression of E2F-1 (Agah et al., 1997). Additionally, E2F-1-induced apoptosis has been found to be independent of p53 (Agah et al., 1997) and was suppressed by E1B (Kirshenbaum et al., 1996) or by the presence of insulin-like growth factor 1 (IGF-1) (von Harsdorf et al., 1999).

1.2.4 TARGETING THE G2/M BOUNDARY IN THE CARDIAC CELL CYCLE

The focus has so far mainly concentrated on the dissection of regulatory factors involved in the G1/S transition in cardiomyocytes. To get a complete picture of the cardiac cell cycle, it is thus indispensable to expand the view beyond the G1/S transition. The necessity to identify the critical role of G1/S-downstream cell cycle molecules becomes evident from the expression pattern of such molecules during heart development. It has been suggested that the permanent withdrawal of cardiomyocytes from the cell cycle be directly correlated with the disappearance of the mitotic cyclin A, while the expression of D-type cyclins still persists beyond the proliferation stop (Yoshizumi et al., 1995). Downregulation of both mRNA and protein expression of the mitotic cyclin B1 to undetectable levels and the accompanying decrease in Cdc2 kinase activity only a few days after birth (Brooks et al., 1997; Kang et al., 1997) point to a significant involvement of molecules present in the G2/M boundary in the cell cycle block.

Targeting the G2/M boundary in the cardiac cell cycle is getting even more attractive in term of the outcome of molecular investigations intervening at the first cell cycle checkpoint. Recent findings in the transgenic mice overexpressing Cdk2 (Liao et al., 2001) give rise to the existence of an additional block in the G2/M boundary, since forced expression of Cdk2 was not sufficient to reactivate the expression of Cdc2 and cyclin B1. Moreover, induction of cell cycle re-entry by E1A and E2F-1 was strictly limited to an increase in DNA synthesis in cardiomyocytes (Kirshenbaum and Schneider, 1995; Liu and Kitsis, 1996; Kirshenbaum et al., 1996; Agah et al., 1997; Bishopric et al., 1997; Akli et al., 1999; von Harsdorf et al., 1999). Resetting the cardiac cell cycle at the
G1/S checkpoint (Agah et al., 1997) may not be the target of choice, because the cells at this stage have already undergone an S phase before they acquire binucleation and polyploidy due to endoreduplication.

The absence of M phase entry reported in the studies mentioned above emphasizes the G2/M cell cycle region as an additional critical checkpoint in the cardiac cell cycle progression. In this respect, it is not surprising that the role of large T antigen in cardiomyocytes has been intensively investigated (Field, 1988; Sen et al., 1988; Katz et al., 1992; Miller et al., 1994; Jahn et al., 1996), since its target is not only G1/S- but also the G2/M transition, leading to an increase in mitotic cyclin expression and enhanced Cdc2 activity in diploid human cells when overexpressed (Chang et al., 1997). The reported immortalization of cardiomyocytes by large T antigen is still a matter of ongoing debate due to the open questions left regarding the remaining cardiospecific characteristics of the transformed cells.

In summary, there is a significant lack of knowledge regarding the regulation of the cardiac cell cycle downstream of the G1/S checkpoint, thus asking for intensive experimental investigations at the G2/M boundary in order to receive a more comprehensive view of the proliferation arrest in cardiomyocytes.

1.3 Genetic Engineering of Cardiomyocytes

The treatment of cardiomyopathies and heart failure after myocardial infarction is an enormous therapeutic challenge; the missing repair capacity of cardiomyocytes is the major obstacle. In severe cases, allograft heart transplantation represents the last option to prolong the patient’s life. The improved graft acceptance due to successful developments in immunosuppressive therapy makes heart transplantation the most effective therapy for the failing heart today. However, it is estimated that the limited supply of donor hearts will prevent in the United States alone heart transplantation for most of the estimated 40000 patients 65 years and younger who could benefit annually (Jankowski and Wagner, 1999).

The shortage of transplantable hearts, the morbidity associated with chronic immunosuppression and the existing danger of graft rejection clearly limit the utility of cardiac transplantation and ask for alternative therapeutic strategies. In this respect, the
approach of transplanting single cells would circumvent many of the problems associated with entire organ transplantation. Another potential therapy implicates the genetic engineering of heart cells eventually leading to an improved cellular function by replacing lost or defective genes or adding genes known to produce proteins with beneficial effects. Even a combination of these two strategies is conceivable for the development of the ultimate cure to repair a ‘broken heart’. An overview is here provided of the potential possibilities of genetically engineered cardiomyocytes for prospective therapies and the use of long-term culture systems of adult cardiomyocytes as a model system to investigate such novel approaches.

1.3.1 APPROACHES FOR CELLULAR CARDIOMYOPLASTY

1.3.1.1 Cellular transplantation using cardiomyocytes

Irreversible cellular damage in the heart and the resulting deficits in cardiomyocyte number lead to a loss of contractile function. An appropriate therapy may implicate the replacement, repair or the enhancement of the biologic function of damaged cells in order to strengthen the weakened heart (Kessler and Byrne, 1999). For this goal, the idea of transplanting single cells instead of an entire heart has become an attractive approach (see Figure 1-6 on page 23). The strategy assumes that grafted cells will normally respond to physiological stimuli and thus support organ function. This would under optimal condition mean that implanted cells within an infarcted scar should possess the same electrical and mechanical characteristics as a native cardiomyocyte and enhance cardiac performance by directly contributing to contraction. Cardiomyocytes are electromechanically coupled by specialized cell-cell junctions, the so-called intercalated disks containing adherens and gap junctions for mechanical and electrical coupling, respectively. The major adherens junction protein in the mature mammalian heart is N-cadherin (Volk and Geiger, 1984) and the major gap junction protein is connexin-43 (Beyer et al., 1987). The electromechanical coupling between cardiomyocytes is fundamental for a coordinated contraction of the myocardium. Theoretically, the most appropriate cell type to choose for cellular transplantation is thus the cardiomyocyte itself.
Figure 1-6: Potential sources of muscle cells used for cellular transplantation. Advances in molecular biology and gene transfer techniques have opened the door for the development of new therapeutic approaches, including cellular transplantation, to assist the failing myocardium.

The possibility of transplanting cardiomyocytes was first reported in the pioneering studies of Field, Soonpaa, Koh and associates (Soonpaa et al., 1994; Koh et al., 1995b). They demonstrated for the first time that fetal cardiomyocytes could be grafted into the normal myocardium of mice. Intercalated disk formation, gap junctions and connexin-43 staining were observed at interfaces between grafted cells and host myocardium confirming incorporation into the heart (Soonpaa et al., 1994). This group reported also cardiomyocyte graft formation over more than 10 weeks after engraftment of fetal cardiomyocytes in the heart of dystrophic mice and dogs (Koh et al., 1995b). Similar findings showing intercalated disk formation were detected in grafting studies using human fetal cardiomyocytes and neonatal porcine cardiomyocytes transplanted into porcine myocardium (Van Meter et al., 1995) as well as by using murine fetal cardiomyocytes, transduced with an adenoviral vector prior to transplantation into hearts of syngeneic adult mice (Gojo et al., 1997).

After these promising results, the use of fetal cardiomyocytes in the infarcted and failing myocardium was tested. The first studies had demonstrated that the cardiac environment was favorable to the engrafted fetal cardiomyocytes (Leor et al., 1996) and even a marked increase in angiogenesis had been observed both within the grafts and adjacent host myocardium (Watanabe et al., 1998). Grafting of fetal cardiomyocytes in a coronary occlusion/reperfusion model had been found to be associated with improved left ventricular function (Scorsin et al., 1997).

An improved systolic function in cryoinjured rat hearts had been also observed after transplantation of fetal cardiomyocytes in the scar tissue, although the grafted cells did not contract synchronously with the host adult myocardium (Li et al., 1996b).
formation of gap junctions between grafted fetal cardiomyocytes and the injured myocardium allowing electrical coupling had been described (Connold et al., 1997). Regarding the role of electromechanical junctions for the integration of the grafted cardiomyocytes, Reinecke and colleagues had found that the principal requirements for a functional improvement could be achieved by transplantation of fetal and neonatal cardiomyocytes (Reinecke et al., 1999). In the same study, however, implantation of isolated adult ventricular cardiomyocyte failed. Finally, Sakai and colleagues have demonstrated the possibility to enhance heart performance of rats by reimplanting adult cardiomyocytes previously harvested from the left atrial appendage (Sakai et al., 1999).

1.3.1.2 Strategies using stem-cell derived cardiomyocytes

A serious impediment for a widespread application of cardiomyocytes for cellular transplantation is the difficulty to obtain sufficient numbers of donor cells. The use of embryonic stem (ES) cells which are totipotent cells derived from the inner cell mass of blastocytes as an alternative source for cardiomyocytes has been first described by Klug and colleagues (Klug et al., 1996). They stably transfected a construct encoding the aminoglycoside phosphotransferase under the control of the α-cardiac MHC promoter into pluripotent ES cells. The resulting cells had differentiated in vitro and were subjected to neomycin selection to select stem cell-derived cardiac myocytes. These cells exhibited spontaneous, rhythmic contractile activity and were suitable for cardiac transplantation and formation of intracardiac grafts (Klug et al., 1996).

The isolation of a cardiomyogenic cell line from murine bone marrow stromal cells has been suggested as another possibility to substitute fetal cardiomyocytes (Makino et al., 1999). Some concerns regarding electrical coupling remain since there are no data available about intercalated disk proteins in these cardiac-like cells. In one study, the cardiomyogenic differentiation of bone marrow cells and their survival in myocardial scar tissue resulting in an improved myocardial function was evaluated (Tomita et al., 1999).

A recent report by Orlic and colleagues described the possibility to inject multipotent cells derived from the bone marrow of healthy mice into the undamaged region surrounding the infarct (Orlic et al., 2001). They demonstrated that the bone marrow cells infiltrate into the damaged region, where they started to express characteristics of
cardiomyocytes, smooth muscle cells and endothelial cells resulting in improved contractile function. Regarding the new formation of cardiomyocytes in the infarcted heart, the authors presented only rather scarce evidence that these cells had become cardiomyocytes. More recently, it could be shown that a distinct fraction of bone marrow-derived cells or their progeny were incorporated into ischemically damaged myocardium after injection into lethally irradiated mice (Jackson et al., 2001). The cells expressed proteins typical for myogenic cells such as α-actinin. In addition, the efficiency of the bone marrow-derived stem cell conversion is very low, so it seems only hardly possible that this technique could significantly contribute to repair a damaged heart. It is certainly of great interest that no tissue specific myocardial stem cells could be identified so far.

1.3.1.3 Cellular transplantation using cells different from cardiomyocytes

A feasible approach to increase the amount of contractile tissue in a failing heart could be the transplantation of skeletal muscle cells. One advantage, making this cell type especially attractive, is its regenerating capacity. Each mature skeletal myofiber contains a few myogenic cells known as satellite cells that remain in a less differentiated state. After injury, they are able to enter the mitotic cycle and later fuse with each other and with injured myofibers restoring intact myofibers. Several groups have raised the hypothesis that transplanted myogenic cells obtained from skeletal muscle could undergo ‘milieu-influenced differentiation’ and thus become cardiac muscle cells (Leor et al., 1997). This phenomenon has been supported by dynamic cardiomyoplasty, an experimental surgical procedure, in which vascularized grafts of left latissimus dorsi muscle is wrapped around the epicardial surface of the heart. After preconditioning with repeated electrical stimulation, a conversion of fast to slow (cardiac-like) twitch fibers could be observed (Hooper and Stephenson, 1993).

First experiments using myogenic cells focused on the fate of these cells after cellular transplantation into the hearts of syngeneic animals (Koh et al., 1993a; Koh et al., 1995a; Robinson et al., 1996) or of autologous satellite cells grafted into heart (Marelli et al., 1992; Chiu et al., 1995; Yoon et al., 1995; Taylor et al., 1997; Atkins et al., 1999). Murry and colleagues demonstrated that neonatal skeletal myoblasts can be engrafted into cryoinjured rat heart forming multinucleated myotubes which then differentiate into
mature myofiber (Murry et al., 1996b). The different electrical properties are a drawback for cardiac repair. Although electromechanical coupling between skeletal and cardiac myocytes has been demonstrated *in vitro* (Reinecke et al., 2000), it still remains uncertain if implanted myoblasts are actively responsible for force generation during contraction (Jain *et al.*, 2001).

The fact that heart function can be enhanced with cells without cardiomyocyte characteristics has been described by Li and coworkers (Li *et al.*, 1999). They demonstrated that transplanted smooth muscle cells formed smooth muscle tissue in myocardial scar tissue showing an improved contractile function. The authors assumed an increase in myocardial wall tension and elasticity which minimized ventricular dilatation.

### 1.3.1.4 Alternative cellular transplantation strategies

The difficulties to obtain human fetal and neonatal donor hearts for a prospective therapy and the need to learn more about the fate of implanted stem cells in the hearts have initiated a number of alternative strategies regarding cellular transplantation to ‘rejuvenate’ the damaged heart.

One approach in direction of a cellular cardiomyoplasty using skeletal muscle cells was to convert cardiac fibroblasts into muscle cells. This transdifferentiation approach has been investigated by Murry and colleagues by inducing skeletal muscle differentiation in heart lesions by introducing the muscle-specific transcription factor MyoD (Murry *et al.*, 1996a), but myofibrillogenesis could not be observed and the authors did not evaluate the experimental outcome concerning the extent of myocardial function.

Another strategy implied the induction of angiogenesis by angiogenic factors and other growth factors to augment contractility of the remaining cardiomyocytes. Such therapeutic peptides would be secreted from genetically engineered cells being transplanted into the damaged myocardium. The usefulness of implanted cells into the heart acting as a platform for protein delivery was demonstrated using the genetically modified myogenic cell line C2C12 expressing recombinant TGF-β1 (Koh *et al.*, 1995a).

The potential advantage of using genetically engineered cells could be to control the amount of gene expression. This could be achieved by a selective elimination of some of
the implanted cells using suicide genes engineered into the cells (Kessler and Byrne, 1999).

The most logical, and possibly the most attractive method would be the reversal of the terminal differentiation of uninjured cardiomyocytes for manipulating the cardiac repair process. One strategy in this direction would imply the induction of cell proliferation in adult cardiomyocytes by genetic engineering (Figure 1-7).

![Figure 1-7: Ultimate strategy for cell-mediated myocardial repair](image)

After harvesting intact cardiomyocytes from a patient biopsy, the number of adult cardiomyocytes would be expanded in vitro by induction of cell proliferation using recombinant DNA technology. Subsequent reimplantation of these cells would finally lead to an improved contractile function of the diseased heart.

The cells would be obtained ex vivo and then propagated in vitro up to a sufficient number and would be reimplanted into the damaged myocardium. This strategy may have several advantages. First, grafts of autologous cardiomyocytes would definitely minimize the risk of an immune rejection associated with allogeneic or xenogeneic cells or at least avoid the costly therapy with conventional immunosuppressive agents to induce donor-specific tolerance. Second, the use of adult cardiomyocytes would provide the properties of mature functional cells that could integrate with adjacent cells in the tissue and enhance cardiac performance by directly contributing to contraction. Furthermore, induction of cell proliferation in vitro would allow cardiomyocyte growth under controlled conditions in contrast to an induction in situ, thereby facing the risk of neoplasia initiation in other cell types. The safety profiles of these in vitro modified cells could be stringently assessed prior to administration to the patient. Such an approach assumes that the reimplanted cardiomyocytes would maintain their adult cardiac
phenotype, which is coupled to the inability to proliferate. Induction of cell proliferation \textit{in vitro} has to be thus transient.

The possibility of engrafting cardiac myocytes grown in culture had first been demonstrated in the work of Steinhelper and Delcarpio (Steinhelper \textit{et al}., 1990; Delcarpio \textit{et al}., 1991). Atrial tumors isolated from transgenic mice atrial expressing the SV40 large T antigen were propagated as subcutaneous tumors in syngeneic mice to generate the cell line AT-1 that bears some characteristics of atrial cardiomyocytes. Grafts of these cells did, however, not form gap junctions with the native myocardium while the mitotic activity of these cells \textit{in vivo} remained (Koh \textit{et al}., 1993b). Introduction of HL-1 cells, a derivative of AT-1 (Claycomb \textit{et al}., 1998), allowed the formation of stable grafts within healthy porcine myocardium together with formation of adherens junctions and gap junctions, but these cells failed to survive in the infarction model (Watanabe \textit{et al}., 1998).

In summary, cell transplantation would be an exciting approach for myocardial repair. The most practical source for cells is provided by the use of the patient’s own cells (El Oakley \textit{et al}., 2001). Nevertheless, the strategy for cellular transplantation using autologous cardiomyocyte still faces significant hurdles as for instance the induction of cell proliferation. Before such methods and other strategies become available for therapy, several fundamental problems regarding cardiobiology and the experimental procedure need to be solved. The use of a long-term culture system of adult cardiomyocytes may be a practicable way and is discussed next.

1.3.2 ADULT CARDIOMYOCYTES IN LONG-TERM CULTURE AS A MODEL SYSTEM

1.3.2.1 Establishments of primary cultures of adult cardiomyocytes

Cultures of cardiomyocytes allow to study cardiomyocyte behavior without taking into account existing influences from the large number of other stimuli originating from interstitial tissue and other cell types present \textit{in vivo}. Generally, \textit{in vitro} systems represent an alternative to investigations on the whole heart, which is not easily accessible for experiments. Heart tissue had first been put in culture 90 years ago by Burrows, placing pieces of explanted embryonic chick hearts in culture (Burrows, 1912). Proteolytic enzymes to dissociate heart tissue to obtain single cardiomyocytes for cell
culture had been used by Cavanough for chick embryo myocytes (Cavanough, 1955) and by Harary and Farley for mammalian cardiomyocytes (Harary and Farley, 1960). Not before 1976, a technique enabling the isolation of viable adult ventricular cardiomyocytes had been reported. This was due to the strong physical connections of this cell type caused by intercalated disks and the extracellular matrix (Powell and Twist, 1976). The first ventricular adult cardiomyocytes had been cultured in 1977 by Jacobson (Jacobson, 1977), followed by Claycomb and Palazzo (Claycomb and Palazzo, 1980), and Nag and Cheng (Nag and Cheng, 1981), while Cantin and colleagues reported the culture of atrial cardiomyocytes (Cantin et al., 1981).

Despite the fact that the majority of long-term studies using myocytes have been performed on fetal and neonatal cardiomyocytes, it would be preferable to use adult cardiomyocytes, if one is interested in the in vivo state. The advantage of adult cardiomyocytes is that the cells are fully differentiated and retain main characteristics of adult cardiomyocytes in the intact heart. There exist two methods for the cultivation of adult ventricular cardiomyocytes as defined originally by Jacobson and Piper (Jacobson and Piper, 1986). The ‘rapid attachment model’ was introduced by Piper and coworkers, in which the adult cardiomyocytes attach within 3 hours to serum-pretreated culture dishes in a medium supplemented with 4% serum (Piper et al., 1982). Afterwards, the cells are cultured in serum-free medium. Using this technique, the cells retain their rod-shaped morphology and striated appearance of newly isolated cardiomyocytes for a few days but are unable to contract spontaneously. In the ‘redifferentiation model’ (Jacobson, 1977; Claycomb and Palazzo, 1980; Nag and Cheng, 1981; Borg et al., 1984; Eppenberger et al., 1988), the cells are cultured in the presence of high serum (10 – 20%). Under these conditions, the cardiomyocytes begin to attach to the culture surface within 2 days and start to spread on the substrate, sending out pseudopodia-like projections in different directions. The cells undergo a morphologic transition from an elongate in vivo shape to a spherical shape within several days. These changes are accompanied by the degradation and eventual regeneration of structures of the contractile apparatus as well as of the cytoskeleton (Eppenberger et al., 1988). The result of this redifferentiation process in culture is an electrically coupled beating tissue-like network consisting of adult cardiomyocytes. The use of such long-term cultures of adult cardiomyocytes for basic and clinical research is the topic of the following section.
1.3.2.2 Long-term cultures of ventricular adult rat cardiomyocytes and their application.

Ventricular adult rat cardiomyocytes (ARC) pass through several morphologic states in the redifferentiation model of culture. During cultivation, it has been demonstrated that proteins of the contractile apparatus and the cytoskeletal structure go through several steps of degradation and regeneration, offering a convenient model for studying regeneration of myofibrils (Eppenberger et al., 1988). Several aspects of this structural remodelling taking place \textit{in vitro}, are reminiscent of mechanisms occurring during physiologic and pathologic hypertrophy \textit{in vivo} (Eppenberger-Eberhardt et al., 1990; Eppenberger-Eberhardt et al., 1993; Schaub et al., 1997) including an increase in cardiomyocyte volume (Messerli et al., 1993). Therefore, ARC in primary culture represent a suitable \textit{in vitro} model for detailed analysis of the hypertrophic reaction at the cellular level (Eppenberger et al., 1994).

Furthermore, cultured ARC allow the investigation of the molecular basis of cell-cell interactions during contact formation between individual cardiomyocytes under various experimental conditions, events that are almost impossible to investigate \textit{in vivo} (Hertig et al., 1996a; Hertig et al., 1996b; Zuppinger et al., 2000b). With regard to propose novel therapeutic approaches for the treatment of damaged myocardium, such cell-cell contact studies are of importance. They clearly demonstrated that adult cardiomyocytes are able to form \textit{de novo} intercalated disk-like structures being a prerequisite for the electric coupling with the myocytes \textit{in vivo}. It was also shown in co-cultures of adult atrial and ventricular rat cardiomyocytes that these two cell types are able to form intercalated-disk like structures between one and another (Eppenberger and Zuppinger, 1999). Together with the findings of Sakai (see page 23) and the successful establishment of cultures of atrial human cardiomyocytes (Dr. J. Feucht, personal communication), transplantation of atrial human cells into ventricular tissue seems very well possible (Zuppinger et al., 2000a).

It has been shown, that ARC maintained in primary culture allow easily the study of influences of growth and differentiation factors on isolated cardiomyocytes (Donath et al., 1994; Harder et al., 1996; Eppenberger-Eberhardt et al., 1997). These kind of experiments reveal new findings regarding the function of growth factors and also
provide a model system for the application of factors as possible therapeutic peptides. Genetically engineered cardiomyocytes could eventually secrete particular proteins. The implementation of recombinant DNA technology in culture systems of adult cardiomyocytes combines powerful tools to study problems of cell and molecular biology of basic and applied nature (Figure 1-8).

For instance, effects of the expression of specific genes can be monitored in vitro, thus possibly providing information about the physiological and pathological role of such gene products in the myocardium. The dissection of molecular pathways by genetic engineering in a tissue-culture environment represents a useful experimental platform for the study of cardiobiology and for a better understanding of the complex physiological processes encountered in vivo. One should think that using the system of genetically engineered adult cardiomyocytes have become a standard experimental procedure in cardiovascular research in the meantime. However, the difficulties to use standard transfection procedures in adult cardiomyocytes (Rust et al., 1998) have prevented a thorough application of recombinant DNA technology in those culture systems.

