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TGFβ signaling regulating neural stem cell development

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1. ZUSAMMENFASSUNG

Die Charakterisierung von zellulären und molekularen Mechanismen, welche die Entwicklung von Stamm- und Vorläuferzellen regulieren, ist ein zentrales Anliegen meiner Dissertation. Die Entstehung zellulärer und morphologischer Vielfalt während der Entwicklung wird durch diverse intrazelluläre und extrazelluläre Faktoren beeinflusst. Die Analyse individueller Signalwege, sowie deren komplexes Zusammenspiel in zeitlich- und räumlich konzertierten Prozessen, ist ein aktueller Schwerpunkt in der Entwicklungs- und Stammzellbiologie. Aufgrund ihres breiten Entwicklungspotentials und ihrer Manipulierbarkeit bilden Stammzellen der Neuralleiste ein geeignetes Modellsystem, um experimentelle Daten und damit neue Erkenntnisse in der Stammzellentwicklung zu gewinnen. Neuralleistenstammzellen lösen sich nach ihrer Entstehung vom Neuralrohr und wandern in verschiedene embryonale Strukturen ein. Dort beteiligen sie sich maßgeblich an der Bildung verschiedener neuraler und nicht-neuraler Zelltypen und Gewebe. Das Schicksal der Neuralleistenstammzelle kann *in vitro* durch verschiedene Signalwege beeinflusst werden, z.B. durch den TGFβ Signalweg. Basierend auf konditioneller Gen-Inaktivierung im Mausmodell ist es möglich, die *in vivo* Funktion des TGFβ Signalweges in der Entwicklung von Neuralleistenstammzellen und neuroepithelialer Stammzellen des Mittelhirns zu untersuchen.

Im ersten Teil meiner Dissertation beschreibe ich, wie die Neuralleisten-spezifische Ablation des TGFβ Signalweges zu schweren Entwicklungsdefekten führt, die an ein DiGeorge Syndrom-Krankheitsbild erinnern (Wurdak et al., 2005). Die Hauptursache dieser vererbbaren Krankheit ist eine interstitielle Mikrodeletion 22q11, die bei etwa 95 % der Betroffenen nachweisbar ist. Die aus der gestörten Entwicklung embryonaler Strukturen, vor allem des Kiemenbogensystems resultierenden Symptome sind unter anderem Spaltbildung (Lippen-Kiefer-Gaumen-Spalte) in Kombination mit Herzfehlern (conotruncale Defekte) und Hypo- oder Aplasie der Nebenschilddrüse und des Thymus mit T-Zelldefekt und Immunschwäche. Wir haben herausgefunden, dass mutierte Neuralleistenzellen, die eine gezielte Inaktivierung des TGFβ receptor type 2 (*Tgbr2*) Gens aufweisen, ebenso wie Wild-Typ-Neuralleistenzellen in das Kiemenbogensystem einwandern können. Dort angekommen zeigen sie allerdings Differenzierungsdefekte, welche die Bildung diverser nicht-neuraler Gewebe nachhaltig stören. Desweiteren konnten wir zeigen, dass Crkl, ein signal-leitendes Adapterprotein, in

Neuralleistenzellen nur phosphoryliert und somit aktiviert werden kann, wenn der TGFβ Signalweg aktiviert ist. Interessanterweise ist das Crkl-Gen ein DiGeorge-Syndrom-Kandidatengen und in der Region 22q11 lokalisiert. Insgesamt weisen unsere Resultate darauf hin, dass eine Verbindung zwischen der Modulation des TGFβ Signalweges und der Ätiologie der Erkrankung besteht.

Neben dem DiGeorge Syndrom-Phänotyp weisen die *Tgfbr2*-Mutanten Entwicklungsdefekte in den Augen auf (Ittner et al., 2005). Im zweiten Teil meiner Dissertation beschreibe ich die detaillierte Analyse der Augenanomalie, die wiederum nicht mit gestörter Migration von Neuralleistenzellen, dafür aber wieder auf Differenzierungsdefekte zurückzuführen ist. Wir konnten die Kontribution von Neuralleistenzellen zur Entwicklung mesenchymaler Strukturen im Auge verfolgen und zeigen, dass der TGFβ Signalweg das Schicksal von postmigratorischen Neuralleistenzellen beeinflusst. Insbesondere konnten wir demonstrieren, dass TGFβ-abhängige Differenzierungsprozesse mit Expression der Transkriptionsfaktoren Foxc1 und Pitx2 assoziiert sind. Beide Transkriptionsfaktoren sind in der Entstehung der menschlichen Augenkrankheit Axenfeld Rieger`s Syndrom involviert. Deshalb liefert unser Mausmodell eine weitere molekulare Basis für experimentelle und klinische Ursachenforschung einer menschlichen Krankheit.

Zusammengefasst weisen unsere Resultate darauf hin, dass der TGFβ Signalweg in Kombination mit dem zellulären Kontext, das Schicksal von Stamm- und Vorläuferzellen entscheidend beeinflussen kann (Wurdak et al., Übersichtsartikel, zur Publikation eingereicht). Diese Hypothese wird durch weitere Resultate, welche im 3. Teil dieser Dissertation beschrieben werden, gestärkt. *Tgbr2*-Mutanten zeigen eine drastische Expansion des Mittelhirns, welche auf eine so genannte Überproliferation und auf Kosten von Differenzierung neuroepithelialer Zellen, zurückzuführen ist (Wurdak et al., Manuskript zur Publikation eingereicht). Mutiertes Gewebe aus dem dorsalen Mittelhirn weist eine signifikant erhöhte Anzahl von neuralen Stammzellen in Kultur auf, die ein signifikant erhöhtes Selbsterneuerungspotential zeigen. Interessanterweise ist die erhöhte Selbsterneuerung der mutanten Neuroepithelzellen mit erhöhter Aktivität des kanonischen Wnt Signalweges assoziiert. Weiterhin kann TGFβ, der von Wnt induzierten Proliferation von Neuroepithelzellen aus dem Mittelhirn, entgegenwirken. Deshalb könnte der TGFβ Signalweg ein Schlüsselfaktor in der Stammzellentwicklung sein, der die Expansion von Stamzellen

negativ reguliert und somit die Balance zwischen Selbsterneuerung und Differenzierung entscheidend kontrolliert.

2. SUMMARY

My doctoral research has focused on mechanisms regulating the fate of progenitor and stem cells during vertebrate development. The generation of multiple cell types in the correct spatiotemporal manner involves complex interactions of extracellular and intracellular signals that are poorly understood. These issues can be addressed in neural stem cells of the brain, as well as in neural crest stem cells (NCSCs) that arise from the dorsal neural tube. NCSCs migrate in the growing embryo to various locations, giving rise to diverse neural and non-neural cell-types and tissues. One of the factors able to instruct NCSCs to adopt specific lineages *in vitro* is transforming growth factor β (TGFβ). To elucidate the role of TGFβ signaling in neural crest development *in vivo* and to address its impact on neural crest-related disease, I have used conditional inactivation of TGFβ signaling in a mouse model. In addition, I have studied molecular mechanisms regulating the broad developmental potential of NCSCs in cell culture. Finally, I have investigated the role of TGFβ signaling on neuroepithelial stem cells during midbrain/hindbrain development *in vivo* and *in vitro*.

In the first part of my thesis, I show that NCSC-specific ablation of TGFβ signaling results in all the developmental malformations characteristic of DiGeorge syndrome (Wurdak et al., 2005). DiGeorge syndrome is a human congenital microdeletion syndrome characterized by cardiovascular, craniofacial, and thymus and parathyroid gland anomalies. The disease is attributed to disturbed formation of the pharyngeal apparatus, a transient vertebrate-specific structure. We could show that mutant neural crest cells migrate normally into the pharyngeal apparatus but are unable to acquire non-neural cell fates. Moreover, we found that TGFβ signaling is required for phosphorylation of the signal adaptor protein Crkl, which is associated with development of human DiGeorge syndrome. Importantly, this finding indicates a link of TGFβ signal modulation to the etiology of DiGeorge syndrome.

In addition to the DiGeorge syndrome-like phenotype, we found that neural crest-specific TGFβ signal ablation resulted in severe developmental eye defects (Ittner et al., 2005). In the second part of my thesis I illustrate how malformations of anterior eye structures are again associated with impaired differentiation, but not with migration of mutant neural crest-derived progenitor cells. We have revealed distinct contributions of neural crest cells to anterior and posterior mesenchymal murine eye structures, and show that TGFβ signaling in these cells is

crucial for normal eye development. Moreover, we have demonstrated that TGFβ-dependent differentiation is mediated by the transcription factors Foxc1 and Pitx2. Both transcription factors are implicated in human Axenfeld-Rieger`s anomaly. Thus, our findings shed new light onto the origin of eye disorders that can give rise to glaucoma and blindness.

Taken together, our findings indicate an important role for TGFβ signaling in the context-dependent regulation of progenitor and stem cell fate (Wurdak et al., invited review, manuscript submitted). This hypothesis is strengthened by the fact that *Tgfbr2*-mutant embryos also display a drastic midbrain phenotype, as illustrated in part 3 of my thesis. This phenotype is due to the significant expansion of proliferating neuroepithelial cells, while differentiation is reduced (Wurdak et al., manuscript submitted). Strikingly, mutant tissue harbors an increased number of self-renewing, multipotent neural stem cells as assessed in neurosphere culture. The self-renewing proliferation of mutant neural stem cells is associated with enhanced activation of canonical Wnt signaling *in vitro* and *in vivo*. Moreover, TGFβ signaling can antagonize Wnt-induced neuroepithelial cell proliferation. In summary, TGFβ signaling may be a key factor negatively controlling stem cell pool expansion, and the balance of neural stem cell proliferation and differentiation during midbrain development.

3. INTRODUCTION

3.1. Stem cells

Stem cells are undifferentiated cells which have the unique potential to produce multiple cell types and tissue in the body. They are characterized by prolonged self-renewal capacity and multipotency (Figure 3.1). These features allow stem cells to fulfill multiple functions, for instance during embryogenesis and organogenesis. Moreover, adult or somatic stem cells are involved in tissue homeostasis and, in addition, in regeneration and repair, at least of certain tissues. Because of these characteristics stem cells offer the opportunity to treat many degenerative diseases, including Parkinson`s disease, Alzheimer`s disease, and stroke, by replacing or restoring damaged or missing tissue. Treatment strategies might range from transplantation of stem cells or their derivatives, to mobilization and manipulation of endogenous stem cell pools.

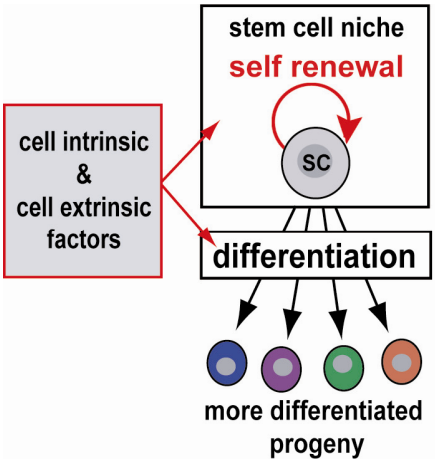

Figure 3.1. Characteristics of stem cells.
Stem cells are multipotent, giving rise to a diverse set of differentiated progeny. They exist in a specific environment, termed stem cell niche. The niche attracts stem cells and keeps them in an undifferentiated state by supporting self-renewing cell divisions. The balance of self-renewal and differentiation is critically influenced by numerous cell-intrinsic and cell-extrinsic factors.

The stem cells with the broadest range of differentiation potential are cells isolated from the inner cell mass of the blastocyst (Lerou and Daley, 2005). These pluripotent embryonic stem cells can be propagated almost indefinitely in culture, and are able to differentiate into any cell type of the three germ layers ectoderm, endoderm and mesoderm. Compared to embryonic stem cells, adult stem cells are only found in small number in their appropriate tissue environments in the adult body, termed stem cell niches (Fuchs et al., 2004). For example, hematopoietic stem cells exist in the bone marrow and can give rise to distinct blood

cell types (Kondo et al., 2003). Moreover, the stromal fraction of bone marrow-derived cells contains pluripotent mesenchymal stem cells that can be induced to differentiate into various mesenchymal cell lineages, as well as non-mesenchymal lineages including brain derivatives (Jiang et al., 2003; Jiang et al., 2002; Mezey et al., 2000). In addition, multipotent adult stem cells have been identified in various other niches including the subventricular zone of the brain, hair follicle structures, and intestinal crypts (Fuchs et al., 2004).

Interestingly, upon transplantation, adult stem cells have been observed to undergo multilineage differentiation even across germ layers (Jiang et al., 2003; Jiang et al., 2002; Krause et al., 2001; LaBarge and Blau, 2002; Mezey et al., 2000; Toma et al., 2001). However, the mechanisms underlying this unexpected 'plasticity' of somatic stem cells have been debated (Frisen, 2002; Wagers and Weissman, 2004). It has been hypothesized that tissue injury can increase the conversion of, for instance, a bone marrow-derived hematopoietic stem cell into a cell type of another lineage (Jiang et al., 2002; LaBarge and Blau, 2002). The notion of a so-called transdifferentiation event is confronted with other potential explanations for somatic stem cell 'plasticity', such as de-differentiation of a mature cell into a more naive state, fusion of stem cells with differentiated endogenous cells, or heterogeneity of a stem cell pool used for transplantation (Mikkers and Frisen, 2005; Wagers and Weissman, 2004). Despite this ambiguity, different types of stem cells might be collected, grown, and stored to provide a plentiful supply of replacement tissue for transplantation into any body site. However, from a clinical perspective, the development of stem cell-based therapies is still at an early stage. The determination of the most suitable tissue source of stem cells for possible therapeutic applications is one important issue to be addressed. Further basic research in stem cell biology will be necessary to understand how stem cells interact with each other and with their niche, and to control the balance between endogenous stem cell proliferation and the differentiation into specific cell types (Figure 3.1). Moreover, molecular and cellular mechanisms that facilitate the efficient induction of beneficial stem cell integration into existing tissues still remain to be elucidated.

3.2. The balance between stem cell self-renewal and differentiation

Mechanisms regulating stem cell number and maintenance have become fundamental issues to decode the complexity of stem cell biology. A stem cell may self-renew, differentiate or undergo programmed cell death (Figure 3.2). Self-renewal is the process allowing stem cells to replicate themselves without restricting their broad developmental potential. Stem cells may undergo symmetrical divisions to generate two identical daughter stem cells (symmetric self-renewal), or alternatively they may undergo asymmetric divisions, which generate one daughter cell that is identical to the mother cell and a second, more differentiated cell type (asymmetric self-renewal). Hence, the total number of stem cells depends on cell fate decisions. Symmetric self-renewal will increase the stem cell pool, while asymmetric self-renewal is associated with stem cell maintenance and differentiation (Figure 3.2). In contrast, differentiation of stem cells, as well as apoptosis will decrease or deplete the stem cell pool. Consequently, expansion or elimination of stem cells will have a marked effect on the final number of terminally differentiated cells in tissue and organs. Thus, the balance between stem cell maintenance and differentiation, and the mechanisms involved in keeping this balance, have to be tightly controlled.

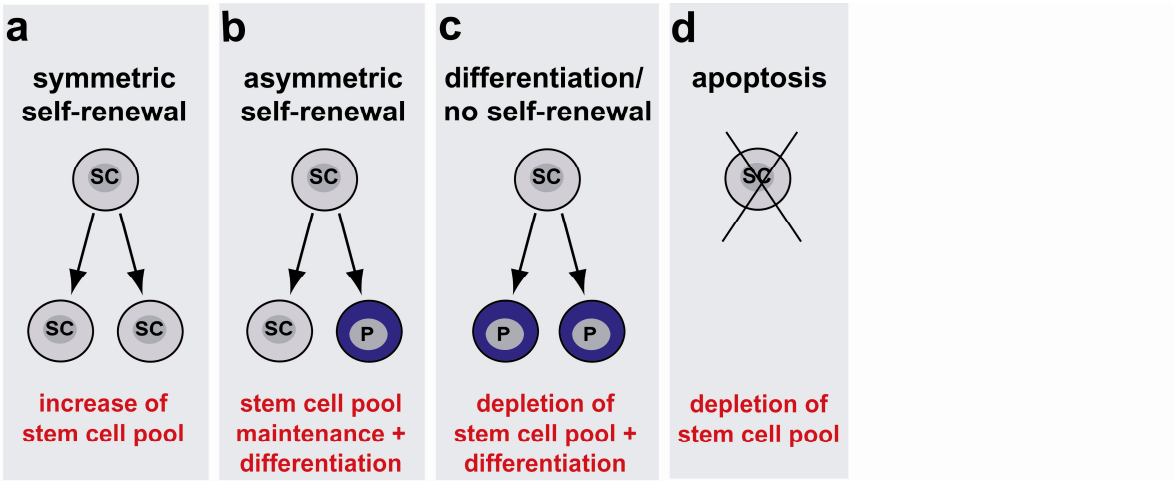

Figure 3.2. Mechanisms regulating stem cell number.
A stem cell can self-renew by dividing symmetrically to generate two stem cells (a), or asymmetrically to generate a stem cell and a more restricted progenitor cell (b), or a terminally differentiated cell type. The generation of differentiated progeny concomitant with lack of self-renewal (c), as well as programmed cell death (d) will finally cause depletion of the stem cell pool.

A key feature in the maintenance of a stem cell-like state is the interaction of the stem cell with the immediate environment, forming the stem cell niche. It is thought that the niche provides the appropriate signaling cues, allowing stem cells to self-renew and to respond to differentiation programs. The coordinate activities of multiple signaling pathways are likely required for the regulation of stem cell fate decisions. The Notch, Wnt, Hedgehog, bone morphogenetic protein (BMP), and transforming growth factor β (TGFβ) signaling pathways have been implicated in the control of stem cell maintenance and differentiation (Molofsky et al., 2004; Sommer and Rao, 2002). Moreover, components of the extracellular matrix, for instance several integrins, can modulate the effects of multiple growth factors on stem cells (Dutton and Bartlett, 2000). In order to find common genes controlling the stem cell state, several groups have performed a comparative characterization of the transcriptome of different stem cell populations, including embryonic stem cells, hematopoietic stem cells, neural stem cells, and neural crest stem cells (NCSCs) (Buchstaller et al., 2004; Ivanova et al., 2002; Ramalho-Santos et al., 2002). However, these studies show minimal overlap, and hence can not uncover a unique genetic fingerprint of stem cells *per se*. This may be explained by different experimental approaches, or by substantial intrinsic differences between distinct types of stem cells.

The common observation that not any cell introduced into a stem cell niche behaves as a stem cell in terms of self-renewal and multipotency shows the importance of the intrinsic cellular state. The intrinsic properties of a stem cell critically determine how it integrates and interprets signaling cues provided by its environment. Along the course of differentiation, the variety of cell fate options open to a differentiating cell become more and more restricted. Concomitantly, genes attributed to the chosen cell lineage should be activated, while transcription of genes associated with other lineages should be decreased. The transcriptional accessibility of different genes is regulated through the chromatin status. Therefore, it is conceivable that epigenetic events are crucially involved in the determination of stem cell fate. Several studies provide increasing evidence for the link between mechanisms of chromatin remodelling and neuronal plasticity (Hsieh and Gage, 2005). Intriguingly, chromatin remodelling and histone modification have also been implicated in the conversion of oligodendrocyte precursors to neural stem cells and in the modulation of *Sox2* activity, a gene strongly associated with a neural stem cell-like state (Kondo and Raff, 2004).

In summary, stem cell development is regulated by intrinsic cellular properties, as well as by the extracellular environment and the combinatorial activity of multiple signaling pathways. This implies that signaling cues have to be viewed as parts of a complex and dynamic network that changes with time and location. Depending on the cellular context stem cells have to integrate signaling events to ensure the generation of the appropriate number of self-renewing stem cells and of differentiating progeny. Future studies will have to focus on how such signaling networks are established, as well as on the contribution of individual signaling pathways. In addition crosstalk and modulation between signal transduction pathways is an important issue to be addressed on the molecular level.

3.3. Neural stem cells

Neural stem cells are a subtype of stem cells in the nervous system, which can self-renew and can generate both neurons and glia. They can be isolated from the embryonic central nervous system (CNS) (Cattaneo and McKay, 1990; Kilpatrick and Bartlett, 1993; Reynolds et al., 1992), the peripheral nervous system (PNS) (Stemple and Anderson, 1992), and also from adult brain (Lois and Alvarez-Buylla, 1993; Reynolds and Weiss, 1992). Embryonic neural stem cells of the CNS are derived from the neuroepithelium. Neuroepithelial stem cells show typical epithelial features, and are highly polarized along their apical-basal axis. Upon the onset of neurogenesis, neuroepithelial cells successively give rise to so-called radial glial cells, which exhibit neuroepithelial as well as astroglial properties (Götz and Huttner, 2005). Most of the neurons in the brain are derived from radial glial cells, which represent a more mature type of stem cell than neuroepithelial cells (Malatesta et al., 2000; Williams and Price, 1995). In contrast to the broad distribution of embryonic neural stem cells during brain development, the existence of neural stem cells in the adult brain is associated with distinct locations. In particular, adult neural stem cells have been found in the two principal adult neurogenic regions, the subventricular zone (SVZ), and the hippocampus, and in non neurogenic regions, including spinal cord (Gage, 2000; Temple, 2001). Moreover, a recent study describes the isolation of neural stem cells from the postnatal cerebellum (Lee et al., 2005). Though neural stem cells are usually identified on the basis of their *in vitro* behaviour after isolation, these studies shed new light on cellular mechanisms involved in adult

neurogenesis as well as neural regeneration. In addition, markers used to isolate stem cell-like populations from the adult brain, for instance the transmembrane protein prominin-1 (CD133) (Lee et al., 2005; Uchida et al., 2004; Weigmann et al., 1997) or the carbohydrate LeX (Capela and Temple, 2002; Capela and Temple, 2006), might be promising candidates to identify and characterize brain stem cells *in vivo*. However, further research will be necessary to define the specificity and functional role of potential neural stem cell markers.

PNS neural progenitor cells are derived from NCSCs, which are characterized by their immunoreactivity for the low-affinity nerve growth factor p75 (Lee et al., 1992), nestin (Lendahl et al., 1990) and the high mobility group (HMG)-box family transcription factor Sox10 (Paratore et al., 2001). Because of their broad developmental potential, NCSCs represent an ideal model system to study stem cell biology (Figure 3.3). During development neural crest cells emerge from the dorsal part of the neural tube and emigrate to various locations within the embryo to generate most structures of the PNS, as well as non-neural tissues such as pigmented melanocytes, smooth muscle cells in the outflow tract of the heart, and craniofacial bones, cartilage, and connective tissues (Le Douarin et al., 2004). By using primary neural crest culture systems and clonal analysis, it was possible to show multipotency of NCSCs and of progenitor cells in targets of the neural crest, and to identify several growth factors that are able to promote the differentiation of neural crest cells into specific lineages *in vitro* (Le Douarin et al., 2004; Sommer, 2001). Upon changing environmental conditions, NCSCs are competent to respond to several instructive growth factors including BMP2, Wnt, Notch, or TGFβ signaling (Kleber and Sommer, 2004; Sommer, 2001). Interestingly, TGFβ signaling is interpreted in a dosage- and context-dependent manner by NCSCs in culture. In cell-clusters, TGFβ applied at low concentrations promotes autonomic neurogenesis, while promoting a non-neural, smooth-muscle-like fate when applied to single cells. However, the nature of these cells still remains to be elucidated. Apoptosis is an alternative fate of TGFβ-treated communities, and can be observed when the factor is applied at higher concentrations (Hagedorn et al., 2000). Thus, short-range cell-cell interactions can modulate cell fate decisions. These so-called community effects might change the genetic programs of interacting cell clusters responding to instructive growth factors. The molecular basis of the context-dependent interpretation of TGFβ by NCSCs remains to be investigated. To address

this issue we have compared single NCSCs and interacting communities of NCSCs -in presence or absence of a TGFβ ligand- by differential gene expression analysis (Wurdak, Niederer and Sommer, unpublished data). Evaluation of the data *in silico* revealed several interesting candidate genes, which may be functionally investigated in future studies (Niederer, diploma thesis). The central issue of my present thesis is the hitherto unclear role of TGFβ signaling in NCSC development *in vivo*.

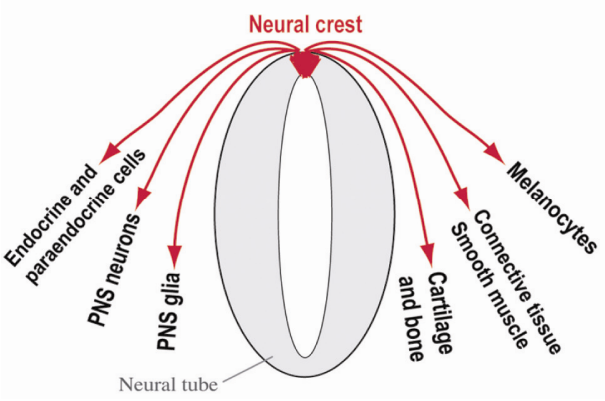

Figure 3.3. Multipotency of the neural crest. NCSCs are a unique multipotent vertebrate cell type arising from the dorsal part of the neural tube. Subsequently, they migrate through the tissue of the developing embryo and differentiate into an array of cell types.

3.4. TGFβ signaling

TGFβ proteins are members of the TGFβ superfamily of secreted signaling molecules with pleiotropic functions in diverse cellular processes including proliferation, differentiation, apoptosis, and cell fate specification during development (Hogan, 1996; Piek et al., 1999; Shi and Massague, 2003). The TGFβ superfamily of cytokines includes three major subfamilies: the TGFβs, the activins, and the BMPs. Although the TGFβ ligands elicit different cellular responses, they are structurally related and dimerize as active forms. Two distinct transmembrane receptors (type 1 and 2) containing an intracellular kinase domain carry the TGFβ-like signal into the nucleus via a conserved pathway (Shi and Massague, 2003). Ligand binding induces the type 1 and type 2 receptors to associate, followed by the unidirectional phosphorylation of the type 1 receptor by the type 2 receptor (Figure 3.4). The activated type 1 receptor then in turn phosphorylates particular members of the Smad family of intracellular mediators, called receptor activated Smads (R-Smads). Smad2 and -3 were shown to transduce activin/TGFβ signals, while Smad1, -5, and -8 preferentially transduce BMP

signals. Upon phosphorylation a conformational change is induced in R-Smads, which are released from the receptor and are now able to interact with the common partner Smad (Co-Smad), Smad4. R-Smad/Smad4 complexes enter the nucleus and regulate gene expression by binding to DNA sequences termed Smad-binding elements, and by interaction with many general and tissue-specific transcription factors and transcriptional coactivators. Growing biochemical and developmental evidence suggests that TGFβ receptors can also phosphorylate non-Smad proteins, supporting the notion of alternative, non-Smad pathways that might cooperate with Smad-dependent signal transduction (Moustakas and Heldin, 2005). Three different isoforms, TGFβ1, TGFβ2 and TGFβ3, have been described to 'trigger' the TGFβ signal transduction. The isoforms are highly similar in their biological activities *in vitro* but differ in their *in vivo* expression patterns reflected by isoform-specific phenotypes displayed by *TGFβ*-knockout mice (Roberts and Sporn, 1992). *TGFβ1* as well as *TGFβ2* null-mice display developmental cardiac defects (Letterio et al., 1994; Sanford et al., 1997). Lineage determination, migration, or maturation of a neural crest subpopulation, the so called cardiac neural crest populating the outflow tract of the heart might, be linked to abnormal cardiac development (Srivastava et al., 1997). *TGFβ3* null-mice die shortly after birth, displaying delayed pulmonary development (Kaartinen et al., 1995; Leveen et al., 2002).

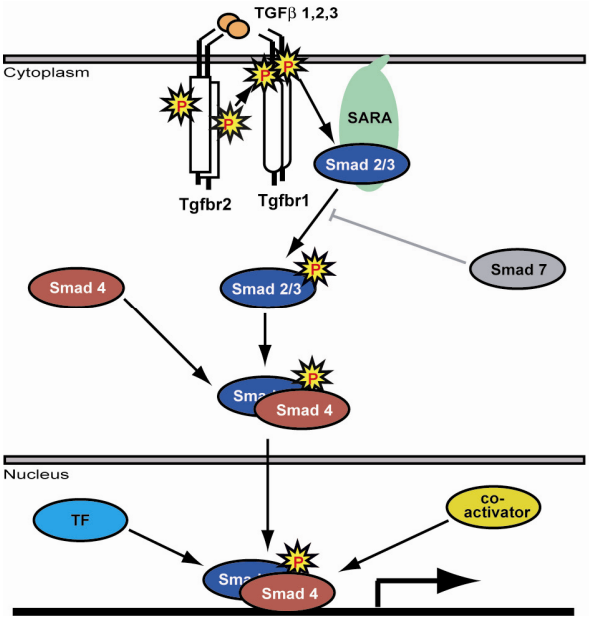

Figure 3.4. The Smad-dependent TGFβ signaling pathway.
The dimeric TGFβ ligands interact with heterotetrameric complexes of type 2 (Tgfbr2, also termed TGFβRII) and type 1 (Tgfbr1, also termed TGFβRI) receptors, which leads to phosphorylation and activation of Tgfbr1 by the constitutively ative kinase domain of Tgfbr2. Tgfbr1 kinase phosphorylates and activates receptor-activated (R)-Smads, Smad2 and Smad3, which may be recruited by a membrane bound protein, termed Smad Anchor for Receptor Activation (SARA). (R)-Smads form complexes with the common mediator, Smad4, and enter the nucleus, where they bind to DNA and interact with transcription factors and co-activators to regulate gene expression.

Overall, the multifunctional character of TGFβ family members implies a need for tight control of their activities mediated by feedback mechanisms and cross talk with other pathways. Understanding this complex interplay on the molecular level will be a goal of future research efforts.

3.5. Conditional inactivation of TGFβ signaling

In order to circumvent early embryonic lethality, we have conditionally inactivated TGFβ signaling in neural stem cells *in vivo*. The *Cre/loxP* system has been used to generate a *Tgfbr2* 'loss-of-function' mutation specifically in NCSCs and neuroepithelial stem cells of the midbrain/hindbrain region. Since *Tgfbr2* is essential for binding the TGFβ isoforms 1-3, the gene ablation is proposed to result in the total and specific loss of TGFβ signaling. Therefore, I have crossed mice carrying *Cre*-recombinase under the control of the *Wnt1* promotor (Danielian et al., 1998), and heterozygous for a *Tgfbr2* floxed allele (Leveen et al., 2002) with mice homozygous for the *Tgfbr2* floxed alleles (Figure 3.5). *Wnt1* is expressed specifically in the neural plate, in the dorsal neural tube and in early migratory neural crest population at all axial levels excluding the forebrain. It has also been shown to be expressed in the midbrain/hindbrain region until embryonic day 15 playing an essential role in midbrain/hindbrain development (Brault et al., 2001; McMahon et al., 1992; Panhuysen et al., 2004). In order to monitor expression of the *Wnt1-Cre* transgene, *Wnt1-Cre* mice have been crossed into the R26R reporter line (Soriano, 1999). The ROSA26 locus has been shown to be ubiquitously and uniformly expressed at all developmental and postnatal times (Zambrowicz et al., 1997). In the R26R reporter line, the transcript produces a functional β-galactosidase (β-Gal) protein only after *Cre*-mediated recombination. The resulting β-Gal activity allows monitoring of the activity of the *Cre*-recombinase and *in vivo* fate mapping of mutant cells. The analysis of mutant mice was combined with primary cell culture systems established for PNS/CNS neural stem cells (Hari et al., 2002; Kleber et al., 2005; Lee et al., 2004; Reynolds and Rietze, 2005). Additionally, an immortalized NCSC line (Rao and Anderson, 1997) mimicking the differentiation potential of primary NCSCs has been used to investigate TGFβ signal modulation on the molecular level.

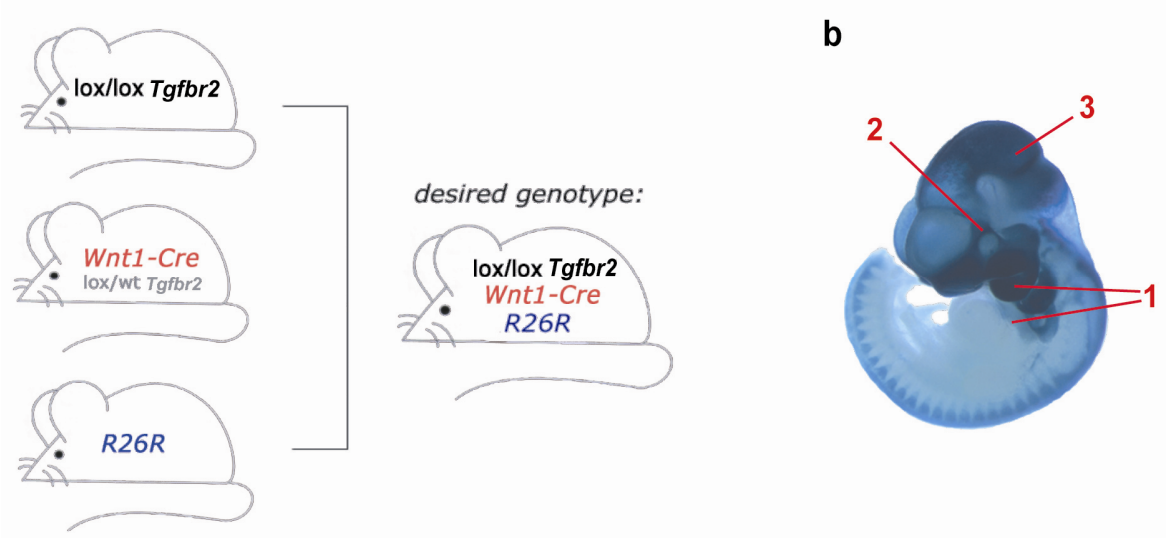

Figure 3.5. Breeding strategy for the conditional ablation of *Tgfbr2*.
(a) In order to obtain the genotype of interest, animals homozygous for the floxed allele of exon 4 of *Tgfbr2* (Leveen et al., 2002) are crossed with the *Wnt1-Cre* mouse line (Danielian et al., 1998) and the R26R reporter line (Soriano, 1999). (b) *In vivo* fate mapping at E10.5 showing the migration of β-Gal-positive NCSCs into structures of the pharyngeal system and the cardiac outflow tract (1), which are the focus of part 1 of my thesis. Furthermore, NCSCs localize to the developing eye (2), which is analyzed in part 2. Finally, *Wnt1-Cre*-activity is also prominently detected in neuroepithelial cells of the midbrain/hindbrain region (3), which is investigated in part 3.

In part 1 and 2 of the present thesis, I will focus on the *in vivo* function of TGFβ signaling, as well as TGFβ signal modulation in NCSC development and NCSC-related disease.

In addition, the chosen approach allowed me to determine the role of TGFβ signaling during midbrain/hindbrain development. Hence, part 3 of my thesis focuses on TGFβ signaling regulating the maintenance of neuroepithelial-derived neural stem cells of the midbrain.

Inactivation of TGFβ signaling in neural crest stem cells leads to multiple defects reminiscent of DiGeorge syndrome

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Specific inactivation of TGFβ signaling in neural crest stem cells (NCSCs) results in cardiovascular defects and thymic, parathyroid, and craniofacial anomalies. All these malformations characterize DiGeorge syndrome, the most common microdeletion syndrome in humans. Consistent with a role of TGFβ in promoting non-neural lineages in NCSCs, mutant neural crest cells migrate into the pharyngeal apparatus but are unable to acquire non-neural cell fates. Moreover, in neural crest cells, TGFβ signaling is both sufficient and required for phosphorylation of Crkl, a signal adaptor protein implicated in the development of DiGeorge syndrome. Thus, TGFβ signal modulation in neural crest differentiation might play a crucial role in the etiology of DiGeorge syndrome.

4.1. INTRODUCTION

During development, neural crest cells emerge from the dorsal part of the neural tube and emigrate to various locations within the embryo to generate most of the peripheral nervous system and a variety of other structures (Le Douarin and Dupin, 2003). In particular, neural crest cells localized in the pharyngeal apparatus contribute to non-neural tissues, such as craniofacial bone and cartilage, thymus, parathyroid glands, and cardiac outflow tract and septum (Graham, 2003; Jiang et al., 2000; Kirby and Waldo, 1995). The function of neural crest cells in the generation of these tissues, however, has been debated (Graham, 2003).

Formation of the pharyngeal apparatus involves complex interactions of neural crest, ectoderm, endoderm, and mesoderm whose development must be co-ordinated (Graham, 2003). Significantly, alterations to the development of this region are often associated with congenital human birth defects such as DiGeorge or Velocardiofacial syndrome. DiGeorge syndrome is the most common microdeletion syndrome in humans characterized by cardiovascular defects plus thymic, parathyroid and craniofacial anomalies (Lindsay, 2001; Vitelli and Baldini, 2003). Approximately 80% of the patients carry a variably sized deletion on chromosome 22 (del22q11). Ablation of genes affected by the microdeletion indicated two pathophysiological mechanisms causing DiGeorge syndrome: Mutations of the transcription factor Tbx1 lead to disturbed pharyngeal arch patterning. Consequently, neural crest cells are unable to populate the pharyngeal apparatus (Jerome and Papaioannou, 2001; Lindsay et al., 2001; Merscher et al., 2001). However, upon deletion of the gene encoding the signal adaptor protein Crkl neural crest cells localize to the pharyngeal apparatus, but they do not form non-neural derivatives from this structure (Guris et al., 2001). In both cases, aberrant neural crest cell migration, survival, or differentiation might contribute to DiGeorge syndrome (Lindsay, 2001; Vitelli and Baldini, 2003). A direct role of neural crest cells in the development of the pharyngeal apparatus, however, was not demonstrated by the analysis of these mutants. Furthermore, the signals specifying non-neural fates from neural crest cells in the pharyngeal apparatus have not yet been identified.

Previously, cell culture experiments allowed the identification of several growth factors able to instruct migratory and postmigratory neural crest cells to adopt specific lineages (Lee et al., 2004; Morrison et al., 2000; Shah et al., 1996; Shah et al., 1994). One of these factors is transforming growth factor (TGF)β that elicits multiple responses in cultured NCSCs. Depending on the cellular context, it can promote the generation of non-neural smooth muscle-like cells or autonomic neurons, or induce apoptosis (Hagedorn et al., 2000; Hagedorn et al., 1999; Shah et al., 1996). Here we investigated the role of TGFβ signaling in NCSCs and their derivatives in vivo, with an emphasis on migration, non-neural fate decision, and differentiation processes of neural crest cells in the pharyngeal apparatus.

4.2. RESULTS AND DISCUSSION

To address the role of TGFβ signaling in neural crest development *in vivo,* we have used the *Cre/loxP* system to conditionally inactivate the *TGFβ receptor type II (Tgfbr2)* gene essential for TGFβ signal transduction (Leveen et al., 2002). *Wnt1-Cre*-mediated recombination of a floxed allele of *Tgfbr2* resulted in loss of TGFβRII protein in neural crest cells isolated from mutant embryos and in mutant neural crest derivatives *in vivo* (Figure 4.1).

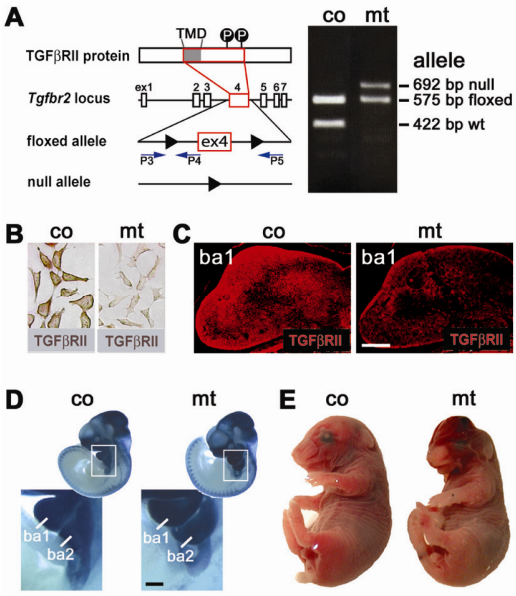

Figure 4.1. Neural crest-specific conditional ablation of *Tgfbr2*.
(A) Exon 4 (ex4) of the *Tgfbr2* locus, encoding the transmembrane domain (TMD) and the intracellular phosphorylation sites (P) of the TGFβRII protein, is flanked by loxP-sites and deleted in NCSCs upon breeding with *Wnt1-Cre* mice. Identification of floxed *Tgfbr2* allele in control (co) and of recombined *Tgfbr2*-null allele in mutant (mt) animals by PCR with indicated primers P3, P4 and P5 (Leveen et al. 2002). (B-C) Presence and absence of TGFβRII (brown/red) in primary NCSC explants (B), and in neural crest cells populating the branchial arch (ba) 1 in control and mutant embryos at E11.5 (C). (D) Normal distribution of β-Gal-expressing neural crest cells in the pharyngeal apparatus of E10.5 mutant mice, as assessed by *in vivo* fate mapping. ba1 and ba2 in enlarged areas marked by boxes. (E) Overall appearance of control and mutant mice at E18.5. Note the craniofacial anomalies in the mutant. Scale bars, 200 µm.

This is consistent with the previously reported activity of *Wnt1-Cre* in virtually all neural crest cells (in addition to its expression in the mid/hindbrain area) (Lee et al., 2004). At embryonic day (E) 10.5, mutant embryos did not exhibit obvious malformations, and *Tgfbr2*-deficient neural crest cells migrated correctly into the pharyngeal apparatus, as assessed by *in vivo* fate mapping using the ROSA26 Cre reporter mouse line (Figure 4.1D) (Hari et al., 2002; Jiang et al., 2000). Mutants were recovered at the expected Mendelian frequency until E18.5 but they died perinatally, displaying multiple developmental defects including mid/hindbrain abnormalities not covered in this study (Figure 4.1E). Control littermates carrying a non-recombined *Tgfbr2* allele showed no anomalies.

Malformations of cranial bones and cartilage as well as cleft palate were found in all mutant mice examined at E18 (Figure 4.2A,B), as described (Ito et al., 2003). As cleft palate together

with craniofacial dysmorphism are typical features of DiGeorge syndrome in humans, we examined mutant mice at different developmental stages for other defects characterizing DiGeorge syndrome. Patients suffering from DiGeorge syndrome have absent or hypoplastic parathyroid and thymus glands (Kirby and Waldo, 1995). Similarly, in all *Tgfbr2*-mutant mice at E18, staining of serial cross sections of the neck for parathyroid hormone indicated either hypoplastic (3 of 5) or undetectable (2 of 5) parathyroid glands (Figure 4.2C). Likewise, the size of the thymus glands was reduced in mutant mice at E18 (58±7% of control size; n=8; $p<0.01$). In normal mouse development, mesenchymal derivatives of neural crest cells are transiently detectable in the cortical region of the developing thymus and parathyroid glands (Figure 4.2D) (Jiang et al., 2000). Moreover, elimination of cephalic premigratory neural crest in chicken and absence of neural crest in the pharyngeal apparatus of transgenic mice results in hypoplastic parathyroid and thymic glands (Conway et al., 1997; Kirby and Waldo, 1995; Ohnemus et al., 2002). This suggests that development of pharyngeal arch-derived glands might depend on interactions between neural crest-derived cells and pharyngeal endoderm. Consistent with the hypothesis that neural crest cells are important for proper thymus gland formation, neural crest-specific inactivation of TGFβ signaling resulted in a reduction of neural crest cells found by *in vivo* fate mapping in the cortex of the developing thymus at E13.5 (Figure 4.2D).

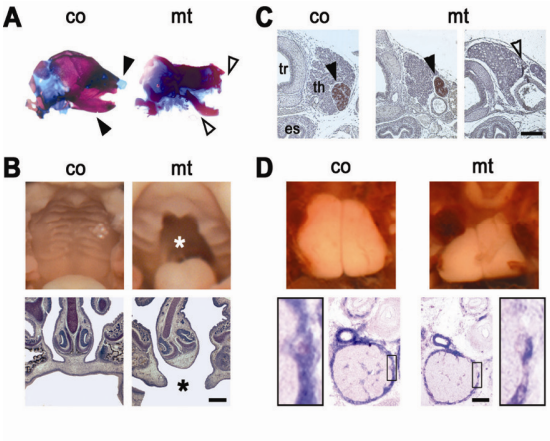

Figure 4.2. Features of DiGeorge syndrome in *Tgfbr2*-mutant mice.
(A) Hypoplastic or absent bone (red) and cartilage (blue) structures (open arrowheads) in mutant (mt) mice at E18. co, control. (B) Cleft palate (*) in macroscopic view (top) and on frontal sections (bottom) in mutant mice at E18. (C) Parathyroid glands stained for parathyroid hormone (brown; arrowhead) were hypoplastic or undetectable in mutant embryos at E18 (open arrowhead). tr, trachea; es, esophagus; th, thyroid gland. (D) Hypoplastic thymus in mutant mice at E18 (top), correlating with fewer cortical β-Gal-expressing neural crest cells (blue) compared to control at E13.5 (bottom). Scale bars, 200 µm (B, C), 100 µm (D).

The main cause of mortality in DiGeorge patients are congenital heart defects such as a single truncus arteriosus and a ventricular septum defect (VSD) (Kirby and Waldo, 1995). Aberrant development of cardiac neural crest that normally contributes to the heart and its outflow tract

is thought to cause these defects. This assumption is based on genetic manipulations in mouse models and on cardiac neural crest ablation in chicken, which lead to a truncus arteriosus and a VSD (Conway et al., 1997; Kioussi et al., 2002; Kirby and Waldo, 1995; Ohnemus et al., 2002). Recently, neural crest-specific inactivation of the type I bone morphogenic protein (BMP) receptor Alk2 resulted in cardiac outflow tract malformations (but not in other tissue anomalies also associated with DiGeorge syndrome), indicating a role of TGFβ family factors in cardiovascular development (Kaartinen et al., 2004). To determine whether the prenatal lethality in the *Tgfbr2*-mutant mice might result from heart defects, we examined the hearts of mutant mice at E18 histologically and by intracardial ink injection. All mutant hearts (n=18) displayed a truncus arteriosus together with a VSD (Figure 4.3). In addition, we found abnormal patterning of the arteries arising from the aortic arch, as also found in DiGeorge syndrome patients (Figure 4.3D) (Kirby and Waldo, 1995).

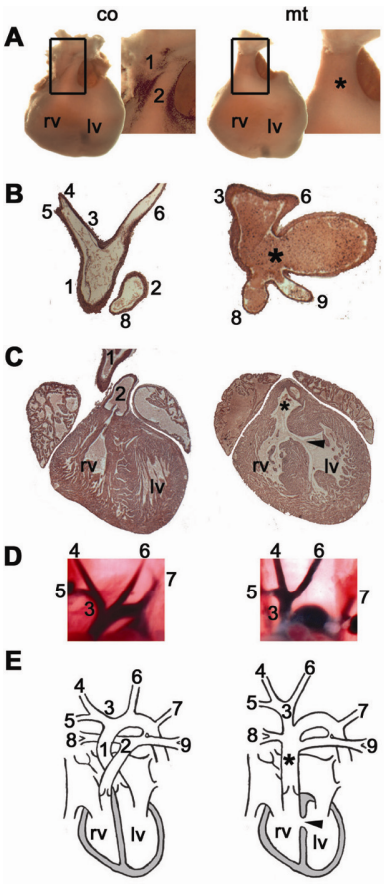

Figure 4.3. Malformations in the heart of *Tgfbr2*-mutant mice.
(A) Ventricular heart at E18 with detailed view of the paired outflow tract in control (co) and of the truncus arteriosus (*) in mutant (mt) mice. rv, right, lv, left ventricle; 1, aorta; 2, pulmonary trunk. (B) Frontal section of the normal outflow tract and the truncus arteriosus (*). (C) Frontal section of the heart in control and mutant with defective septum between rv and lv (arrowhead). (D) Abnormal branching of the left carotid artery from the brachiocephalic trunk in mutant mice visualized by intracardial ink injection. (E) Schematic view of abnormal branching and heart defects in the mutant compared to wild type anatomy. The mutant's left carotid artery (6) arises from the brachiocephalic trunk (3) and the pulmonary arteries (8, 9) originate in the truncus arteriosus (*). 4, right carotid artery; 5, right subclavian artery; 7, left subclavian artery.