1.3.3 GENE TRANSFER METHODS FOR CARDIOMYOCYTES

Progresses in human genetics, genetic engineering of animals, molecular and cellular technologies and developments in gene transfer have led cardiovascular medicine to the dawn of a new therapeutic era. The concept of gene therapy aims to transfer and to express specific genes to relieve the consequences of a disease (Mulligan, 1993). Two gene therapy strategies have been proposed. One is the gene transfer ex vivo, which implies the removal of cells, followed by gene transduction in vitro and the in vivo
reimplantation of the modified cells. The second to mention is the direct gene transfer in vivo, employing a variety of gene delivery approaches and vectors that have been applied in animal models. The vector is the agent that carries the gene to the desired site of action. Two general categories of vectors exist, viral or nonviral, but also a combination of the delivery methods is possible. The ideal vector would be relatively safe, highly efficient, and of tissue- or cell type specificity. It should provide high but controllable levels of long lasting expression. Such a vector, however, does not exist and it will not be constructed in the near future (Nabel and Leiden, 1999). A number of different vectors have been developed instead, each providing unique properties for basic research in cardiology and for a prospective treatment of heart diseases (Eppenberger, 1996).

1.3.3.1 Viral gene transfer systems

Retrovirus-mediated gene transfer is widely used for experiments in vitro and in vivo, because of the capability of this kind of virus to stably integrate into the host chromosome, potentially leading to long-term expression of the transgene (Yee, 1999). The drawback of retroviruses is their inability to infect nonproliferating cells rendering them useless for an application in adult cardiomyocytes. In contrast to retroviral vectors, adenoviruses infect a wide variety of replicating and nonreplicating cell types and have been shown to be very efficient in delivering the transgene into cardiomyocytes in vitro and in vivo (Kass-Eisler et al., 1993; Guzman et al., 1993; Kirshenbaum et al., 1993). However, the strong immune response adenoviral vectors evoke will make clinical applications unlikely (Hajjar et al., 2000). Adeno-associated virus (AAV) vectors have also been considered in cardiovascular application. They are based on a nonpathogenic parvovirus with a broad host range and are able to transduce a variety of both dividing and nondividing cell types (Flotte et al., 1993; Podsakoff et al., 1994). Kaplitt et al. have reported in vivo gene transfer into myocardium via infusion of AAV vectors into pig coronary arteries, resulting in the expression of the transgene in cardiomyocytes without apparent toxicity or inflammation (Kaplitt et al., 1996). An efficient gene transfer has also been demonstrated for neonatal rat cardiomyocytes in culture (Maeda et al., 1998). As shown for retroviral and adenoviral vectors, there is a number of problems appearing when AAV vectors are used and which have to be solved before these vectors can be
employed in gene therapy for humans; the size of the transgene is limited to a few kilobases and there exist considerable difficulties to generate large quantities of pure viral particles (Rolling and Samulski, 1995). A lentivirus-derived vector systems, based on a modified retroviral HIV genome, has been suggested as well for the transduction of nondividing cells allowing integration of the transgene into the host genome (Naldini et al., 1996). The application of these vectors has been demonstrated in embryonic and neonatal cardiomyocytes in vitro (Mochizuki et al., 1998; Rebolledo et al., 1998; Sakoda et al., 1999) but the use in adult cardiomyocytes in vitro and in vivo has not been reported yet. Recombinant herpes simplex viruses infecting rat cardiomyocytes in vitro and in vivo (Coffin et al., 1996; Wright et al., 2001) and vectors based on Semliki Forest virus transducing cultured neonatal rat cardiomyocytes (Roks et al., 1997) have been applied too. Both systems need further evaluation regarding their safety and usefulness for cardiobiology and for their prospective application in vivo because of their potential cytotoxicity.

1.3.3.2 Nonviral gene transfer and hybrid systems

The problems with the use of viral vectors have led to optimize and improve nonviral gene delivery, e.g. direct gene transfer using uncomplexed plasmid DNA has been demonstrated to produce a low but detectable rate of transgene expression in cardiac muscle in vivo (Lin et al., 1990; Wolff et al., 1990; Acsadi et al., 1991). Microinjection of ‘naked DNA’ for gene transfer into adult cardiomyocytes in vitro has also been used but clearly lacked efficiency, especially when applied in freshly isolated adult cardiomyocytes (Zuppinger et al., 2000b). In general, delivery of plasmid DNA to adult cardiomyocytes by conventional means such as calcium phosphate precipitation (Chen and Okayama, 1988) and lipofection (Felgner et al., 1987) has not worked (Antin et al., 1988; Rust et al., 1998), although there have been reports of highly successful variations of these techniques in cultured neonatal cardiomyocytes (Xu et al., 1992). A relatively high plasmid transfection in vivo has recently been achieved by the formation of plasmid DNA complexes with stearyl-poly-L-lysine low-density lipoproteins. This has resulted in a prolonged expression of the transgene up to 30 days after injection (Affleck et al., 2001).
Great promise for gene transfer into cardiomyocytes has been demonstrated by hybrid strategies combining efficiency of virus-mediated transfection and the convenience of a plasmid-based approach. One of these strategies for an efficient gene transfer into the rat heart has made use of the enhancement of liposome-mediated transfer by the incorporation of fusogenic viral proteins from the Sendai virus (Aoki et al., 1997a; Aoki et al., 1997b). Another method to transfect cardiomyocytes in culture with remarkable success has used trinary complexes containing replication-deficient adenoviruses, poly-L-lysine, and expression plasmids encoding a gene of interest. Using this method, a transfer rate of 70% in neonatal cardiomyocytes (Kohout et al., 1996) and an efficiency of up to 10% in adult cardiomyocytes had been achieved (Wei et al., 2000).

1.3.3.3 The quest for the vector of choice

The field of gene transfer is receiving a great deal of attention, and substantial progress has been made in a relatively short time (Kass-Eisler and Leinwand, 1997), but gene transfer into cardiomyocytes, particularly into adult cardiomyocytes, is still a difficult task. Each of the above reviewed methods for gene delivery has particular advantages and disadvantages. Therefore, the vector of choice primarily depends on the nature of the biologic question to be addressed. For instance, microinjection may be a useful method for adult cardiomyocytes in long-term culture when the localization of the gene product within the cell is of interest. However, the number of adult cardiomyocytes expressing the transgene may be too low for further biochemical and cellular analyses. Based on its high efficiency, adenovirus-mediated gene transfer could circumvent this problem. In case the function of many genes needs to be screened, the adenoviral vector would on the other hand not be useful; the necessary effort to produce recombinant constructs would be too high. The evaluation of the appropriate vector plays an import role in the establishment of the experimental setup and will be thus inevitably linked with the success of the experiment.
1.4 THE AIM OF THIS STUDY

Although the irreversible exit of cardiomyocytes from the cell cycle has received much attention by scientists, it is not yet known which factors are responsible for the arrest. The current understanding of the function of cell cycle-dependent molecules as well as the complex regulation of the cardiac cell cycle itself is still at a preliminary stage of development. While most studies have concentrated on the role of cell cycle molecules in the G1/S transition phase, molecules downstream of this point in the cardiac cell cycle have been solely characterized with respect to their expression pattern during heart development. A direct correlation between regulatory factors involved in the G2/M transition and the permanent withdrawal from the cell cycle in cardiomyocytes had been suggested, but a functional characterization of the factors and potential ways to reactivate the cell cycle by genetic means is still missing. The rationale to focus on the G2/M boundary was provided by indications from transgenic approaches at the G1/S control point. These investigations had disclosed evidences that supported a further strong cell cycle arrest at the G2/M transition.

The aim of the work presented in this dissertation was to get a better idea what the strong proliferation arrest of adult cardiomyocytes is based on and to identify novel ways to induce cell cycle progression in postmitotic cardiomyocytes. Major attention has been therefore paid to the G2/M checkpoint. In particular, the role of the cyclin-dependent kinase Cdc2 forming together with its regulatory subunit cyclin B1 the mitosis-promoting factor, abbreviated MPF, the key player in the molecular network for M phase entry, was studied. The experimental platform used for our investigations has been the long-term culture system of ARC. This in vitro system represents a convenient model and certainly allows extrapolation to the in vivo situation, its experimental handling, however, is rather fastidious. As a consequence, one major milestone to achieve in this study has been the establishment of experimental procedures which permitted reliable statements about the cardiac cell cycle. The exploration of molecular techniques specifically including gene transfer technologies and their application has been crucial to shift experiments from mainly descriptive to more functional nature using ARC culture systems. The application of these acquired techniques should allow to induce MPF
activity, which would provide first information about the functional recovery of the MPF complex and its influence on postmitotic cardiomyocytes.
2 RESULTS

2.1 ESTABLISHMENT OF A NOVEL VIRUS-BASED GENE TRANSFER METHOD FOR CULTURED VENTRICULAR ADULT RAT CARDIOMYOCYTES (ARC)

Isolated cardiomyocytes maintained in primary culture allow the study of cardiac function without the need to consider the intrinsic complexity encountered in vivo. The majority of these in vitro investigations have been performed on embryonic and neonatal cardiomyocytes. However, adult cardiomyocytes in culture provide a more accurate model of the adult heart because these cells are derived from a terminally differentiated myocardium. The use of recombinant gene technology on adult cardiomyocytes has still been little effective due to the poor efficiency of standard gene transfection methods (Rust et al., 1998). Highly efficient gene transfer with several virus-based gene delivery systems had been demonstrated in cultured embryonic and neonatal cardiomyocytes (Kass-Eisler et al., 1993; Kohout et al., 1996; Roks et al., 1997; Maeda et al., 1998; Mochizuki et al., 1998). For cultured ventricular ARC, only recombinant adenoviruses were reported to transduce these cells in culture efficiently (Kass-Eisler et al., 1993; Kirshenbaum et al., 1993). Although a high infection rate has been observed, the time-consuming generation of adenoviral particles is still a major concern, especially if a large number of transgenes is to be analyzed. New methods for gene delivery into adult cardiomyocytes will have to be developed, which not only facilitate basic research using adult cardiomyocyte culture systems but may be of importance for prospective gene therapy in the heart too.

2.1.1 EFFICIENT TRANSDUCTION OF CULTURED ARC BY RECOMBINANT SINDBIS VIRUS (SIN)

In this study, the potential of a novel viral system to transduce ARC in culture was investigated, namely by using Sindbis virus (SIN)-based vectors. SIN is a positive-strand RNA virus, which belongs to the alphavirus genus (Schlesinger and Schlesinger, 1996). It has a wide host range and is able to amplify its genome to high levels resulting in the expression of large amounts of viral proteins. Wild-type strains cause acute encephalitis
in mice as a result of induced neuronal apoptosis (Lewis et al., 1996). In recent years, SIN-based vectors have undergone an impressive development to achieve efficient heterologous gene expression in various cells. The advent of replication-deficient versions made SIN vectors attractive as a possible tool for gene therapy (Bredenbeek et al., 1993). The potential of SIN vectors to deliver genes into organ-specific cells was reported for neural cells in vivo (Altman-Hamamdzie et al., 1997; Gwag et al., 1998) and in vitro (Gwag et al., 1998; Ehrengruber et al., 1999).

The feasibility of SIN as a gene transfer vehicle for cultured ARC was tested by using a replication-deficient SIN encoding the β-galactosidase gene, termed SINrep5/lacZ. To support further gene expression analysis in ARC, a replication-deficient SIN encoding the green fluorescent protein (GFP) was additionally created, termed SINrep19/GFPwt. The recombinant viral particles generated were based on the principle of the SINrep5 system, which was introduced by Bredenbeek and colleagues (Bredenbeek et al., 1993) (Figure 2-1).

The RNA templates for SINrep5/LacZ, SINrep19/GFPwt and the defective helper DHBB were obtained by in vitro transcription using the corresponding cloned DNA plasmids (see section 4.2.1 on page 108). Complementation between the SIN replicon and the defective helper was accomplished by coelectroporation into baby hamster kidney (BHK) cells, resulting in the production of infectious SIN particles. Maximum viral concentration achieved was $5 \times 10^7$ infectious units per milliliter (IU/ml) for SINrep5/lacZ and $1 \times 10^8$ IU/ml for SINrep19/GFPwt. These values refer to the gene
transfer efficiency determined in BHK cells, meaning that one milliliter of the viral stock solution, for instance of SINrep5/lacZ, leads to detectable levels of transgene expression in $5 \times 10^7$ cells. However, it does not consider the number of viral particles that is able to infect but unable to achieve transgene expression. This may be due to inappropriate packaging or inefficient yield of full length RNA previously generated in vitro (RNA transcripts reached a size of more than 12 kb depending on the transgene inserted into pSINrep5). But the number of BHK cells displaying SIN-induced cell rounding without visually detectable transgene expression level was generally low (Figure 2-2). This observation pointed to a homogenous population of recombinant viral particles in the titers generated.

Figure 2-2: BHK cells infected by recombinant SIN encoding the reporter gene GFP (SINrep19/GFPwt) (30 hours post-infection). A hallmark of SIN infection in BHK cells is the inhibition of host cell protein synthesis within a few hours and cytopathic effects that occur within 12 - 16 hours after infection. (A) SIN-induced cytotoxicity was manifested by cell rounding and detachment from the substrate. (B) The SIN characteristic to achieve high gene transfer rate into BHK cells was easily recognizable by the strong fluorescence signal derived from GFP. The vast majority of cells exhibiting cytopathic effects expressed also the transgene product. This correlation revealed the homogeneity of the viral particles produced with respect to their recombinant genome content. Scale bar 50µm

For quantitative determination of SIN-mediated gene transfer, cultured ARC were exposed to SINrep5/lacZ. The evaluation was performed with this recombinant SIN due to the high sensitivity of the β-galactosidase assay (X-Gal staining) permitting even the detection of only small traces of transgene product in infected cells. ARC were kept 7 days in culture before infection took place with various concentrations of SINrep5/lacZ, and after 24 hours, infection efficiency was assessed by X-Gal staining. The number of ARC expressing the reporter gene increased in a dose-dependent manner (see Figure 2-3 on page 40). Exposure of $5 \times 10^4$ cells to $5 \times 10^7$ IU/ml of SINrep5/lacZ resulted in transgene expression in up to 80% of cultured ARC. This corresponds with the amount of
viral titer applied to a multiplicity of infection (MOI) of 320, meaning that 80% of cultured ARC in the dish are transduced by applying 320 IU per cell on average.

Figure 2-3: Infection of cultured ARC by recombinant SIN coding for the β-galactosidase gene (SINrep5/lacZ). Gene transfer efficiency was determined using the β-galactosidase assay 24 hours post-infection. Dosage-dependent expression of the SIN-mediated transgene in ARC became obvious, displaying a gene delivery efficiency of up to 80% when highest viral concentration was applied. Each bar represents the mean of data derived from three separated dishes (5.5 – 6.0 x 10⁴ cells/dish); error bars represent standard error of the mean (SEM).

Additionally, SIN was tested for its ability to accomplish transgene expression in ventricular cardiomyocytes freshly isolated from the adult rat heart. Subsequent to the isolation procedure, ARC were kept in suspension culture and infected with SINrep5/lacZ before being seeded and allowed to attach to the culture substrate. After 6 hours, a time in which transgene expression could be already observed in infected BHK cells, β-galactosidase activity test for ARC was negative. Transgene expression was readily detected 24 hours after the infection procedure; 48 hours post-infection, the staining of infected cardiomyocytes became even more prominent. However, the maximal achieved infection rate was not as high as in the case of cultured ARC, ranging between 10% and 20% (see Figure 2-4 on page 41).

The general experimental observation was that ARC kept in culture for a couple of days were more prone to SIN-mediated gene transfer than ARC kept only for a few hours in culture before infection. This difference was likely due to a potential damage of viral receptors on the cell surface during ARC preparation, in which the cells are dissociated from the tissue by enzymatic digestion. The receptors necessary for viral endocytosis may therefore not be fully reconstituted yet, leading to a reduced uptake of viral particles.
in cardiomyocytes infected shortly after isolation. One of these receptors may be the laminin receptor which has been described as a SIN receptor (Wang et al., 1992).

**Figure 2-4:** Expression of β-galactosidase in ARC after infection with SINrep5/lacZ (48 hours post-infection). Infected cells were identified by their blue appearance after X-Gal staining. (A) ARC were kept 7 days in culture before exposed to recombinant SIN. Asterisks indicate ARC not expressing the transgene product. (B) ARC were exposed to recombinant SIN shortly after isolation. The micrograph shows the morphologic heterogeneity of ARC kept 2 days in culture. Prior to flattening, most rod-shaped cells acquired a round morphology as a transition state during cytoskeleton reorganization. Infection efficiency was found to be lower for freshly isolated ARC than for ARC kept in culture for longer time before infection. Arrow points to an infected ARC still in its rod-shaped morphology; arrowhead points to an infected ARC that had started to spread. Scale bar 100µm

### 2.1.2 SIN INFECTION CAUSES SELECTIVE CYTOPATHOGENIC EFFECTS

SIN infection and its effects regarding cytotoxicity on adult cardiomyocytes were compared to those on other heart-derived cells (see Figure 2-5 on page 42). For this purpose, cytosine arabinoside was omitted from the culture medium permitting the overgrowth of proliferation-competent cells, such as fibroblasts, endothelial, and smooth muscle cells, which under that condition contaminated ARC preparations. Only a small number of cells expressed the transgene product upon exposure to low SINrep5/lacZ concentrations. However, an increasing number of dying non-cardiomyocytes, revealing strong cytopathogenic effects by SIN, were observed with increasing number of infectious units applied, while ARC appeared to be less affected. A selective survival of ARC, most of them exhibited transgene expression, were detected, whereas almost all other cell types detached from the culture substrate 24 hours after infection with high SINrep5/lacZ concentration (> 10^7 IU/ml).
Selective cytopathogenic effects caused by SINrep5/lacZ. Culture condition without cytosine arabinoside resulted in the overgrowth of proliferation-competent cells, remainders of the ARC isolation procedure. After 7 days in culture, the cells were infected with various concentrations of SINrep5/lacZ and examined 24 hours later. (A) Infection with low viral concentrations (5 x 10^5 IU/ml) led to only a few cells positive for β-galactosidase expression (blue appearance). (B) Infection with high viral concentrations (5 x 10^7 IU/ml) revealed that the majority of non-cardiomyocytes rounded up and detached from the culture dish within one day. SINrep5/lacZ did not visibly affect ARC leaving noninfected or infected ARC attached to the substrate. Scale bar 200µm

It appeared that ARC were more resistant to SIN infection than other cells types of the heart. This characteristic was also seen in experiments when using low viral doses and transduction was likely to be a result of infection by single infectious units. The observed selective survival was therefore rather an intrinsic property of ARC than a consequence of differences in the viral uptake by the different cell types. Infection with SINrep5/lacZ was also found to be cytopathic in cultured neonatal rat cardiomyocyte (NRC), provoking cell rounding and detaching from the substrate already 24 hours post-infection (data not shown).

Since it had been reported that the alphavirus-specific nonstructural protein nsP1 was found to cause disassembly of the actin cytoskeleton in various cell lines shortly after infection (Laakkonen et al., 1998), putative ultrastructural alterations in the morphology of infected ARC by SINrep5/lacZ were analyzed using light and immunofluorescence microscopy (see Figure 2-6 on page 43). As judged from F-actin staining, no abnormality and displacement in the cytoarchitecture compared to noninfected ARC were detected 48 hours after infection. Additionally, the nuclei of infected ARC displayed no apoptotic blebbing. Though ARC were not visibly affected within this period, the spontaneous beating activity of ARC decreased for several days when exposed to SINrep5/lacZ, and finally ceased 4 days post-infection. After more than 6
days post-infection, morphologic changes in infected cardiomyocytes became apparent, and cells started to round up.

![Image](image_url)

**Figure 2-6:** Tolerance of cultured ARC to SIN infection. Micrographs show ARC infected with SINrep5/lacZ 48 hours after infection (2.5 x 10^7 IU/ml). (A-D) Triple fluorescence-staining of such infected ARC. (A) Phase-contrast of infected ARC. (B) DAPI staining. The binucleation seen is a typical characteristic of ventricular ARC. (C) Immunofluorescence labeling for β-galactosidase revealed infected ARC. (D) Staining with phalloidin-rhodamine demonstrated that F-actin fibers of infected ARC remained intact for at least 2 days post-infection. Scale bar 50µm

### 2.1.3 Analysis of Transgene Expression in Cultured ARC Infected by SIN

The duration of transgene expression and its dependence on the gene delivery system used is an important issue; it defines the kind of transgenic experiment that is possible. In case of SIN-mediated gene transfer, expression in a vast majority of cells is confined to a few hours due to rapid shut off of host mRNA translation resulting in cell death (Frolov et al., 1996).

To determine the period of transgene product expression, 7-day-old cultured ARC were exposed to SINrep5/lacZ and SINrep19/GFPwt, respectively, and examined the following days. Insight into protein synthesis of these infected cells was gained by pulse-labeling with ^35^S-methionine during cultivation (see Figure 2-7A on page 44). The metabolic labeling disclosed ongoing synthesis of reporter gene products in infected ARC at day 4 after infection. In contrast, BHK cells had only to be pulse-labeled at 14
hours post-infection in order to prevent major cytotoxic effects. Additionally, time course experiments revealed an accumulation of transgene products in infected ARC on the first days after exposure (see Figure 2-7B).

These findings unveiled the comparably high tolerance of ARC to SIN infection. Reducing, however, or even diminishing the cytopathic effects of SIN could lead to prolonged transgene expression in these cells, thus offering a larger time window for studying transgene function. With this in mind, attempts were undertaken to generate SIN particles based on the RNA template SINrep19/GFP, which harbors the nonstructural protein mutation nsP2-726L. This mutation had been described to establish persistent replication of SIN RNA without affecting cell growth and morphology of BHK cells (Agapov et al., 1998). Several attempts to produce in BHK, HeLa and HEK 293 cells such noncytopathic infectious particles failed, presumably due to the low
replication efficiency of the mutated SIN replicon. The impracticality of this specific mutation to generate infectious particles was confirmed later by a report by Frolov and colleagues (Frolov et al., 1999).

In summary, SIN vectors transduced adult cardiomyocytes efficiently and transgene expression was readily detectable 24 hours post-infection. SIN vectors therefore allowed short-time expression experiments in these transfection-resistant cells. Thus, in addition to the data derived from immunofluorescence analysis, localization studies in the cytoarchitecture or myofibrillar apparatus of ARC using SIN became attractive.