Overall, these heart defects led to functional right-sided heart failure with venous congestion, resulting in a vasodilatation of the jugular veins (data not shown), a characteristic clinical feature of patients with severe cardiac insufficiency.

Taken together, our findings show that mice with a mutation of *Tgfbr2* in NCSCs develop all the morphological features of DiGeorge syndrome. The question arises, whether these developmental defects are due to impaired migration, programmed cell death and/or a failure in fate decision or differentiation of neural crest cells. *In vivo* fate mapping revealed that, as in the control, mutant neural crest cells were able to populate the pharyngeal apparatus and the forming aortico-pulmonary septum at E10.5 (Figures 4.1 and 4.4). Likewise, expression of the NCSC-marker Sox10 (Paratore et al., 2001) and migration was not affected in *Tgfbr2*-deficient neural crest cells emanating from neural tubes in explant cultures (data not shown). As in the control, neural crest-derived cells in the pharyngeal apparatus of mutant mice lost sox10 expression while expressing the neural crest markers pax3 and ehand (data not shown). In addition, we did not observe differences in the number of apoptotic cells in the pharyngeal apparatus of control and mutant mice at E10.5 (data not shown).

Our data indicate that neural crest cell differentiation in the pharyngeal apparatus rather than migration or cell survival is defective in the absence of TGFβ signaling. To test this hypothesis, we analyzed the expression of smooth muscle α-actin in neural crest derivatives, since TGFβ is known to induce a smooth muscle-like cell fate in NCSCs *in vitro* (Shah et al., 1996). While in the control at E10.5, β−Gal-expressing neural crest derivatives in the prospective aortico-pulmonary septum stained for smooth muscle α-actin, *Tgfbr2*-mutant neural crest cells in this region did not contribute to the development of the smooth musculature (Figure 4.4B). We therefore suggest that the non-neural, smooth muscle α-actin-positive cells (which have also been termed ‘myofibroblasts’ and ‘smooth muscle–like cells’) (Hagedorn et al., 1999; Morrison et al., 1999) generated from TGFβ-treated NCSCs in culture indeed represent smooth muscle cells. At later developmental stages, neural crest cells that failed to adopt a smooth muscle cell fate underwent apoptotic cell death (data not shown). The absence of neural crest-derived smooth muscle cells in mutants explains the defective

separation of the aorta from the pulmonary trunk, leading inevitably to a truncus arteriosus (Figure 4.3).

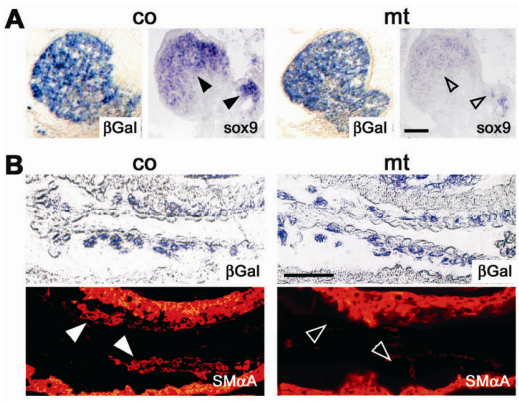

Figure 4.4. *Tgfbr2*-mutant neural crest cells fail to acquire non-neural fates.
(A) Equal distribution of β-Gal-expressing neural crest cells (blue) in the first and second branchial arch of control (co) and *Tgfbr2*-mutant (mt) mice at E10.5. Presence (arrowheads) and absence (open arrowheads) of sox9, required for normal craniofacial bone and cartilage development, in branchial arches. (B) Presence of β-Gal-expressing neural crest cells (blue) in the developing aorto-pulmonary septum of mutant embryos at E10.5. Mutant neural crest cells fail to differentiate into smooth muscle α-actin (SMαA)-expressing cells (red) forming the septum. Scale bars, 100 μM.

The transcription factor sox9 is required for the specification of an osteochondroprogenitor cell from neural crest cells, and conditional inactivation of *sox9* in NCSCs results in craniofacial anomalies (Mori-Akiyama et al., 2003). During normal development sox9 is expressed in neural crest cells in the first and second pharyngeal arches, which give rise to the craniofacial skeleton. In contrast, sox9 expression was markedly reduced in *Tgfbr2*-mutant neural crest cells populating the first and second pharyngeal arches (Figure 4.4A). These data indicate that neural crest cells are unable to acquire an osteochondroprogenitor cell fate in the absence of TGFβ signaling, which leads to the malformations of cranial bones and cartilage observed in *Tgfbr2*-mutant mice (Figure 4.2A).

The microdeletion on chromosome 22 (*del22q11*) found in most of the DiGeorge syndrome patients encompasses about 30 genes but does not include either genes encoding TGFβ receptors or its ligands (Lindsay, 2001; Vitelli and Baldini, 2003). Thus, gene products eliminated by *del22q11* may functionally interact with TGFβ signaling in neural crest cells. For instance, orthologs of *PCQAP*, a gene within the microdeleted region (Berti et al., 2001), have been shown to modulate TGFβ signaling (Kato et al., 2002), although the role of *PCQAP* in the development of the pharyngeal apparatus is unknown. Inactivation of two of the genes deleted in *del22q11*, *Tbx1* and *Crkl*, phenocopies symptoms of DiGeorge syndrome (Lindsay, 2001; Vitelli and Baldini, 2003). In *Tbx1* mutants, early steps in pharyngeal arch

patterning are disrupted, which, as a secondary effect, interferes with neural crest migration into the pharyngeal arch complex (Vitelli and Baldini, 2003; Vitelli et al., 2002a). Similarly, manipulation of genes that genetically interact with *Tbx1*, such as *Fgf8* and *Vegf*, affect migration and/or survival of neural crest cells into the pharyngeal apparatus (Frank et al., 2002; Stalmans et al., 2003; Vitelli and Baldini, 2003). Inactivation of any one of *Tbx1*, *Fgf8*, or *Vegf* results in abnormal patterning of the aortic arch arteries, leading to conotruncal heart defects (Frank et al., 2002; Stalmans et al., 2003; Vitelli and Baldini, 2003). In contrast, we found that *Tbx1* expression was not altered in mice carrying a neural crest-specific mutation of *Tgfbr2* (data not shown) and that the development of DiGeorge-like syndrome in these mice is due to a failure in non-neural fate acquisition and differentiation rather than in migration or survival of mutant neural crest cells. Moreover, intracardial ink injection at E10.5 revealed normal formation of the aortic arch arteries in *Tgfbr2*-mutant mice (data not shown). Thus, although we cannot exclude it at present, the differences in phenotypes do not support an association between a *Tbx1* genetic pathway and TGFβ signaling in neural crest cells.

Ablation of the *del22q11* gene *Crkl* in mice, however, generates a phenotype reminiscent of that obtained by TGFβ signal inactivation in NCSCs, although with a lower penetrance (Guris et al., 2001). While pharyngeal arch patterning is normal, *Crkl*-mutant mice develop cardiovascular and mild craniofacial anomalies, as well as hypoplastic thymic and parathyroid glands. These deficiencies conceivably result from a postmigratory defect of neural crest cells lacking *Crkl* (Guris et al., 2001), similar to the deficiencies observed in mice with *Tgfbr2*-mutant neural crest cells (Figures 4 and 5). The gene product of *Crkl*, Crkl, belongs to the Crk family of adaptor proteins that are involved in different signaling pathways (Feller, 2001). In particular, CrkII –which shares 60% identical residues with Crkl– mediates oncogenic transformation induced by epidermal growth factor and TGFβ, and overexpression of a dominant negative mutant of CrkII prevents TGFβ-induced cell differentiation *in vitro* (Ota et al., 1998). Therefore, Crkl might be involved in TGFβ-dependent differentiation of neural crest cells. To address this hypothesis, we first assessed the status of Crkl phosphorylation during development of the pharyngeal apparatus. Tyrosine-phosphorylated Crkl was readily

detectable in several structures of the wild-type pharyngeal apparatus at E10, including the

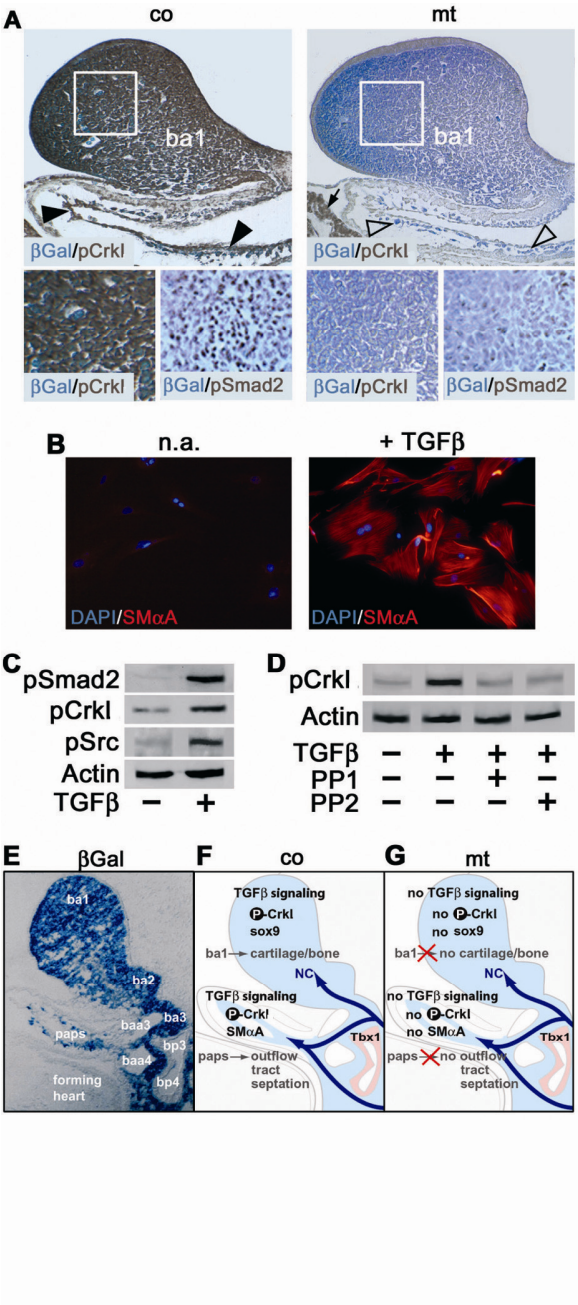

Figure 4.5. TGFβ-dependent Crkl phosphorylation and neural crest differentiation. (A) Mutant (mt) neural crest cells expressing β-Gal (blue) in branchial arch 1 (ba1), branchial arch 2 (Supplementary Fig. 1), and the developing aortico-pulmonary septum (arrowheads) at E10 show strongly reduced phosphorylation of Crkl (pCrkl) (brown; gross and detailed view) and lack nuclear phospho-Smad2 (brown; detailed view) compared to control (co). Note comparable staining intensity for phosphorylated Crkl in β-Gal-negative tissue (arrow) in mt and co. (B) Immortalized NCSCs (Monc1 cells) express smooth muscle α-actin (SMαA) upon treatment with TGFβ while untreated (n.a.) cells are SMαA-negative. (C) Western blot analysis reveals increased Smad2-, Crkl- and Src-phosphorylation by TGFβ in Monc1 cells. (D) Crkl phosphorylation in TGFβ-treated Monc1 cells is reduced to levels of untreated cells in the presence of Src kinase inhibitors PP1 and PP2, respectively. (E) β-Gal-expressing neural crest cells populate the pharyngeal apparatus (saggital section; ba1-3, branchial arches 1-3; bp3/4, branchial pouches 3/4; baa3/4, branchial arch arteries 3/4; paps, prospective aorto-pulmonary septum). (F) Neural crest cells (NC, blue) present in the pharyngeal apparatus require TGFβ-signaling for Crkl phosphorylation and for expression of the non-neural markers sox9 in the first branchial arch and SMαA in the forming septum of the heart outflow tract. (G) *Tgfbr2*-deficient neural crest cells migrate normally into the pharyngeal apparatus. Here, the signal adaptor protein Crkl fails to be phosphorylated in response to TGFβ, and mutant neural crest cells fail to express sox9 and SMαA. *Tbx1* expression in the branchial pouch endoderm and early pharyngeal arch patterning is not affected. The failure of mutant neural crest cells to acquire non-neural fates in the early pharyngeal apparatus impairs the development of tissues derived from the pharyngeal apparatus and leads to a DiGeorge-like phenotype.

pharyngeal arches 1 and 2 and the prospective aorto-pulmonary septum (Figure 4.5A; Supplementary Figure 4.1). However, phosphorylation of Crkl was restricted to a narrow, early time window during development of the pharyngeal apparatus and was no longer observed from E11.5 onwards (data not shown). Intriguingly, Crkl phosphorylation was

impaired in *Tgfbr2*-deficient neural crest cells in the pharyngeal apparatus at E10, concomitant with absence of TGFβ-dependent phosphorylation of Smad2 in the mutant (Figure 4.5A; Supplementary Figure 4.1). Moreover, TGFβ signaling promoted Crkl phosphorylation in an NCSC line that gives rise to smooth muscle-like cells in response to TGFβ (Figure 4.5B,C) (Chen and Lechleider, 2004; Sommer et al., 1995). This demonstrates that Crkl phosphorylation is downstream of TGFβ signaling in neural crest cells. Thus, Crkl might mediate TGFβ-dependent non-neural fate acquisition of neural crest cells at early stages of pharyngeal apparatus development. TGFβ elicits a wide range of cellular processes, involving activation and cross-talk of multiple signaling pathways (Derynck and Zhang, 2003; Massague and Chen, 2000). For instance, TGFβ signaling has been reported to induce phosphorylation and activation of Src tyrosine kinase regulating TGFβ-dependent apoptosis, cell migration, or tumor cell invasiveness (Fukuda et al., 1998; Kim and Joo, 2002; Park et al., 2004; Tanaka et al., 2004). Interestingly, a recent report identified Crkl as a downstream signaling component of Src kinase in integrin-induced migration (Li et al., 2003).

In conclusion, we propose that TGFβ isoforms expressed in the pharyngeal apparatus (Millan et al., 1991) promote non-neural fate decisions in postmigratory neural crest cells, allowing these cells to contribute to multiple craniofacial, cardiovascular, and glandular structures (Figure 4.5E-G). Accordingly, inactivation of TGFβ signaling in neural crest cells prevents development of these structures and recapitulates all morphological features of DiGeorge syndrome.

Compound developmental eye disorders following inactivation of TGFβ signaling in neural crest stem cells

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Development of the eye is dependent on the interaction of epithelial and neuroectodermal cells with periocular mesenchyme. Here we reveal distinct neural crest contributions to both anterior and posterior mesenchymal eye structures and show that TGFβ signaling in these cells is crucial for normal eye development. In the anterior eye, TGFβ released from the lens is required for the expression of transcription factors *Pitx2* and *Foxc1* in neural crest-derived cornea and eye chamber angle structures known to control intraocular pressure. As in patients carrying mutations in *PITX2* and *FOXC1*, TGFβ signal inactivation in neural crest cells leads to ocular defects characteristic of Axenfeld-Rieger's anomaly. Moreover, TGFβ enhances Foxc1 and induces Pitx2 expression in fibroblasts and embryonic *ex vivo* eye cultures. In the posterior eye segment, neural crest cell–specific inactivation of TGFβ signaling results in a condition reminiscent of human persistent hyperplastic primary vitreous. As a secondary effect, retinal patterning is also disturbed in mutant mice.

5.1. INTRODUCTION

Normal function of the eye is dependent on a variety of highly specialized structures in the anterior eye segment. These include the cornea and lens necessary for light refraction, the iris protecting the retina from excess light, and the ciliary body and ocular drainage structures that provide aqueous humor required for cornea and lens nutrition and for the regulation of intraocular pressure (Figure 5.1a). Development of these tissues involves the coordinated interactions between surface and neural ectoderm with the periocular neural crest (NC)-derived mesenchyme. Failure of these interactions results in multiple developmental eye disorders, such as Axenfeld-Rieger's anomaly, which comprises microphtalmia, hypoplastic iris, polycoria, and abnormal chamber angle patterning, and is associated with a high prevalence of glaucoma (Alward, 2000).

Development of the anterior eye segment depends on the proper function of two transcription factors in the periocular mesenchyme, the forkhead/winged helix factor FOXC1 and the paired-like homeodomain factor PITX2. In humans, hypomorphic and overactivating mutations in either gene leads to Axenfeld-Rieger's anomaly (Alward, 2000), and mutation of either *Foxc1* or *Pitx2* in mice results in defective anterior eye segment formation, much like in human Axenfeld-Rieger's anomaly (Holmberg et al., 2004; Kume et al., 1998; Lu et al., 1999). While downstream targets of FOXC1 expressed in the eye are supposedly involved in modulating intraocular eye pressure and ocular development (Tamimi et al., 2004), PITX2 target genes have been associated with extracellular matrix synthesis and stability (Hjalt et al., 2001). In contrast, the upstream regulators of both FOXC1 and PITX2 remain to be determined. Moreover, the identity of cells expressing FOXC1 and PITX2 during anterior eye patterning is unclear.

It is conceivable that aberrant development of mesenchymal NC cells contribute to the malformations observed in Axenfeld-Rieger's anomaly. Indeed, portions of the anterior eye segment including corneal endothelial cells and collagen-synthesizing keratocytes, as well as iris melanocytes, were proposed to originate from the NC (Graw, 2003; Hari et al., 2002; Wehrle-Haller and Weston, 1997). The defined contribution of NC, however, has been debated, as most of the data comes from avian models in which ocular development appears

to be slightly different from that in mammals (Cvekl and Tamm, 2004). Moreover, mechanisms controlling ocular NC migration and differentiation remain to be elucidated.

Transforming growth factor (TGF) β represents a candidate factor to control ocular NC cell development. TGFβ signaling is required for the generation of multiple non-neural derivatives of the NC (Wurdak et al., 2005). Interestingly, TGFβ signaling during eye development is critical, as ligand inactivation and overexpression lead to defective ocular development in mice (Flugel-Koch et al., 2002; Sanford et al., 1997). In both models normal development of the anterior eye segment is affected, possibly due to impaired NC migration and/or differentiation. In particular, the phenotype upon disruption of the *Tgfbr2* gene recapitulates certain features observed in *Foxc1* and *Pitx2* mutant mice. However, the cellular role of TGFβ signaling in ocular NC development is unknown, and a link between TGFβ signaling and activation of the transcription factors FoxC1 and PITX2 in ocular development has not been established so far (Sanford et al., 1997).

Here, we performed *in vivo* cell fate mapping to define in detail the contribution of NC to the forming eye in mice. In addition, we used conditional gene targeting to inactivate TGFβ signaling in NC stem cells and, as a result, in ocular NC derivatives to assess TGFβ actions on these cells during eye development.

5.2. RESULTS

5.2.1. Neural crest cells contribute to multiple structures derived from the eye mesenchyme

NC cell-specific constitutive expression of β-galactosidase in corresponding transgenic mice allows monitoring of NC cell migration and fate during development *in vivo* (Chai et al., 2000; Hari et al., 2002). This approach was used in the present study to define the ocular structures originating from the NC. *Rosa26* Cre reporter *(Rosa26R)* mice, expressing β-galactosidase upon Cre-mediated recombination, were mated with transgenic mice expressing Cre recombinase under control of the *Wnt1* promoter. While *Wnt1* is not expressed in any structure of the developing eye (data not shown), it is expressed in the dorsal neural tube, allowing *Wnt1-Cre*-mediated recombination in virtually all NC stem cells (Lee et al., 2004; Wurdak et al., 2005). In *Wnt1-Cre*/*Rosa26R* double transgenic mice, β−galactosidase-expressing NC-derived cells can be visualized by X-gal staining. Previously, a contribution of NC-derived cells to ocular development in mice has been proposed to occur after embryonic day (E) 12 (Cvekl and Tamm, 2004). Interestingly, NC-derived cells were already detectable at E10 surrounding the optic cup and the lens vesicle (Figure 5.1b). Until E13.5, the NC-derived cells were predominantly found in the periocular mesenchyme, while the overlying epithelium, the lens, and the retina were consistently X-gal-negative. In addition, we observed that structures of the primary vitreous, located between lens and retina, are NC-derived (Figure 5.1b). At later stages, X-gal-positive cells contributed to corneal stroma and endothelium and to structures of the chamber angle (Figure 5.1a). In mature eyes, the stroma of the iris, the ciliary body, and the trabecular meshwork, as well as cells of the choroid and primary vitreous, are all of NC origin (data not shown). Taken together, NC-derived cells contribute to eye development as early as the eye vesicle is formed, and subsequently to various structures of the maturing eye.

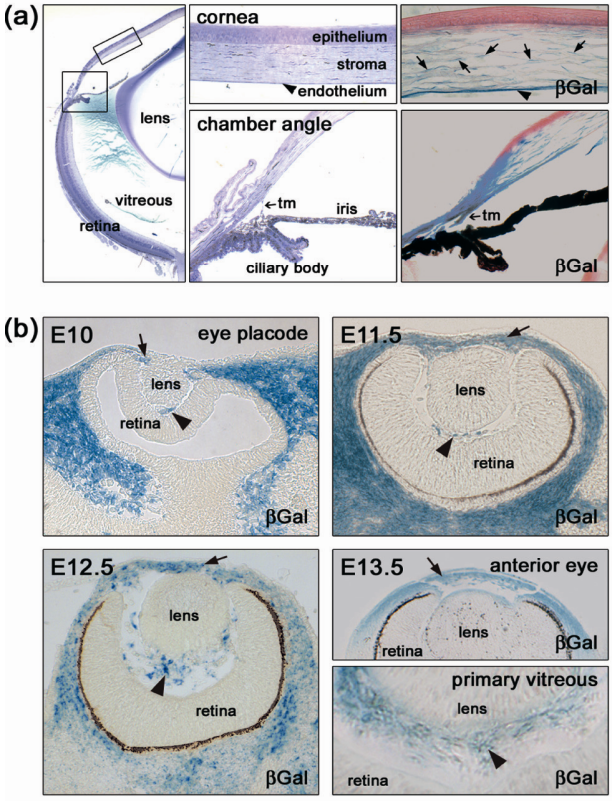


Figure 5.1. Neural crest (NC)-derived cells contribute to ocular development.
(a) Toluidine blue staining of an adult eye with detailed view of the corneal assembly, including outer epithelium, stroma, and inner epithelium. The chamber angle at the irido-corneal transition includes the trabecular meshwork (tm). *In vivo* fate mapping of NC-derived, β-galactosidase (βGal; blue) expressing cells revealed NC origin of corneal keratocytes (arrows) and of corneal endothelium (arrowheads). In addition, structures of the chamber angle, including the trabecular meshwork are NC-derived. (b) At E10, the optic cup is surrounded by NC-derived cells expressing β-galactosidase (βGal; blue). Until E13.5 the majority of the cells in the periocular mesenchyme (arrows), which forms the anterior eye segment, are of NC origin. In addition, the primary vitreous (arrowheads) shows a strong NC contribution.

5.2.2. Multiple ocular anomalies upon inactivation of TGFβ signaling in NC-derived periocular mesenchyme

The expression pattern of TGFβ ligands and their receptors during eye development was visualized by immunohistochemistry at different developmental stages (E10.5 to E18). TGFβ2 expression peaked in the forming lens at E13.5 and E15, but decreased towards E18 (Figure 5.2b; data not shown), whereas TGFβ isoforms -1 and -3 were undetectable (Supplementary Figure 5.1; data not shown). At E13.5, TGFβ receptor type 2 (Tgfbr2) was expressed in periocular mesenchyme, lens, retina, and the primary vitreous. Because *in vivo* fate mapping revealed substantial NC contribution to periocular mesenchyme, TGFβ signaling may be important for development of ocular NC derivatives. Therefore, we analyzed eyes of mouse embryos after NC-specific inactivation of TGFβ signaling (Leveen et al., 2002; Wurdak et al., 2005). Tissue-specific signal inactivation was achieved by *Wnt1-Cre*-mediated deletion of exon 4 of the *Tgfbr2* gene (Figure 5.2a), which leads to loss of Tgfbr2 protein expression in NC stem cells (Wurdak et al., 2005) (Supplementary Figure 5.2). In such *Tgfbr2*-mutant mice both Tgfbr2 expression and TGFβ-induced phosphorylation of the

downstream signaling molecule Smad2 (pSmad2) remained undetectable in the periocular mesenchyme (Figure 5.2c).

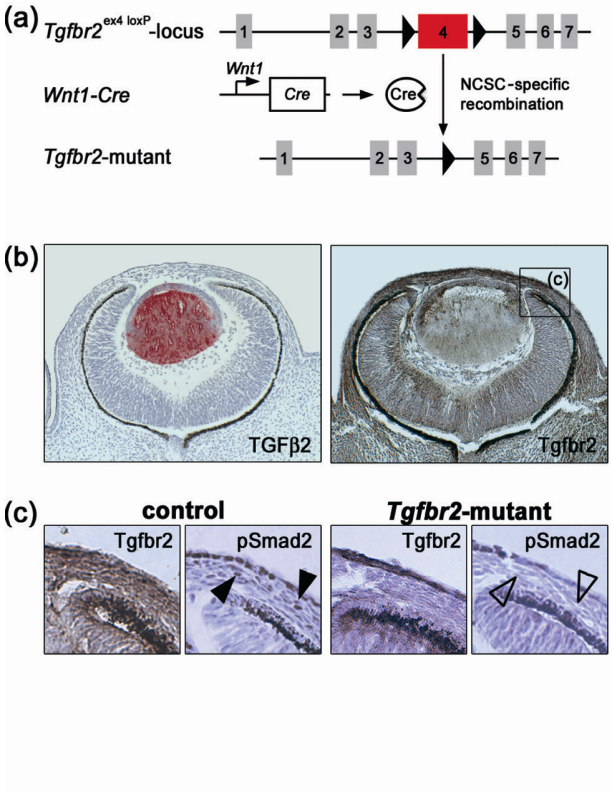


Figure 5.2. Inactivation of TGFβ-signaling in ocular neural crest (NC)-derived cells.
(a) Strategy for Cre/loxP-mediated deletion of exon 4 of the *Tgfbr2* locus in NC stem cells (NCSC). Exon 4 (red), encoding the transmembrane domain and the intracellular phosphorylation sites of the Tgfbr2 protein, is flanked by loxP-sites (triangles) and deleted in NCSCs upon breeding with *Wnt1-Cre* mice. (b) TGFβ ligand and receptor expression in the developing eye. At E13.5, immunoreactive TGFβ2 (red) is predominantly expressed in the lens, whereas Tgfbr2 immunostaining (brown) shows a broad expression of the receptor in the forming eye, including the periocular mesenchyme, lens, primary vitreous, and retina. (c) Detailed view of the forming anterior eye segment (box in b) emphasizes the strong expression of Tgfbr2 (brown) in the prospective chamber angle, corneal stroma and endothelium of control embryos. Upon deletion of *Tgfbr2* in NCSC, Tgfbr2 is undetectable in corresponding structures. Moreover, defective TGFβ signaling in these structures is also reflected by the absence of phosphorylated (p) Smad2 in *Tgfbr2*-mutant (open arrowheads) as compared to control embryos (arrowheads).

At E18, main structures of the anterior eye segment, including the forming ciliary body, iris and trabecular meshwork, were well defined in control animals. Therefore, eye development in the absence TGFβ signaling in NC-derived cells was first analyzed at E18. Most impressively, eyes from *Tgfbr2*-mutant embryos were 26 ± 1% smaller than eyes from control littermates (Figure 5.3a; Figure 5.4). The cornea in control eyes was properly structured into epi- and endothelium covering a thick stroma (Figure 5.3b). However, in *Tgfbr2*-mutant mice the cornea lacked an endothelial layer and no normal stroma was formed. In control mice, corneal structures and the lens were clearly separated to form the anterior eye chamber (Figure 5.3c). In contrast, cornea and lens of *Tgfbr2*-mutant eyes failed to separate and no proper anterior eye segment was built. Moreover, normal formation of the trabecular meshwork and the ciliary body, indicated by a wrinkle of the iris primordium in control eyes, was not observed in *Tgfbr2*-mutants (Figure 5.3c). In addition, eye sections from E18 *Tgfbr2*-

mutant embryos revealed a remarkable accumulation of cells between lens and retina (Figure 5.3d), while vessels of the hyaloid vascular system were present in corresponding structures of control eyes. Finally, the retina of control mice was clearly structured into an inner and an outer layer of cells, whereas the retina of *Tgfbr2*-mutant mice presented a diffuse patterning (Figure 5.3e). Thus, *Tgfbr2*-mutant embryos show microphthalic eyes with anomalies of the anterior segment much like in human Axenfeld-Rieger's anomaly, and additional defects of the posterior eye segment.

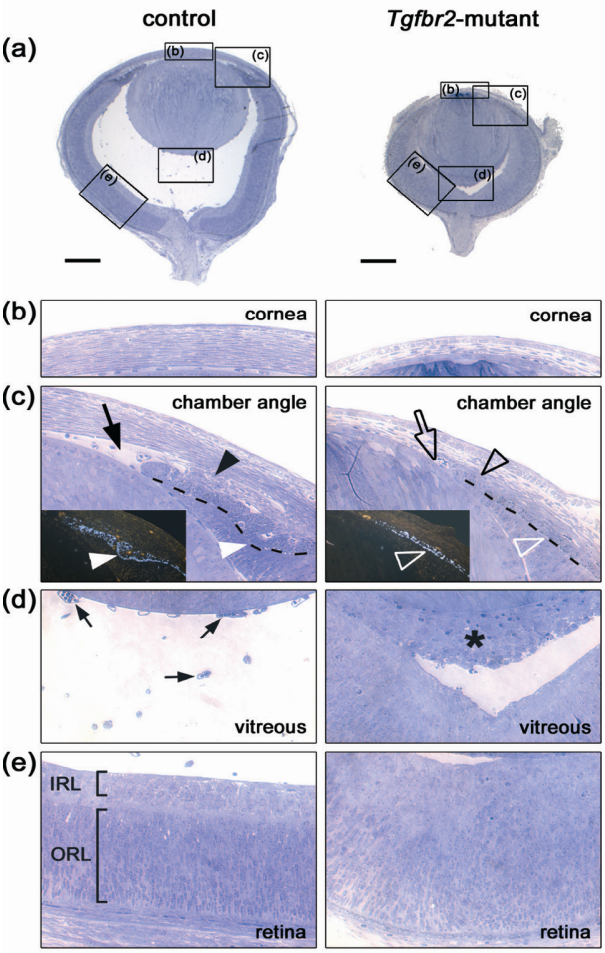


Figure 5.3. Compound ocular anomalies in *Tgfbr2*-mutant mice.
(a) Toluidine blue staining of semi-thin sagittal sections of eyes at E18 reveals a smaller size with absent anterior chamber and a retrolental cellular infiltration in *Tgfbr2*-mutant as compared to control embryos (boxes for details described below). Scale bars: 250 µm. (b) Abnormal corneal stroma in *Tgfbr2*-mutant embryos. (c) Structures of the forming chamber angle with the trabecular meshwork (black arrowhead) in control eyes are absent in *Tgfbr2*-mutant eyes (open black arrowhead). Here, lens and cornea fail to separate to form the anterior eye chamber (open arrow). In addition, dark field pictures (inserts) visualizing the pigment of the forming iris (broken line on toluidine blue-stained sections) reveal initiation of ciliary body formation (white arrowheads) in control eyes, and its absence in *Tgfbr2*-mutant eyes (open white arrowheads). (d) At E18 in control eyes, the primary vitreous consists of loosely arranged vessels of the hyaloid vascular system (arrows). In contrast, *Tgfbr2*-mutant mice show a dense cell mass between lens and retina (asterix), reminiscent of human persistent hyperplastic primary vitreous. (e) The retina of control eyes displays typical patterning with clear separation into an inner (IRL) and an outer layer (ORL). In *Tgfbr2*-mutant mice, however, there is no apparent patterning of the retina.

5.2.3. Persistent hyperplastic primary vitreous in *Tgfbr2*-mutant mice

In normal mice, the primary vitreous, including the hyaloid vascular system, persists until P30. Its regression starts postnatally around P14 to form the avascular and transparent secondary vitreous (Ito and Yoshioka, 1999). In patients with congenital persistent

hyperplastic primary vitreous, developmentally abnormal primary vitreous becomes a fibro-vascular membrane, formed behind the lens (Amaya et al., 2003). Much like in human persistent hyperplastic primary vitreous (Haddad et al., 1978), irregular retrolental structures present in *Tgfbr2*-mutant mice consisted of several different cell types (Figure 5.5a). These included fibroblast-like cells, prospective melanocytes expressing dopachrome tautomerase (dct; also termed trp-2), smooth muscle α-actin-positive pericytes, and vessels of the hyaloid vascular system. Moreover, Ki-67 staining revealed proliferative cells in the retrolental tissue.

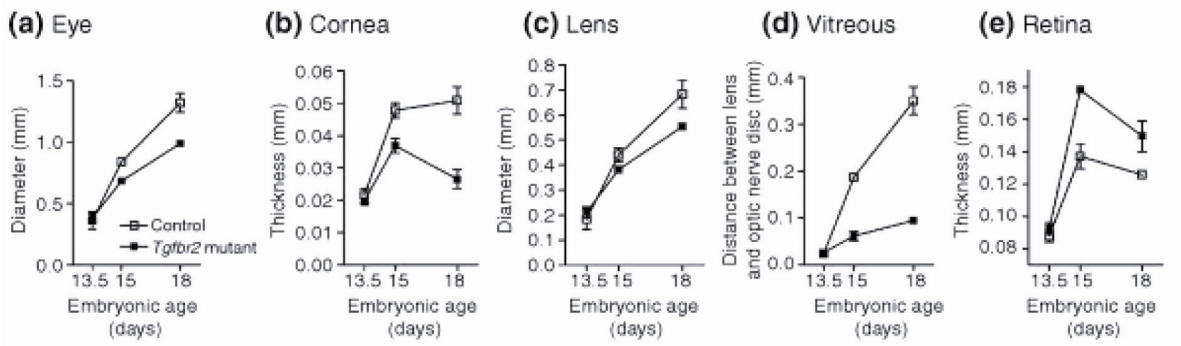


Figure 5.4. Impaired ocular growth in *Tgfbr2*-mutant mice leads to microphthalmia.
The developing eyes and its compartments from Tgfbr2-mutant and control embryos are of comparable size at E13.5. Subsequently, the eyes from *Tgfbr2*-mutant mice show reduced size compared to controls. The growth of the lens is comparable, but the thickness of the cornea and vitreous (measured as distance between lens and optic nerve disc) is drastically decreased in *Tgfbr2*-mutant mice. In contrast, the thickness of the retina is increased in the mutant. For each time point, midorgan sagittal sections of both eyes were analyzed for at least three mice.

Effects on the retina have been reported in patients with persistent hyperplastic primary vitreous (Goldberg, 1997). Moreover, as instructive signals from the lens promote normal patterning of the retina (Yamamoto and Jeffery, 2000), the irregular retrolental structures in *Tgfbr2*-mutant mice may alter normal interaction of lens and retina. To test whether retinal development in *Tgfbr2*-mutant mice was affected, retinas from embryos of different ages were stained for factors known to be expressed at distinct stages during development (de Melo et al., 2005). At E15 the inner parts of the retina from control mice strongly expressed the transcription factors Brn3A in retinal ganglion cells and Pax6 in amacrine cells of the ganglion cell layers (Figure 5.5b). In contrast, *Tgfbr2*-mutant embryos displayed lower numbers of both Brn3A- and Pax6-positive retinal cells. Moreover, at E15 the number of TUNEL-positive cells was increased in the retina of *Tgfbr2*-mutants as compared to control

embryos (13.3 ± 2.5 /5 µm section (mt) vs. 5.6 ± 0.5 (co); $p<0.01$). At E18, expression of Brn3A, Pax6 and neurofilament defines distinct layers of the developing retina in control eyes (Figure 5.5c). However, in *Tgfbr2*-mutant mice patterning into cell layers was disturbed. Moreover, the retina thickness was increased in the mutants (Figure 5.4). Eyes of *Tgfbr2*-mutant mice are therefore affected by anomalies similar to persistent hyperplastic primary vitreous and by disturbed retinal patterning.

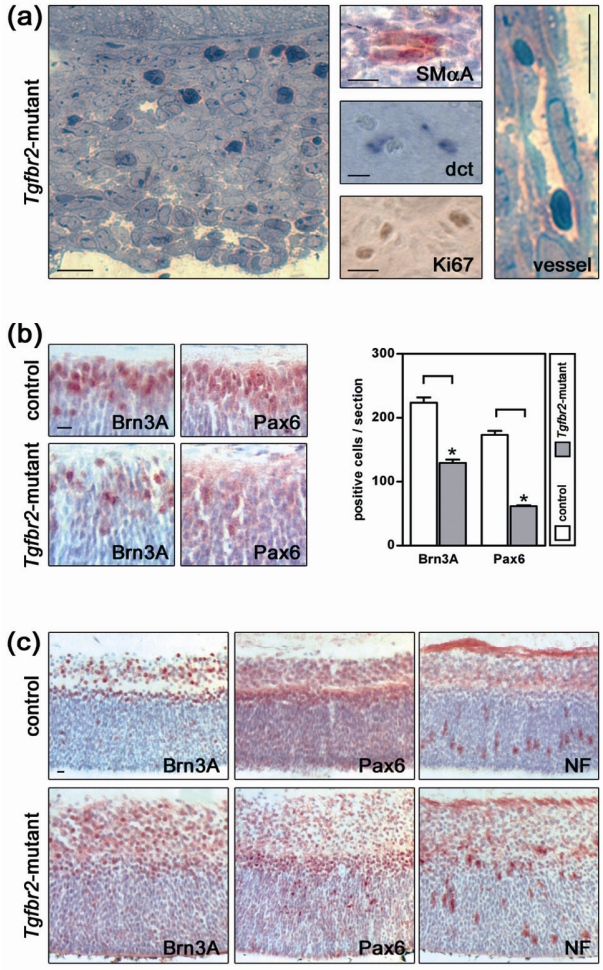


Figure 5.5. Persistent hypertrophic primary vitreous and disturbed retinal patterning in *Tgfbr2*-mutant mice.
(a) Detailed view of the persistent hypertrophic primary vitreous at E18 (left) with a dense retrolental cell mass, composed of different cell types, including smooth muscle α-actin (SMαA; red) positive pericytes and prospective melanocytes expressing dct (blue). Ki67 staining indicates cell proliferation (brown). Moreover, the persistent hypertrophic primary vitreous contains vessels of the hyaloid vascular system (right). (b) Expression of Brn3A and Pax6 is readily detectable at E15 in the inner retinal layers of control eyes. In *Tgfbr2*-mutant eyes, however, cells expressing these markers are less frequent (*, $p<0.001$). (c) At E18, staining for Brn3A, Pax6, and neurofilament (NF) reveals the expected patterning of the retina in control eyes and a diffuse distribution in *Tgfbr2*-mutant embryos. Thus, retinal patterning is disturbed in *Tgfbr2*-mutant embryos with persistent hypertrophic primary vitreous. Scale bars: 10 µm.

5.2.4. Expression of Foxc1 and Pitx2, which are both implicated in Axenfeld-Rieger's anomaly, is TGFβ-dependent in NC-derived ocular cells

Anterior eye segment anomalies in *Tgfbr2*-mutant mice were reminiscent of human Axenfeld-Rieger's anomaly (Figure 5.3). *In vivo* fate mapping revealed unaffected migration of TGFβ–dependent NC cells to the corneal stroma, endothelium, and trabecular meshwork in

Tgfbr2-mutant mice (Figure 5.6a). This indicates that the ocular malformations arise from impaired differentiation rather than from NC cell migration defects. Interestingly, the anomalies observed in the *Tgfbr2*-mutant embryos recapitulate aspects of ocular defects found in *Foxc1*- or *Pitx2*-null mice (Kume et al., 1998; Lu et al., 1999). Therefore, loss of TGFβ responsiveness in the cells of the periocular mesenchyme may affect expression of the transcription factors Foxc1 and Pitx2. To test this hypothesis, we analyzed eyes from *Tgfbr2*-mutant and control embryos at different developmental stages for the presence of Foxc1 and Pitx2. Both factors have been reported to be expressed in the periocular mesenchyme during early development (Figure 5.6b) (Hjalt et al., 2000; Kume et al., 1998), whereas at E15 Foxc1 localizes to the corneal endothelium and structures of the forming trabecular meshwork (Figure 5.6d), and Pitx2 to the corneal stroma (Figure 5.7a).

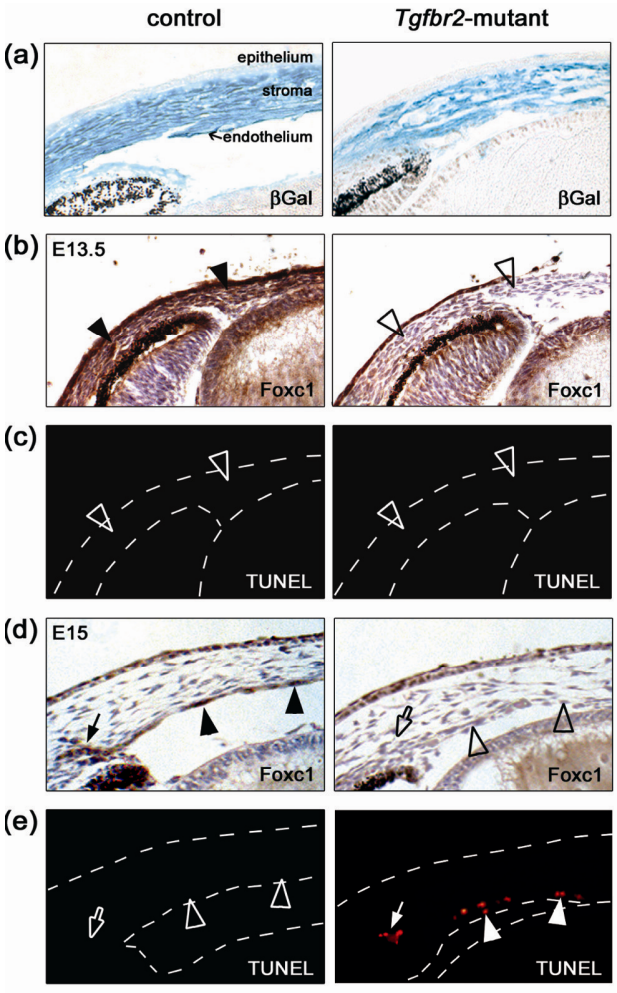


Figure 5.6. *Tgfbr2*-mutant mice lack corneal expression of the transcription factor Foxc1. (a) In vivo fate mapping at E15 (βGal; blue) demonstrates that NC-derived cells have correctly migrated into control and *Tgfbr2*-mutant eyes, contributing to corneal stroma and endothelium. (b) At E13.5, the periocular mesenchyme of control eyes is positive for Foxc1 (brown; arrowheads), whereas Foxc1 is undetectable in corresponding structures of *Tgfbr2*-mutant eyes (open arrowheads). (c) No apoptotic cells are found in control and *Tgfbr2*-mutant eyes at E13.5 by TUNEL analysis (open arrowheads). (d) At E15, the eyes of control embryos show strong expression of Foxc1 (brown) in the forming trabecular meshwork (arrow) and in corneal endothelial cells (arrowheads). In *Tgfbr2*-mutant eyes, NC-derived cells localize to the cornea, but Foxc1 is undetectable in prospective endothelial cells (open arrowheads) and in the forming trabecular meshwork (open arrow). (e) At E15, cells that fail to express Foxc1 in *Tgfbr2*-mutant eyes appear to undergo apoptosis, unlike in control eyes, as revealed by TUNEL analysis (red).

In contrast, in eyes of *Tgfbr2*-mutant embryos Foxc1 was hardly detectable in the periocular mesenchyme at E13.5 and in the forming chamber angle and corneal endothelium at E15. Moreover, *Tgfbr2*-mutant cells which failed to express Foxc1 appeared to subsequently undergo apoptosis around E15, as revealed by TUNEL staining (Figure 5.6e).

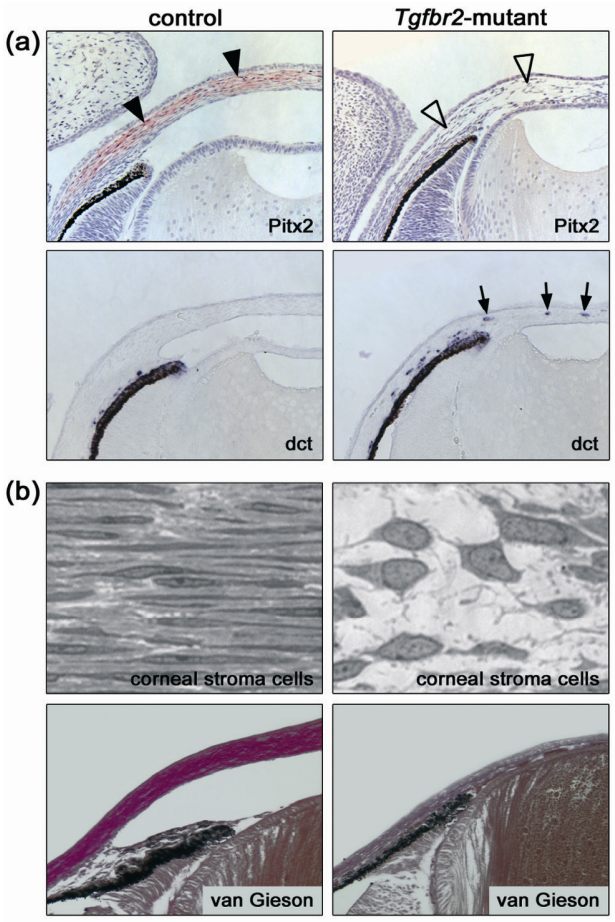


Figure 5.7. Absence of the transcription factor Pitx2 and of collagen formation in the corneal stroma of Tgfbr2-mutant mice.
(a) At E15, Pitx2 expression (red; arrowheads) is restricted to corneal stromal cells in control animals, but undetectable in the corresponding structures of *Tgfbr2*-mutant mice (open arrowheads). In situ hybridization for dct that marks prospective melanocytes reveals atypical expression in the corneal stroma of *Tgfbr2*-mutant embryos (arrows). (b) High magnification of the corneal stroma depicts typical appearance of thin keratocytes in a parallel orientation and a dense extracellular matrix in control eyes at E18. In contrast, corneal stroma of *Tgfbr2*-mutant embryos lacks extracellular matrix and presents cells with large nuclei and a polygonal shape. Van Gieson's staining reveals normal collagen matrix in the corneal stroma of control embryos (purple) that is absent in *Tgfbr2*-mutant embryos.