2.2 Expression of fluorescent protein-tagged contractile proteins in cultured cardiomyocytes using SIN

Epitope tagging has become an important technique for the analysis of intracellular localization of proteins. It implies the in-frame fusion of a specific epitope to the protein sequence by altering the cDNA coding for the protein of interest. The method had been successfully established in the case of cardiomyocytes in culture allowing the demonstration of protein interaction within sarcomeres (Soldati and Perriard, 1991; Auerbach et al., 1999). Furthermore, GFP as an epitope marker is frequently used to monitor the expression, localization and interaction of proteins in living cells (Chalfie et al., 1994), and has also turned out to be an indispensable tool for the study of cardiomyocyte cytoarchitecture in function too (Auerbach et al., 1997; Dabiri et al., 1997).

In order to investigate the potential of SIN for ectopic expression of fluorescent protein-tagged contractile proteins, two types of replication-deficient SIN were generated. The first, which is SINrep5/MLC3f-EGFP and encoding the myosin-light chain domain (MLC3f) fused to the enhanced GFP (EGFP), was produced by coelectroporation of RNA derived from in vitro transcription of pDHBB and the cloned vector pSINrep5/MLC3f-EGFP as well. The second one, termed SINrep5/α-actinin-DsRed and encoding the α-actinin fused to the red fluorescent protein DsRed, was produced in the same way but using RNA derived from the cloned DNA template pSINrep5/α-actinin-DsRed. Maximum titer obtained was 3 x 10^6 IU/ml for SINrep5/MLC3f-EGFP, and 0.5 x 10^6 IU/ml for SINrep5/α-actinin-DsRed, respectively.
2.2.1 RECOMBINANT SIN ALLOWS LOCALIZATION STUDIES IN CULTURED NRC

One day after isolation, cultured NRC were exposed to SINrep5/MLC3f-EGFP. Eight hours after infection, the fluorescence signal derived from EGFP already became detectable in living NRC. The percentage of NRC displaying EGFP fluorescence increased during the next 16 hours to a maximum of >70% (see Figure 2-8 on page 47). Analysis by confocal microscopy of NRC at an early stage after infection (12-20 hours post-infection) revealed a correct localization of the MLC3f-EGFP fusion protein to the A-band in the sarcomere (see Figure 2-8 on page 47). A strong overexpression of exogenous proteins was possibly responsible for the background observed around the nuclei in these infected cells. In contrast, NRC infected by SINrep19/GFPwt showed a diffuse distribution of GFP in the cytoplasm and around the nucleus (see Figure 2-8 on page 47). No alteration of the myofibrillar structure was observed in NRC either expressing the MLC3f-EGFP or GFP as judged by antibody staining against the M-band protein myomesin. This indicated that none of these genes exerted a dominant-negative effect on the sarcomeric assembly. Regarding the case of NRC exposed to SINrep5/α-actinin-DsRed, red fluorescence from α-actinin in living NRC could not be detected, probably due to cytotoxic effects caused by SIN within 24 hours. The time period seemed to be too short for accumulation of detectable amounts of the red fluorescent fusion protein.

Nevertheless, the high transduction efficiency and intense green fluorescence signal suggested that the SIN expression system be used as an alternative to other transfection methods used for the ectopic expression of EGFP-tagged myofibrillar components in cultured NRC.
Figure 2-8: NRC infected by SINrep5/MLC3f-EGFP (A-D) and SINrep19/GFPwt (E-F). Infected NRC were easily recognized by exhibiting green fluorescence (A/C/E). NRC were stained for F-actin (B) and the M-band protein myomesin (D/F). SIN infection was highly efficient in NRC resulting in the expression of MLC3f-EGFP fusion protein in the majority of cultured cells (A/B). Confocal laser scanning microscopy revealed the incorporation of MLC3f-EGFP in the A-band (C), whereas GFP alone did not specifically bind (E). The anti-myomesin staining demonstrated that infected cells had well-developed sarcomeres at 20 hours post-infection (D/F). Scale bars 100µm (A), 2µm (C), 5µm (E)
2.2.2 **RECOMBINANT SIN ENABLES EXPRESSION AND PRECISE TARGETING OF PROTEINS OF THE CONTRACTILE APPARATUS IN ARC**

In cultured ARC, incorporation of the exogenous fusion protein was observed after infection with SINrep5/MLC3f-EGFP, thus demonstrating the usefulness of the SIN system as a vector to express EGFP-tagged proteins also in these cells (see Figure 2-9 on page 49). To test the applicability of the red fluorescent protein DsRed, ARC were infected with SINrep5/α-actinin-DsRed. Costaining with anti-myomesin antibody unveiled that the α-actinin-DsRed fusion protein targeted into myofibrils of transduced ARC at the correct positions resulting in a well-defined M-band/Z-band staining pattern. The fluorescence derived from DsRed became detectable later than that of EGFP in living ARC but developed into a strong signal with a low signal-to-noise ratio after 36 hours post-infection. Maximum titer concentrations were applied to cultured ARC, sufficient to obtain gene transfer rates of 5% - 20% that corresponded to the dose-dependent transfection efficiency previously established (see Figure 2-3 on page 40). ARC were identified by their well-organized sarcomeric structure as judged by antibody staining against myomesin. The vast majority of these cells displayed a correct localization of the exogenous fusion protein in the contractile apparatus.

After these promising results, double-infection of cultured ARC using SINrep5/α-actinin-DsRed and SINrep5/MLC3f-EGFP was attempted. However, simultaneous expression of α-actinin-DsRed and MLC3f-EGFP in ARC could not be accomplished despite a high infection efficiency. In contrast, a preferential expression of the exogenous MLC3f-EGFP fusion protein was observed in these cultures, suggesting a better replicon amplification of SINrep5/MLC3f-EGFP than of SINrep5/α-actinin-DsRed in infected ARC.

Expression of an ectopic contractile protein in rod-shaped, freshly isolated adult cardiomyocytes was achieved by infecting ARC with SINrep5/α-actinin-DsRed shortly after the isolation procedure. As in the case for ARC in long-term culture, the fusion protein was accurately incorporated into the contractile apparatus in infected rod-shaped ARC. This allowed the assumption that in living freshly isolated cardiomyocytes, the exogenous protein uses the same binding sites to assemble into myofibrils as the endogenous counterpart. The accurate localization of the fusion protein proved that
overexpression by the SIN system was suitable for localization studies also in rod-shaped ARC.

Figure 2-9: Confocal laser scanning microscopic analysis of ARC infected with recombinant Sindbis viruses (SIN). ARC in culture were infected with SINrep5/MLC3f-EGFP (A/B) and SINrep5/α-actinin-DsRed (C). Freshly isolated ARC were infected with SINrep5/α-actinin-DsRed (D-F'). The MLC3f-EGFP fusion protein incorporated into the sarcomeres (A) and costaining for myomesin showed no myofibrillar disorganization in cultured ARC at 32 hours post-infection (B). The α-actinin-DsRed fusion protein assembled in the Z-disc (red), as revealed by the antibody staining (green) against the M-band marker protein myomesin (40 hours post-infection; C). ARC kept in short-term culture were successfully infected by SINrep5/α-actinin-DsRed (D-F), leading to specific binding and localization of the α-actinin-DsRed fusion protein in the contractile apparatus (D'-F'). Regarding cell morphology, the documentation was
done after ARC had been cultured for 62 hours. This was sufficient to initiate first adaptations of cardiomyocytes to the culture environment that became obvious from the slight rounding of the former edges of the rod-shaped cells. Overlay of the two fluorescent signals (F/F') originating from α-actinin-DsRed (D/D') and antibody staining against myomesin (E/E') demonstrated correct binding of the exogenous protein to the Z-band. Scale bar 10 µm (A), 2 µm (C), 10 µm (D), 2 µm (D')

2.2.3 USE OF RECOMBINANT SIN BESIDES LOCALIZATION STUDIES OF MYOFIBRILLAR COMPONENTS

The SIN gene delivery system enabled efficient expression and direct, rapid localization of engineered sarcomeric proteins in cultured and freshly isolated rat cardiomyocytes. The SIN system was the first viral gene delivery system to achieve that, but concerns using SIN for cell cycle-regulated studies remained due to the virus-associated cytotoxic effects narrowing the experimental time-window.

The application of SIN in primary cultures of other vertebrate cardiomyocytes was additionally investigated by applying SINrep19/GFPwt to cultured embryonic chicken cardiomyocytes (Figure 2-10). High transduction efficiency was found, thus revealing the known wide host range ability of SIN, but cytopathic effects became evident starting around 36 hours after infection in this cell type too.

![Figure 2-10: Embryonic cardiac chicken cells infected with 2 x 10^7 IU/ml of SINrep19/GFPwt (30 hours post-infection). (A) Efficient transduction was achieved resulting in GFP expression in a large number of these cultured cells. B) Chicken cardiomyocytes were identified by their striated appearance after anti-myomesin staining. Scale bar 25µm](image)

From the results stated above, it was obvious that SIN expression of heterologous proteins in cultured cardiomyocytes was rapid and efficient, therefore offering a convenient way regarding localization studies. Though reduced, interference with host cell protein synthesis in ARC could not be neglected, rendering SIN not attractive for transgenic approaches to recover cell cycle-regulating activities. As a consequence, novel
ways to accomplish transgene expression without causing cytotoxic effects in ARC were further evaluated.

### 2.3 **Plasmid Transfection in Cultured Cardiomyocytes Using Receptor-Mediated Strategies**

Construction of DNA plasmids encoding the transgene to be expressed is generally more straightforward than generation of recombinant viral vectors. In addition, transgene size is not limited by the packaging constraints of the virus, and cytotoxic effects eventually caused by viral proteins involved in transgene expression can be ruled out.

The use of gene transfer into ARC by the receptor-mediated pathway was evaluated as an alternative to microinjection, which requires great technical experiences and yields only a few cells expressing the transgene. The anticipated gene delivery strategy involves DNA binding to a polycationic polymer covalently coupled to a receptor ligand that enables the uptake of the whole transfection complex into the cell via the endogenous pathway (Cotten and Wagner, 1999). Transferrin has been proven to be a useful ligand for this approach since its receptor is ubiquitously expressed enabling straightforward internalization. Additionally, conjugated polylysine allows electrostatic binding to the DNA (Wagner et al., 1990). The polycationic polymer polyethyleneimine (PEI) is also used for DNA attraction and exhibits furthermore endosomolytic function (Boussif et al., 1995). The ability of PEI to actively disrupt the endosome, thereby releasing the transfection complex into the cytoplasm before lysosomal degradation occurs, contributes to a better efficiency of DNA delivery. Coupling of other endosomolytic agents such as adenoviral particles to transferrin-polylysine/DNA complexes greatly enhances receptor-mediated gene transfer (Wagner et al., 1992; Cotten et al., 1992). This adenovirus-enhanced receptor-mediated transferrinfection of DNA, abbreviated AVET (see Figure 2-11 on page 52), had been demonstrated to be of great use for different cell types in a variety of experimental set ups (Buschle et al., 1995; Wollenberg et al., 1997; Kircheis et al., 1999; Schreiber et al., 1999; Wightman et al., 1999; Brunner et al., 2000; Huber et al., 2000).
3.3.1 GENE TRANSFER INTO CULTURED CARDIOMYOCYTES USING TRANSFERRIN-POLYETHYLENIMINE (Tf-PEI)

DNA complexed with PEI having a molecular weight of 800 kDa and coupled to transferrin (Tf-PEI) (Kircheis et al., 1997) were evaluated for their ability to transfect cultured ARC. In a first series of experiments, optimal ratios of PEI amine to DNA phosphates were determined for transfection efficiency and cell toxicity. The reporter construct used for the optimization encoded EGFP under the CMV promoter (pEGFP-N3) permitting the detection of transgene expression in living cells. It also enabled observation of the cell morphology and spontaneous beating activity of the transfected cells over several days.

DNA-Tf-PEI complexes were incubated for 30 min before they were applied to cultured ARC; transgene expression was investigated during the following days. PEI amine to DNA phosphate ratios ranging from 2 to 10 were tested, whereas free amine groups in
transferrin are not considered in these specifications. The overall transfection efficiency achieved with this approach was rather low, ranging below 0.05%. Indeed, highest gene transfer was accomplished with PEI amine to DNA phosphate ratios between 6 – 8. This led to the expression of the transgene reporter product in only a couple of ARC per dish. At least, the few ARC displaying EGFP fluorescence were not morphologically affected and they still showed contraction several days after the transfection procedure (Figure 2-12). Even though gene transfer efficiency was insufficient, it was possible to transfect cultured ARC without any remarkable disturbance of the glycocalyx compared to other transfection procedures (e.g. calcium phosphate precipitation or lipofection-based approaches; data not shown).

**Figure 2-12:** Cultured ARC transfected with a plasmid encoding EGFP by the PEI-transferrinfection. Cells were kept 7 days in culture before transfection was performed, and recordings were made 5 days later. (A) Phase contrast of cardiomyocytes still beating in culture. (B) EGFP fluorescence of transfected ARC. Scale bar 50µm

Tf-PEI was further tested in cultured NRC under the conditions established for cultured ARC. Gene transfection using pEGFP-N3 was also accomplished in these cells without distortion of cell morphology as judged by light microscopy. The efficiency was significantly higher compared to that determined for ARC, being in the one-digit percent range. The PEI-transferrinfection became thus the method of choice for experiments in cultured NRC for the ectopic expression of cell cycle-regulating factors (see section 2.5.4 on page 74).
2.3.2 THE AVET SYSTEM ALLOWS GENE TRANSFECTION OF CULTURED ARC

The results obtained with PEI-transferrin infection revealed one important characteristic of receptor-mediated gene delivery for cultured ARC, namely the absence of severe cytotoxicity. Nevertheless, gene transfer efficiency was not satisfying, particularly since the plasmid used did not include genetic elements like a weak promoter, or an oversized transgene, which could infringe transgene expression.

Based on published data regarding the ability of adenovirus to infect cultured ARC (Kass-Eisler et al., 1993; Kirshenbaum et al., 1993), the AVET system was expected to promote uptake of plasmid DNA into ARC. AVET of cultured ARC was optimized by varying the ratios of biotinylated, psoralen-inactivated adenovirus and streptavidin-polylysine conjugates (STAV-pL) to plasmid DNA and polylysine-transferrin (Tf-pL). Highest gene transfection efficiency was obtained by a transfection mix consisting of 3µg of plasmid DNA, 1.2 x 10^{10} inactivated adenoviral particles, 3µg Tf-pL and 1.5 – 3µg STAV-pL. A further significant transfection improvement was achieved by centrifuging the transfection mix in the culture dishes. This procedure enriched the amount of attached AVET complexes on the cell surface of cultured ARC. Using the conditions described (see section 4.4.2 on page 114), gene expression could be thus observed in more than 20% of cultured ARC after transfection with pEGFP-N3 (see Figure 2-13 on page 55). Immunofluorescence analysis of non-transfected cells as well as of cells expressing the transgene present within the same dish allowed the comparison. An intact myofibrillar apparatus detected several days after the transfection procedure, and no changes in the nuclei shape in transfected ARC revealed a high tolerance for the AVET system.

Despite that transfection of freshly isolated cardiomyocytes using the transfection parameters optimized for cultured ARC was possible, only a few cells per thousand showed transgene expression (data not shown). It was anticipated that the uptake of transfection complexes using the receptor-mediated pathway could be blocked due to an impaired cell surface caused by ARC preparation as suggested above for SIN infection (see page 40).
Figure 2-13: EGFP expression in cultured ARC after gene transfection using the AVET system. ARC kept 7 days in culture were transfected with pEGFP-N3 and analyzed the following days. (A) Phase contrast picture of cultured ARC. (B) EGFP fluorescence was detected in 22 ± 6% of the cultured cells (determined from three independent transfection experiments). (C-E) Immunofluorescence analysis of such cultured ARC. (C) The transgene product (EGFP) was strongly expressed in transfected ARC, tending to accumulate in the perinuclear region of the cell. (D) Myomesin staining did not disclose any differences in the contractile apparatus of transfected and untransfected cells. (E) DNA staining with DAPI unveiled no abnormalities in the morphology of nuclei. The AVET complexes were easily identified as the little spots distributed over transfected cells. Scale bar 100µm (A), 50µm (C).

Subsequently, the use of the AVET system to express sarcomeric proteins was assessed by transfecting pMLC3f-EGFP encoding the EGFP-tagged myosin light chain isoform 3f (see Figure 2-14 on page 56). Transgene expression was estimated to be around 1% in cultured ARC by semi-quantitative determination of the number of ARC exhibiting green fluorescence. Plasmid-dependent factors could be responsible for the reduced efficiency of complex uptake as well as a lower efficacy of transgene transcription. The myosin light chain fusion protein localized into sarcomeres resulting in the characteristic staining pattern of the contractile apparatus previously also found in cardiomyocytes infected by recombinant SIN encoding MLC3f-EGFP (see section 2.2.2 on page 48). In contrast to the latter, the myofibrillar apparatus was still properly organized five days after transfection, and the cell morphology was not affected.
Figure 2-14: ARC transfected with pMLC3f-EGFP encoding the myosin light chain-EGFP fusion protein using the AVET system (3 days post-transfection). The transgene product assembled into the myofibrils, clearly visible from the fluorescence pattern displayed. This record was directly taken from living cells in culture. The shown cell demonstrated unrestricted beating activity revealing unaffected viability and the low cytotoxicity of the gene transfer method used. Scale bar 50\(\mu\)m.

Although the nuclei of transfected ARC did not show any sign of apoptosis-induced blebbing, TdT-mediated dUTP nick-end labeling (TUNEL) assay was performed to exclude the possibility of DNA fragmentation in transfected ARC (Figure 2-15). The TUNEL assay indicated no significant increase of TUNEL-positive nuclei in transfected cells above the background level (<1%) of untransfected ARC.

Figure 2-15: Absence of DNA fragmentation in cultured ARC. ARC were transfected with pEGFP-N3 using the AVET system and analyzed 4 days later. (A) Phase contrast of ARC. (B) EGFP signal revealed ARC expressing the transgene product. (C) DAPI staining supported localization of the nuclei in these cells. (D) No fluorescence signal due to non-incorporation of tetramethyl-rhodamine-5-dUTP suggested that no apoptotic processes be initiated by the transfection method used. (E/F) Positive control for the TUNEL assay. Cells were treated with DNase I, provoking DNA fragmentation in untransfected and EGFP-expressing ARC (E). This enabled the incorporation of tetramethyl-rhodamine-5-dUTP in the nuclei (F). Scale bar 100\(\mu\)m (A), 50\(\mu\)m (E).
2.3.3 TOWARDS GENETIC ENGINEERING OF CULTURED ARC USING THE AVET SYSTEM: EVALUATION OF METHODS FOR A RELIABLE IDENTIFICATION OF TRANSFECTED CELLS

The results obtained so far allowed the conclusion that plasmid-based procedures for gene transfer could be successfully implemented for cultured ARC. The relatively high transfection efficiency of ARC achieved with the AVET system by using the reporter gene product EGFP was close to what could be when standard transfection methods are applied for cell lines. In order to optimize the expression of exogenous proteins in transfected ARC, the use of genetic elements of the expression vector were evaluated. Only sparse information existed about their application in cultured ARC until then.

One approach to distinguish between transfected and untransfected ARC was to use dicistronic mammalian expression vectors (Dirks et al., 1993; Dirks et al., 1994) containing EGFP as the marker for transfection. The gene of interest in such constructs would have to be inserted in front of an internal ribosomal entry site (IRES), which allows start of translation from an internal AUG within the mRNA as described (Pelletier and Sonenberg, 1988; Jang et al., 1988). The coding region for EGFP is placed downstream of the IRES element, permitting both, the gene of interest and EGFP, to be translated from the same mRNA transcript.

The application of such expression vectors was tested in cultured ARC. For that purpose, several bicistronic expression plasmids were generated, of which are mentioned here as examples, pcycA-IRES-EGFP and pTri-cycA-EGFP. The latter is based on the tricistronic mammalian expression vector pTrident2 (Dätwyler et al., 1997; Fussenegger et al., 1998), which was rendered bicistronic by deleting one IRES element in the cloning procedure. It was of interest to determine the number of EGFP-positive ARC transfected with these constructs to estimate the use of such expression vectors as a marker for cell cycle studies. Here, the human cyclin A encoded on both vectors served as a model gene. Transfection of the constructs led to detectable EGFP fluorescence signals in HEK 293 cells, but unfortunately, the constructs were useless in ARC (see Figure 2-16 on page 58); ARC exhibiting green fluorescence were not observed after transfection using pcycA-IRES-EGFP, and only very sporadically encountered using pTri-cycA-EGFP. It was anticipated that besides lower gene transfection in ARC, the
function of such IRES elements, which is known to depend on cellular factors (Martinez-Salas, 1999), was restricted.

![Figure 2-16: Fluorescence micrographs of HEK 293 cells (A/B) and ARC (C) transfected with bicistronic expression vectors enabling cap-independent translation of the reporter gene product. Pictures were taken from cells still in culture. (A/B) Bright fluorescence signal from expressed EGFP were observed in a large number of HEK 293 cells 40 hours after calcium phosphate transfection with pIRES-EGFP (A) and pTri-cycA-EGFP (B). (C) Cultured ARC displayed green fluorescence 4 days after transfection with pTri-cycA-EGFP using the AVET system. Transfected cell was contracting spontaneously, but such EGFP-positive ARC were hardly observed. Up to several transfection approaches were required to yield one transfectant. Scale bar 50 µm (A-C)

Another way to identify transfected cells implied direct epitope tagging of the transgene product. This presumes that ectopic expression of the heterologous protein would reach levels high enough for convenient detection and thus for the straightforward identification of transfected cardiomyocytes. This approach was tested by transfecting ARC with pMHcycA-HA that encodes a HA-tagged version of human cyclin A under the control of the CMV promoter. The transgene product could be readily detected by indirect immunofluorescence analysis using an antibody against the HA epitope (see Figure 2-17 on page 59). This suggested that ectopic expression of cell cycle proteins and their subsequent verification in ARC using the AVET system be feasible.
2.4 CHARACTERIZATION OF CULTURED CARDIOMYOCYTES IN RESPECT OF THE CELL CYCLE

Besides the establishment of genetic means to express cell cycle-regulating molecules for manipulation and possible induction of the cardiac cell cycle, one had to consider the additional conditions set by the experimental environment. This implicated better knowledge of the state of the cell cycle in cultured ARC under the culture conditions used. Such information could provide essential details to work out transgenic strategies to induce well directed ways to deblock cell cycle progression in these postmitotic cells.