Pitx2 was strongly expressed in the corneal stroma at E15 in control eyes, but was undetectable in the eyes of *Tgfbr2*-mutant embryos (Figure 5.7a). Interestingly, some *Tgfbr2*-mutant cells of the corneal stroma expressed dct rather than Pitx2, pointing to incorrect fate acquisition towards melanocytes or misguidance during migration. At E18, the corneal stroma of control embryos consisted of thin keratocytes organized in a lamellar structure and embedded in extracellular matrix, which provides corneal stability and transparency (Figure 5.7b). Moreover, high levels of collagen were detectable in the corneal stroma of control mice. In contrast, collagen staining was negative in the malformed cornea of E18 *Tgfbr2*-

mutant mice, and stromal cells had an abnormal polygonal shape. In summary, NC-derived ocular cells that lack TGFβ responsiveness fail to express Foxc1 and Pitx2 and to undergo correct differentiation into corneal endothelial cells and collagen-synthesizing keratocytes of the corneal stroma.

5.2.5. TGFβ induces Foxc1 and Pitx2 expression in fibroblasts and in *ex vivo* eye cultures

The absence of Foxc1 and Pitx2 expression in developing eyes of *Tgfbr2*-mutant mice raises the question of whether TGFβ signaling is able to regulate Foxc1 and/or Pitx2 expression. To address this issue, cultured rat embryonic fibroblasts were treated with TGFβ, followed by Western blot analysis for the presence of Foxc1 and Pitx2 (Figure 5.8a). In the absence of TGFβ, cells showed weak expression of Foxc1 whereas Pitx2 expression was undetectable. TGFβ treatment, however, strongly increased Foxc1 expression and induced Pitx2 expression, concomitant with increased levels of pSmad2 (Figure 5.8a).

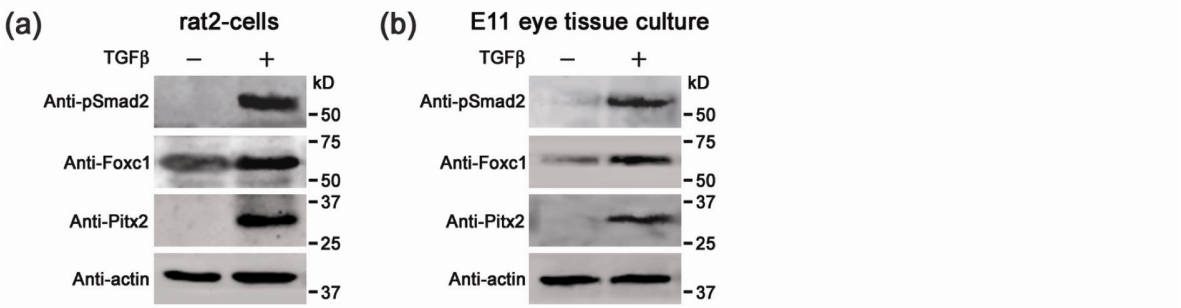


Figure 5.8. TGFβ regulates expression of Foxc1 and Pitx2.
(a) Rat embryonic fibroblasts (rat2) were treated with TGFβ (5ng/mL), which results in increased levels of phosphorylated (p)Smad2, a TGFβ downstream signaling component. Furthermore, TGFβ signaling enhances expression of Foxc1 and induces Pitx2 expression, as revealed by Western blot analysis. (b) In *ex vivo* short term tissue culture of eyes, including periocular mesenchyme from E11 embryos, TGFβ is able to strongly upregulate both Foxc1 and Pitx2 expression.

In addition to fibroblasts, postmigratory NC-derived cells of the periocular mesenchyme were also responsive to TGFβ, as shown in short term tissue cultures of eyes from E11 embryos (Figure 5.8b): Again, treatment with TGFβ resulted in elevated Foxc1 expression. Moreover, Pitx2 expression, which was undetectable in untreated samples, was induced upon addition of TGFβ. In summary, TGFβ treatment upregulates both Foxc1 and Pitx2 expression in a fibroblast cell line and in embryonic eye tissue cultures. Hence, TGFβ signaling is not only

required for the expression of transcription factors associated with developmental eye disorders, but it is also sufficient to regulate their expression.

5.3. DISCUSSION

This study demonstrates that targeted inactivation of TGFβ signaling in NC stem cells perturbs proper development of NC-derived structures in the eye, leading to malformations similar to those found in human Axenfeld-Rieger's anomaly and persistent hyperplastic primary vitreous. The importance of inductive signals from the lens for correct development of the anterior eye segment as well as for retinal patterning has previously been proposed (Genis-Galvez, 1966; Yamamoto and Jeffery, 2000). Mutation in genes causing lens anomalies and subsequent abnormal eye formation have further supported this notion (Jamieson et al., 2002; Semina et al., 2001). Here, we propose that one of the key signaling molecules involved in these processes is TGFβ2, which is highly expressed in the lens at early stages of eye development. Among the signal-receiving cell types, NC-derived cells play a major role in ocular development. According to earlier studies in avian models, NC cells contribute to the developing anterior eye segment (Johnston et al., 1979). Using *in vivo* fate mapping of NC cells, we have extended these findings to a mammalian model and demonstrate that NC-derived cells already contribute to the forming eye vesicle. Later, corneal endothelium and stromal keratocytes, and structures of the chamber angle all originate from the NC. In addition, we found a contribution of NC to the primary vitreous, which normally contains a transient network of vessels that supports the inner eye during development. Intriguingly, all these NC-derived tissues fail to develop properly in the absence of TGFβ signaling although NC cell migration into the forming eye remains unaffected (Figure 5.9). Moreover, we show that transcription factors implicated in anterior eye development are targets of TGFβ signaling. Thus, our data indicate that ocular anomalies in mutant mice are due to the absence of a postmigratory response of NC-derived cells to ocular TGFβ.

5.3.1. NC cell-specific TGFβ signal inactivation leads to defects of the posterior eye segment

The primary vitreous is situated directly behind the lens, containing the hyaloid vascular system beneath NC-derived cells. Normally, the primary vitreous regresses during postnatal eye maturation through tissue remodelling by apoptosis and phagocytosis, thereby generating the avascular, transparent secondary vitreous (Ito and Yoshioka, 1999). In patients suffering

from persistent hyperplastic primary vitreous, a dense cell membrane persists between lens and retina. This congenital disorder is often accompanied by cataract, secondary glaucoma, and a variable degree of microphtalmia (Amaya et al., 2003; Reese, 1955). Similarly, the primary vitreous in the eyes of *Tgfbr2*-mutant mice appears as a dense cellular membrane, and mutant eyes are smaller than those in control mice. Much like in human persistent hyperplastic primary vitreous (Haddad et al., 1978), the persistent retrolental cell mass in *Tgfbr2*-mutant mice contains fibroblast-like cells, pigmented cells, and vessels of the hyaloid vascular system. In addition, proliferating cells were seen. Other gene mutations in mice have been reported to be associated with a persistent hyperplastic primary vitreous-like phenotype, including *Arf1, Bmp4,* and *p53* mutations (Chang et al., 2001; McKeller et al., 2002; Reichel et al., 1998). In these models, normal postnatal regression of the primary vitreous fails, resulting in a variable degree of anomalies reminiscent of persistent hyperplastic primary vitreous. Similarly, a dense cell mass in the posterior eye has also been observed in *Tgfbr2*-mutant mice, but this has not been further analyzed (Sanford et al., 1997). Moreover, treatment of pregnant mice with retinoic acid, which is known to interfere with TGFβ signaling (Pendaries et al., 2003), induces anomalies similar to persistent hyperplastic primary vitreous in offspring (Ozeki et al., 1999). Thus, we conclude that TGFβ signaling in NC-derived cells constituting the primary vitreous is important for tissue morphogenesis.

In the posterior eye segment, retinal development is also disturbed upon ablation of *Tgfbr2* in neural crest cells, apart from the generation of persistent hyperplastic primary vitreous. In particular, we observed increased retinal apoptosis at E15 and abrogated retinal patterning, as shown by histology and layer-specific tissue marker expression. Because there is no NC contribution to the retina, this phenotype is likely due to a secondary, non-cell autonomous effect. Conceivably, the dense persistent primary vitreous in *Tgfbr2*-mutant mice may constrain instructive signals from the lens to the retina. However, such putative signals remain to be identified.

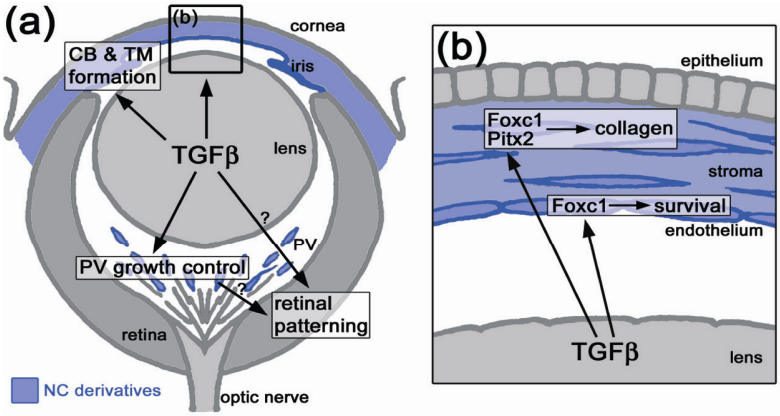


Figure 5.9. TGFβ-dependent development of anterior and posterior ocular structures.
(a) Neural crest (NC)-derived cells (blue) contribute to structures of the anterior eye segment and the primary vitreous (PV). TGFβ signaling is involved in the formation of the ciliary body (CB) and the trabecular meshwork (TM), and in growth control of the PV. Moreover, normal PV development and/or TGFβ signaling are important for correct retinal patterning. (b) In the cornea, prospective stromal keratocytes and endothelial cells are of NC origin. Here, TGFβ signaling is needed for the expression of the transcription factors Foxc1 and Pitx2 and for normal differentiation of NC-derived cells into collagen-synthesizing stromal keratocytes. Moreover, in forming corneal endothelial cells (and in the TM), expression of Foxc1 and cell survival requires TGFβ signaling.

5.3.2. TGFβ signal-dependent transcription factors and the generation of Axenfeld-Rieger`s anomaly

In addition to the defects reminiscent of persistent hyperplastic primary vitreous, all *Tgfbr2*-mutant mice display several developmental defects in the anterior eye. The anterior chamber of the eye is absent in the mutant because cornea and lens fail to separate. Furthermore, normal formation of the ciliary body and of the chamber angle with the trabecular meshwork requires TGFβ signaling, as these structures are defective in the mutant mice. The abnormalities presented by *Tgfbr2*-mutant mice are characteristic of the disorders found in patients with Axenfeld-Rieger's anomaly (Cvekl and Tamm, 2004). There, anterior segment dysgenesis impairs the regulation of the intraocular pressure, which frequently leads to developmental glaucoma. Other mouse mutants have also been implicated as models for developmental anterior eye disorders. Mice homozygous for an inactivating mutation of *Pax6*, a candidate for human Peter's anomaly, lack eyes (Graw, 2003). Heterozygous mice have defects in the anterior eye segment, although less severe than those found in *Tgfbr2*-mutants (Collinson et al., 2001). However, the expression of Pax6 in eyes of *Tgfbr2*-mutant mice is not affected (data not shown), suggesting that their defects do not depend on Pax6 modulation. In human Axenfeld-Rieger's anomaly, mutations have been found in the genes

encoding the transcription factors FOXC1 and PITX2 (Alward, 2000). Deletion of either *Foxc1* or *Pitx2* (Kume et al., 1998; Lu et al., 1999) in mice leads to defects in the anterior eye segment, very similar to those in *Tgfbr2*-mutant mice described in this study. In the eye, Foxc1 is expressed in the forming corneal stroma and endothelium and, at later stages, in the structures of the prospective trabecular meshwork (Kume et al., 1998). Intriguingly, these structures express Foxc1 in a TGFβ signal-dependent manner, and *Tgfbr2*-mutant prospective corneal endothelial and trabecular meshwork cells undergo apoptosis not observed in control eyes. Furthermore, TGFβ upregulates Foxc1 expression in fibroblasts and cultured eye tissue, in agreement with a previous report that described *Foxc1* as a target gene of TGFβ in human cancer cell lines (Zhou et al., 2002). Thus, the data suggest that lens-derived TGFβ signaling controls survival and development of the NC-derived periocular mesenchyme that gives rise to corneal endothelium and trabecular meshwork by regulating Foxc1 expression in these cells (Figure 5.9).

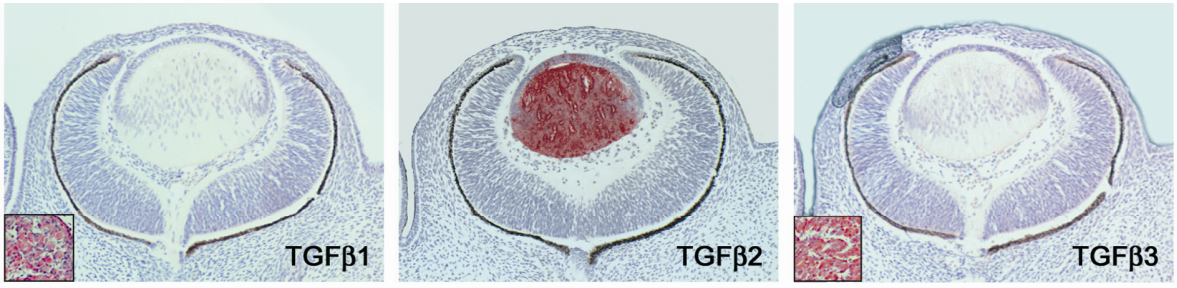
Pitx2 is predominantly expressed in NC-derived corneal stromal cells that become collagen-synthesizing keratocytes. In *Tgfbr2*-mutant mice, however, corneal stromal cells do not express Pitx2, and consequently, fail to develop into collagen-synthesizing keratocytes. Recently, mutations in the human *Tgfbr2* gene have been reported to cause Marfan's syndrome, a disorder also associated with defective extracellular matrix synthesis (Mizuguchi et al., 2004). Thus, we conclude that corneal NC-derived cells have to undergo TGFβ-dependent expression of Pitx2 and differentiation to become stromal keratocytes that produce the collagen matrix (Figure 5.9). In support of this hypothesis, Pitx2 expression is strongly induced in fibroblasts and eye tissue upon TGFβ signal activation. In Axenfeld-Rieger's anomaly patients who have a disease-linked mutation in the *PITX2* gene, ocular anomalies appear to be accompanied by additional defects, including tooth abnormalities, redundant periumbilical skin, and heart defects (altogether referred to as Rieger's syndrome) (Alward, 2000). Beside its expression in NC-derived cells of the forming eye, Pitx2 is expressed in several other tissues during development, including the teeth, umbilicus, and the heart (Hjalt et al., 2000). In contrast to the mesenchymal expression pattern in the eye, in other organs the expression of Pitx2 is restricted to structures that are not NC derived. However, these structures, and especially tooth anlagen, are surrounded by or are in close contact to NC-

derived cells (Chai et al., 2000). Nevertheless, *Tgfbr2*-mutant embryos display no anomalies of tooth anlagen and umbilicus at E18 (data not shown). Therefore, Pitx2-dependent anomalies in *Tgfbr2*-mutant mice seem to be restricted to the eyes, although because of embryonic lethality we could not determine whether there are additional Pitx2-dependent defects at a developmental stage later than E19.

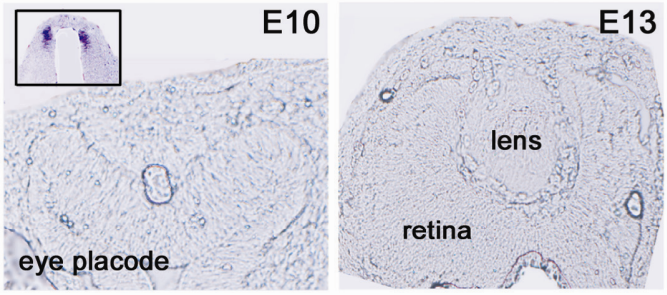
We recently reported that inactivation of TGFβ signaling in NC stem cells also leads to cardiac and craniofacial defects, and parathyroid and thymic gland anomalies, reminiscent of human DiGeorge syndrome (Wurdak et al., 2005). Moreover, depending on the cellular context, TGFβ promotes non-neural cell fates in cultured NC cells (Hagedorn et al., 1999; Shah et al., 1996). Hence, together with the findings from the present study, there is good evidence that TGFβ is a key modulator of non-neural differentiation of postmigratory NC cells during development of multiple tissues, including the eye.

In conclusion, we show extensive NC contribution to the developing anterior eye segment and, to our knowledge for the first time, to the primary vitreous. Moreover, proper differentiation of NC-derived ocular cells is TGFβ dependent (Figure 5.9). In detail, TGFβ is involved in growth restriction of the primary vitreous, and consequently, *Tgfbr2*-mutant mice suffer from persistent hyperplastic primary vitreous. In the anterior eye segment, anomalies in *Tgfbr2*-mutant mice are reminiscent of human Axenfeld-Rieger's anomaly. Ocular expression of *Pitx2* and *Foxc1*, which when mutated can cause Axenfeld-Rieger's anomaly, is TGFβ-dependent, suggesting that both transcription factors are involved in mediating TGFβ signaling in ocular cells during development. Interestingly, a report of a family suffering from both Axenfeld-Rieger's anomaly and persistent hyperplastic primary vitreous suggested a common linkage between genes for Axenfeld-Rieger's anomaly and persistent hyperplastic primary vitreous (Storimans and Van Schooneveld, 1989). Thus, our findings may lead to further understanding of the pathophysiology of Axenfeld-Rieger's anomaly and persistent hyperplastic primary vitreous.

5.4. SUPPLEMENTARY MATERIAL



Supplementary Figure 5.1. Expression of TGFβ isoforms during eye formation.
While TGFβ1 (left panel) and TGFβ3 (right panel) are not expressed in the developing eye, the expression of TGFβ2 (red; middle panel) peaks at E13.5 and is restricted to the lens. Insets serve as control and illustrate areas in the trigeminal ganglia positive for TGFβ1 and 3 on same sections.



Supplementary Figure 5.2. Absence of Wnt1 expression during eye formation.
Wnt1 in situ hybridization analyses at E10 (left panel) and E13 (right panel) revealed that wnt1 expression is readily detectable in the dorsal neural tube (blue; insert), leading to *Wnt1-Cre*-mediated recombination in virtually all neural crest stem cells (Wurdak et al., 2005). However, on same sections wnt1 is not expressed in ocular structures

TGFβ signaling regulates midbrain size and self-renewal of neural stem cells

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Regulating the choice between stem cell maintenance versus differentiation crucially determines growth and size of organs during embryonic development. However, signaling networks and molecular mechanisms controlling proliferation, self-renewal, and differentiation are poorly understood. Here we demonstrate a crucial role of TGFβ signaling in these processes. Tissue-specific deletion of *TGFβ receptor type II* (*Tgfbr2*) in the midbrain/hindbrain region results in ectopic Wnt/β-catenin expression and subsequently in a drastic increase of dorsal midbrain size. This phenotype is due to horizontal expansion of the proliferating neuroepithelial cell population at the expense of differentiation, while patterning of the midbrain/hindbrain area remains unaffected. Moreover, self-renewing proliferation of *Tgfbr2*-deficient neuroepithelial stem cells of the mutant dorsal midbrain is strongly enhanced and associated with persistent activation of β-catenin. Thus, TGFβ signaling antagonizes canonical Wnt signaling and negatively regulates self-renewal and stem cell pool expansion during midbrain development.

6.1. INTRODUCTION

The central nervous system (CNS) develops from self-renewing, multipotent neural stem cells that give rise to neural progenitor cells and eventually to neurons, astrocytes, and oligodendrocytes (Temple, 2001). Neural stem cells are present throughout the developing nervous system, but their frequency rapidly decreases with time until they become restricted to specific areas in the adult brain (Alvarez-Buylla et al., 2001). The balance between neural stem and progenitor cell proliferation versus differentiation is a crucial factor determining growth and size of the developing brain (Ohnuma and Harris, 2003). Neural stem cells can undergo symmetric, non-neurogenic divisions that produce two neuroepithelial stem cells and cause horizontal expansion of the proliferative population. Alternatively, they engage in asymmetric divisions that are associated with the generation of neuronal progenitors (Götz and Huttner, 2005). Moreover, symmetric neurogenic divisions on the basal side of the neuroepithelium can contribute to rapid expansion of neuronal cells. Thus, the number of neural stem cells and of their progeny critically depends on when the cells switch from symmetric to asymmetric divisions and on whether the cells re-enter or exit the cell cycle (Ohnuma and Harris, 2003).

Multiple signaling pathways have been implicated in the regulation of neural stem and progenitor cell proliferation. In cell culture, fibroblast growth factor 2 (FGF2) and epidermal growth factor (EGF) act as mitogenic signals (Temple, 2001). *In vivo* and in cell culture, inactivation of various Notch signaling components leads to a depletion of the neural stem cell pool both in the developing CNS and in the adult subventricular zone (SVZ) (Hitoshi et al., 2002; Nyfeler et al., 2005; Ohtsuka et al., 2001). Similarly, sonic hedgehog (Shh) has been proposed to control neural stem cell maintenance in the adult SVZ (Machold et al., 2003) and proliferation of neural stem and progenitor cells in parts of the developing CNS and in the adult hippocampus (Lai et al., 2003; Ruiz i Altaba et al., 2002). Manipulation of the canonical Wnt signaling pathway also leads to dramatic changes in neural stem and progenitor populations (Kleber and Sommer, 2004). In particular, overexpression of Wnts or persistent activation of the downstream signaling component β-catenin in the CNS promotes cell cycle progression and negatively regulates cell cycle exit, resulting in horizontal expansion of precursor populations (Chenn and Walsh, 2002; Megason and McMahon, 2002). However, *in*

vivo overexpression of *Wnt1* in the midbrain of transgenic mice leads to increased cell proliferation only in the dorsal but not ventral midbrain (Panhuysen et al., 2004), while in cultures of dissociated ventral midbrain cells Wnt1 is able to enhance proliferation concomitant with downregulation of cell-cycle inhibitor molecules such as p27 (Castelo-Branco et al., 2003). Thus, the signaling pathways regulating neural stem and progenitor cell proliferation might be brain area-specific and influenced by the cellular context.

While the aforementioned signaling factors positively control maintenance and expansion of neural stem and progenitor pools, transforming growth factor (TGF)β upregulates cell cycle inhibitors and counteracts cell cycle progression in neuroepithelial cells in a context-dependent manner, at least in cultures of dissociated cells or in cortical slice cultures (Seoane et al., 2004; Siegenthaler and Miller, 2005). TGFβ, which signals through activation of TGFβ receptor type I and II (TGFβRI and TGFβRII) and phosphorylation of the signaling effectors Smad2 and Smad3, elicits multiple cellular responses, including a cytostatic effect on various cell types (Siegel and Massague, 2003). Ligand and receptor as well as phosphorylated Smad2 are expressed at early developmental stages in the ventricular zone (VZ) (de Sousa Lopes et al., 2003; Miller, 2003), consistent with a role of TGFβ signaling in controlling neural stem and progenitor cell numbers. Although TGFβ has been associated with neuronal differentiation and survival in the CNS (Brionne et al., 2003; Farkas et al., 2003; Krieglstein et al., 2000), the function of TGFβ signaling in neural stem and progenitor cells *in vivo* remains to be determined. Here we show that in the midbrain, TGFβ represses Wnt/β-catenin expression and controls neural stem cell self-renewal in a region-specific manner.

6.2. RESULTS

6.2.1. Loss of TGFβ signaling leads to enlargement of the dorsal midbrain

To elucidate the role of TGFβ signaling during midbrain/hindbrain development *in vivo*, we have used the *cre/loxP* system to conditionally ablate the *TGFβ receptor type II* (*Tgfbr2)* gene (Figure 6.1a). Since Wnt1-expressing neural progenitor cells are major contributors to midbrain/hindbrain formation (Dymecki and Tomasiewicz, 1998), *Wnt1-Cre*-mediated deletion of *Tgfbr2* was used as previously described (Wurdak et al., 2005). *In vivo* fate mapping of neural cells harboring *Wnt1-Cre* activity was performed using the ROSA 26 (R26R) reporter mouse line (Hari et al., 2002; Wurdak et al., 2005). X-Gal staining was readily detectable in the forming midbrain and hindbrain of both control and mutant specimen at embryonic stage (E) 12.5 (Figure 6.1b-d**,** data not shown). Examination of X-Gal-stained sagittal brain sections revealed a major contribution of β-galactosidase-expressing cells to dorsal midbrain, isthmus and cerebellum (Figure 6.1c). Ventral midbrain and pons displayed fewer β-galactosidase-expressing cells. These were intermingled with a large number of non-recombined cells, preventing in particular the analysis of dopaminergic neuron formation in the ventral midbrain upon TGFβ signal inactivation (Figure 6.1c). Overall X-Gal staining intensities and distribution of β-galactosidase-positive cells were not changed in mutant compared to control brains at E12.5 (Figure 6.1c, data not shown).

Wnt1-Cre-mediated deletion of *Tgfbr2* resulted in undetectable TGFβRII protein in mutant cells populating the midbrain/hindbrain area at E12.5 (Figure 6.1d). In addition, levels of phosphorylated-Smad2 (pSmad2) were strongly reduced, indicating loss of TGFβ signal transduction to downstream signaling components (Supplementary Figure 6.1). Intriguingly, in control embryos pSmad2 was not yet detectable at E10.5, reached a peak level at around E12, and drastically decreased at E13, indicating a narrow time window of TGFβ signal activity during formation of the isthmus and adjacent midbrain and cerebellar structures (Supplementary Figure 6.1).

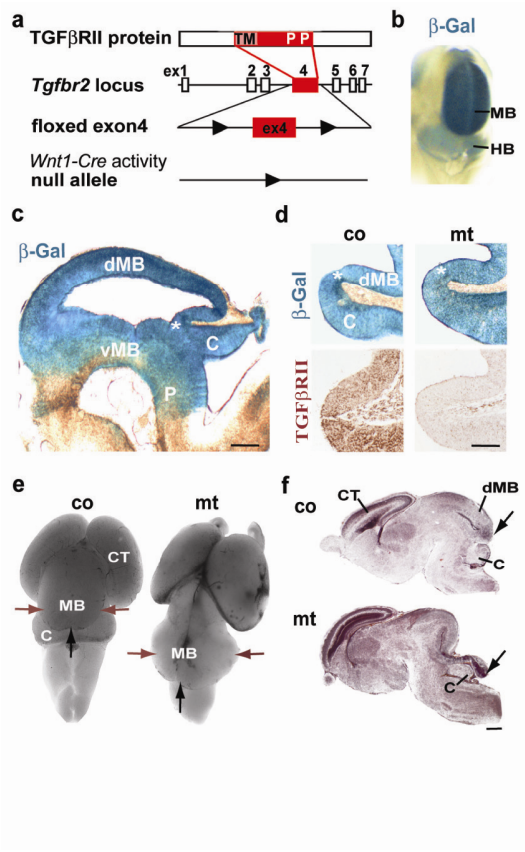


Figure 6.1. Conditional ablation of *Tgfbr2* during midbrain/hindbrain development.
(a) Exon (ex) 4 of the *Tgfbr2* locus, encoding the transmembrane domain (TM) and the intracellular phosphorylation sites (P) of the TGFβRII protein, is flanked by loxP-sites and deleted upon breeding with *Wnt1-Cre* mice. (b) Dorsal view of midbrain (MB) and hindbrain (HB) area of E12.5 control embryo displaying Cre-mediated β-galactosidase expression (blue). (c) X-Gal stained sagittal brain section of E12.5 control embryo showing contribution of β-galactosidase-expressing cells to distinct midbrain/hindbrain structures including dorsal midbrain (dMB), ventral midbrain (vMB), cerebellum (C), pons (P), and isthmus (asterisk). (d) Presence and absence of TGFβRII (brown) in β-galactosidase expressing cells (blue) populating the isthmus (asterisk) and adjacent structures of cerebellum (C) and dorsal midbrain (dMB) of E12.5 control (co) and mutant (mt) embryos. (e,f) Gross morphology of E18 control and mutant brains showing a drastic expansion of the mutant midbrain (e, dorsal view, CT: cortex, MB: midbrain, C: cerebellum). Haematoxylin/Eosin-stained sagittal brain sections (f) reveal expansion of the mutant dorsal midbrain (dMB) in lateral (brown arrows) and caudal (black arrows) direction covering the normal appearing cerebellum (C). Secondary effects on cortex (CT) development are due to neural crest-specific defects (Wurdak et al., 2005). Scale bars: c,d, 100 µM; f, 500 µM.

In the mutant, morphological alterations became first detectable macroscopically at E13.5. In particular, the *Tgfbr2*-deficient midbrain was enlarged as compared to the control brain. This aberrant expansion increased with embryonic age and culminated in a drastic lateral and caudal tissue enlargement, fully covering the cerebellum at E18 (Figure 6.1e,f, data not shown). In contrast, the morphology of the mutant cerebellum appeared normal throughout embryonic development. Mutant embryos died before birth due to multiple developmental defects, including severe cardiac anomalies (Wurdak et al., 2005).

6.2.2. Mutant embryos display overproliferation of midbrain neuroepithelial structures

A local organizing center located at the midbrain/hindbrain boundary termed isthmus organizer is implicated in controlling early development and identity of adjacent midbrain/hindbrain areas. The question arises whether alterations in the *Tgfbr2*-mutant midbrain may originate from a disturbed patterning of the isthmus region. However, we found no alteration in the expression of the homeobox transcription factor otx2, a key factor

positioning the isthmus organizer (Broccoli et al., 1999), in mutant compared to control embryos at E12.5 (Supplementary Figure 6.2). Consistent with this finding, no aberrant morphology of the mutant midbrain/hindbrain junction was observed, indicating correct positioning of the isthmus and adjacent areas.

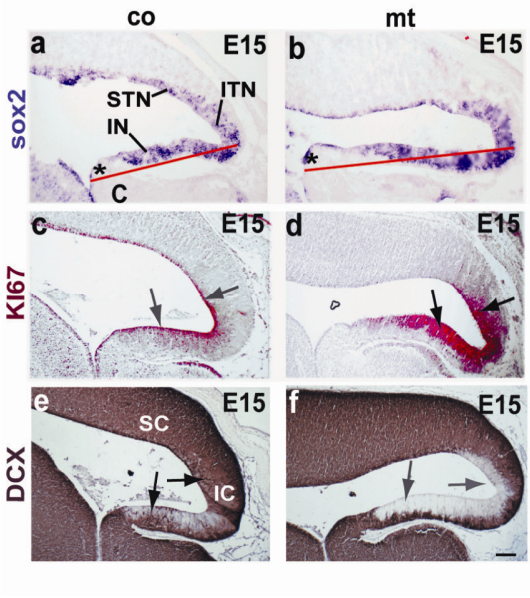


Figure 6.2. Overproliferation and reduced differentiation in the mutant dorsal midbrain at E15. (a,b) sox2 in situ hybridization analysis (blue) of sagittal brain sections visualizes the isthmal neuroepithelium (IN), inferior tectal neuroepithelium (ITN) and superior tectal neuroepithelium (STN) of control and mutant dorsal midbrains. Red lines indicate the distance between isthmus (asterisk) and the caudal limit of the sox2-positive neuroepithelium of control (line: 0.59 ± 0.04 mm) and mutant (line: 0.92 ± 0.08 mm) dorsal midbrains, demonstrating caudal tissue extension in mutants ($p<0.01$, n=3, 2 sections/animal). (c,d) KI67 staining (red) reveals massively increased proliferation of the mutant isthmal and inferior tectal neuroepithelium. Corresponding control tissue shows restricted KI67-positive staining. (e,f) Doublecortin (DCX) immunostaining (brown) is detected in control structures (black arrows) but absent in corresponding proliferative mutant structures (grey arrows). Scale bar 100 µM.

Alternatively, midbrain expansion in *Tgfbr2*-mutant embryos might originate from decreased apoptosis. Programmed cell death was examined by TUNEL staining at different developmental stages. However, only few apoptotic cells were detected in the normal midbrain/hindbrain area between E13 and E15, and no decrease in the number of apoptotic cells was apparent in the mutant (data not shown). Thus, expansion of the mutant midbrain cannot be explained by reduced cell death. To test whether absence of TGFβ signaling affects neuroepithelial cell proliferation, we analyzed expression of the proliferation marker KI67 and of the neuroepithelial marker sox2, a transcription factor associated with maintenance of neural progenitor features (Graham et al., 2003). Unlike in the control, proliferation in the so-called isthmal and inferior tectal neuroepithelium strongly increased during development of mutant embryos from E13 to E15, and mutant dorsal midbrains at E15 displayed massively enhanced proliferation within the sox2-positive neuroepithelium, concomitant with a notable tissue expansion in the caudal direction (Figure 6.2a-d). In contrast, immunostaining for

doublecortin (DCX), a marker for immature neuronal cells (Rao and Shetty, 2004), revealed decreased differentiation in mutant as compared to control neuroepithelial structures (Figure 6.2e,f). In particular, at E15 the DCX-negative domain only constituted 11.8 ± 0.2% of the prospective inferior colliculus area in controls, while in mutants the DCX-negative area appeared to match the KI67-positive area and constituted 42.5 ± 0.8% of the total inferior colliculus area (n=3 animals, analyzing 2 sections/animal; $p < 0.01$). Thus, increased proliferation and horizontal expansion in *Tgfbr2*-deficient midbrains occurred at the expense of differentiation.

6.2.3. Mutant dorsal midbrains show ectopic canonical Wnt signaling

Canonical Wnt signaling via the intracellular signaling component β-catenin is a key signaling pathway implicated in cell fate determination and proliferation. Wnt/β-catenin signal activation regulates expansion of progenitor cells in the developing cortex and other brain areas (Chenn and Walsh, 2002; Kleber and Sommer, 2004; Panhuysen et al., 2004). To test whether midbrain neuroepithelial expansion in the absence of TGFβ signaling is linked to Wnt signaling, we first examined the expression of wnt1 mRNA in control and mutant dorsal midbrains by in situ hybridization analysis at E12.5, around the stage of peak TGFβ signaling activity (Supplementary Figure 6.1). Intriguingly, we found ectopic expansion of the wnt1 domain in the mutant isthmal neuroepithelium (Figure 6.3a). Additionally, staining for cyclin-dependent cell cycle regulator p27Kip1 (p27) was reduced in the domain of ectopic wnt1 expression (Figure 6.3a). Canonical Wnt signaling leads to stabilization of β-catenin and its translocation into the nucleus (Reya and Clevers, 2005). Immunostaining combined with confocal microscopy demonstrated nuclear accumulation of β-catenin specifically in cells populating the region of ectopic wnt1 in E13 mutant embryos (Figure 6.3b). Corresponding control cells lacked nuclear β-catenin and exhibited only the form of the protein associated with cell-cell adhesion complexes (Reya and Clevers, 2005). Furthermore, Western blot analysis using an antibody specific for dephosphorylated β-catenin confirmed the presence of activated protein in E12.5 mutant dorsal midbrains, while it could not be detected in controls (Figure 6.3c). Thus, TGFβ signal inactivation in the midbrain/hindbrain region results in nuclear accumulation of activated β-catenin in the dorsal midbrain.

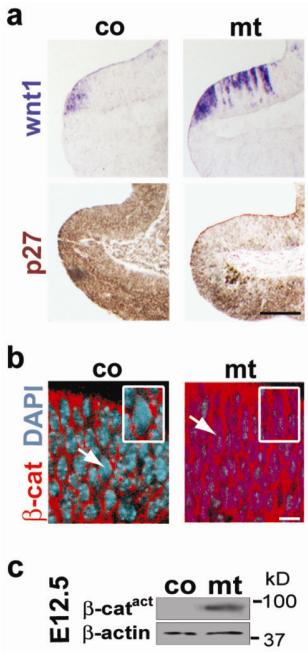


Figure 6.3. Ectopic canonical Wnt signaling in the dorsocaudal mutant midbrain.
(a) wnt1 in situ hybridization analysis (blue) and p27Kip1 (p27) immunostaining (brown) of E12.5 adjacent sagittal sections show ectopic expression of wnt1 in the mutant isthmal neuroepithelium concomitant with strongly reduced p27. Scale bar: 100 µM. (b) β-catenin immunostaining (red) combined with DAPI nuclear labelling (blue) demonstrates nuclear localization of the protein (purple) in mutant tissue at E13. Single confocal planes focusing on the region of ectopic wnt1 in the mutant and the corresponding control region are shown. Insets show higher magnification of cells indicated by arrows. Scale bar: 10 µM. (c) Western blot analysis demonstrates presence of activated β-catenin (β-cat^{act}) in the mutant dorsal midbrain at E12.5. Activated β-catenin was below detection level in cells isolated from control dorsal midbrains.

6.2.4. Absence of TGFβ signaling affects self-renewal capacity but not multipotency of neural stem cells

Horizontal expansion of *Tgfbr2*-mutant dorsal midbrain epithelium at the expense of differentiation might result from persistent non-neurogenic divisions of neural progenitor cells, while normally many neuroepithelial cells switch to neurogenic divisions during development and eventually exit the cell cycle and differentiate. Transition from proliferative progenitor cell divisions to neurogenic divisions is accompanied by a lengthening of the cell cycle (Calegari et al., 2005). Thus, if the neuroepithelial expansion in *Tgfbr2*-deficient midbrain is indeed due to continuous non-neurogenic divisions, cell cycle should be shorter in the mutant than in the control. To estimate the cycling time of proliferating cells, we performed cell cycle FACS analysis in combination with KI67 staining of dissociated cells from control and mutant dorsal midbrains (Figure 6.4). S phase stays relatively constant in mammalian cells while the length of G1 regulates cell cycle time (Takahashi et al., 1995). Hence, the relative fraction of proliferating progenitor cells passing through S phase is higher in cell populations with shorter cell cycle time. At E10.5, proliferation and the fraction of cells in S phase was similar in mutant and control embryos (data not shown), consistent with TGFβ peak activity occurring after this stage (Supplementary Figure 6.1). However, in the

dorsal midbrain at E13, the relative fraction of proliferative cells in S phase was significantly higher in the mutant as compared to the control (difference control vs. mutant: 14.3±6.3%; n=4; p=0.028) (Figure 6.4b). Thus, a significant population of *Tgfbr2*-mutant neuroepithelial cells divide with a shorter cell cycle time than do control cells, suggesting maintained non-neurogenic divisions of mutant progenitor cells.

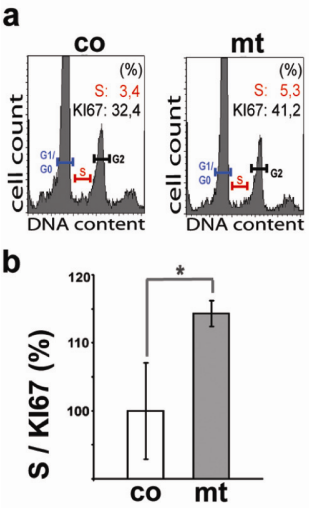


Figure 6.4. Increased fraction of proliferative S phase cells in the mutant dorsal midbrain.
(a) FACS analysis of KI67 and cell cycle distribution of dissociated E13 dorsal midbrain cells. A representative panel of control and mutant cell cycle FACS analysis with percentages of G0/G1, S, and G2 phase, as well as the percentage of proliferative cells determined by KI67 FACS analysis is shown. Note that consistent with the phenotypic dorsal midbrain expansion in mutant embryos, the percentage of KI67-positive cells is significantly increased (n=4, p<0,01) in mutant (41,5±0,5%) as compared to control (35,9±2,6%) dorsal midbrains. (b) The percentage of the total fraction of proliferating cells present in S phase (S/KI67; mean of control is defined as 100%) is significantly increased in mutants (p=0.028, n=4), indicating a shorter cell cycle time in mutant dorsal midbrains.

The above findings conceivably reflect an influence of TGFβ on stem cell self-renewal, proliferation of progenitors, or cell lineage decision. To address on the cellular level how loss of TGFβ signaling and concurrent activation of the Wnt pathway might influence neural stem and progenitor cells, we cultured neurospheres (Reynolds and Weiss, 1996) derived from dissociated dorsal midbrains of E12.5 and E15 control and *Tgfbr2*-mutant embryos. Recombination efficiency within primary, secondary, and tertiary neurospheres was assessed by X-Gal staining (Figure 6.5a). In both control and mutant cultures virtually all spheres were positive for X-Gal staining in agreement with the major contribution of *Wnt1-Cre*-recombined cells to dorsal midbrain structures *in vivo* (Figure 6.1). Both at E12.5 and E15 formation of primary spheres was significantly increased in mutant compared to control cultures (Figure 6.5b, Supplementary Figure 6.3b). Interestingly, increased formation of mutant spheres was observed in EGF-deficient/FGF-containing medium but not in FGF-deficient/EGF-containing medium, suggesting an initial expansion mainly of FGF-responsive neural stem/progenitor

cells in mutant tissue (Supplementary Figure 4). In order to compare the self-renewal capacity of mutant and control stem/progenitor cells, single primary spheres were dissociated and repropagated at clonal density. The number of secondary spheres per primary sphere was significantly increased in mutant cultures, suggesting a higher number of self-renewing neural stem/progenitor cells present in mutant dorsal midbrains at E12.5 as well as at E15 (Figure 6.5b, Supplementary Figure 6.3b). Moreover, consistent with increased levels of β-catenin *in vivo* (Figure 6.3), activated β-catenin was prominently detected in mutant but not in control cultures of secondary neurospheres (Figure 6.5a, Supplementary Figure 6.3a).

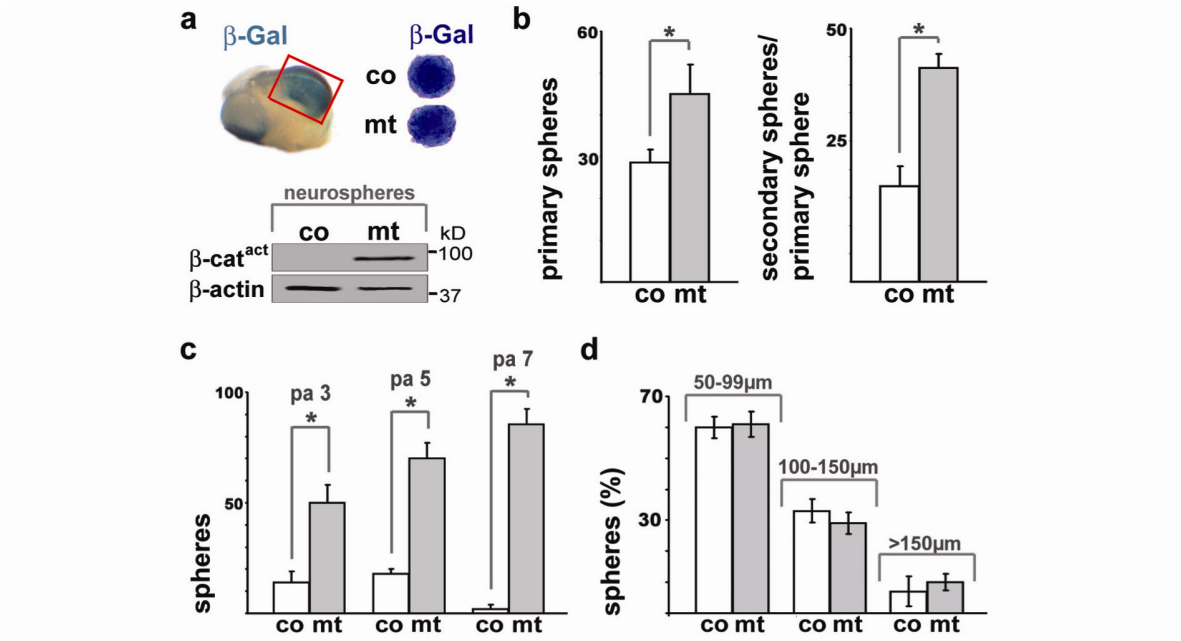


Figure 6.5. Mutant neural stem cells show increased self-renewal while progenitor cell proliferation is unaffected.
(a) Cells isolated from E12.5 control and mutant dorsal midbrains (top, left, red box) give rise to β-Gal-positive neurospheres (top, right). Western blot analysis demonstrates increased level of activated β-catenin (β-cat^{act}) in secondary neurospheres (bottom). (b) Neurosphere assays at E12.5 show a significantly increased number of mutant primary spheres and of mutant secondary spheres per primary sphere (asterisk; $p<0.01$, n=5). (c) Successive increase in number of E12.5 clonally-derived mutant spheres over seven serial passages (pa). The third, fifth and seventh passages are shown (asterisk; $p<0.01$, n=3). (d) Similar percentages of small diameter, medium diameter and large diameter control and mutant primary neurospheres at E12.5 (n=3).

To analyze the self-renewal activity of mutant compared to control neural stem cells over time, primary neurospheres generated from dorsal midbrains at E12.5 and E15 were

dissociated and serially propagated at clonal density (Figure 6.5c, Supplementary Figure 6.3c). Strikingly, mutant sphere formation steadily increased with each successive passage. In particular, in neurosphere cultures derived from mutant dorsal midbrains at E12.5 the frequency of spheres at passage 7 exceeded that at passage 3 by a factor of 1.6±0.16 ($p<0.01$; n=3). In contrast, in cultures from E12.5 and E15 dorsal midbrains formation of control spheres remained relatively constant at significantly lower levels over six passages and decreased towards zero in the seventh passage (Figure 6.5c, Supplementary Figure 6.3c). These data indicate not only a greater number of neural stem cells in primary mutant tissue, but also enhanced self-renewal of mutant neural stem cells over time.

Besides their ability to self renew, the key characteristic of neural stem cells is tripotency (Reynolds and Rietze, 2005). Upon differentiation, about 70% of control and mutant spheres were tripotent, giving rise to neurons, oligodendrocytes and astrocytes and no significant difference in the overall differentiation potential of control and mutant spheres was observed (Figure 6.6). To determine whether loss of TGFβ signaling may influence cell lineage specification within individual differentiating spheres, the percentage of cells that had acquired a neuronal versus glial fate was calculated. Similar percentages of neurons, oligodendrocytes and astrocytes were found in mutant and control secondary spheres (Figure 6.6b). Neurons and oligodendrocytes together constituted about 10% to 15%, whereas astrocytes constituted the majority of cells present in differentiated spheres.

We next addressed whether loss of TGFβ signaling not only affects the self-renewal of stem cells but also the proliferation of stem cell-derived transient amplifying progenitor cells. Since increased proliferation correlates with sphere size, we compared the diameters of control versus mutant neurosphere populations. Interestingly, the average diameter of mutant primary and secondary spheres derived from either E12.5 or E15 dorsal midbrains was not altered as compared to control spheres (Figure 6.5d, Supplementary Figure 6.3d, Supplementary Table 6.1). Additionally, control and mutant spheres consisted of a similar number of cells throughout several passages (Supplementary Table 6.1) indicating that proliferation of transient amplifying progenitor cells is not affected by the absence of TGFβ signaling.