2.4.1 G1/S TRANSITION IN CULTURED CARDIOMYOCYTES

In order to study the influence of the culture conditions on the G1/S transition phase in cardiomyocytes, the rate of DNA synthesis was determined by bromodeoxyuridine (BrdU)-labeling. Cultured ARC and NRC were incubated for 48 hours with BrdU enabling its incorporation in newly synthesized DNA (see Figure 2-18 on page 61). The sustained exposure to BrdU was chosen because of the unknown kinetics for DNA synthesis in these cells. In NRC, cultured in maintenance medium (see section 4.1.1 on page 107) consisting of 1% serum, the percentage of BrdU-positive nuclei of cardiomyocyte was around 0.5%. However, the number of BrdU-positive nuclei in these...
cells increased significantly up to 10% when medium commonly used for ARC
cultivation containing 10% fetal calf serum (FCS), supplemented with 200 mM L-
glutamine, but without cytosine arabinoside was applied (see section 4.1.1 on page 107).
The observed difference indicated that medium optimization by adjusted serum
concentration played presumably a decisive role. Using the same culture medium for
ARC, only about 0.5% BrdU-positive nuclei were found in ARC (see Figure 2-18 on
page 61). These results suggested that mechanisms controlling the S phase entry be more
susceptible to cell cycle-inducing stimuli in cultured NRC than in ARC.

2.4.2 MITOSIS AND CYTOKINESIS IN CULTURED NRC

The high number of BrdU-positive nuclei of cultured NRC in medium containing 10% 
serum proposed analysis with regard to additional cell cycle-related events.
Cardiomyocyte culture systems were screened for mitotic figures by
immunofluorescence microscopy. Mitotic figures, including late telophase coupled with
cytokinesis, were sporadically observed (<0.1%) in NRC cultured with 10% serum (see
Figure 2-19 on page 62). However, mitotic figures implying cytokinesis were detected
neither in NRC cultivated with 1% serum nor in cultured ARC. These evidences further
strengthened that NRC are probably more prone than ARC to respond to hormones and
growth factors. Considering that NRC were isolated in a developmental stage in which
their cell cycle is supposably still active, one could assume that a certain susceptibility
remained also in vitro. It should be mentioned here, that cultured NRC were very
heterogenous regarding their nuclei number, varying between one and two nuclei
depending on the time of isolation, whereas cultured ARC were more homogenous, most
of them being binucleated. The uniformity of cultured ARC could refer to the long
lasting cell cycle arrest at the time of isolation.
Figure 2-18: Ongoing DNA synthesis in cultured cardiomyocytes determined by the extent of BrdU incorporation within 48 hours of culture time. Labeling experiments were performed in triplicate, and at least 2000 nuclei were counted per dish for quantitative assessment. NRC cultured in medium consisting of 1% serum incorporated BrdU in $0.4 \pm 0.2\%$ of their nuclei. Significant increase was detected by changing to medium containing 10% serum, leading to DNA synthesis in $9.5 \pm 1.3\%$ of the myocardiac nuclei. Entry into S phase was sporadic for ARC cultured with 10% serum, displaying only $0.5 \pm 0.2\%$ BrdU-positive nuclei. This revealed the resistance of ARC to mitogenic stimuli present in the serum. (A-I) Representative immunofluorescence pictures of NRC cultured in medium with 1% serum (A-C), with 10% serum (D-F), and of ARC cultured in medium with 10% serum (G-I). Cardiomyocytes were recognized by antibody staining against the contractile protein titin (A/D/G), and nuclei were identified by DAPI (B/E/H). BrdU incorporation (C/F/I) was detected by indirect immunofluorescence using an anti-BrdU monoclonal antibody. BrdU incorporation was readily seen in nuclei of NRC cultured with 10% serum (F), whereas for
the other two cases, incorporation (C/I) was mainly attributed to nuclei of cells which did not have striated appearance (A/G). Antibody staining with the anti-titin M8 antibody regularly resulted in additional nuclei staining (A/D/G). Scale bar 25µm

**Figure 2-19:** Course of mitosis in NRC cultured with medium consisting of 10% serum (see page 63). Micrographs show immunofluorescent pictures of NRC displaying mitotic figures; nuclei were stained with DAPI (A-D), microtubules were visualized by antibody staining against alpha-tubulin (E-H), phalloidin-rhodamine was used to visualize F-actin (I-L) and anti-titin antibody was used to identify cardiomyocytes (M-P). Nuclei of cardiomyocytes in early prophase (A) were recognized by the loss of diffuse chromatin appearance. In this stage, mitotic spindle began to assemble outside the nucleus (E). Metaphase nuclei in which the chromosomes are aligned across the middle of the spindle could be easily distinguished from interphase nuclei (B/F). Further mitotic figures found in NRC under this culture conditions showed chromatid separation, a characteristic for cells in anaphase (C). Also recognizable was the spindle apparatus in this phase (G/K), whereby polar and astral microtubules were clearly visible (G). Additionally, cardiomyocytes (P) in late telophase were present, in which daughter nuclei reformed (D). In rare cases, late telophase was even coupled with cytokinesis, characterized by forming of the cleavage furrow and the midbody, which is the region of microtubule overlap (H/L). Interphase nuclei of two cardiomyocytes (B/D) and one non-cardiomyocyte (C) were included in the micrographs to appreciate better the mitotic figures. Interestingly, anti-titin M8 antibody staining which resulted in nuclear staining of cells in interphase, did not stain the nuclei in later stages of the M phase (N-P), probably due to accomplished nuclear envelope breakdown, eliminating the epitope recognized by this antibody. Scale bar 10µm
**2.4.3 IMMUNOFLUORESCENCE ANALYSIS FOR ENDOGENOUS Cdc2 AND CYCLIN B1 IN CULTURED ARC**

The tight mechanism regulating entry into S phase had been reported to be overcome in primary culture systems of ARC by ectopic expression of the transcription factor E2F-1 (Agah et al., 1997), whereas control mechanisms involved in the G2/M transition in these cells still remained to be unveiled. As a prelude to establish genetic strategies intervening at the G2/M boundary, endogenous expression of Cdc2 and cyclin B1, which form a complex called the mitosis-promoting factor MPF, was investigated in cultured ARC.

A major problem appeared considering the small but nevertheless existing heterogeneity of the primary cardiomyocyte culture harboring other cell types able to divide. Only single-cell analysis using immunofluorescence microscopy allowed a direct discrimination between cardiomyocytes and other cell types while e.g. Western blot based on total protein lysate was not appropriate for the analysis of cell cycle components.

Only minor expression of cyclin-dependent kinase Cdc2 was manifested in the nuclei of ARC cultured for 9 days as suggested by the low immunofluorescence signal observed (see Figure 2-20 on page 65). No specific nuclei staining for cyclin B1 was observed in ARC as expected from *in vivo* data previously published by other groups (see section 1.2.1 on page 13). Faint staining, however, of the nuclei by the anti-cdk1/cdc2 antibody was somewhat surprising. The relevance of this result concerning MPF activity was further investigated and described in detail in the following.
Figure 2-20: Expression of MPF components in ARC kept 9 days in culture and analyzed by immunofluorescence microscopy. Cultured ARC stained with anti-cdk1/cdc2 antibody (A) and anti-cyclin B1 antibody (B). (C/D) DAPI staining revealed nuclei in the cells. (E/F) Staining with anti-titin antibody distinguished cardiomyocytes and non-cardiomyocytes. That small amount of Cdc2 protein may be present in cultured ARC was proposed by the weak signal obtained from the indirect immunofluorescence microscopy (A). Nuclei in non-cardiomyocytes (D), which were identified by their non-striated appearance (F), stained positive for cyclin B1, whereas no nuclear signal was observed in cardiomyocytes (B). The diffuse signal occurring in a perinuclear fashion seen in the cardiomyocyte was judged to be unspecific (B). Scale bar 25µm

2.5 RECOVERY OF MPF ACTIVITY IN CULTURED NRC AND ARC

2.5.1 CLONING OF CYCLIN B1-HA AND ITS ECTOPIC EXPRESSION IN CULTURED ARC

Due to the assumed presence of Cdc2 in the nuclei, one tested whether functional recovery of MPF in cultured ARC could be accomplished by the sole expression of exogenous cyclin B1 in order to complement the absence of endogenous cyclin B1. The cDNA encoding cyclin B1 was cloned by reverse transcription of mRNA derived from HeLa cells being mainly enriched in late G2 phase (see Figure 2-21 on page 66). Additional HA-tagging at the carboxy terminus of the transgene provided a convenient way to detect the heterogenous gene product by indirect immunofluorescence
microscopy. The CMV promoter in the mammalian expression vector pcDNA3.1cycB1-HA, ultimately used for transfection, ensured further strong cyclin B1-HA mRNA transcription. Expression of the transgene product and its correct size was confirmed by Western Blot analysis in transiently transfected HEK 293 cells (data not shown; correct size of cloned cyclin B1-HA and its functionality were confirmed in plasmid derivatives of pcDNA3.1cycB1-HA, see section 2.5.3 on page 71).

Figure 2-21: Mammalian cells downstream of the G1/S cell cycle checkpoint. HeLa cells were arrested in the G1/S transition phase by the double-thymidine block. Recordings of cells were made 2 hours (A-C), 4 hours (D-F), 6 hours (G-I), 8 hours (J-L), and 10 hours (M-O) after release from the block. Phase contrast micrographs of these cells enriched in cell cycle stages beyond the restriction point are shown in A/D/G/J/M. Anti-BrdU antibody staining revealed ongoing DNA synthesis in the cells, labeled for 20 min with BrdU (B/E/H/K/N). Morphologic alterations in the nuclei shape were visualized by DAPI staining. DNA synthesis could be readily detected after 2 and 4 hours (B/E) but decreased after 6 hours (H) indicating that cells moved out of S entering G2 phase. BrdU-incorporation could be hardly observed after 8 to 10 hours (K/N) suggesting cells in G2 and M phase. After 10 hours, a vast number of cells entered M phase, seen by the characteristic morphology of cells dividing (M) and the condensed nuclei observed in these cells (O). Such synchronized cell cultures served as a mRNA source of genes whose transcription fluctuates strongly during the cell cycle. The cyclin B1 cDNA was cloned from the mRNA pool of cells harvested 8 hours (J-L) after the release. Scale bar 25 μm

Cultured ARC transfected with pcDNA3.1cycB1-HA were analyzed 72 hours post-transfection for the expression of the transgene product, nuclei and general morphologic appearance (see Figure 2-22 on page 67). Expression of the transgene product together with endogenous Cdc2 was confirmed by anti-HA antibody- and anti-cdk1/cdc2 antibody staining. No obvious abnormalities in cell architecture were seen in transfected ARC compared to nontransfected ones. Special attention was paid to the nuclei of transfectants in order to detect potentially induced chromosome condensation due to
The presence of active MPF complexes. No changes in chromatin structure were found in these cells; they still exhibited interphase-like nuclei. An initiation of MPF activity could not be completely ruled out by immunofluorescence analysis, but evidence did not really support cell cycle re-entry at the G2/M boundary.

Further aspects of MPF regulation had to be thus considered to achieve its functional recovery.

Figure 2-22: Cultured ARC transfected with pcDNA3.1cycB1-HA. Triple immunofluorescence analysis was performed 72 hours after the transfection procedure using the AVET system. (A) Expression of cyclin B1-HA in ARC was confirmed by anti-HA antibody leading to nuclei and cytoplasm staining, and allowing clear distinction between untransfected and transfected ARC. (B) Low expression of endogenous Cdc2 in the nuclei of cultured ARC was suggested by anti-cdk1/cdc2 antibody staining. (C) DAPI staining showed no morphologic differences between untransfected and transfected ARC in culture. The little spots seen were assumed to derive from the AVET complexes consisting huge amounts of plasmid DNA. The contractile apparatus was still well organized in ARC expressing cyclin B1-HA as demonstrated by staining against the sarcomeric protein titin (D). Scale bar 25 µm
2.5.2 Determination of Mitotic Cyclin Degradation Activity in Cultured ARC

Cyclin proteolysis performed by the 26S proteasome is a key event in the anaphase onset during M phase and still persists in G1 phase (see section 1.1.3 on page 9). Regarding the situation in cardiomyocytes, the activity of this destruction pathway remains yet to be defined. It was assumed that the reported decrease of cyclin B1 protein levels during heart development (Brooks et al., 1997; Kang et al., 1997) may not be ascribed only due to downregulated transcription but could also be under the influence of post-transcriptional control. Such a regulatory mechanism, including active degradation by the proteasome pathway, would clearly not facilitate the recovery of MPF function in cultured ARC.

To detect a potentially active cyclin degradation machinery, ectopic expression of cyclin B1 in cultured ARC was investigated more closely. Even though cyclin B1-HA protein was detected in transfected ARC, the percentage of such cells in the dishes was estimated below 0.1%. The low number may be attributed to the low transfection efficiency depending on the plasmid used and/or a rapid destruction of the transgene product occurring in transfected cells. Assessment of the number of transfectants with undetectable amounts of cyclin B1 protein could thus provide clues about the ongoing mitotic cyclin proteolysis.

An additional reporter gene in pcDNA3.1cycB1-HA was included enabling the determination of transfection efficiency independently from cyclin B1-HA expression. As shown above referring to IRES expression plasmids (see section 2.3.3 on page 57), a second promoter on the plasmid was necessary to allow both reporter gene and cyclin B1-HA transcription from the same construct. Subsequently, the reporter gene EGFP was put under the control of the SV40 promoter and was assembled with pcDNA3.1cycB1-HA leading to pcDNA3.1cycB1-HA/EGFP. The second construct generated was pcDNA3.1cdc2-HA/EGFP, which differed from pcDNA3.1cycB1-HA/EGFP in encoding HA-tagged Cdc2 in spite of cyclin B1-HA. Expression of Cdc2-HA served as a negative control in the following experiment. It was used to investigate mitotic cyclin degradation activity since Cdc2 had been reported not to be influenced by the proteasome pathway (McGowan et al., 1990; Nishiyama et al., 2000). Additionally, the antibiotic lactacystin, a highly specific proteasome inhibitor (Fenteany and Schreiber,
was applied to cell cultures to inhibit degradation of exogenous cyclin B1-HA among other proteins.

The reliability of the proposed experimental set up to determine cyclin degradation activity in cultured ARC was first tested in unsynchronized HEK 293 cells (see Figure 2-23A on page 70). This cell line was transiently transfected with pcDNA3.1cycB1-HA/EGFP and showed a significantly higher ratio of transfectants expressing cyclin B1-HA when treated with lactacystin compared to transfectants untreated with the inhibitor. Transfectants in the cell cultures were recognized by EGFP fluorescence, and expression of exogenous cell cycle protein was visualized by antibody staining against the HA-tag. Omitting lactacystin enabled the cells to degrade cyclin B1-HA efficiently. Transfectants treated with lactacystin expressed 20% more cyclin B1-HA compared to untreated transfectants. This also demonstrated that HA-tagging of cyclin B1 at the carboxy terminus did not lead to a noticeable stabilization regarding proteolytic degradation of the transgene product.

It must be kept in mind that mitotic cyclin degradation occurs predominantly in late M and G1 phase (Brandeis and Hunt, 1996). Lactacystin-mediated inhibition of cyclin B1-HA degradation was thus only of use in cells being in these cell cycle stages. The observed difference of 20% in transfectants displaying exogenous cell cycle protein expression could therefore directly indicate the percentage of late M- and G1 phase cells in the unsynchronized cell population. The outcome confirmed the inhibitory function of lactacystin in the experimental setup and cyclin B1-HA as a useful marker for proteasome activity.

HEK 293 cells transiently transfected with pcDNA3.1cdc2-HA/EGFP showed no significant difference between ratio of transfectants positive for Cdc2-HA expression when treated and untreated with the proteasome inhibitor. The lack of lactacystin-dependent effects in cells transfected with pcDNA3.1cdc2-HA/EGFP allowed experimental artifacts due to unspecific inhibition of protein degradation by the proteasome inhibitor to be excluded.
Figure 2-23: Proteasome activity assay in proliferating HEK 293 cells (A) and cultured ARC (B). Cells were transiently transfected either with pcDNA3.1cycB1-HA/EGFP or pcDNA3.1cdc2-HA/EGFP, both constructs encoding EGFP under the SV40 promoter to assess transfectants. HA-tagging of cyclin B1 and Cdc2, encoded under the CMV promoter on pcDNA3.1cdc2-HA/EGFP and pcDNA3.1cycB1-HA/EGFP, respectively, allowed straightforward detection of these exogenous cell cycle proteins in transfectants by anti-HA antibody staining.

One day after transfection, incubation with lactacystin followed where required, lasting 24 hours for HEK 293 and 72 hours for ARC. Ratios of transfectants positive for anti-HA antibody staining, noted transfectants(HA) to transfectants defined by their EGFP expression were quantitatively determined and compared to each other. Error bars representing SEM were derived from transfections performed in triplicates (see section 4.6.3 on page 119 for more details concerning statistics).

Representative immunofluorescence pictures used for determination are shown in each case below the corresponding bar. (A) Degradation of cyclin B1-HA could be significantly blocked by lactacystin in HEK 293 cells transiently transfected with pcDNA3.1cycB1-HA/EGFP, in which 58 ± 6% of the transfectants were positive for anti-HA staining compared to 41 ± 3% when untreated with lactacystin. Amount of detectable Cdc2-HA levels in HEK 293 cells transiently transfected with pcDNA3.1cdc2-HA/EGFP was not significantly changed by lactacystin, denoted in percentages 56 ± 8% and 52 ± 5% for cells treated and untreated with the inhibitor, respectively. (B) Almost all transfected ARC displaying distinct EGFP fluorescence showed additionally positive staining against the HA-tag with and without lactacystin. This was true for each adult cardiomyocyte transfected with pcDNA3.1cycB1-HA/EGFP or pcDNA3.1cdc2-HA/EGFP. Differences in the ratios of HA-positive transfectants due to lactacystin treatment were not significant, determined by quantitative analysis of the values obtained, which were 92 ± 7% (+) and 85 ± 6% (-) for ARC transfected with pcDNA3.1cycB1-HA/EGFP, and 86 ± 4% (+) and 91 ± 3% (-) for ARC transfected with pcDNA3.1cdc2-HA/EGFP (+ meaning in the presence and - in the absence of lactacystin). This suggested that mitotic cyclin degradation by the proteasome pathway not be active in cultured ARC.
The same experimental approach was used for ARC, and did not result in a significant difference in lactacystin-treated and untreated cells regarding expression of cyclin B1-HA and Cdc2-HA, respectively (see Figure 2-23B on page 70). For Cdc2-HA, one expected no great effects by the inhibitor on the detectable levels of the exogenous cell cycle protein in transfectants. No abnormalities in the morphologic appearance of transfectants could be demonstrated by the exogenous Cdc2-HA. The presence of cyclin B1-HA being detectable in almost all transfected ARC, which were identified by EGFP fluorescence, was also independent of lactacystin treatment.

The conserved nine amino acid destruction box for cyclin B1 responsible for its recognition and subsequent proteolysis (Brandeis and Hunt, 1996) was checked using the SWISS-PROT protein database and found to be 100% homologue in humans and rats, composed of RTALGDIGN. The possibility that the human cyclin B1-HA could not be detected by the cyclin degradation machinery existing in rat cells was ruled out by the complete identity.

In summary, these findings proposed the absence of an active mitotic cyclin proteolysis machinery in adult cardiomyocytes under the experimental conditions used. Active cyclin B degradation should therefore not play a major obstacle in the de novo initiation of MPF activity in these cells.

2.5.3 Generation of MPF Expression Vectors and Their Transfection in Cell Lines

A potential involvement of the Wee1/Myt1 kinases, which phosphorylate amino residues T14 and Y15 on Cdc2 thereby inactivating its catalytic function (see section 1.1.2 on page 6; Heald et al., 1993; McGowan and Russell, 1995; Watanabe et al., 1995), was taken into account. Experimental evidences of Wee1 existence in lysates taken from the ventricles of adult rat hearts had been published (Kim et al., 1998) and upregulation of Wee1 mRNA in freshly isolated ARC had been shown in our laboratory (Dr. Christian Weikert, unpublished observations).

The inhibitory pathway controlled by Wee1 is known to be overridden by a dominant mutant form of Cdc2, which is Cdc2AF, where T14 and Y15 are changed to alanine and phenylalanine, respectively (Krek and Nigg, 1991; Heald et al., 1993). This mutant form of Cdc2 was subsequently considered for the generation of expression constructs aimed
to induce MPF activity (Figure 2-24). Plasmids encoding one MPF component, namely pcDNA3.1cycB1-HA, pSBC2cdc2-FLAG, and pSBC2cdc2AF-FLAG were generated, as well as double expression vectors, termed pcDNA3.1cycB1-HA/cdc2-FLAG and pcDNA3.1cycB1-HA/cdc2AF-FLAG, which encode both MPF components on one plasmid. Transfections using two different plasmids, which sometimes lacks efficiency, could thus be circumvented by these double expression constructs. Different tagging at the carboxy terminus by the HA and FLAG epitope allowed to determine expression of the exogenous cell cycle proteins and distinguish them from each other.

Figure 2-24: Mono- and double expression cassettes generated with the aim to restore MPF activity. The vectors pcDNA3.1cycB1-HA, pSBC2cdc2-FLAG, and pSBC2cdc2AF-FLAG are precursors of the MPF expression vectors pcDNA3.1cycB1-HA/cdc2-FLAG and pcDNA3.1cycB1-HA/cdc2AF-FLAG, which had been constructed by assembly of the mono expression vectors indicated in the scheme. The SV40 promoter drives transcription of the wild-type cdc2 and cdc2AF gene, respectively, both FLAG-tagged, thus allowing separate detection of the corresponding transgene products from cyclin B1-HA by indirect immunofluorescence. Cdc2AF encodes the phosphorylation site mutant of Cdc2 (T14A, Y15F) that cannot be downregulated by members of the Wee1 kinase family. HA-tagged cyclin B1 is under the control of the CMV promoter system that allows strong transcription of the transgene.

To examine the reliability of pcDNA3.1cycB1-HA/cdc2-FLAG and pcDNA3.1cycB1-HA/cdc2AF-FLAG, HEK 293 and COS cells were transiently transfected with these plasmids. Main purpose was to test the DNA constructs in term of transgene product expression and their straightforward detection by immunofluorescence analysis. Correct
sizes of transgene product encoded on the two vectors were confirmed by Western blot (Figure 2-25).

**Figure 2-25:** Western blot analysis of HEK 293 cells transiently transfected with the MPF vectors pcDNA3.1cycB1-HA/cdc2-FLAG and pcDNA3.1cycB1-HA/cdc2AF-FLAG. Cells were harvested 40 hours after transfection and Western blot analysis of electrophoretically fractionated total cell extracts were probed for the expression of cyclin B1-HA and Cdc2-FLAG, using anti-HA and anti-FLAG antibodies, respectively. Endogenous Cdc2 and cyclin B1 detected in lysates of untransfected cells by the appropriate antibodies served as size markers for their exogenous counterparts.