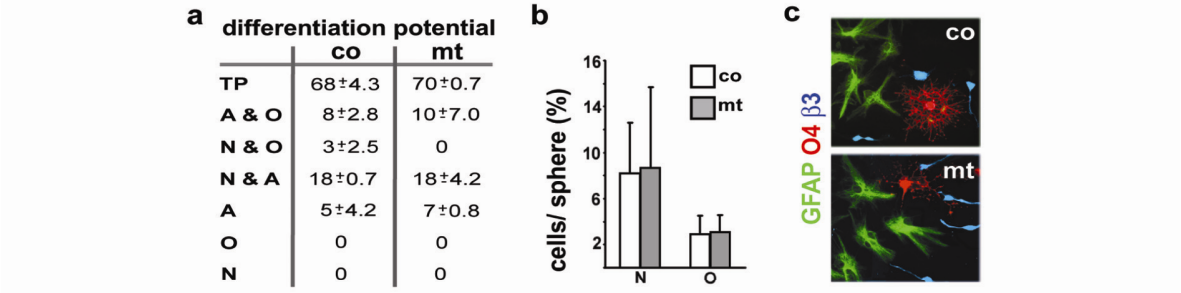


Figure 6.6. TGFβ signal inactivation does not affect differentiation potential of neurosphere cells. (a) Similar multi-lineage potential of E15 control and mutant secondary spheres in differentiating culture conditions (n=3). Markers used: GFAP for astrocytes (A), O4 for oligodendrocytes (O), β3-tubulin for neurons (N). TP, tripotent (positive for all three markers). (b) Loss of TGFβ signaling does not alter percentage of neurons (N) versus oligodendrocytes (O) within single differentiated secondary spheres (n=3). The majority of control and mutant sphere cells (~85%) are astrocytes. (c) Differentiation of secondary control and mutant neurosphere into astrocytes (green), oligodendrocytes (red), and neurons (blue), as assessed by triple immunostaining.

6.2.5. TGFβ decreases sphere-forming potential and counteracts Wnt signal-dependent proliferation of midbrain neuroepithelial stem cells

Since loss of TGFβ signaling significantly enhanced self-renewal of mutant neural stem cells, increased TGFβ may elicit the opposite effect. To test this hypothesis, we examined the sphere-forming potential of wild-type dorsal midbrain cells at E12.5 upon treatment with TGFβ1. Strikingly, the number of primary spheres and of secondary spheres per primary sphere was significantly reduced in the presence of TGFβ1, while sphere sizes remained unaffected (Figure 6.7a, Supplementary Table 6.1). Moreover, in neuroepithelial cells (Kalyani et al., 1997) obtained from dorsal midbrains at E12.5, TGFβ1 did not affect the potential to differentiate into neuronal and glial cells (Figure 6.7b). Hence, TGFβ was able to decrease the number of sphere-forming units without affecting cell fate decisions and progenitor cell proliferation *per se*.

Both the *Tgfbr2*-mutant dorsal midbrain *in vivo* and neurosphere cultures derived from mutant midbrains displayed elevated levels of activated β-catenin (Figures 6.3, 6.5, Supplementary Figure 6.3). These findings raise the question of whether TGFβ can directly counteract cellular responses elicited by Wnt ligands. To address this issue, neuroepithelial cells dissociated from wild-type dorsal midbrains at E12.5 were cultured at clonal density on

Wnt1-expressing feeder layers in presence or absence of TGFβ1. We found that the number of cells per clone was significantly increased in presence of Wnt1 as compared to control conditions (Figure 6.7c). Strikingly, this Wnt-mediated mitotic effect was reduced to control levels upon treatment with TGFβ1, indicating that TGFβ efficiently antagonizes Wnt signal-dependent proliferation.

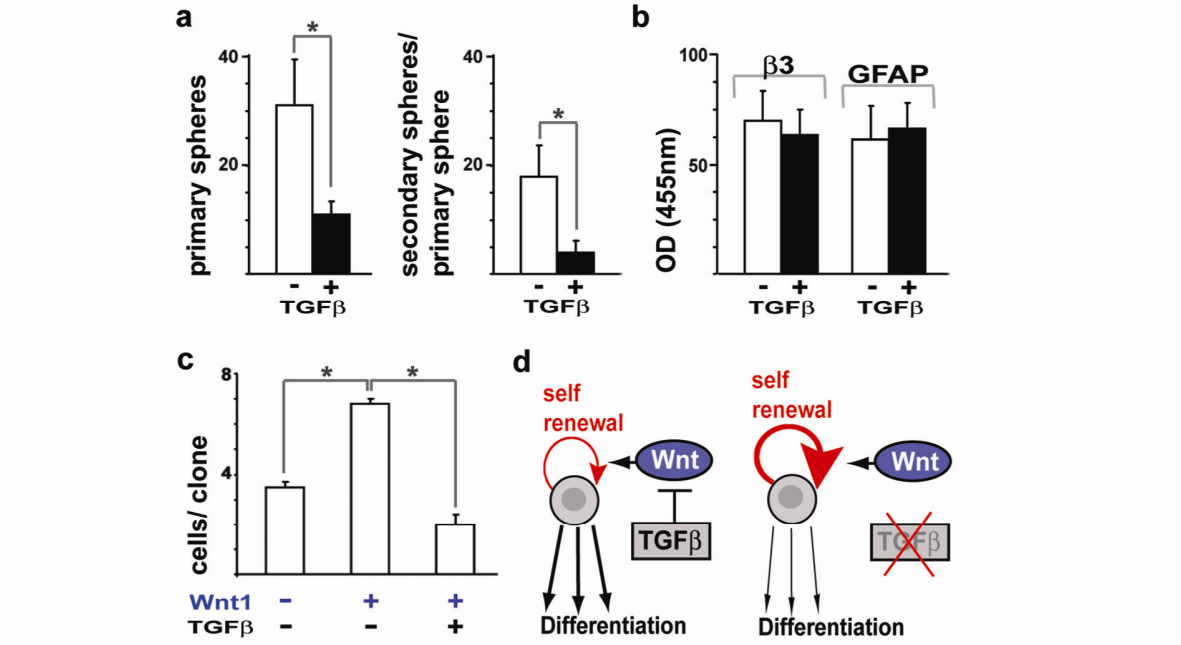


Figure 6.7. TGFβ decreases the sphere-forming potential of midbrain cells without influencing neuronal versus glial fate decisions.
(a) Significantly decreased number of E12.5 wild-type primary spheres and of secondary spheres per primary sphere in cultures treated with 0.5 ng/mL TGFβ1 as compared to untreated cultures (asterisk; $p<0.01$, $n=6$). (b) Comparable neuronal versus glial differentiation of neuroepithelial midbrain cells in presence or absence of TGFβ1 (markers used: GFAP for glial differentiation, β3-tubulin (β3) for neuronal differentiation). The graph illustrates the staining intensity of marker-positive cells in mass culture ($n=3$). (c) TGFβ counteracts Wnt signal-dependent proliferation of midbrain neuroepithelial cells in clonal culture conditions (asterisks; $p<0.01$, $n=3$; cell death in all conditions <10%.). (d) Model of TGFβ signaling regulating maintenance versus differentiation of midbrain neural stem cells by antagonizing Wnt-mediated self-renewal. In the absence of TGFβ signaling, Wnt signal activity is increased, leading to enhanced self-renewal at the expense of differentiation.

6.3. DISCUSSION

Stem cell maintenance, progenitor proliferation, and timely differentiation play important roles in regulating brain size. Though it has become evident that distinct signaling pathways are implicated in these processes (Ohnuma and Harris, 2003), little is known about functional interactions and integration of distinct signaling events. Here, we demonstrate that inactivation of TGFβ signaling leads to overproliferation of midbrain neuroepithelial structures at the expense of differentiation, and consequently to a drastic horizontal expansion of the mutant dorsal midbrain. TGFβ negatively regulates self-renewal of midbrain neural stem cells and their maintenance during development without affecting multipotency *per se.* Intriguingly, loss of TGFβ signaling causes region-specific, ectopic expression of activated Wnt/β-catenin. In addition, TGFβ is sufficient to counteract Wnt-induced proliferation of midbrain neuroepithelial cells. Taken together, our results provide new evidence for a functional crosstalk between TGFβ and Wnt signaling in controlling the fate switch of neural stem cells in the dorsal midbrain from non-neurogenic self-renewing proliferation to differentiation.

6.3.1. TGFβ signaling negatively regulates self-renewal of midbrain neuroepithelial stem cells

Several findings presented here support our hypothesis that TGFβ signaling influences specifically non-neurogenic stem cell expansion in the dorsal midbrain rather than neurogenic progenitor proliferation. The transition from uncommitted self-renewal to neuronal lineage commitment is thought to depend on the switch from symmetric proliferative divisions, generating two identical daughter cells, to asymmetric neurogenic divisions that maintain one identical daughter cell plus a more differentiated neuronal progenitor (Götz and Huttner, 2005). Upon inactivation of TGFβ signaling, proliferation in the sox2-positive neuroepithelium of the mutant dorsal midbrain was massively enhanced as compared to the control, concomitant with horizontal expansion of neuroepithelial structures and reduced neuronal differentiation, while apoptosis was not changed (Figure 6.2**,** data not shown). Normally, the transition from proliferative divisions to neurogenic divisions is accompanied by cell cycle lengthening (Calegari et al., 2005). However, proliferative cells from the mutant dorsal midbrain displayed a relatively shorter cell cycle length compared to their control

counterparts (Figure 6.4). These data suggest that at least a fraction of *Tgfbr2*-mutant neuroepithelial cells fail to efficiently switch from symmetric proliferative divisions to neurogenic divisions.

The neurosphere assay provides a quantitative *in vitro* model system to study stem and progenitor cell proliferation, self-renewal, and multipotency (Reynolds and Rietze, 2005). A neurosphere is a mixture of self-renewing neural stem cells, differentiated neurons and glia, and proliferating progenitor cells (Reynolds and Weiss, 1996). The latter represent the most frequent cell type within a sphere, and thus the proliferation rate of these cells will crucially determine sphere size and cell number. *Tgfbr2*-mutant compared to control neurosphere cultures showed neither a difference in the average sphere size, nor in the size distribution of primary and secondary neurosphere populations (Figure 6.5d, Supplementary Figure 6.3d, Supplementary Table 6.1). Additionally, mutant and control neurospheres contained comparable numbers of cells, indicating similar rates of progenitor cell proliferation during spherogenesis. This is in contrast to inactivation of other regulators of neural stem/progenitor cells, such as Notch1, Bmi-1 or Pten, which leads to an increased number of self-renewing stem cells in neurosphere assays, but in addition also to altered sphere size (Groszer et al., 2001; Molofsky et al., 2003; Ohtsuka et al., 2001).

Although neurosphere size and cell number was not changed upon TGFβ signal inactivation, dissociated cells isolated from mutant dorsal midbrains at E12.5 gave rise to significantly more neurospheres as compared to control tissue (Figure 6.5, Supplementary Figure 6.3). Complementary to these results, the presence of TGFβ in wild-type cultures strongly decreased primary and secondary neurosphere formation (Figure 6.7). Given that a neural stem cell has a greater self-renewal potential than a transient amplifying progenitor cell, serial passage experiments of clonally-derived neurospheres are an appropriate tool to compare mutant versus control stem cell activity over time (Reynolds and Rietze, 2005). Strikingly, mutant spheres were successfully propagated over more than seven passages, while propagation of control spheres was limited to six passages. Moreover, neurosphere formation in mutant cultures increased with each successive passage, while it occurred at a significantly lower level in control cultures (Figure 6.5c, Supplementary Figure 6.3c).

Importantly, neither TGFβ signal activation nor inactivation influenced multipotency of midbrain neural stem cells and changed the cellular composition in clonal and mass cultures (Figures 6.6, 6.7b). This is in contrast to loss of the neural stem cell factor *Bmi-1*, which alters the composition of differentiated cells derived from neural progenitors (Zencak et al., 2005). Thus, TGFβ does not suppress neural stem cell features by instructively or selectively promoting the generation of a specific cell lineage.

In sum, these data provide strong evidence for enhanced self-renewing activity of *Tgfbr2*-mutant neural stem cells that persists over time. Thus, during normal development TGFβ signaling appears to allow differentiation by negatively regulating stem cell self-renewal. Interestingly, NCSCs lacking TGFβ signaling also fail to give rise to properly differentiated progeny in diverse embryonic structures (Ittner et al., 2005; Wurdak et al., 2005). Accordingly, TGFβ signaling might be a crucial factor in the transition of diverse types of stem/progenitor cells to a fully differentiated phenotype.

6.3.2. Crosstalk between TGFβ and canonical Wnt signaling in neural stem cell development

Immunohistochemistry of pSmad2 indicated that activation of canonical TGFβ signaling is limited to a narrow time window around E12 during dorsal midbrain and cerebellar development (Supplementary Figure 6.1). The peak level of wild-type TGFβ signal activity coincides with ectopic expression of wnt1 and elevated levels of activated β-catenin in mutant embryos (Figure 6.3). Ectopic canonical Wnt signaling is region-specific, extending the normal wnt1 expression domain at the midbrain/hindbrain junction caudally into the isthmal neuroepithelium of the mutant dorsal midbrain. Therefore, canonical TGFβ signaling controls Wnt/β-catenin in a spatially restricted manner during midbrain development. Intriguingly, absence of transient TGFβ signaling also leads to ectopic Wnt signaling in mutant neurosphere cultures at E12.5 and E15 (Figure 6.5a, Supplementary Figure 6.3a). Thus, presence of Wnt/β-catenin strongly correlates with persistence of neural stem cells in the *Tgfbr2*-mutant dorsal midbrain. In good agreement with our results, a recent report demonstrated that ectopic expression of *Wnt1* at the midbrain/hindbrain junction enhanced proliferation of neuroepithelial precursor cells (Panhuysen et al., 2004). This resulted in

enlargement of the dorsal midbrain, a phenotype much resembling the enlargement of *Tgfbr2*-mutant midbrains (Figure 6.1e). Similar to *Tgfbr2* deletion, *Wnt1* misexpression did not affect the patterning activity of the isthmus organizer and the regional identity of the midbrain/hindbrain area (Supplementary Figure 6.2) (Panhuysen et al., 2004).

Inactivation of TGFβ signaling and subsequent region-specific, ectopic Wnt/β-catenin activation was concomitant with strongly decreased levels of the cyclin-dependent kinase inhibitor p27 (Figure 6.3a). Both Wnt1 signaling and loss of TGFβ signaling have been shown to result in downregulation of p27 protein expression, which has been implicated in normal cell proliferation and tumorigenesis (Castelo-Branco et al., 2003; Go et al., 2000; Mishra et al., 2005). Intracellular accumulation of p27 causes cell cycle arrest and initiation of differentiation (Durand et al., 1998), and p27 overexpression in the cortical neuroepithelium enhances the proportion of cells exiting the cell cycle (Tarui et al., 2005). Moreover, TGFβ1 promotes cell cycle exit in the developing cortex through cyclin-dependent kinase inhibitors (Siegenthaler and Miller, 2005). Conversely, canonical Wnt/β-catenin promotes cell cycle reentry in neural precursors (Chenn and Walsh, 2002). Hence, impaired cell cycle exit in TGFβ-deficient neuroepithelial cells may be a key mechanism underlying the phenotypic development of *Tgfbr2*-mutant embryos. Alternatively, TGFβ might promote neurogenesis by directly extending the cell cycle length, which causes neural stem cells to adopt a neurogenic fate (Calegari and Huttner, 2003). Although we cannot exclude this possibility at the moment, a recent report demonstrated that TGFβ did not affect cell cycle time in slice cultures of the developing cortex (Siegenthaler and Miller, 2005), supporting the hypothesis that TGFβ does not primarily affect cell cycle length in undifferentiated neural progenitors but controls cell cycle re-entry of neural stem cells undergoing symmetric, non-neurogenic divisions.

Taken together, the results described here provide several lines of evidence for antagonistic crosstalk between TGFβ and Wnt signaling, regulating self-renewal during dorsal midbrain development. First, TGFβ signaling directly controls expression of *Wnt1* on the transcriptional level (Figure 6.3a). Consequently, activity of the canonical Wnt pathway via β-catenin is modulated by TGFβ in the dorsal midbrain. Second, TGFβ is able to efficiently counteract Wnt-induced proliferation of midbrain neuroepithelial cells in culture, indicating

that an antagonistic crosstalk between TGFβ and Wnt signal transduction pathways might also occur at the level of protein interaction. Regulatory Smads such as Smad2 and Smad3, as well as the common Smad4, a downstream component shared between TGFβ, BMP and Activin signaling, can physically interact with β-catenin/Lef1 complexes (Kleber and Sommer, 2004). In addition, physical association and functional interactions between the inhibitory Smad7 and β-catenin have recently been demonstrated (Edlund et al., 2005). However, the details of the coordinated TGFβ/Wnt signaling network in neural stem cell development remain to be elucidated. Functionally, combinatorial signaling of TGFβ family members with Wnt has been implicated in cancer and in maintenance and fate decisions of intestinal crypt stem cells, NCSCs, and embryonic stem cells (He et al., 2004; James et al., 2005; Kleber et al., 2005; Takaku et al., 1998). In the CNS, Wnt and a TGFβ family factor cooperatively regulate telencephalon expression of Emx2 (Theil et al., 2002), which itself promotes symmetric cell divisions in the developing cortex (Heins et al., 2001). Thus, the question arises whether the orchestrated balance of these signaling pathways plays a general role in controlling stem cell pools and self-renewal, for example in the developing cortex and the adult brain. It has to be noted, though, that TGFβ signal inactivation (this study) and Wnt signal activation (Panhuysen et al., 2004) in the midbrain/hindbrain region affects stem cell numbers only in a particular area of the dorsal midbrain, suggesting that combinatorial TGFβ/Wnt signaling in neural stem cell development is restricted to certain brain regions. This might involve tissue-specific modulators of TGFβ signaling. In cultured telencephalic neuroepithelial cells, exogenously added TGFβ can only exert its antiproliferative effect in the absence of the transcription factor FoxG1 (Seoane et al., 2004), a factor intrinsic to the developing cortex. Thus, self-renewal and proliferation of neural stem cells from different brain areas are likely to be regulated by region-specific signaling networks.

6.4. SUPPLEMENTARY MATERIAL

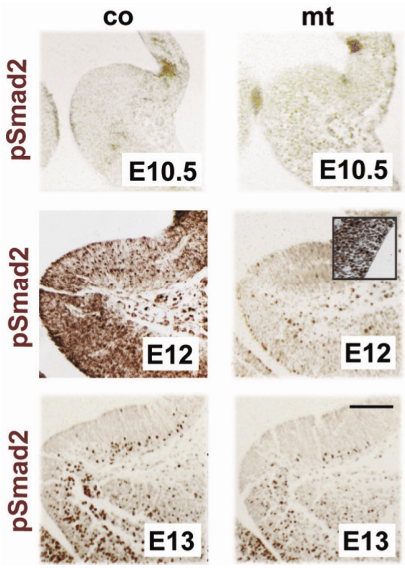
a

E12.5	primary spheres	secondary spheres
co	112±39	109±45
mt	99±44	98±40
+TGFβ	95±40	102±53
E15		
co	119±10	98±45
mt	116±15	96±50

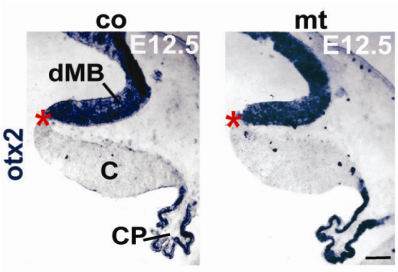
b

passage	co	mt
2	1237±158	1353± 65
3	1252±143	1287±102
4	1330±114	1196± 55
5	1306± 69	1319± 48

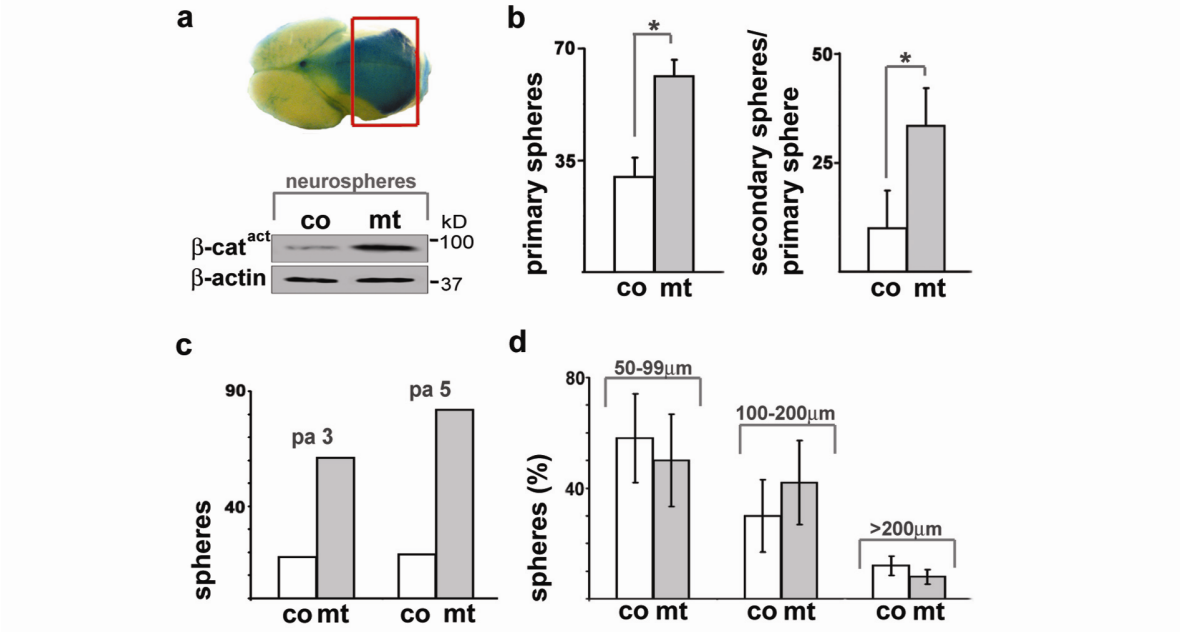
Supplementary Table 6.1. Gain and loss of TGFβ signaling do not influence average size and cell number of neurospheres.
(a) Mutant and TGFβ-treated primary and secondary neurospheres from dorsal midbrains at indicated stages do not differ in size compared to control spheres (n=3; average diameters in µM ± standard deviations are shown). (b) Comparable average cell number of E12.5 control and mutant neurospheres throughout 5 successive passages. The data are expressed as number of cells per sphere (mean ± standard deviations), counting 30 – 60 spheres per condition.



Supplementary Figure 6.1. Activity of canonical TGFβ signaling.
Immunostaining for phosphorylated Smad2 (pSmad2) of E10.5, E12 and E13 control and mutant embryos, focusing on the isthmus and adjacent midbrain/hindbrain areas. In controls, pSmad2 is not detectable at E10.5, reaches a peak level at E12, and is downregulated at E13. In mutants compared to controls, pSmad2 levels are strongly reduced in midbrain/hindbrain structures affected by the recombination. Note that pSmad2 is detectable in non-recombined areas of the ventral midbrain in mutants at E12 (inset). Scale bar: 100 µM.



Supplementary Figure 6.2. Positioning of the isthmus organizer is control-like in mutant embryos.
Sagittal sections display unaltered morphology of mutant dorsal midbrain (dMB), cerebellum (C) and choroid plexus (CP) at E12.5. In situ hybridization analysis demonstrates normal positioning of the otx2 expression domain and of the isthmus (red asterisk) of control and mutant embryos. Scale bar: 100 µM.



Supplementary Figure 6.3. Increase of mutant neural stem cells from E15 dorsal midbrains in neurosphere culture.

(a) Western blot analysis shows increased levels of activated β-catenin (β-cat^{act}) in secondary neurospheres derived from control and mutant dorsal midbrains (red box) at E15. (b) Formation of primary spheres, as well as formation of secondary spheres per primary sphere is significantly increased in mutant compared to control cultures (asterisk; $p<0.01$, $n=5$). (c) Increased number of clonally-derived mutant spheres over five passages (pa). Third and fifth passage are shown (bars represent the average of 2 experiments). (c) Comparable distribution of small diameter, medium diameter and large diameter control and mutant primary neurospheres.