Indirect immunofluorescence microscopy unveiled cells expressing cyclin B1-HA and Cdc2-/Cdc2AF-FLAG simultaneously, albeit the consistent was not uniform in all transfectants (see Figure 2-26 on page 74). Though not determined quantitatively, different effects between cells transfected with pcDNA3.1cycB1-HA/cdc2-FLAG and pcDNA3.1cycB1-HA/cdc2AF-FLAG became apparent. Cells expressing cyclin B1-HA and Cdc2-FLAG did not exhibit a unique phenotype, some of them showing interphase nuclei and others even mitotic figures. The mutant version Cdc2AF-FLAG induced in the vast majority of cells an abnormal chromosome condensation, a so-called premature chromosome condensation. Such a behavior concerning the non-phosphorylatable mutant of Cdc2 causing unscheduled mitotic entry was in accordance with several reports (Krek and Nigg, 1991; Heald et al., 1993; Jin et al., 1996; Hagting et al., 1998; Jin et al., 1998). These effects are particularly noteworthy because they proved the function of the cloned vectors and thus their potential use for further experiments in cultured cardiomyocytes.
2.5.4 Ectopic Expression of Cell Cycle Proteins in Cultured NRC

The successful application of the two MPF vectors in cell lines allowed experiments in cardiomyocytes. The cloned constructs and possible effects when overexpressed were first elucidated in NRC. Cyclin B1-HA, Cdc2-FLAG, and Cdc2AF-FLAG were investigated on their ability to exert cell cycle-related events (see Figure 2-27 on page 75). The role of the expressed proteins was determined in NRC kept under high serum conditions, which was anticipated to provide the suitable environment for cell cycle induction. Using the Tf-PEI transfection system, cultured NRC were transfected.
with pcDNA3.1cycB1-HA, pSBC2cdc2-FLAG, and pSBC2cdc2AF-FLAG. Although NRC tended to be more susceptible to cell cycle-inducing stimuli than ARC see section 2.4.1 on page 59, the vast majority of transfectants were not markedly perturbed concerning their nuclei morphology. The time span to express the exogenous cell cycle proteins was restricted to 48 hours, which was assumed to be sufficient for detection of completed or still ongoing cell cycle-related events in transfectants. Possible effects occurring after 48 hours could not be totally ruled out because of the undefined kinetic behavior of the cardiac cell cycle. Changes in the nuclei due to the presence of the exogenous cell cycle proteins could so far not be shown under the given experimental conditions. It was concluded that MPF activity could not be induced by a forced expression of one of the two MPF components in this cell type.

Figure 2-27: Ectopic expression of MPF components in NRC cultured with 10% serum. NRC were transfected with pSBC2cdc2-FLAG (A-C), pSBC2cdc2AF-FLAG (D-F), pcDNA3.1cycB1-HA (G-I), and pHM6-lacZ (J-L) using the Tf-PEI system and analyzed 48 hours later. Transgene products in NRC were identified by antibody staining against the FLAG epitope (A/D), and the HA epitope (G/J). Nuclei were visualized by DAPI staining (B/E/H/K) and cardiomyocytes were identified by staining against the sarcomeric protein titin (C/F/I/L). Neither Cdc2-FLAG (A) nor the mutant Cdc2AF-FLAG (D) when expressed were able to induce nuclear alterations (B/E). The same was also true for cyclin B1-HA (G), causing no significant changes of the nuclei shape to those found in untransfected ones (H). The vector pHM6-lacZ encoding the HA-tagged β-galactosidase served as control to exclude potential transfection artifacts. Scale bar 10µm
2.5.5 Transfection of MPF Vectors and Their Effects in Cultured NRC and ARC

The experiment with NRC served as a means to examine the potential impact of the double expression vectors pcDNA3.1cycB1-HA/cdc2-FLAG and pcDNA3.1cycB1-HA/cdc2AF-FLAG in cultured ARC. NRC cultured with high serum were transfected with the two constructs and eventual effects were checked by using indirect immunofluorescence analysis 48 hours later (Figure 2-28). Transgene expression was determined by antibody staining against the corresponding epitopes used for their tagging. As earlier described for COS cells, simultaneous expression of both transgene products was not encountered in every transfectants. In these cells, detection signal for Cdc2-FLAG and Cdc2AF-FLAG were not above the background level as seen in untransfected cells, whereas cyclin B1-HA presence could be readily verified. Such NRC showed no differences to NRC untransfected or transfected with pcDNA3.1cycB1-HA. This corroborated the findings previously made, suggesting that a unique expression of cyclin B1 not be sufficient to induce MPF activity-related events.

Figure 2-28: MPF double expression vectors transfected in NRC cultured with 10% serum (see page 77). NRC were transfected using the Tf-PEI system with pcDNA3.1cycB1-HA/cdc2-FLAG (A-H) and pcDNA3.1cycB1-HA/cdc2AF-FLAG (I-P), and incubated for 48 hours before analyzed using triple immunofluorescence microscopy. Cyclin B1-HA was visualized by anti-HA antibody staining (A/E/I/M). Anti-FLAG antibody was used to verify expression of Cdc2-FLAG (B/F) and Cdc2AF-FLAG (J/N). Cells were stained with DAPI to unveil chromosome morphology (C/G/K/O). Muscle striation in cells was assessed by anti-titin antibody staining (D/H/L/P). NRC transfected with pcDNA3.1cycB1-HA/cdc2-FLAG and pcDNA3.1cycB1-HA/cdc2AF-FLAG, respectively, and staining positive only for cyclin B1-HA (A/I), did not disclose any induced effects on cell structure (C/D/K/L). Immunofluorescence signal specific for Cdc2-FLAG (B) and Cdc2AF-FLAG (J) could not be detected in these cells despite prolonged exposure time while recording. Transfectants expressing cyclin B1-HA (E/M) and Cdc2-FLAG (F)/Cdc2AF-FLAG (N) at the same time were characterized by apparent smaller nuclei, likely as a consequence of induced chromosome condensation (G/O). Scale bar 10µm
Striking differences became evident in transfectants expressing both gene products. They showed highly condensed nuclei, easily recognizable by the intense DAPI signal. In this respect, transfection performed with pcDNA3.1cycB1-HA/cdc2-FLAG and pcDNA3.1cycB1-HA/cdc2AF-FLAG did not result in noticeable differences when compared to each other. Using pcDNA3.1cycB1-HA/cdc2AF-FLAG on the other hand did not yield as many transfectants as pcDNA3.1cycB1-HA/cdc2-FLAG. In several independent transfection experiments, an altered nuclei appearance was shown for more than 90% of such transfectants. Cultured NRC expressing both transgene products to detectable levels were rarely found; only a few dozens cells at the most per culture dish could be detected. Nevertheless, the changes in nuclei shape correlated so strongly with the simultaneous expression of cyclin B1-HA and Cdc2-FLAG or Cdc2AF-FLAG, respectively, that a direct relation could eventually be proposed. It could be proven that the protein products of the genes encoded on the constructs were well functioning, and particularly that the tags applied did not interfere with the ability to bind and activate Cdc2.

The same experimental approach was subsequently conducted in NRC cultured with only 1% serum. Equal results as seen under high serum conditions could be obtained. NRC expressing both cyclin B1-HA and Cdc2AF-FLAG is shown in Figure 2-29 as an example. The ability to induce nuclei condensation revealed the potential to interact directly in the nuclei causing cell cycle-related effects, apparently independent of the need for extracellular stimuli provided by high serum.

Figure 2-29: NRC cultured with 1% serum and transfected with pcDNA3.1cycB1-HA/cdc2AF-FLAG. The impact of the presence of Cyclin B1-HA (A) and Cdc2AF-FLAG (B) in NRC was seen also under low serum conditions, resulting in a highly condensed nuclei (C). Striation in cardiomyocytes was detected by anti-titin staining (D). Scale bar 10µm
The hypothesis to target precisely the cell cycle in ARC using the MPF vectors were addressed next. For that purpose, cultured ARC were transfected with the two above mentioned double expression plasmids using the AVET system (Figure 2-30). With regard to the difficult detection of transgene products when using the AVET system and assuming a more inert cell cycle response, ARC were allowed to express the exogenous cell cycle proteins for 72 hours after the transfection procedure. As already seen for NRC, transfection experiments per dish yielded only a few ARC that expressed both genes encoded on each construct to levels readily noticeable in immunofluorescence microscopy. Similar to the situation in NRC, nuclei of such transfected ARC displayed a highly compact appearance associated with an abnormal clumped morphology. The almost absolute match observed (>90%, determined from several transfection experiments) between altered nuclei and the presence of cyclin B1-HA together with Cdc2-FLAG or Cdc2AF-FLAG, respectively, suggested the functional recovery of MPF in ARC by the double expression vectors. A special preference to induce MPF activity could not be accredited to pcDNA3.1cycB1-HA/cdc2AF-FLAG, since ARC coexpressing the wild type version Cdc2-FLAG and cyclin B1-HA underwent nuclei transformation in the same manner. It became now feasible to examine MPF activity more closely thus providing some clues of its profound effects on the behavior of cardiomyocytes.

Figure 2-30: MPF recovery in ARC (see page 80). Cultured ARC were transfected with the double expression vectors pcDNA3.1cycB1-HA/cdc2-FLAG (A/C/E/G), pcDNA3.1cycB1-HA/cdc2AF-FLAG (B/D/F/H), and the control vector pHM6-lacZ (I-K). Immunofluorescence staining was conducted 72 hours after transfection. Expression of the exogenous gene products cyclin B1-HA (A/B), Cdc2-FLAG (C), Cdc2AF-FLAG (D), and β-galactosidase-HA (I) was confirmed by antibody staining against the corresponding tag epitopes. Nuclei were visualized by DAPI labeling (E/F/J). Cardiomyocytes were identified by anti-titin (G/H) and anti-myomesin (K) antibody staining. Simultaneous expression of cyclin B1-HA (A/B) and Cdc2-FLAG (C)/Cdc2AF-FLAG (D) led to chromosome condensation (E/F). Such nuclei had a reduced size with unusual morphology that was associated with an eye-catching bright DAPI signal compared to their counterparts in untransfected cells (E). ARC expressing β-galactosidase (I) exhibited normal interphase-like nuclei (J), ruling out the possibility of nonspecific, transfection-dependent effects on the nuclear structure. Scale bar 10µm
2.5.6 Induction of Myofibrillar Decay and Histone H3 Phosphorylation

Tightly packed nuclei induced by MPF activity in both NRC and ARC were mainly concomitant with myofibrillar distortion. The degree of myofibrillar disorganization in transfectants was variable, ranging from minor to heavily affected sarcomeric structures, in which an organized pattern was barely observable (see Figure 2-31 on page 82). A strong correlation between the degree of nuclei condensation and degradation of the striated structure was not obvious. A different behavior of NRC and ARC with regard to their susceptibility to MPF activity was also not evident. However, one could conclude that Cdc2 kinase activity was the direct or eventually the indirect cause of myofibrillar decay in these cells. An interesting feature in such cells appeared when the contractile apparatus was assessed by immunofluorescence staining with the polyclonal anti-titin M8 antibody. As earlier mentioned (see Figure 2-19 on page 62), nuclear staining by the titin antibody disappeared in NRC undergoing M phase. These findings held also true for the condensed nuclei in NRC and ARC caused by MPF activity thus proposing M phase-related karyoskeletal changes, like nuclear envelope breakdown, which had been reported to be the direct consequence of lamin subunit phosphorylation caused by MPF (Peter et al., 1990).

Chromosome condensation is accompanied by phosphorylation of histones (Nigg, 2001). Histone H3 phosphorylation has been strongly linked with mitotic chromosome condensation (Koshland and Strunnikov, 1996). It was therefore of interest to test whether chromosome condensation in cardiomyocytes expressing MPF would correlate with H3 phosphorylation. Site specific phosphorylation was asserted using an antibody highly specific for the phosphorylated form of the amino terminus of histone H3 at serine 10 (Hendzel et al., 1997). Histone H3 phosphorylation was first investigated in NRC transfected with pcDNA3.1cycB1-HA/cdc2-FLAG and pcDNA3.1cycB1-HA/cdc2AF-FLAG, respectively (see Figure 2-32 on page 84). Proof of active MPF complexes could be derived by the altered nuclear morphology found in transfectants, and phosphorylation of histone H3 was analyzed by specific antibody staining against this epitope. Induced mitotic figures in NRC transfected with either pcDNA3.1cycB1-HA/cdc2-FLAG or pcDNA3.1cycB1-HA/cdc2AF-FLAG were clearly positive in term of the phosphorylated state of histone H3. This observation could be made without exception in
all transfectants investigated, emphasizing the phosphorylation of histone H3 as a result of induced MPF activity.

Subsequently, the potential of MPF activity eventually leading to H3 phosphorylation in cultured ARC was examined. Transfection of cultured ARC with pcDNA3.1cycB1-HA/cdc2-FLAG and pcDNA3.1cycB1-HA/cdc2AF-FLAG was accomplished by the AVET system, and analysis was performed in the same way as for NRC (see Figure 2-33 on page 85). Phosphorylated H3 was pervasive in all transfectants displaying abnormal nuclei shape, independently of the expression vector used. This allowed to conclude that recovered MPF was able to trigger a typical mitosis-related event in these postmitotic cells. Although such an indication of a re-entry into the cell cycle was convincing, the impact of MPF recovery on the cells was limited in respect of their cell cycle progression, since neither proper chromosome segregation nor cytokinesis could be demonstrated. This suggested the necessity of additional mechanisms controlling cell cycle progression and arrest in these cells. Nevertheless, the outcome regarding H3 phosphorylation was exciting since the role of histone H3 kinase has not been attributed
to Cdc2 kinase so far (Fry et al., 1998; Adams et al., 2001; Murnion et al., 2001). Targeting the cell cycle in ARC with forced expression of MPF thus led to the activation of other cell cycle-regulating molecules. This in turn revealed the potential of these cells to undergo also cell cycle events, which are not directly conducted by the re-established MPF activity.
Figure 2-32: Histone H3 phosphorylation in cultured NRC. Cardiomyocytes were analyzed 48 hours after transfection with pcDNA3.1cycB1-HA/cdc2-FLAG (A/D/G/J), pcDNA3.1cycB1-HA/cdc2AF-FLAG (B/E/H/K), and pHM6-lacZ (C/F/I/L). Transfectants were recognized by anti-HA antibody staining (A-C) and phosphorylation status of histone H3 was determined by anti-phospho-histone H3 antibody (D-F) staining. DAPI was used to detect morphologic alterations in the nuclei shape (G-I), and sarcomeric proteins were visualized by anti-myomesin antibody staining (J-L). MPF activity was confirmed in transfectants (A/B) by checking their nuclei shape (G/H). Histone H3 proteins were found to be phosphorylated in transfected NRC having one or more condensed nuclei (D/E). NRC expressing β-galactosidase (C) did not reveal any sign of a condensed nucleus (I) associated with phosphorylated H3 (F; extended exposure time). Scale bar 10µm.
Figure 2-33: Histone H3 phosphorylation in cultured ARC. Cardiomyocytes were analyzed 72 hours after transfection with pcDNA3.1cycB1-HA/cdc2-FLAG (A/D/G/J), pcDNA3.1cycB1-HA/cdc2AF-FLAG (B/E/H/K), and pHM6-lacZ (C/F/I/L) using the AVET system. Transfectants were identified by anti-HA antibody staining (A-C), and phosphorylation status of histone H3 was determined by anti-phospho-histone H3 antibody staining (D-F). DAPI was used to detect morphologic alterations in the nuclei shape (G-I), and sarcomeric proteins were visualized by anti-myomesin antibody staining (J-L). Highly compact nuclei (G/H) were observed in transfectants identified by cyclin B1-HA expression (A/B). Condensation was associated with phosphorylation of histone H3 (D/E). Control transfection with pHM6-lacZ was needed to exclude potential artifacts under the experimental regime used: ARC expressing β-galactosidase (C) did not exhibit characteristics regarding their nuclei (F; extended exposure time/I) as it was observed for ARC transfected with pcDNA3.1cycB1-HA/cdc2-FLAG or pcDNA3.1cycB1-HA/cdc2AF-FLAG. Scale bar 10μm.
3  DISCUSSION

3.1  POTENTIAL AND LIMITATIONS OF SIN-BASED VECTORS IN HEART RESEARCH

The results regarding viral gene transfer have demonstrated transgene expression for the first time using recombinant SIN in cardiomyocytes isolated from neonatal and adult rat heart, and from embryonic chicken heart as well. The object of the investigations was to find an appropriate method for convenient genetic engineering of cultured ARC with special emphasis on the ectopic expression of cell cycle genes. Priority was given to viral vectors mainly because of their known high infection efficiency, but also because no nonviral transfection method to accomplish reliable gene transfer into cultured ARC was available at that time.

One major experimental advantage of SIN especially attractive in the context of this work was the ability to generate recombinant infectious particles within one day once the recombinant RNA had been established. The subsequent application in ARC of such recombinant particles demonstrated the high infectivity of SIN in postmitotic cells, revealing a transgene expression in 80% of ARC exposed to SINrep5/lacZ for one day. A comparable high infection rate in cultured ARC had been only obtained so far with adenoviral gene delivery systems (Kass-Eisler et al., 1993; Kirshenbaum et al., 1993). Our high infection rates also observed in cultured NRC are in good accordance with rates reported in the meantime by McWhinney and colleagues; they have mentioned an infection rate of 90% using recombinant SIN encoding the β-galactosidase gene (McWhinney et al., 2000).

Synthesis and presence of the reporter proteins GFP and β-galactosidase were observed in cultured ARC even 4 days after infection, while the spontaneous beating activity decreased over several days, and cell death occurred after prolonged cultivation suggesting apparent cytotoxicity of this viral vector. Nevertheless, cytotoxic effects of SIN infection on host protein synthesis and metabolism were less severe in ARC at first, than those, which occurred in cultured NRC, fibroblasts, and BHK cells in particular. The differences between cultured NRC and ARC concerning the viability after SIN infection may point to an age-dependent mortality, which had already been described for
primary cultures of embryonic dorsal root ganglia neurons (Levine et al., 1993). The delay in cytopathogenicity observed in different cell types could be due to differences in cellular factors, influencing the viral RNA replication and thus the shut off of host protein synthesis.

The successful implementation of the SIN system in rat hippocampal slice cultures to deliver transgenes into neurons has been reported (Maletic-Savatic et al., 1999; Ehrengruber et al., 1999; D’Apuzzo et al., 2001). These studies had shown that the infected neurons appear normal and viable for up to five days post-infection, which is even longer than the time period seen for cultured ARC. The time frame offered by SIN may be suitable for specific experimental approaches in primary culture systems of ARC. For cell cycle studies, however, a less restricted time window would have been preferred. Of major concern was the impact of the viral polymerase on the expression of cell cycle regulators and the host protein synthesis per se.

Presumably useless for cell cycle studies, the SIN system may emerge as a useful tool for another important research field in cardiology, namely the exploration of the structure-function relationship concerning the myofibrillar sarcomere. This research field has attracted respectable interest in recent years, in particular due to the identification of sarcomeric protein mutants and their association with cardiomyopathy (for a review: Seidman and Seidman, 1998). Expression of contractile proteins harboring such mutations by the SIN system in adult cardiomyocytes may turn out to be helpful to assess whether the mutant protein exerts a dominant-negative effect on the sarcomere or acts as a null mutation that has lost the ability to assemble into myofibrils. Gene transfer of exogenous contractile proteins to study the localization and function in cultured adult cardiomyocytes has been mainly achieved by recombinant adenovirus (Westfall et al., 1997; Rust et al., 1998; Rust et al., 1999). By using the SIN system, expression of fluorescent protein-tagged myofibrillar components in freshly isolated ARC could be demonstrated for the first time (see Figure 3-1 on page 88). The ectopic expression of fluorescent protein-tagged myofibrillar proteins in cultured NRC and ARC mediated by the SIN system represents a novelty. Besides high infection efficiency of SIN, high expression levels in cardiomyocytes are characteristic. The observed strong signal from the fluorescent marker protein allowed not only a qualitative assessment about the
expression but also the rapid localization of the fusion protein in infected cardiomyocytes.

Figure 3-1: SIN-mediated gene transfer of GFP-tagged contractile proteins into freshly isolated ARC. After cloning and generation of infectious particles encoding the fusion protein, straightforward localization of the protein of interest in rod-shaped cardiomyocytes is possible due to the high transgene expression levels achieved by the SIN system.

However, some restrictions must be made regarding prospective applications. For instance, detection of the fluorescence signal derived from α-actinin-DsRed was not possible in living NRC, probably due to the cytotoxic effects caused by SIN within 24 hours. This time window could have been too narrow for the accumulation of detectable amounts of the red fluorescent fusion protein. In addition, the simultaneous infection of ARC with two different SIN constructs turn out to be difficult. Although exposed to both SINrep5/MLC3f-EGFP and SINrep5/α-actinin-DsRed, cultured ARC expressed preferentially SINrep5/MLC3f-EGFP. This was probably caused by the different sizes between the two SIN replicons, whereby the shorter SIN replicon, namely SINrep5/MLC3f-EGFP with a RNA length of 9.2 kb, was of advantage compared to SINrep5/α-actinin-DsRed with a RNA length of 11.4 kb. Consequently, the viral polymerase amplified predominantly the SINrep5/MLC3f-EGFP. This prevented propagation of the longer construct and the corresponding transgene expression got finally discriminated. It seems evident that the SIN system can only be used for the localization of contractile proteins in short-time expression studies. After longer periods of cultivation, cytopathogenicity became obvious in infected cardiomyocytes. Mutations in the nonstructural proteins of the viral polymerase had been shown to result in a significant reduced cytopathogenicity of the SIN replicon in different cell lines (Agapov et al., 1998; Frolov et al., 1999). Unfortunately, mutation nsP2-726L did not allow the
packaging into infectious viral particles, as we had attempted in this study. Meanwhile, SIN variants had been isolated that are found to be noncytopathic in mammalian cell lines and which can be even packaged efficiently (Perri et al., 2000). Investigations are currently under way to clear the potential of such noncytopathic SIN particles in ARC. First results have been encouraging, pointing to reduced cytotoxicity and an increased life span of infected ARC (Dr. Josef Magyar, personal communications). It will be important to study more deeply whether these noncytopathic versions can be used successfully in cardiomyocytes during expanded culture times. One may then be able to explore influences of mutations in sarcomeric proteins on the function of the contractile apparatus.

In freshly isolated adult cardiomyocytes, the SIN-mediated gene transfer allowed a comparatively high expression level of transgene products resulting in a strong fluorescence signal. An uneven distribution of the fluorescence signal derived from the α-actinin-DsRed fusion protein could often be observed, suggesting that distant binding sites be only saturated after closer ones had been occupied. It is assumed that the accumulation of the transgene product takes place preferentially around the nucleus leading to a stronger signal in the perinuclear region. Nonetheless, the appropriate sarcomeric targeting of the ectopically expressed proteins may give information about the situation in vivo, indicating a continuous exchange of contractile proteins in the adult heart. The turnover rate of sarcomeric proteins must play an essential role with regard to the perpetual work to be done and the longevity of each one cardiomyocyte. Since adult cardiomyocytes have lost the ability to divide, they must possess other mechanisms to ensure functionality through life: a rapid and everlasting replacement of components of the contractile apparatus is likely to be one of those. The results regarding the correct binding of α-actinin-DsRed represent thus a good indication of such a highly active process in adult cardiomyocytes.

There are some interesting features, which make SIN a conceivable vector for in vivo applications. One is certainly the high infection efficiency together with a strong expression of the heterologous protein. Additionally, infectious particles can be generated that do not contain the complete set of all viral genes, thus rendering them replication-deficient and providing protection against widespread infection of other tissues. Finally, SIN viruses do not integrate into the host chromosomal DNA. The risk of
causing cell transformation is thus minimal. Whether SIN will prove to contribute to the expanding field of molecular cardiobiology or cardiovascular gene therapy will strongly depend on the development of noncytopathic SIN vectors. Until then, exploitation of the SIN system will remain restricted to specific applications as shown e.g. for cardiomyocytes in culture.

3.2 CELL CYCLE ENGINEERING OF CULTURED ADULT CARDIOMYOCYTES

The riddle of the cell cycle of differentiated cardiomyocytes has provoked many research projects with the ultimate goal to solve it. Here, by intervening at the G2/M boundary and by aiming to a functional recovery of MPF, a more or less untouched facet of the cell cycle was explored. It was of special interest to perform the investigations in the primary culture system of ventricular adult cardiomyocytes, representing a convenient model for the adult cardiac phenotype in vivo. Nevertheless, cultured adult cardiomyocytes are often treated as matter of peripheral importance among research groups. One reason might simply be a more straightforward handling of embryonic and neonatal cardiomyocytes, which is based in addition on a less costly isolation procedure. Only one report has so far been published using cultured adult cardiomyocytes to analyze the effect of cell cycle-regulating factors by genetic means (Agah et al., 1997), eventually giving evidences for the difficult gene transfer into ARC. The AVET system has emerged in this context as a true tool for an easier way to the genetic manipulation of the adult cardiac cell cycle, which will shortly be discussed next.

3.2.1 NOVEL WAYS TO TRANSFECT CULTURED ARC

To avoid the demonstrated limitations of Sindbis virus-mediated gene transfer (see section 3.1 on page 86), transfection strategies were evaluated, which used the receptor-mediated pathway for the genetic modification of cultured ARC. Absence of any major cytotoxic effects in cardiomyocytes was observed when using such an approach under the optimized conditions (see section 4.4.2 on page 114). Cardiomyocytes expressed readily reporter gene products without any visible damage to the cells for several days after the transfection procedure. An unrestricted beating activity could be seen in living
cardiomyocytes, which expressed the prime reporter gene product EGFP over 5 and more days, demonstrating a convincing capacity to function properly.

Tf-PEI turned out to be an appropriate vector for the forced expression of cell cycle molecules in NRC, whereas it failed in terms of directing efficient gene delivery into cultured ARC. Although the transfection rate was only in the lower range when EGFP had been used as the marker protein, most transfectants were unaffected in their viability, which allowed to use Tf-PEI for NRC in culture.

The implementation of the AVET system for transfecting ARC was a breakthrough: reaching in some cases a gene transfer rate found otherwise only for cell lines. Gene transfer efficiency of up to 20% was achieved with a construct encoding EGFP driven by the strong CMV promoter. On the other hand, transfection of plasmids generated for cell cycle studies often resulted in only a few dozens of transfectants per cell dish. Despite the high variations observed in transfection efficiency for the different constructs, the AVET system remains the method of choice for primary cultures of ARC. When first applied, AVET represented a real novelty, which was to achieve a gene delivery efficiency that was until then solely attributed to viral vectors.

In the meantime, a first application of an adenoviral-component gene delivery method to transfect ventricular adult cardiomyocytes has been reported (Wei et al., 2000). The trinary complex used contained replication-deficient adenoviruses, polylysine and the expression plasmids encoding the gene of interest. This technique had already been shown to reach a 70% efficiency in cultured NRC (Kohout et al., 1996). The efficiency reported by Wei et al. in cultured adult cardiomyocytes was between 1 - 10% using an EGFP construct similar to that one we used in this study. The considerable difference in efficiency between NRC and ARC again demonstrates the exceptional difference between the two cell types. Wei et al. have reported an apparent decrease in transfection efficiency with increasing age of the rats, with no successful transfection in cells from 10-week-old rats (Wei et al., 2000). Consequently, they used 4- to 6-week-old rats for their study, whereas we used 6- to 8-week-old rats. They furthermore performed their experiments in a short-time culture of ARC focusing on electrophysiology. This makes it very difficult to assess the potential of the two transfection methods to each other.

Both gene delivery systems, the AVET system and the reported trinary complex system, are partly based on the same components and thus almost identical in their way to enter
the cell. The AVET system provides some more features, like the incorporation of transferrin, enabling the additional binding of the complexes to the corresponding receptor. Furthermore, the use of inactivated adenoviral particles by the AVET system is a great advantage. It circumvents handling with fully competent viral particles though replication-deficient. Today, the AVET system represents the only transfection method, which offers convenient gene delivery into cultured ARC without the use of active viral particles.

As it has been shown here, it is only the AVET system, which enabled reliable experimentation regarding the re-establishment of MPF activity in adult cardiomyocytes. Transfections performed with control vectors ruled out the possibility of transfection-derived artifacts. The presented results showing the expression of EGFP-tagged myosin light chain (MLC3f-EGFP) reflected the application of the AVET system not only in cell cycle studies but also in studies involving the cytoarchitecture. The lack of a significant number of TUNEL-positive ARC transfected by AVET allows the assumption that apoptosis causes no problem.

Based on the findings made in vitro, it seems conceivable to test the AVET system in vivo. However, plasmid-based approaches have normally resulted in a patchy expression pattern in the heart after intramyocardial injection as reported recently (Wright et al., 2001). It is clear that one has to analyze very carefully the performance of the AVET system in vivo in terms of efficiency, duration of transgene expression and possible host inflammation reactions. The usefulness of the AVET system for a prospective in vivo application remains open before a rigid assessment has been performed.

In summary, our results achieved with the AVET system have revealed that the receptor-mediated pathway is a promising route to introduce foreign DNA into adult cardiomyocytes. Refinement of the known adenoviral-component complexes will continue to further facilitate gene transfer into adult cardiomyocytes.

### 3.2.2 TRANSCRIPTIONAL AND TRANSLATIONAL REGULATORY ELEMENTS IN ARC

Once the transfection method had been established, the attention was turned to appropriate genetic elements enabling optimal transcription and translation of the gene of interest. Only little was known concerning the functionality in cultured ARC of such
elements integrated in the expression vector. The objective to reinitiate the activity of a particular cyclin-dependent kinase implied the ectopic expression of two proteins, the cyclin-dependent kinase and its regulatory cyclin subunit, or even more. Another issue was the need for identification of transfected cells by a marker protein. It called for the transfection of one ARC with several different constructs at the same time. Since uncertainty existed concerning the cotransfection, the use of tandem constructs seemed a better idea. Therefore, the use of IRES elements, allowing the translation of up to four genes from the same mRNA in mammalian cells (Fussenegger et al., 1997), was investigated. Descendants of such multicistronic expression vectors, as pscyA-IRES-EGFP and pTri-cycA-EGFP, worked readily in HEK 293 cells, but did not yield the expected results in ARC, leading to no or barely detectable expression of the marker protein whose translation was driven by IRES. Such an inefficiency of the expression plasmids observed in cultured ARC corresponds to findings made by other groups about the IRES function and its application. The activity of IRES elements has been considered to be dependent on certain cellular factors, e.g. on the polypyrimidine tract binding protein (PTB) (Martinez-Salas, 1999). In this context, expression of PTB mRNA level had been shown to be downregulated during heart development in the mouse (Patton et al., 1991), thus potentially inhibiting IRES function. Furthermore, it had been demonstrated in transgenic mice encoding a bicistronic expression construct that IRES-driven translation decreases with increasing age of the animal (Shaw-Jackson and Michiels, 1999). Taken together, these outcomes urge a thorough investigation before an application of different IRES elements in adult cardiomyocytes should be considered, especially if one thinks of a prospective gene therapy.

Nevertheless, tandem constructs were still favored to accomplish ectopic expression of more than one cell cycle protein. Consequently, double expression vectors encoding the gene of interest under the control of two different promoters, namely the CMV and SV40, were generated. The use of the same promoter twice in the constructs was avoided in order to reduce the risk of homologous recombination within the plasmids during their propagation in bacteria. The CMV promoter was used for the double expression vectors pcDNA3.1cycB1-HA/cdc2-FLAG and pcDNA3.1cycB1-HA/cdc2AF-FLAG to drive transcription of the cyclin B1-HA gene, whereas the genes cdc2-FLAG and cdc2AF-FLAG were put under the control of the SV40 promoter. However, simultaneous
expression of both MPF components was not always observed in each cardiomyocyte transfected with pcDNA3.1cycB1-HA/cdc2-FLAG and pcDNA3.1cycB1-HA/cdc2AF-FLAG. Levels of Cdc2-FLAG and Cdc2AF-FLAG were very often not detected, while cyclin B1-HA was clearly present in transfectants. This proposed a relatively low performance of the SV40 promoter in this cell type.

The use of cardiac-specific promoters to drive cell cycle proteins in ARC could not be evaluated within the scope of this study. One advantage of such promoters would be the directed transgene expression in cardiomyocytes, avoiding expression in non-cardiomyocytes. The verification of the cardiomyocyte phenotype of transfectants using a sarcomeric marker, as it was done here, is inevitable since e.g. the ventricle-specific MLC-2 promoter and the α-MHC promoter had only little promoted gene expression in the left ventricle of neonatal rats as compared to a viral promoter (Franz et al., 1997). We assumed that such promoters would have possibly failed to provide sufficient transgene expression for MPF recovery and thus were not considered.

Regulated expression of foreign genes in ARC will be of outmost importance. Regulation by hypoxia response elements had already been demonstrated in cultured NRC and in vivo (Prentice et al., 1997). Additional systems allowing controlled expression of transgenes are the tetracycline-repressible system (Gossen and Bujard, 1992) and more recently the streptogramin-based gene regulation system for mammalian cells (Fussenegger et al., 2000). For the study of the function of cell cycle proteins in cultured ARC, this kind of regulation systems may be appropriate to assess more precisely kinetics and stability of the transgene products.

3.2.3 CARDIAC CELL CYCLE AND ITS ACTIVITY UNDER IN VITRO CONDITIONS

It was obvious that the environment of the cardiomyocytes in vitro would play a substantial role what concerns their behavior. The role of the culture serum and its impact on the cell cycle was thus investigated. We first focused on the G1/S transition by testing ongoing DNA synthesis via BrdU-incorporation. Up to 10% of the nuclei in NRC cultured with 10% FCS were BrdU-positive, compared to only about 0.5% of nuclei in NRC kept under low serum conditions. The rise observed in the presence of high serum of BrdU-positive nuclei in NRC indicated the presence of susceptibility to mitogenic
stimuli in at least a fraction of the cells. Sadoshima and colleagues reported similar findings, namely an increase in the number of BrdU-positive NRC from 1.8% to 6.2% after stimulation with 20% FCS (Sadoshima et al., 1997). However, no marked increase in BrdU-positive nuclei of NRC have also been reported when treated with 10% serum (Tamamori et al., 1998). The inconsistency among the reports may be due to different kinds of serum, which have been used.

Only marginal incorporation of BrdU was detected in cultured ARC despite high serum in the medium. This clearly reflects the strong refusal of ARC to enter the cell cycle even under optimized culture conditions. The difference between cultured NRC and ARC may also be explained by the fact that ARC are predominantly binucleated, whereas cultured NRC represent a heterogeneous population with a large number of cell having only one nucleus. Mononucleated NRC have to replicate their DNA first before they can acquire the binucleated state of the adult. This probably implies the ability of NRC to respond to appropriate signals for S phase entry that could thus account at least in part for the increased BrdU incorporation observed in cultured NRC. In contrast, binucleated ARC have already crossed the G1/S transition point and the S phase, and may therefore have no obvious reason to keep the G1/S barrier open. In regard to that, the reported reactivation of the cell cycle in ARC by forced expression of E2F-1 (Agah et al., 1997), which is a main player for S phase entry, may seem somewhat far-fetched.

Further examinations by immunofluorescence microscopy were intended to unveil additional cell cycle-related events in cultured cardiomyocytes under the prevailing culture conditions. Cytokinesis was absent in cultured NRC under low serum conditions and in cultured ARC, which is in agreement with a statement made by Soonpaa and Field (Soonpaa and Field, 1998). In case of NRC stimulated with high FCS concentration, an increase in MPF activity besides a slight induction of DNA synthesis has been reported (Sadoshima et al., 1997), suggesting that a relevant portion of the cells be beyond the G2/M boundary, maybe even in late M phase. Indeed, our analysis of NRC cultured in 10% FCS disclosed the existence of cardiomyocytes in late telophase associated with cytokinesis. Although the number of such cells was generally reduced to only a few figures thus lacking significance in terms of an overall effect, it revealed the potential of NRC to undergo cell division in culture. The recordings made look very convincing and we are not aware of comparable immunofluorescence pictures showing that clearly NRC
exerting cytokinesis. The cytoskeletal organization of NRC during the M phase has repeatedly been demonstrated in cells freshly isolated from 2-day-old to 8-day-old animals (Li et al., 1997b; Li et al., 1997a). The authors have reported that cardiomyocytes from 2-day-old rats undergoing cytokinesis showed completely dispersed myofibrils, whereas cardiomyocytes from animals older than 2 days exhibited myofibrils in the equator region where cleavage furrows potentially form. They also concluded that the presence of myofibrils may impede cytokinesis, eventually resulting in binucleated cardiomyocytes. This corresponds to the observation seen in Figure 3-2 on page 97, where no striated pattern was present in the equator region in dividing cardiomyocytes. However, a total breakdown of the myofibrillar organization within dividing NRC could not be demonstrated. We investigated the course of microtubule reorganization typically accompanying formation of the mitotic apparatus and noticed accordance with Li and coworkers (Li et al., 1997b). Remnants of microtubules in the mitotic spindle concentrated in the midbody with a narrow bare zone in the center could be confirmed. It is obvious that the pictures so far published and presented here represent only a collection of different cells at different cell cycle stages, and no exact fate of the single cardiomyocyte shown in M phase has been followed. Indeed, scoring mitotic figures in early M phase cannot distinguish between cells that acquire binucleation by just performing karyokinesis and cells that will finally separate by cytokinesis. It is expected that only a small number of cultured NRC displaying mitotic figures will undergo cytokinesis. Time-course studies of living cultured NRC, e.g. expressing an EGFP-tagged version of tubulin, may be very helpful and would allow a more quantitative estimation.
Figure 3-2: Late telophase in cultured NRC. Daughter nuclei reform (A) and cytokinesis is almost complete, with no apparent sarcomeric structure (titin) in the cleavage zone (B), but with the midbody (α-tubulin) persisting between the daughter cells (C). Scale bar 10 µm.

3.2.4 Control of MPF activity and its induction in postmitotic cardiomyocytes

Ectopic expression of wild-type versions of cyclin B1 and wild-type Cdc2 was sufficient to re-establish MPF activity in cultured NRC and ARC (see table 3-1). Several factors potentially impeding the function of Cdc2 kinase had to be taken into account to find appropriate strategies. A single ectopic expression of cyclin B1-HA did not succeed despite the putative presence of endogenous Cdc2, though only weakly detectable by immunofluorescence microscopy.

<table>
<thead>
<tr>
<th>MPF vectors and their impact in cultured cardiomyocytes. Analysis was performed 48 hours (NRC) and 72 hours (ARC), respectively, after the transfection procedure. + nuclei condensation; - no obvious effects on nuclei morphology; nd not determined; * it was assumed that ectopic expression of Cdc2-FLAG and Cdc2AF-FLAG would not have caused reactivation of MPF in ARC, since it already failed in NRC. Also no effects were previously observed when Cdc2-HA was present in ARC transfected with pcDNA3.1cdc2-HA/EGFP.</th>
<th>NRC</th>
<th>ARC</th>
</tr>
</thead>
<tbody>
<tr>
<td>pcDNA3.1cycB1-HA</td>
<td>-</td>
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</tr>
<tr>
<td>pSBC2cdc2-FLAG</td>
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<td>nd*</td>
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<tr>
<td>pSBC2cdc2AF-FLAG</td>
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<tr>
<td>pcDNA3.1cycB1-HA/cdc2-FLAG</td>
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<tr>
<td>pcDNA3.1cycB1-HA/cdc2AF-FLAG</td>
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The result implicated that the amount of endogenous Cdc2 could have been too low to generate enough kinase activity or an inhibitory mechanism could have been fully active.
One could think of proteolytic degradation influencing the possible post-transcriptional regulation of cyclin B1. This is based on the outcome of transfection experiments with pcDNA3.1cycB1-HA, which resulted in only low numbers of ARC expressing the exogenous cyclin B1-HA after anti-HA antibody staining. Transfection with the tandem constructs pcDNA3.1cdc2-HA/EGFP and pcDNA3.1cycB1-HA/EGFP allowed to determine the expression of Cdc2-HA and cyclin B1-HA, respectively, with regard to the transfection rate. Possible degradation activity of the two proteins was studied in unsynchronized HEK 293 cells before the method was applied in cultured ARC. The vast majority of EGFP-positive ARC displayed a sustained immunofluorescence signal for Cdc2-HA and in particular for cyclin B1-HA. It was then concluded that the protein degradation machinery seemed at least not to be active enough to exceed transgene product expression.

Active degradation of mitotic cyclin takes place in proliferating cells from late M to the end of G1 phase (Brandeis and Hunt, 1996). We could show degradation of cyclin B1-HA in a subpopulation of HEK 293 cells. Addition of lactacystin, a specific proteasome inhibitor, caused a significant inhibition of cyclin B1-HA degradation in those cells, while ARC treated with lactacystin showed no effect regarding the detection levels of cyclin B1-HA. One may speculate that ARC are not arrested in late M to G1 phase, at least from the view of mitotic cyclin degradation (Figure 3-3).

![Figure 3-3](image-url)

**Figure 3-3:** Mitotic cyclin degradation during the mammalian cell cycle. Proteolysis of cyclins is initiated at the metaphase to anaphase (M→A) transition and switched off at the end of G1 phase. If the cell cycle is arrested prior to the G1/S transition, as occurs in differentiating cells, cyclin proteolysis remains active according to Brandeis and Hunt (Brandeis and Hunt, 1996). Under this criteria, the absence of cyclin proteolysis would suggest a cell cycle status of ARC between S and early M phase.

Proteolytic destruction of mitotic cyclins is mediated by APC, which itself becomes activated by MPF (Morgan, 1999; Hershko, 1999; see section 1.1.3 on page 9). Nothing is known so far regarding the role of APC in cardiomyocytes. The absence of cyclin B1-
HA degradation in cultured ARC may thus be an indirect indication for inactive APC. Reinitiation of APC by induced MPF activity in ARC transfected with pcDNA3.1cycB1-HA/cdc2-FLAG or pcDNA3.1cycB1-HA/cdc2AF-FLAG was not examined. However, a strong induced mitotic cyclin degradation by MPF in transfected ARC could be ruled out, since cyclin B1-HA was clearly detected by immunofluorescence analysis. It cannot be excluded that MPF activated APC, but it would be necessary to investigate first the endogenous expression of the proteins, which form the APC complex, and to prove their existence in cultured ARC. Bearing in mind that APC is the key player for anaphase progression, as already suggested by its name, such an investigation in cardiomyocytes would be worthwhile.

We did not examine whether phosphorylation of the conserved threonine residue 161 on Cdc2 by endogenous proteins, which is involved in MPF activation, occurs (Desai et al., 1992). One report had mentioned that Cdk7, the kinase presumably responsible for this action (Fisher and Morgan, 1994), is expressed in ARC (Kim et al., 1998), whereas no data is currently available about the corresponding regulatory subunit cyclin H.

A more serious hurdle to be overcome on the way to recover MPF activity in ARC was the potential inhibition by the Wee1 kinase pathway. Experimental evidences propose the presence of Wee1 kinase in ARC (Kim et al., 1998; Dr. Christian Weikert, unpublished observations), so this inhibitor could have been the reason for the failure of exogenous cyclin B1-HA together with endogenous Cdc2 to induce MPF activity. Wee1 kinase had been reported to be unable to prevent MPF activity in mammalian cells when Cdc2AF, which mimics dephosphorylation on T14 and Y15 of wild-type Cdc2, was coexpressed (Krek and Nigg, 1991; Heald et al., 1993). It was thus suggested to use the mutant version in cultured ARC. The double expression vector pcDNA3.1cycB1-HA/cdc2AF-FLAG transfected in unsynchronized COS cells appeared to be a strong inducer of premature mitosis; such premature events in mammalian cells are well known from several reports (Heald et al., 1993; Hagting et al., 1998; Jin et al., 1998). Since the asynchronous COS cells transfected with pcDNA3.1cycB1-HA/cdc2AF-FLAG displayed predominantly condensed nuclei after 24 hours, it could be concluded that these cells were blocked in a mitotic state. This would be consistent with the notion that exit from mitosis is defective when inhibitory phosphorylation on Cdc2 is reduced (Jin et al., 1998). In contrast, by using pcDNA3.1cycB1-HA/cdc2-FLAG, which encodes wild-
type versions of cyclin B1 and Cdc2, COS cells did not exhibit uniformly premitotic figures. A report by Hagting and colleagues supports the differences, showing quantitatively induction of premature mitosis in synchronized HeLa cells (Hagting et al., 1998). They have described that simultaneous expression of cyclin B1 and Cdc2 led to premitotic figures in less than 5% of the cells, whereas almost 60% of the cells showed this nuclear phenotype when they coexpressed cyclin B1 and Cdc2AF or cyclin B1 and a constitutively active version of Cdc25C. It may be that simultaneous coexpression of cyclin B1 and the mutant Cdc25C could result in MPF activity in cultured ARC. This would provide functional evidences of endogenous Cdc2 in these cells, since the Cdc25C mutation would only work in the presence of its substrate, which is Cdc2.

The fact that Cdc2AF induced premature mitosis and a subsequent mitotic arrest in cell lines raised clearly some doubts, namely, if Cdc2AF was a useful promoter of cell division in ARC. However, the aim was not primarily to reinduce cell proliferation in cardiomyocytes, but to obtain knowledge of the cell cycle control patterns specific for this cell type by recovering MPF activity.

MPF activity was established first in cultured NRC employing the double expression vectors pcDNA3.1cycB1-HA/cdc2-FLAG and pcDNA3.1cycB1-HA/cdc2AF. Ectopic expression of cyclin B1-HA, Cdc2-FLAG, and Cdc2AF-FLAG separately did not induce nuclei condensation in NRC. No condensed nuclei occurred until transfectants expressed both MPF components; this was accomplished by transfection with either pcDNA3.1cycB1-HA/cdc2-FLAG or pcDNA3.1cycB1-HA/cdc2AF. The heterogeneity concerning the cell cycle stage of NRC caused some problems for the following reason. MPF was activated in some transfected NRC having one nucleus, presumably being in late G1/S, S or even G2 phase, whereas others had already acquired binucleation. Inducing MPF activity in NRC before genome replication has been like activation of MPF in cell lines at inappropriate cell cycle stages. The result is the appearance of chromosomes displaying an abnormal clumped morphology, a sign of partial condensation. Binucleated NRC showed after transfection a similar mitotic phenotype, indicating that forced MPF induction did not leave NRC untouched even at a later cell cycle stage.

It was unexpected that coexpression of wild-type versions of cyclin B1 and Cdc2 to recover MPF activity in cultured NRC was equally potent as expression of cyclin B1
together with Cdc2AF. Even under low serum conditions, transfection with pcDNA3.1cycB1-HA/cdc2-FLAG or pcDNA3.1cycB1-HA/cdc2AF-FLAG resulted in nuclei condensation in NRC. One may conclude that control mechanisms constrict the activity of MPF in NRC less than in proliferating cells. The same conclusion seemed also to be valid for cultured ARC since transfection with pcDNA3.1cycB1-HA/cdc2-FLAG did not lay behind pcDNA3.1cycB1-HA/cdc2AF-FLAG in obtaining a mitotic phenotype. It should be emphasized here again that coexpression of cyclin B1-HA and Cdc2-FLAG or Cdc2AF-FLAG, respectively, always resulted in condensed nuclei of ARC. Inhibition of MPF by the Wee1 kinase in cultured ARC was not likely, since otherwise wild-type Cdc2-FLAG would have been ineffective. A threshold effect could explain this observation whereby exogenous cyclin B1-HA/Cdc2-FLAG gradually titrated out the G2 phase inhibitor which may be in part responsible for the negative outcome when cyclin B1-HA was expressed alone. This would suggest, together with the absence of a highly active mitotic cyclin degradation machinery, the nonexistence of significant hurdles impeding MPF activity in postmitotic cardiomyocytes once the two components, cyclin B1 and Cdc2, are present. These results allow the statement that ARC at least provide a favorable environment on the post-transcriptional level to induce MPF activity.

That gives rise to focus on factors involved in the transcriptional regulation of endogenous cyclin B1 and cdc2 in cardiomyocytes. It had previously been demonstrated by Western blot that forced expression of E2F-1 in cultured ARC leads only to a small protein amount of Cdc2 (Agah et al., 1997), implying the presence of multiple repression factors. Another report had stated that transcription of the cdc2 gene in fetal ventricular cardiomyocytes is activated by the nuclear factor Y (NF-Y) (Liu et al., 1998). This transcription factor had been shown to be of importance in the p53-dependent repression of the cdc2 promoter region in mammalian cell lines (Yun et al., 1999). The tumor suppressor p53 has also been known to decrease the activity of the cyclin B1 promoter that would accentuate a central function of p53 also in G2 besides that one in the G1 phase (Innocente et al., 1999). If one combines these findings with the observation, that p53 protein is heavily upregulated in ARC (Magyar and Eppenberger, 1999), it is likely that p53 may play a significant role in the transcriptional downregulation of cyclin B1 and cdc2 in adult cardiomyocytes. An active p53-mediated repression of the promoter
region could be responsible for the observation, that the SV40 promoter is not as efficient in cultured ARC as expected (see page 93). This assumption is based on a report, in which p53 had been demonstrated to repress SV40 promoter-dependent transcription (Perrem et al., 1995).

A recent study has elucidated the function of p53 in large T antigen-transformed cardiomyocytes (Huh et al., 2001). The report provides plausible evidences that functional abrogation of p53 is needed in order to induce proliferation in these heart-derived tumor cells, otherwise large T antigen expression would result in cell death. It is tempting to assume that the inactivation of p53 in this case did not just prevent the cells from undergoing apoptosis, but that another direct positive impact on cell proliferation exists, when p53-mediated transcriptional repression of the cdc2 and cyclin B1 genes is eliminated. Such a function, however, of p53 within the G2/M phase of the cardiac cell cycle has yet to be verified by appropriate experiments.

3.2.5 ACTIVITIES IN POSTMITOTIC CARDIOMYOCYTES DOWNSTREAM OF MPF

Chromatin compaction was the most obvious effect of simultaneous expression of cyclin B1-HA and Cdc2-FLAG/Cdc2AF-FLAG. A proper segregation, however, of chromatids could not be observed. This has led to the conclusion that the cells did not undergo a complete M phase program concomitant with cytokinesis. Nuclear condensation was encountered in practically all the observed cases, indicating in fact a mitotic arrest but also suggesting presence of further checkpoints, which may join in the withdrawal from the cardiac cell cycle.

Concerning cell division, a previous report has suggested that decreased Cdc2 kinase function during the binucleation process be sufficient to form nuclear division but not to induce cytoplasmic division in cardiomyocytes after birth (Kang et al., 1997). With regard to that, our attempt to reset the cardiac cell cycle by forced expression of MPF in order to induce cell proliferation at the G2/M transition failed (see Figure 3-4 on page 103).

Binucleated ARC have already replicated their genome and are thus meant to be ready for action downstream the G1/S checkpoint; they would not need the G1/S site as the prime target. If this was the case, intervention at the G2/M checkpoint, as suggested by a
number of authors (Agah et al., 1997; Soonpaa et al., 1997; MacLellan and Schneider, 1998; Magyar and Eppenberger, 1999; Liao et al., 2001) and as was conducted here would be nevertheless questionable, because binucleated ARC have already undergone karyokinesis. The only event that remains unfinished for cell cycle completion is cytokinesis. However, cyclin B1 degradation in cultured ARC was found to be nonexistent by the assay used in this study. The available experimental data so far do not argue for an arrest being present in late M to late G1 phase, including cytokinesis, since proteolytic degradation of mitotic cyclin was shown to be in progress within these stages of the mammalian cell cycle as mentioned above (see Figure 3-3 on page 98).

![Figure 3-4](image-url)

**Figure 3-4:** Resetting the cardiac cell cycle towards cytokinesis. Neither reactivation of G1/S- nor G2/M key players led to a complete cycle in ventricular ARC (Agah et al., 1997; this work). Regarding the phenotype of unstimulated ARC, only cytokinesis is missing for cell cycle completion. Functional recovery of molecules directly involved in this stage might be more successful with the aim to accomplish proliferation (see main text for further details).

The abnormal clumped morphology of chromosomes observed after ARC transfected with either pcDNA3.1cycB1-HA/cdc2-FLAG or pcDNA3.1cycB1-HA/cdc2AF-FLAG points to an inappropriate induction of MPF. The reason is very probably prematurely induced chromosome condensation by MPF. The diploid nuclear DNA content of one of the two nuclei caused by endomitosis is not prepared for a regular M phase, because no DNA replication has previously taken place to generate two sister chromatids from each chromosome. The result may thus be mitotic catastrophe and cell death. This may lead to the somewhat naive interpretation that the two nuclei are acting independently, meaning that they do not ‘sense’ each other, because ARC as a cell had already completed S phase. The restored MPF activity in the cell was not able to establish a link between the DNA content of the two independent nuclei. It should be kept in mind that these
statements are made on the assumption of a diploid nuclear DNA content in cultured ARC, which has been reported to be true for about 93% of ventricular ARC (Kellerman et al., 1992).

The observed loss of nuclear integrity was accompanied with histone H3 phosphorylation; this revealed the potential of MPF to initiate cell cycle-related events in its vicinity, but which lie outside of MPF’s direct phosphorylation competence. Though not capable to induce cell division, reformation of MPF made it possible for ARC and NRC at least on the molecular level to re-enter the cell cycle. The fact of histone H3 phosphorylation had demonstrated the activation by MPF of the corresponding kinase. The kinase responsible for histone H3 phosphorylation has not yet been determined for mammalian cells, whereas it has been recently identified as the B-type aurora kinase in drosophila (Adams et al., 2001; Murnion et al., 2001). Whether MPF is a direct regulator of the B-type aurora kinase activity is not known at present. The finding that overexpression of a dominant negative version of the B-type aurora kinase in mammalian cells led to failure of cytokinesis and to formation of multinucleated cells (Terada et al., 1998) would thus resemble to the situation encountered in postmitotic cardiomyocytes. It would be worthwhile exploring the regulation and function of B-type aurora kinase in cardiomyocytes, in order to find out more on the role of this factor in late M phase and potential cytokinesis in these cells. For the latter, of interest may be the further functional recovery of Plk1 activity, which has been found to be absent in adult cardiomyocytes (Georgescu et al., 1997) and is not only involved in MPF activation but appears to play also a role in cytokinesis (Nigg, 2001).

In both cultured NRC and ARC, MPF-induced condensation of the nuclei was accompanied by a variable degree of myofibrillar disorganization and decay in cardiomyocytes. One could hypothesize that the occurrence of any disorganization in the contractile apparatus might be the consequence of the reinduced MPF activity. Reiss and colleagues have attributed a role to reactivated MPF in adult cardiomyocytes concerning the regeneration potential of these cells (Reiss et al., 1996). They have demonstrated in the remaining cardiomyocytes after myocardial infarction in the rat model system an increase of MPF activity up to a level comparable to the one in the neonatal stage. Our in vitro results suggest that MPF activity lead to a decrease in the contractile function of cardiomyocytes due to myofibrillar decay and not do take part in the putative
regenerative process of an injured heart as proposed by Reiss (Reiss et al., 1996). Moreover, it appears that MPF exerts a pathophysiological effect on the cardiomyocyte itself, and may well contribute in part to the reduced heart performance observed in pathological hypertrophy after infarction. But how important such an involvement of MPF may be in terms of clinical relevance is very difficult to judge.

One may argue that the function of cell cycle proteins stimulating cell cycle progression might be ‘poisonous’ for postmitotic cardiomyocytes, if one considers the obtained results on MPF recovery and on the ectopic expression of other cell cycle regulator proteins known to trigger cardiomyocyte death (Kirshenbaum and Schneider, 1995; Liu and Kitsis, 1996; Kirshenbaum et al., 1996; Agah et al., 1997; Bishopric et al., 1997; von Harsdorf et al., 1999). A recent report supports this proposition, suggesting a role of cyclin-dependent kinase Cdk2 as the mediator in hypoxia-induced apoptosis in cardiomyocytes (Adachi et al., 2001). Therefore, it is no surprise that expression of cell cycle inhibitors might have a potentially protective effect on the myocardium. First evidence for such a course had been demonstrated by overexpression of the cyclin-dependent kinase inhibitor p16, which suppressed left ventricular hypertrophy induced by aortic banding in rats (Nozato et al., 2001). Based on a supposedly beneficial effect of p16, the idea of the cardiac cell cycle as a new target for the treatment of cardiac hypertrophy has subsequently been promoted by Nagai and colleagues (Nagai et al., 2001). It is clear that this hypothesis has to be confirmed in future studies, thus the cardiac cell cycle remains very tempting as an object for novel therapeutic approaches.

### 3.3 Outlook: Proliferative Adult Cardiomyocytes at Last?

One has to admit that the goal to reinduce cell proliferation in adult cardiomyocytes by overexpression of positive cell cycle regulators has not yet been reached, or only at best in part. Neither G1/S nor G2/M as targets as conducted in this study turned out to be really successful. Transformation of cardiomyocytes using SV40 large T antigen had in fact been reported, but a thorough characterization of the resulting cells regarding their cardiac phenotype is still missing (Sen et al., 1988; Field, 1988; Katz et al., 1992; Miller et al., 1994).
It appears that cardiobiology of higher vertebrates from an evolutionary point of view has preferred the concept of a perpetually functioning contractile unit (cardiomyocyte) with a extremely low regenerative potential (blocked cell cycle) instead of an active regenerative mechanism (working cell cycle). One reason for such a preference might be the serious problem to undergo cell division thereby maintaining the ability to contract at the same time. Cell death accompanied by the disorganization of the myofibrillar apparatus after induction by a cell cycle-positive stimulus, as it has been demonstrated here, could thus be explained as a consequence of an activated cell cycle that is in conflict with the existing cytoarchitecture. This would imply that intervention at any point in the adult cardiac cell cycle would be inappropriate as long as the cell exhibits its cardiac phenotype. In contrast, this study provided for neonatal cardiomyocytes some evidences that cell division may also occur in the presence of organized sarcomeric structures, one of the prime characteristics of this cell type. The known absence, however, of cytokinesis in cultured adult ventricular cardiomyocytes is a clear indication of the extraordinary stable cell cycle arrest in this developmental stage, but to claim that the puzzle of the cell cycle in adult cardiomyocytes will not be solved one day would surely be premature.

Elaboration of even more sophisticated research techniques will definitely boost the discovery of additional cell cycle proteins and their interplay in cardiomyocytes. It is of course highly desirable that this body of acquired knowledge will find one day its way into clinical application. Given the still rather small number of publications in this field, one may postulate that unraveling the mystery of the cardiac cell cycle has just begun.
4 MATERIALS AND METHODS

4.1 CELL CULTURE TECHNIQUES

4.1.1 CARDIOMYOCYTE ISOLATION AND CULTIVATION

Ventricular cardiac myocytes were isolated from 6- to 8-week-old female OFA rats (BRL, Füllinsdorf, Switzerland) by retrograde perfusion of the hearts according to an established method (Eppenberger-Eberhardt et al., 1990). Culture dishes were coated with 0.1% gelatin. ARC were cultured right after the isolation in medium containing Medium M199 (Amimed AG, Basel, Switzerland), 10% FCS (PAA Laboratories GmbH, Linz, Austria), 20mmol/l creatine monohydrate (Sigma, St. Louis, MO, USA) and 1% penicillin/streptomycin (Gibco, Grand Island, NY, USA). Proliferation of fibroblasts and other proliferation-competent cells was inhibited by adding 10μM cytosine arabinoside (araC; ICN Biochemicals, Cleveland, OH, USA) to the medium. After five days in culture, araC was omitted and the medium was supplemented with 200mmol/l L-glutamine (Amimed AG) to eventually provide culture conditions enabling cell proliferation. Regarding SIN infection experiments, araC was present during the whole period except when especially mentioned. The addition did not significantly alter the transduction efficiency of SIN vectors in cardiomyocytes. For short-term cultures, adult rat cardiomyocytes (ARC) were kept in medium containing 1% FCS (PAA) on laminin-coated dishes (Gibco).

Cardiomyocytes from newborn rats were prepared as described (Komiyama et al., 1996). After isolation, neonatal rat cardiomyocytes (NRC) were cultured in plating medium (67% DMEM (Amimed AG), 17% Medium M199 (Amimed AG), 10% horse serum (Gibco), 200mmol/l L-glutamine (Amimed AG) and 1% penicillin/streptomycin (Gibco)). After one day, the medium was changed to the araC-free medium used for ARC cultivation (see above) including L-glutamine supplementation (200mmol/l). Under low serum condition, NRC were cultured in maintenance medium (78% DMEM (Amimed AG), 20% Medium M199 (Amimed AG), 200mmol/l L-glutamine (Amimed AG), 1% penicillin/streptomycin (Gibco), 1% horse serum (Gibco) and 10^{-4}mol/l phenylephrine (Sigma)).
Embryonic chicken cardiomyocytes were isolated from 11-day-old chicken embryos and cultured as described (Agarkova et al., 2000).

### 4.1.2 CELL LINES

COS, HEK 293 and HeLa cells were grown in DMEM (Amimed AG) supplemented with 10% FCS (Biological Industries, Kibbuz Beth Hamaek, Israel), 200mmol/l L-glutamine (Amimed AG), 1% penicillin/streptomycin (Gibco) in a humidified atmosphere at 37°C, with 6% CO₂. BHK-21 cells were cultured the same way but using 5% FCS.

### 4.2 RECOMBINANT DNA CONSTRUCTS

#### 4.2.1 CONSTRUCTION OF pSIN PLASMIDS USED FOR RNA TRANSCRIPTION

The 4.9 kb XbaI/ClaI fragment of pSINrep5 (Bredenbeek et al., 1993) (Invitrogen, Groningen, The Netherlands) encoding a portion of virus wild-type sequence was ligated into the corresponding sites of pSINrep19/GFP (kindly provided by Dr. Charles M. Rice, Washington University of Medicine, St. Louis, Missouri, USA; Agapov et al., 1998) replacing the point mutation at the nsP2 position 726 (nsp2-726L) in the viral coding region leading to pSINrep19/GFPwt.

The pSINrep5/MLC3f-EGFP was cloned by releasing the 1.2 kb fragment encoding the MLC3f-EGFP fusion protein by EcoRI/NotI digestion from pMLC3f-EGFP. Sticky ends were filled-in with dNTP using Klenow polymerase, and the fragment was ligated into the PmlI site of pSINrep5.

The pSINrep5/α-actinin-DsRed was cloned as follows: The 3.4 kb EcoRI/NotI fragment of pα-actinin-DsRed-N3 (kindly provided by Prof. Dr. J.-C. Perriard, ETH Zurich, Switzerland) encoding the DsRed-tagged human α-actinin 2A isoform, was blunt-end cloned into the PmlI site of pSINrep5 giving the final pSINrep5/α-actinin-DsRed vector.

#### 4.2.2 MISCELLANEOUS PLASMIDS USED FOR TRANSFECTION OF ARC

The dicistronic expression vector pcycA-IRES-EGFP was constructed by subcloning at first the 1.5 kb EcoRI/SalI fragment of pBabe-Puro-cyclinA (kindly provided by Dr. Bruno Amati, ISREC, Epalinges, Switzerland) consisting of the human cyclin A gene
into the corresponding sites of pCITE-4a(+) (Novagen, Madison, WI, USA) to give pCITE-cycA. From this vector, the EcoRV/NotI fragment was ligated into the corresponding sites of pIRES-EGFP (Clontech Laboratories, Palo Alto, CA, USA) resulting into pcycA-IRES-EGFP.

The dicistronic expression vector pTri-cycA-EGFP was generated by cloning the EcoRI/NotI fragment coding for cyclin A from pCITE-cycA into the corresponding sites of the tricistronic expression vector pTrident2 (Fussenegger et al., 1998) thereby deleting one IRES element resulting into pTri-cycA. The EcoRI/NotI fragment encoding EGFP from pEGFP-N1 (Clontech Laboratories) was previously cloned into pCITE-4a(+) to give pCITE-EGFP. The BamHI/BglII fragment of pCITE-EGFP was finally cloned into the BglII site of pTri-cycA leading to pTri-cycA-EGFP.

The expression vector pMHcycA-HA was cloned by amplifying human cyclin A from pBabe-Puro-cyclinA using the forward primer 5'-gccaccATGTTGGCAACTCTGCGCCG-3' and the reverse primer 5'-TTTAGTGTCTCTGGTGGGTTGA-3' (gene coding sequences are indicated by capital letters). The cyclin A fragment was subcloned into the pcDNA3.1/V5/His-TOPO vector (Invitrogen, Groningen, The Netherlands) according to the manufacturer’s protocol resulting into pcDNA3.1cycAII. The HindIII/EcoRV fragment of pcDNA3.1cycAII coding for cyclin A was then inserted into the HindIII/PmlI site of the HA-tagging vector pMH (Roche Diagnostics GmbH, Mannheim, Germany) to give pMHcycA-HA. Sequence analysis revealed the presence of a mutation in the coding region of cyclin A leading to exchange of the nonpolar aminoacid isoleucine by the nonpolar aminoacid valine at the non-conserved position 120 (I120V).

The vector pEGFP-N3 (Clontech Laboratories) was used to optimize transfection efficiency of cultured cardiomyocytes by the receptor-mediated pathway. The expression vector pHM6-lacZ encoding the HA-tagged lacZ gene was purchased from Roche Diagnostics. The plasmid pMLC3f-EGFP (Auerbach et al., 1997) was kindly provided by Prof. Dr. J.-C. Perriard.

4.2.3 CONSTRUCTION OF PLASMIDS FOR CELL CYCLE STUDIES

The expression vector pcDNA3.1cycB1-HA was generated in the following way: human cyclin B1 was cloned by the reverse transcription polymerase chain reaction (RT-PCR) using the QIAGEN OneStep RT-PCR Kit (QIAGEN GmbH, Hilden, Germany) from
RNA extracts of synchronized HeLa cells being in G2/M phase. Synchronization was performed by the double thymidine block method (Stein et al., 1998). Exponentially growing HeLa cells were blocked by supplementing 2mmol/l thymidine to the culture medium for 16 hours. Afterwards, the cells were released from the first block for 9 hours by removing the thymidine-containing medium with normal growth medium containing 24µmol/l deoxycytidine. After the release-period, the second thymidine block was imposed by adding thymidine to a final concentration of 2mmol/l in the culture medium. After 15 hours, the second block was released by feeding the cells with normal growth medium containing 24µmol/l deoxycytidine. RNA isolation of synchronized HeLa cells was performed using the Trizol Reagent (Gibco) according to the manufacturer’s protocol. Cyclin B1 was amplified and directly HA-tagged at the C-terminus end using following primers, which were based on published sequences: forward 5’-gccaccATGGCGCTCCGAGTCACCAGGAAC-3’ and reverse 5’-tttaacgcgtagtctgggacgtcgtatgggtaCACCTTTTGCACACAGCCTTTGGCTAAATC-3’. The amplified cyclin B1 fragment was subsequently cloned into the pcDNA3.1/V5/His-TOPO vector resulting into pcDNA3.1cycB1-HA, which was confirmed later by sequencing.

To construct pSBC2cdc2-FLAG, human cdc2 was amplified and FLAG-tagged at the C-terminus by PCR from pCMVcdc2-HA (kindly provided by Dr. Bruno Amati, ISREC, Epalinges, Switzerland) using the forward primer 5’-gccaccATGGAAGATTATACCAAAATAG-3’ and the reverse primer 5’-ctctttctgctctcctcgactatactCTTCTTTAATCTGTGGTCC-3’. The PCR fragment was then subcloned into pcDNA3.1/V5/His-TOPO leading to pcDNA3.1cdc2-FLAG. The XhoI/BamHI fragment of pcDNA3.1cdc2-FLAG encoding cdc2-FLAG was subsequently cloned under the control of the SV40 promoter by ligation into the BamHI/SalI site of pSBC-2 (Dirks et al., 1993) resulting into pSBC2cdc2-FLAG.

In addition, cdc2 was amplified and HA-tagged from pCMVcdc2-HA (forward primer: 5’-gccaccATGGAAGATTATACCAAAATAG-3’; reverse primer: 5’-ctctttctgctctcctcgactatactCTTCTTTAATCTGTGGTCC-3’) and cloned into pcDNA3.1/V5/His-TOPO to give pcDNA3.1cdc2-HA.

The vector pSBC2cdc2AF-FLAG was in the same way cloned as pSBC2cdc2-FLAG. The phosphorylation site mutations (T14A, Y15F) were introduced by PCR amplification from pCMVcdc2-HA using the forward primer 5’-gccaccATGGAAGATTATACCAAAATAG-3’
and the mentioned reverse primer used for FLAG-tagging. The authenticity of all PCR fragments was confirmed by sequencing. The introduced site mutations were also verified by diagnostic KpnI digestion since this restriction site has been deleted by the insertion.

The double expression vector pcDNA3.1cdc2-HA/EGFP was cloned by ligation of the smaller SmaI/AflII fragment of pEGFP-N1 (Clontech Laboratories) into the BsaBI/BstZ17I site of pcDNA3.1/V5/His-TOPO to give pcDNA3.1-SV40-EGFP. Cloning of the PvuI/EcoRV fragment of pcDNA3.1cdc2-HA into the corresponding sites of pcDNA3.1-SV40-EGFP resulted into pcDNA3.1cdc2-HA/EGFP (cloning of this vector was performed by Dr. Christian Weikert, ETH Zurich, Switzerland).

The expression vector pcDNA3.1cycB1-HA/EGFP was cloned by ligation of the HindIII/EcoRV fragment of pcDNA3.1cycB1-HA encoding cyclin B1 with the large HindIII/EcoRV fragment of pcDNA3.1cdc2-HA/EGFP.

To generate the double expression vector pcDNA3.1cycB1-HA/cdc2-FLAG, the small AvrII/BstZ17I fragment of pSBC2cdc2-FLAG was ligated into the large AvrII/BstZ17I fragment of pcDNA3.1cycB1-HA.

Analogously, pcDNA3.1cycB1-HA/cdc2AF-FLAG was cloned by inserting the cdc2AF-FLAG gene derived from pSBC2cdc2AF-FLAG into the large AvrII/BstZ17I fragment of pcDNA3.1cycB1-HA.

### 4.3 SIN GENERATION AND APPLICATION

#### 4.3.1 GENERATION OF RECOMBINANT SIN PARTICLES

The pSIN constructs were linearized by NotI digestion and served as a template for *in vitro* transcription. The genes coding for the structural proteins and therefore necessary for viral packaging are provided on the vector pDHBB (Bredenbeek *et al.*, 1993) (Invitrogen). *In vitro* transcription reaction was performed on linearized pDHBB and the pSIN constructs using the InvitroScript CAP Kit (Invitrogen) according to the manufacturer’s instructions. Linearized pSINrep5/lacZ was already provided with the RNA transcription kit. Coelectroporation of the two RNA pieces into BHK cells was performed as described (Liljeström *et al.*, 1991). Briefly, BHK cells were washed twice
with phosphate buffered saline (PBS: 150mmol/l NaCl, 6.5mmol/l Na₂HPO₄ x 2H₂O, 2.7mmol/l KCl, 1.5mmol/l KH₂PO₄, pH 7.3), trypsinized and collected by centrifugation at 400g for 5 minutes. The supernatant was removed and the pellet was washed and resuspended in ice-cold RNase-free PBS. Three further centrifugation- and wash steps followed. The cells were then resuspended at a concentration of 2 x 10⁷ cells/ml in ice-cold RNase-free PBS. The RNA samples (5µg of each construct) for electroporation were mixed with 500µl cell suspension in a 1.5ml electroporation cuvette on ice. The cells were immediately shocked twice using the following settings: 1.5kV, 25µFD with the pulse controller resistance set on infinity. After the electric shocks, the cells were allowed to sit at room temperature for 10 minutes. After this recovery period, the cells were diluted in culture medium and plated. The culture medium containing the infectious particles was harvested 24 - 30 hours after the electroporation procedure and stored in aliquots at -80°C. The titers were determined by infecting BHK cells with diluted virus stock and counting the cells that displayed fluorescence 24 hours after infection. Regarding recombinant particles of SINrep5/lacZ, titer was determined by 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) staining. The staining solution (1mg/ml X-Gal (Sigma), 15mmol/l potassium ferricyanide, 15mmol/l potassium ferrocyanide, and 1mmol/l MgCl₂ in PBS) was applied for 2 - 4 hours at 37°C resulting in blue staining of infected cells. The indicated virus concentrations refer to the concentrations determined on BHK cells and are expressed in infectious units per milliliter (IU/ml). Multiplicity of infection (MOI) defines how many infectious units are applied per cell in a dish. The following titers were maximally obtained: 5 x 10⁷ IU/ml for SINrep5/lacZ IU/ml, 10⁸ IU/ml for SINrep19/GFPwt, 3 x 10⁶ IU/ml for SINrep5/MLC3f-EGFP and 0.5 x 10⁶ IU/ml for SINrep5/α-actinin-DsRed. In accordance with a report of Frolov and colleagues (Frolov et al., 1999), no infectious particles could be generated from the pSINrep19/GFP transcripts containing nsP2-726L, the mutation that diminishes the cytotoxicity of the SIN vectors in BHK cells.
4.3.2 **INFECTION OF CARDIOMYOCYTES WITH RECOMBINANT SIN AND DETERMINATION OF INFECTION EFFICIENCY**

Though SIN vectors used in this work were based on an avirulent laboratory strain of Sindbis virus, appropriate containment procedures (Biosafety Level 2) were established and high caution was used while working with the infectious particles.

ARC maintained 7 - 10 days in culture were infected by overlaying them with viral stock solution diluted or undiluted in M199 medium containing 1% FCS and 5mmol/l creatine monohydrate (40µl virus solution/1cm² culture dish). After an incubation time of 1 - 4 hours at 37°C, fresh culture medium was added and the cells were kept in culture for the indicated time periods. Infection of rod-shaped freshly isolated adult cardiomyocytes was performed in the same way 1 - 16 hours after the isolation procedure. Infection of NRC and embryonic chicken cardiomyocytes was conducted after one day in culture.

To determine the infection efficiency quantitatively, ARC kept 7 days in culture were infected by SINrep5/lacZ as described above. After an incubation time of one hour at 37°C, complete culture medium was added. After 24 hours, cells were fixed in 3% paraformaldehyde (PFA) and β-galactosidase expression was assayed by X-Gal staining. Records of random regions within the treated cultures were taken by immunofluorescence and conventional bright field microscopy. Infection experiments were performed in triplicate. At least 200 cells were counted for each culture dish. Numbers of infected and noninfected cells were quantified by averaging over the surface area of the culture dishes. The results are expressed as mean ± standard error of the mean (SEM).

4.4 **NONVIRAL GENE TRANSFER**

4.4.1 **TRANSFECTION OF CELL LINES**

COS cells were transiently transfected using the SuperFect Transfection Reagent (Qiagen) according to the manufacturer's instructions. For the transient transfection of HEK 293 cells, the calcium phosphate precipitation method was performed according to a modified protocol (Westfall *et al.*, 1997a). Briefly, the reaction was started by adding 3µg of the plasmid DNA to 75µl 2 x HBS (274mmol/l NaCl, 1.9mmol/l Na₂PO₄ x 2H₂O, 38mmol/l HEPES, pH 7.05) and then adding H₂O to a volume of 142.5µl. After
mixing, 7.5µl of 2.5mol/l CaCl₂ was added and the precipitate was allowed to form at room temperature for 30 - 40 seconds. For transfection, the precipitate was added dropwise to HEK 293 cells that had been grown to semiconfluence. The medium was exchanged with fresh medium after 4 - 6 hours and the cells were analyzed at the indicated time point.

4.4.2 Transfection of cultured NRC and ARC using the receptor-mediated pathway

Transferrin-polyethylenimine (Tf-PEI) conjugates, transferrin-poly(L)lysine conjugates (Tf-pL) with an average chain length of 250 lysine residues, streptavidin-polylysine conjugates (STAV-pL) and biotinylated, psoralen-inactivated adenovirus (AdV) dl1014 were generously provided by Dr. Ernst Wagner, Boehringer Ingelheim R&D, 1121 Vienna, Austria. The PEI-transferrin system was used to transfect cultured NRC. For this purpose, NRC were transfected 24 hours after seeding with a density of 2 - 3 x 10⁵ cells per 35mm dish. To optimize PEI-transfection, pEGFP-N3 (3µg DNA) in 150µl HBS (150mmol/l NaCl, 10mmol/l HEPES, pH 7.3) was added to different amounts of Tf-PEI in 150µl HBS. Best results were obtained using nitrogen (PEI) to phosphate (DNA) ratios of 6 - 8. These ratios correspond to 2.25 - 3µg Tf-PEI for 3µg plasmid DNA. After an incubation of 30 minutes at room temperature, the transfection mix was added dropwise onto NRC. The culture dishes were then immediately centrifuged for 5 minutes at 100g and cultured as described. Transfection complexes were removed 14 – 18 hours later by replacing the medium. Cells were analyzed for gene expression 48 hours after transfection using direct and indirect immunofluorescence microscopy.

Gene transfection of cultured ARC was sporadically achieved by the Tf-PEI using the same conditions mentioned above for NRC. The gene delivery system mainly used for ARC was the AVET system. The cells were generally transfected 6 - 8 days after the isolation procedure. For optimal transfection efficiency of ARC, 3µg STAV-pL in 100µl HBS were mixed with approximately 1.2 x 10¹⁰ inactivated AdVdl1014 in 100µl HB and incubated for 30 minutes at room temperature. After adding 3µg of plasmid DNA in 150µl HBS, a further 30 minutes incubation followed at room temperature. The mixture was combined with 3µg Tf-pL in 150µl HBS and incubated for 30 minutes at room temperature before the transfection mix was added dropwise onto ARC cultures.
Afterwards, the dishes were centrifuged at 100g for 5 minutes, supporting a rapid concentration of the transfection complexes on the cell surface. Cultivation medium was replaced 4 - 16 hours after the transfection and analysis was performed at the indicated time points.

Quantitative determination of the gene transfer efficiency using the AVET system was conducted by transfecting pEGFP-N3. At least 500 cells were counted in each experiment. Experiments were performed in triplicate. The results are expressed as mean ± SEM.

4.5 TECHNIQUES FOR CELL ANALYSIS

4.5.1 ANTIBODIES

The monoclonal mouse (mM) anti-myomesin antibody (clone B4; generated in this laboratory, see Grove et al., 1984) and the polyclonal rabbit (pR) anti-titin M8 antibody (Obermann et al., 1996), generously provided by Dr. M. Gautel, MPI Dortmund, were used as sarcomeric markers. The monoclonal rat anti-HA antibody (clone 3F10) was purchased from Roche Diagnostics. For the detection of the FLAG-epitope, the mM anti-FLAG M2 antibody was used from Sigma. The pR anti-phospho-histone H3 antibody was purchased from Upstate Biotechnology (Lakeplacid, NY, USA). Antibody staining against endogenous cyclin B1 was performed by using the mM anti-cyclin B1 antibody (clone GNS1; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). The mM anti-cdk1/cdc2 antibody (clone 1; C12720) was obtained from Transduction Laboratories (Lexington, KY, USA). The mM anti-α-tubulin antibody (Clone B-5-1-2) was purchased from Sigma. F-actin was stained by rhodamine-conjugated phalloidin and nuclei were stained with DAPI (Molecular Probes, Eugene, OR, USA). The mM anti-β-gal- and the mM anti-GFP antibodies were obtained from Roche Diagnostics. The mM anti-BrdU antibody was a component of the BrdU Labeling and Detection Kit I from Roche Diagnostics.

For immunofluorescence stainings, combinations of fluorescein isothiocyanate (FITC), cyanine-3 (Cy3) or Texas Red (TxRed), and cyanine-5 (Cy5)-conjugated secondary antibodies together with DAPI were used. FITC-conjugated goat anti-mouse IgG (Jackson Immuno Research, Hamburg, Germany) was used for the detection of the anti-
myomesin-, anti-FLAG-, anti-cdk1/cdc2-, anti-cyclin B1- and anti-β-gal antibody. FITC-conjugated goat anti-rabbit IgG (Cappel Research, West Chester, PA) was used to visualize the anti-phospho-histone H3 and anti-titin M8 antibody. The TxRed-conjugated goat anti-mouse IgG and the TxRed-conjugated goat anti-rabbit IgG (both Jackson Immuno Research) were used to detect the anti-myomesin antibody and the anti-titin M8 antibody, respectively. The Cy3-conjugated donkey anti-rat IgG (Jackson Immuno Research) was used throughout the work to detect the anti-HA antibody. Cy5-coupled donkey anti-mouse IgG (Jackson Immuno Research) was applied to visualize the anti-myomesin-, anti-FLAG-, anti-cdk1/cdc2-, and the anti-α-tubulin antibody.

For immunoblotting, horseradish peroxidase (HRPO)-conjugated goat anti-mouse IgG and HRPO-conjugated rabbit anti-rat IgG (both Dako Diagnostics AG, Zug, Switzerland) were used as secondary antibodies.

**4.5.2 IMMUNOFLUORESCENCE ANALYSIS AND MICROSCOPY**

Cells were fixed in 3% PFA in PBS for 15 minutes, washed with PBS, permeabilized in 0.2% Triton X-100/PBS for 12 minutes and preincubated with 0.1% bovine serum albumin (BSA) in PBS for 20 minutes. The cells were incubated with primary antibodies for 2 – 3 hours at RT. After washing with PBS, secondary antibodies were added for 1 hour. The specimens were washed in PBS and mounted in 0.1mol/l Tris buffer pH 9.5, glycerol (3:7) containing 50mg/ml n-propyl gallate as anti-fading reagent (Messerli et al., 1993). For staining with anti α-tubulin antibodies, the cells were rinsed briefly with a microtubule protective buffer as described by Schliwa and colleagues (MP-buffer: 65 mmol/l PIPES, 25mmol/l HEPES, 10mmol/l EDTA, 3mmol/l EGTA, 3mmol/l MgCl₂ pH 6.9; Schliwa et al., 1981) and then fixed for 10 minutes with 3% paraformaldehyde in MP-buffer. After fixing, the antibody stainings were conducted as stated above.

Fluorescently labeled cells were analyzed using a Zeiss Axiohot fluorescence microscope or a Zeiss Microscope type III RS both equipped with Neofluar objectives (Zeiss AG, Feldbach, Switzerland). Live pictures of EGFP-positive cells were taken on a Zeiss Axiovert microscope. Images were captured with a CCD camera and finally processed with Photoshop software (Adobe Systems, San Jose, CA, USA). Confocal analysis of SIN infected cardiomyocytes were carried out on a Leica inverted microscope DM IRB/E (Leica Microsystems AG, Glattbrugg, Switzerland), a Leica true confocal
scanner TCS NT and a Silicon Graphics workstation (SGI S.A., Schlieren, Switzerland). Image processing was done on a Silicon Graphics workstation using Imaris (Bitplane AG, Zurich, Switzerland), a 3D multi-channel image processing software specialized for confocal microscopy images (Messerli et al., 1993).

4.5.3 IMMUNOBLOTTING

Cells were washed twice with 1x PBS and scraped off into lysis buffer (0.1mol/l Tris buffer pH 6.8, 2% sodium dodecylsulfate (SDS), 20% glycerol and protease inhibitors). The samples were mixed and boiled for 5 minutes. They were centrifuged at 13000 rpm for 5 minutes at 4°C and the supernatant was stored at –80°C. Samples were diluted appropriately with lysis buffer, and β-mercaptoethanol was added to a final concentration of 5mmol/l. The SDS-PAGE electrophoresis was done according to Laemmli (Laemmli, 1970) with minigels (Bio-Rad Laboratories AG, Life Science Research, Reinach, Switzerland). Electrophoresis was carried out on a 12.5% SDS-polyacrylamide gel (12.5% acrylamide/bisacrylamide stock solution (30%/0.8%), 375mmol/l Tris buffer pH 8.8, 0.1% SDS, 0.5mg/ml APS and 0.05% TEMED) at 120V. Blotting was performed at 80V in a wet chamber blotting apparatus (Mini-electrophoretic Blotting System; C.B.S Scientific Company Inc., Axon Lab AG) onto a nitrocellulose membrane (Hybond™-C extra, Amersham Pharmacia Biotech). Protein transfer and equal amounts of protein loaded were checked by staining the blots with ponceau red (Ponceau S solution; Serva, Heidelberg, Germany). Unspecific binding sites were blocked with 5% non-fat dry milk (w/v) in low salt (LS) buffer (0.01mol/l Tris buffer pH 7.4, 9g NaCl, 5ml 0.1% Tween20) over night at 4°C. Incubation with first antibody diluted in PBS supplemented with 1% non-fat milk powder was done for 2 hours at room temperature. After washing the blots 3 x 5 minutes with LS buffer, the incubation with the second antibody was performed for one hour at room temperature. The last three washing steps were done in LS buffer, each for 5 minutes. Chemiluminescence detection was performed with SuperSignal (Pierce, Socochim S.A., Lausanne, Switzerland) according to the manufacturer’s protocol and visualized on Fuji Medical X-ray films.
4.6 SPECIFIC ASSAYS FOR CELL ANALYSIS

4.6.1 $^{35}$S-METHIONINE LABELING OF SIN INFECTED CELLS AND AUTORADIOGRAPHY

ARC maintained in 60 mm culture dishes were infected at a MOI of more than 300. The dishes were incubated and then labeled with $^{35}$S-methionine at specific time points. For this, the cells were washed twice with PBS and incubated in M199 medium lacking methionine (Amimed AG) for 30 minutes at 37°C. Each culture dish was then pulsed with 40µCi of $^{35}$S-methionine (Amersham Pharmacia Biotech, Uppsala, Sweden). After 30 minutes, complete M199 medium was added and the incubation continued for another 10 minutes. The medium was removed, and the cells were washed three times with PBS, then scraped and dissolved in lysis buffer. After boiling and centrifugation, incorporation of $^{35}$S-methionine was determined by a liquid scintillation analyzer measuring samples from each supernatant. Protein amounts of equal radioactivity were separated on a 12.5% SDS–polyacrylamide gel. After Coomassie staining, the gel was dried and autoradiographed. In addition, pulse-chase labeling of BHK cells infected by an MOI of 20 was performed and analyzed as described above.

4.6.2 ANALYSIS OF CYTOTOXIC EFFECTS USING TUNEL (TdT-MEDIATED dUTP NICK-END LABELING) ASSAY

DNA fragmentation due to induced apoptosis was detected using the Apoptosis Detection System, Fluorescein (Promega, Madison, WI, USA) according to the manufacturer's protocol. Briefly, cells were fixed and permeabilized as described. Nick-end labeling of DNA strand breaks were conducted by terminal deoxynucleotidyl transferase (TdT; Promega) with tetramethyl-rhodamine-5-dUTP (Roche Diagnostics). After 30 minutes at 37°C, enzyme reaction was stopped by adding 2 x SSC (300mmol/l NaCl, 30mmol/l sodium citrate, pH 7.0) for 15 minutes. As a positive control, cells were preincubated in DNase I buffer (10mmol/l Tris buffer pH 7.4, 10mmol/l NaCl, 5mmol/l MgCl₂, 0.1mmol/l CaCl₂, 25mmol/l KCl) for 15 minutes and incubated with 0.1 U/ml DNase I (Roche Diagnostics) for 10 minutes at room temperature prior to nick-end labeling. After nick-end labeling, the cells were processed as described (see section 4.5.2 on page 116).
4.6.3 PROTEASOME-DEPENDENT DEGRADATION ASSAY

One day after transient transfection using the calcium phosphate method, HEK 293 cells were treated for 24 hours with 10µmol/l lactacystin (Alexis Biochemicals, Laefelingen, Switzerland) before fixing and antibody staining against the HA-tag to detect the transgene product. Analogously, 7-day-old ARC were first transfected using the AVET system. After one day, 10µmol/l lactacystin was added and cells were cultured for additional 72 hours before analysis took place. Controls were treated the same way but without the addition of lactacystin. Transfectants were identified by their EGFP-fluorescence, and the ratios of HA- and EGFP-positive to all EGFP-positive cells were determined. All transfections were performed in triplicates. To determine the ratios, at least 750 cells in case of HEK 293 cells, and at least 50 cells in case of cultured ARC were counted to document the expression of exogenous cell cycle proteins applying the different approaches. The results are expressed as mean ± SEM. The two-tailed Student’s test was used for evaluation of significance. Values of \( p < 0.02 \) were considered to be significant.

4.6.4 DETECTION OF DNA SYNTHESIS

Ongoing DNA synthesis in cardiomyocyte culture systems was monitored as incorporation of 5’-bromo-2’-deoxyuridine (BrdU) using the BrdU Labeling and Detection Kit I (Roche Diagnostics). Cardiomyocyte cultures were incubated with 10µmol/l BrdU for 48 hours (starting at culture day 7 for ARC, and day 1 for NRC) before they were fixed with ethanol as fixative (70% EtOH, 15mmol/l glycine pH 2.0) and processed according to the manufacturer’s instructions. To distinguish cardiomyocytes from non-cardiomyocytes, anti-titin antibody staining using the anti-titin M8 antibody was performed. Labeling experiments were performed in triplicates. For each counting, at least 2000 nuclei of cardiomyocytes were counted per dish from randomly chosen locations. The results are expressed as mean ± SEM.

Synchronization of HeLa cells were monitored by BrdU labeling. Incubating cell cultures with 10µmol/l BrdU for 20 minutes was performed at the indicated time points before incorporation was determined with the BrdU Labeling and Detection Kit I.
5 REFERENCES


**PUBLICATIONS & PRESENTATIONS**

Parts of this thesis have already been published or are in preparation for publication. The author had also the opportunity to personally present this work at national and international meetings.

**Papers**


**Published abstracts**


**Talks**

16th European Society for Animal Cell Technology (ESACT)-Meeting (1999), Lugano, Switzerland
6th Meeting on Cardiovascular Biology and Clinical Implications (1999), Interlaken, Switzerland

Postgraduate Course: Molecular and cellular cardiology (2000), ETH-Hönggerberg, Zurich, Switzerland

Jahrestagung der Schweizerischen Gesellschaft für Kardiologie (2001), Zurich, Switzerland

**Poster presentations**

16th European Society for Animal Cell Technology (ESACT)-Meeting (1999), Lugano, Switzerland

3rd International Workshop on Cardiomyocytes in Culture (2000), Ascona, Switzerland

BIOL ETH Symposium (2000), Davos, Switzerland
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