DiGeorge Syndrome and Pharyngeal Apparatus Development

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DiGeorge syndrome is the most frequent microdeletion syndrome in humans, characterized by cardiovascular, thymic and parathyroid, and craniofacial anomalies. The underlying cause is disturbed formation of the pharyngeal apparatus, a transient vertebrate system, giving rise to multiple endocrine, craniofacial, and cardiac outflow tract structures. The pharyngeal apparatus is composed of derivatives of ectoderm, endoderm, mesoderm and the neural crest. Thus, complex intra- and intercellular interactions have to be orchestrated in the correct spatiotemporal manner to establish proper formation, segmentation, and patterning of the pharyngeal system. In this process the involvement of signaling pathways, the function of single genes and genetic networks, as well as the role of distinct cell types have been discussed. The analysis of engineered mouse mutants developing a DiGeorge syndrome-like phenotype has revealed genes and signaling pathways crucial for pharyngeal apparatus development. Concomitantly, these models have shed new light on the etiology of DiGeorge syndrome at the cellular and molecular level.

7.1. Background

DiGeorge syndrome is a clinically heterogeneous disease caused by a multigene deletion from chromosome 22, resulting from an error in recombination at meiosis (Lindsay, 2001). It is classified along with velo-cardio-facial syndrome and conotruncal anomaly face syndrome as a 22q11 microdeletion. The disease represents the most frequent microdeletion syndrome in humans with an incidence of 1 in 4000 live births (Botto et al., 2003; McDonald-McGinn et al., 1997; Ryan et al., 1997). Approximately 90% of the patients share a common 3Mb hemizygous deletion of 22q11.2, encompassing approximately 30 genes (Lindsay, 2001; Shaikh et al., 2000). Apparently identical deletions can cause a widely differing severity of the disease, with clinical features of high variability (Table 7.1). The majority of DiGeorge syndrome patients have cardiac outflow tract defects, with abnormal patterning of the aortic arch arteries, truncus arteriosus, tetralogy of fallot, and ventricular septum defects. The cardiovascular phenotype, which is the main cause of mortality in DiGeorge syndrome, is often associated with craniofacial anomalies such as cleft palate and facial dysmorphogenesis. Moreover, typical features of DiGeorge syndrome are thymus and parathyroid gland aplasia/hypoplasia, which can result in immune deficiency and hypocalcemia, respectively (Portnoi et al., 2005; Ryan et al., 1997; Scambler, 2000). Many studies have also reported an increased incidence of learning difficulty, behavioural problems, and psychiatric disorders (Scambler, 2000), but the molecular origin of these neurobehavioural defects remains to be elucidated.

Present research efforts aim at elucidating networks of genetic and molecular factors crucial for pharyngeal apparatus development, a process tightly associated with the etiology of DiGeorge syndrome. Formation and patterning of the pharyngeal apparatus is extremely complex, involving organization of different cell types derived from the embryonic tissue layers ectoderm, endoderm, mesoderm, and neural crest-derived mesenchyme (Figure 7.1). The pharyngeal system consists of 5 pharyngeal arches appearing in the lateral wall of the foregut between 8-11 days of embryonic development in the mouse and between 2-7 weeks of gestation in humans. The arches are separated from each other externally by ectoderm-lined pharyngeal clefts and internally by endoderm-lined pharyngeal pouches. The 5 arches, develop in anterior to posterior order, and are numbered from 1 to 6 (the fifth arch, which is

buried, is termed the sixth arch by convention). Pharyngeal arch arteries forming at the core of the pharyngeal arches constitute the primitive vascular supply to pharyngeal structures and connect the aortic sac with the dorsal aortae. Subsequently, the caudal arch arteries four to six undergo extensive remodelling and are primary contributors to the mature aortic arch and its major branches.

Each pharyngeal arch has its own identity and contributes to specific structures according to its anteroposterior position. For example, the first arch forms the jaw and parts of the middle ear, the second contributes to the hyoid apparatus (bone and cartilage structure in the floor of the mouth) and muscles of facial expression, while the posterior arches become incorporated into the throat (Graham, 2003). Moreover, the different cell types within the individual arches give rise to distinct derivatives. The core mesoderm forms craniofacial muscles and endothelial cells of the arch arteries, while the ectoderm gives rise to the epidermis of the pharyngeal apparatus and to neuronal tissues of arch-associated ganglia. The endoderm forms the epithelial lining of the pharynx and the pharyngeal pouches. The latter harbour the endocrine tissue of the thymus, thyroid and parathyroid glands, which develop with contributions both from pharyngeal endoderm and from the neural crest. Neural crest cells within the pharyngeal system derive from multipotent NCSCs that detach from the dorsal neural tube and migrate to various embryonic locations (Graham, 2003; Le Douarin et al., 2004) (Figure 7.1A). In addition to glandular structures of the pharyngeal apparatus, neural crest cells also contribute to cardiovascular development and give rise to connective and skeletal tissue.

The finding that genes within the region of the DiGeorge microdeletion are conserved between the human and mouse genome, as well as gene targeting technologies such as the *Cre/loxP* system, allowed studying deletions of multiple and single genes in mice (Baldini, 2002; Lindsay, 2001). A variety of distinct mouse models phenocopying DiGeorge syndrome have shed new light on crucial genes involved in the etiology of the disease. Moreover, they also revealed the role of genes which are not present in the microdeletion, but which modulate the severity and penetrance of the DiGeorge-like phenotype (Bachiller et al., 2003; Frank et al., 2002; Garg et al., 2001; Niederreither et al., 2003; Stalmans et al., 2003; Wurdak et al., 2005). Interestingly, all gene mutations described so far have been shown to affect

development and morphogenesis of the pharyngeal system or pharyngeal derivatives, raising the possibility of a common genetic network involved in the generation of DiGeorge syndrome. However, recent studies indicate that genetic factors regulate pharyngeal system development in a complex spatial and temporal manner. Morphogenesis of the pharyngeal apparatus involves the initial segmentation and expansion of pharyngeal arches and pouches, and development of pharyngeal derivatives from these structures. The orchestration of these successive developmental phases might depend on distinct genetic pathways. However, the players and molecular interactions involved in these dynamic genetic networks are only partially understood. In this review we focus on recent evidence suggesting a crucial role for transcription factors and major signaling cues in pharyngeal system development. Moreover, intra- and intercellular interactions in the formation of distinct pharyngeal tissues and their derivatives during different developmental stages are discussed.

Table 7.1. Clinical features of DiGeorge syndrome

Craniofacial anomalies

- cleft palate (incomplete closure of the palate)
- micrognathia (small size of the lower jaw)
- ear anomalies (typically low set, deficient vertical diameter, abnormal auricle/pinna folding)
- telecanthus (increased distance between eyes)
- small mouth (associated with hypernasal speech)

Glandular malformations

- hypo- or aplasia of the parathyroid glands (neonatal hypocalcaemia with tetany or seizures due to impaired calcium homeostasis)
- hypo- or aplasia of the thymus (T cell deficiency with susceptibility to infections due to impaired T cell maturation)

Cardiovascular defects

- tetralogy of Fallot (complex of heart malformations: (1) ventricular septum defect (VSD; hole connecting the ventricles), (2) pulmonic stenosis (obstruction of the right ventricular outflow tract), (3) overriding aorta (aorta is positioned over the VSD), (4) hypertrophy of the right ventricle)
- type B interrupted aortic arch (discontinued aortic arch between the left carotid artery and the left subclavian artery)
- truncus arteriosus (ventricles with a common arterial outflow trunk)
- right aortic arch (persistence of the bilateral system of embryonic pharyngeal arch vessels)
- transposition of the great arteries (reversed aorta and pulmonary artery, with the aorta arising from the right ventricle and the pulmonary artery receiving blood from the left ventricle)
- aberrant right subclavian artery (anomalous origin from the proximal descending aorta)
- ventricular septum defects (VSD; hole connecting the ventricles)

Behavioural disorders

- learning difficulties (variable mild to moderate)
- paranoid schizophrenia, major depressive illness

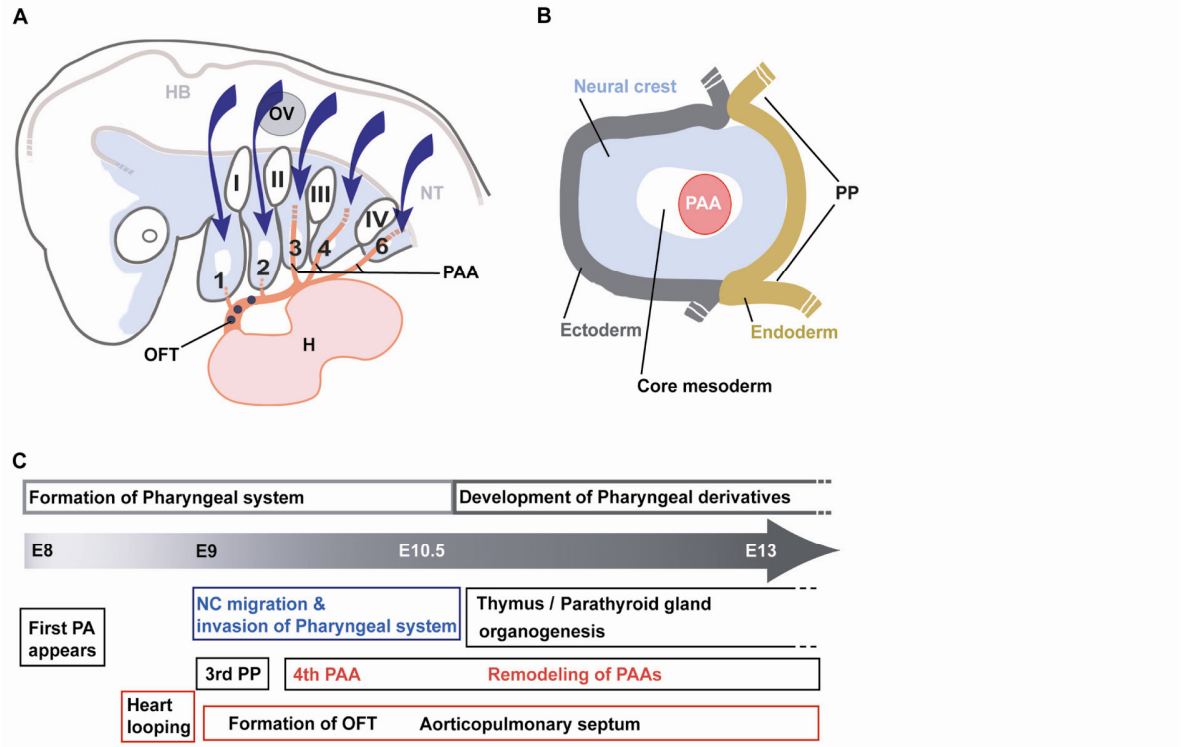

Figure 7.1. Schematic view of pharyngeal system development.
(A) Migrating neural crest cells (dark blue arrows) arising from the hindbrain (HB) and the neural tube (NT) populate the pharyngeal system (blue) and the forming outflow tract (OFT, blue dots) of the heart (H). The complete pharyngeal apparatus at around embryonic day (E) 10.5 consists of pharyngeal arches (1-6) with mesodermal cores (white), pharyngeal pouches (I-IV) and pharyngeal arch arteries (PAA). OV: Otic vesicle. (B) Schematic illustration of pharyngeal arch composition (sagittal view). Pharyngeal epithelia (ectoderm + endoderm) surround the neural crest-derived ectomesenchyme and the mesodermal core. PAA: Pharyngeal arch artery, PP: Pharyngeal pouch. C: Simplified time scale of significant events during pharyngeal system development, categorized into two phases: (i) initial formation of pharyngeal arches and pouches starting at around E8 and (ii) subsequent development of pharyngeal derivatives.

7.2. Early formation of the pharyngeal apparatus

Mechanisms and tissue interactions responsible for the initial formation and growth of the pharyngeal apparatus are still poorly understood. One key molecule implicated in this process is T-box1 (Tbx1). The *Tbx1* gene is located within the 22q11 region and encodes a protein belonging to the T-box family of transcription factors, characterized by a conserved DNA-binding domain. Within the pharyngeal apparatus *Tbx1* is expressed in the ectoderm and endoderm (together referred to as pharyngeal epithelia), as well as in the mesodermal core of the pharyngeal arches, but not in neural crest cells (Lindsay, 2001). In mice, homozygous inactivation of *Tbx1* results in a highly consistent DiGeorge-like phenotype, but embryos

haploinsufficient for *Tbx1* also reflect parts of the DiGeorge syndrome phenotypic spectrum (Jerome and Papaioannou, 2001; Lindsay et al., 2001; Merscher et al., 2001; Taddei et al., 2001). Moreover in human patients, mutations of *TBX1* without the classical 22q11 deletion have been described, stressing the crucial role of this factor in the pathogenesis of DiGeorge syndrome (Yagi et al., 2003).

Several studies provide ample evidence that Tbx1 is required for early pharyngeal system development. Inactivation of *Tbx1* causes abnormal early patterning and hyplasia/aplasia of the pharyngeal arches, plus impaired formation of the second and fourth pharyngeal pouches (Jerome and Papaioannou, 2001; Kelly et al., 2004; Vitelli et al., 2002a). Posterior pharyngeal arches and pouches are more severely affected, in line with a stronger *Tbx1* expression in these structures (Vitelli et al., 2002a). Interestingly, a recent report using inducible inactivation of *Tbx1* in conjunction with *in vivo* cell fate mapping demonstrates that early absence of *Tbx1* at around E7.5 results in a phenotype identical to that displayed by germline *Tbx1* knock-out mice (Xu et al., 2005). In contrast, inactivation of *Tbx1* at around E9 exclusively impairs the development of pharyngeal segments posterior to and including the third pharyngeal pouch, implicating an anterior-to-posterior gradient of Tbx1 activity over time.

Hence, *Tbx1* expression must be tightly regulated in distinct segments during pharyngeal system development, but little is known concerning potential upstream regulators. It has been shown that (i) ablation of the secreted morphogen *sonic hedgehog* (*Shh*) (Garg et al., 2001), (ii) absence of two distinct isoforms of vascular endothelial growth factor (*Vegf*) (Stalmans et al., 2003), or (iii) loss-of-function mutation of *chordin* (*Chrd*), an antagonist of bone morphogenetic protein (Bmp) signaling (Bachiller et al., 2003), can result in reduction of *Tbx1* transcription in pharyngeal epithelia of mutant mice. Both *Vegf* and *Chrd*-mutant mice display the complete spectrum of DiGeorge syndrome-like birth defects with incomplete penetrance (Bachiller et al., 2003; Stalmans et al., 2003,), while mice deficient in *Shh* are born with severe craniofacial anomalies (Chiang et al., 1996; Helms et al., 1997).

Intriguingly, a conserved *cis*-enhancer element responsive to Shh signaling upstream of *Tbx1* has been described (Hu et al., 2004; Yamagishi et al., 2003). This enhancer harbours a

forkhead transcription factor (Fox)-binding element which is required for *Tbx1-lacZ* transgene expression in the pharyngeal endoderm and head mesenchyme. The cognate Fox proteins Foxc1, Foxc2 and Foxa2 can bind the *Tbx1* enhancer but are thought to function independently in different cell-types. Foxc1 and Foxc2 are proposed to regulate *Tbx1* in the head mesenchyme, while Foxa2 might control expression of *Tbx1* in the pharyngeal endoderm (Weinstein et al., 1994; Yamagishi et al., 2003). Moreover, a regulatory loop between *Foxa2* and *Tbx1* is suggested in the pharyngeal core mesoderm (Hu et al., 2004). Overall, these findings suggest a role of Shh signaling and Fox proteins in the transcriptional control of *Tbx1*. However, *Shh* mutant-mice display a milder phenotype than *Tbx1*-mutants and the regulatory region of the *Tbx1* gene harbours diverse putative transcription factor binding sites (Yamagishi et al., 2003). Taking intracellular dynamics in the pharyngeal system into account, it is conceivable that a variety of signaling events and molecular interactions upstream of *Tbx1* remain to be elucidated.

In terms of transcriptional targets of Tbx1, genes encoding molecules engaged in fibroblast growth factor (FGF) signaling are the most promising candidates so far. A cell-autonomous genetic link between *Tbx1* and *Fgf8* has been proposed (Vitelli et al., 2002b) and several data speak in favour of this notion. Hypomorphic *Fgf8* and homozygous *Tbx1*-mutants display comparable phenotypes, and conditional *Fgf8* inactivation in the *Tbx1* expression domain leads to impaired cardiovascular development (Brown et al., 2004). In addition, Tbx1 regulates the expression of *Fgf8* in the pharyngeal endoderm (Abu-Issa et al., 2002; Frank et al., 2002; Kochilas et al., 2002; Vitelli et al., 2002a). Importantly, the putative link between Tbx1 and FGF signaling in pharyngeal tissue is further strengthened by the finding that *Fgf10* expression can be directly activated by T-box transcription factors. Tbx1 and Tbx5 can bind a conserved T-box element located in the 5' region of the *Fgf10* gene (Xu et al., 2004). However, other factors might regulate FGF signaling in addition to Tbx1. For example, transcription of *Fgf8* but not *Tbx1* is reduced in retinaldehyde dehydrogenase 2 (Raldh2)-mutant mice, which also recapitulate a DiGeorge-like phenotype (Niederreither et al., 2003).

In summary, recent data suggest a common, albeit still hypothetical genetic pathway playing a crucial role during early formation of the pharyngeal system (Figure 7.2). Shh and Fox proteins seem to act upstream of *Tbx1* expression, while FGF signaling might act downstream

of Tbx1-controlled transcription. It is possible that the pathway emerging around Tbx1 acts through a general, cell-autonomous mechanism facilitating initiation and expansion of pharyngeal segments in an anterior-to-posterior manner. Consistent with this hypothesis, Tbx1 is expressed in an anterior-to-posterior gradient during pharyngeal segmentation, and deletion of *Tbx1* is associated with reduced proliferation in endodermal, as well as mesodermal cells (Xu et al., 2005; Xu et al., 2004). Accordingly, lack of endodermal tissue expansion leads to impaired early pharyngeal system morphogenesis, hence secondarily interfering with invasion of neural crest cells, which is essential for further pharyngeal development. Concomitantly, cells of the pharyngeal epithelia might provide signaling cues such as FGF signaling to adjacent neural crest cells. Several studies demonstrate that inactivation of *Fgf8* specifically in pharyngeal epithelia leads to partial cell death of the mesenchymal, neural crest-derived cell population within the arches, thus suggesting that epithelial-mesenchymal interactions are critically involved in pharyngeal apparatus development (Abu-Issa et al., 2002; Macatee et al., 2003; Trumpp et al., 1999).

7.3. Development of pharyngeal arch arteries and the cardiac outflow tract

The formation of pharyngeal arch arteries starts at around E8.5 and can be categorized into two phases: (i) development of the bilaterally symmetrical anlage of the pharyngeal arch arteries, and (ii) an extensive reconfiguration process that begins at around E11.5 and involves remodelling of the fourth to sixth pharyngeal arch arteries, plus concomitant regression of the first two arch arteries. By E13 the asymmetric configuration of the aortic arch, the brachiocephalic and common carotid arteries, and the ductus arteriosus is almost of mature type (Hiruma et al., 2002). The exact molecular nature of normal, as well as pathogenic morphogenetic processes involved in pharyngeal arch artery development is still unknown. However, several loss-of-function mutations causing a DiGeorge syndrome-like phenotype can either interfere with initial arteriogenesis, or impair the subsequent remodelling process, inevitably leading to anomalies of the mature aortic arch system.

In good agreement with a central role for *Tbx1* during patterning of the pharyngeal system, the transcription factor is also implicated in the initial phase of pharyngeal arch artery

development. Heterozygous *Tbx1*-mutants have hypoplastic to absent fourth pharyngeal arch arteries (Jerome and Papaioannou, 2001; Lindsay et al., 2001), while homozygous *Tbx1*-mutants also lack pharyngeal arch arteries three to six, indicating that Tbx1 functions in a dosage-dependent manner (Lindsay, 2001). Accordingly, homozygous *Tbx1*-mutants show a severe phenotype that is rarely found in patients, while heterozygous *Tbx1*-mutants have aortic arch and vascular anomalies typical for DiGeorge syndrome such as interrupted aortic arch type B, and aberrant origin of the right subclavian artery and the right aortic arch (Jerome and Papaioannou, 2001; Lindsay et al., 2001; Merscher et al., 2001). Interestingly, a recent study demonstrates that *Tbx1* ablation specifically in the pharyngeal epithelia but not the mesodermal core accounts for aberrant formation of the fourth pharyngeal arch arteries (Zhang et al., 2005). Consistently, genes proposed to interact with *Tbx1* in the pharyngeal epithelia such as *Vegf* and *Fgf8* display abnormalities of pharyngeal arch arteries at around E10 (Macatee et al., 2003; Stalmans et al., 2003), and are at least partially able to modulate the phenotype of heterozygous *Tbx1*-mutant embryos (Stalmans et al., 2003). Overall, this supports a primary role for *Tbx1* and its putative modulators during pharyngeal patterning and arteriogenesis, and a non-cell autonomous role during remodelling of the aortic arch vascular system.

Besides *Tbx1*, *Crkl* is a DiGeorge syndrome candidate gene within the del22q11 region. *Crkl* encodes an adaptor protein that is implicated in response to growth factor and focal adhesion signaling (Feller, 2001). Homozygous ablation of *Crkl* causes a DiGeorge syndrome-like phenotype, including typical aortic arch anomalies, conceivably due to postmigratory neural crest defects (Guris et al., 2001). Unlike *Tbx1*-mutant embryos, *Crkl* null-mutants initially form all symmetrical pharyngeal arch arteries, indicating that aortic arch defects in these mutants occur during reconfiguration of pharyngeal arch arteries. Disturbance of this remodelling process is also associated with aberrant TGFβ signaling, as shown for *Tgfb2*-null mice (Molin et al., 2002). Interestingly, phosphorylation of Crkl, which is highly expressed in neural crest-derived tissues (Guris et al., 2001), can be modulated by TGFβ signaling *in vitro* and *in vivo* (Wurdak et al., 2005). Similar to inactivation of *Crkl*, neural crest-specific ablation of TGFβ signaling affects neither neural crest migration nor initial formation of pharyngeal arch arteries, but rather results in typical defects of the mature aortic arch system

(Choudhary et al., 2006; Wurdak et al., 2005). This indicates a link between TGFβ signaling and Crkl modulation during the specification of postmigratory neural crest cells, which will also be discussed in the context of craniofacial development.

The development of neural crest cells, infiltrating the third, fourth, and sixth pharyngeal arches, and the forming cardiac outflow tract, is crucially involved in cardiovascular patterning (Kirby and Waldo, 1995). So called cardiac neural crest cells were initially identified by surgical ablation from the neural tube in chick embryos, and more recently by *in vivo* cell fate mapping performed in mice (Jiang et al., 2000; Kirby, 1987; Kirby, 1990). Proper development of the cardiac neural crest subpopulation is thought to be essential for configuration of the aortic arch system, as well as for correct development of the aorticopulmonary septum, a transient structure that originates from the dorsal wall of the aortic sac and separates aorta and pulmonary artery (Jiang et al., 2000; Kirby and Waldo, 1995). Impaired septum formation in the heart outflow tract leads to truncus arteriosus, usually accompanied by a ventricular septum defect. Together, these symptoms are an important cause of morbidity in post-natal life of DiGeorge children and require heart surgery to prevent the presence of unoxygenated blood in the systemic circulation. Anomalies of the cardiac outflow tract can be associated with altered or retarded migration, survival, or differentiation of the cardiac neural crest, and disturbance of a variety of major signaling pathways including Wnt/β-catenin signaling, Notch signaling, and signaling of TGFβ superfamily members (Stoller and Epstein, 2005). Recently, we have shown that neural crest cells deficient in *transforming growth factor receptor type 2* (*Tgfbr2*) have the ability to migrate correctly into the future aorticopulmonary septum at E10.5, but fail to adopt a smooth-muscle-like fate in this transient structure and later appear to die (Wurdak et al., 2005). Thus, impaired fate decision of TGFβ-deficient neural crest cells likely results in a truncus arteriosus. However, smooth muscle formation in the aorta and major vessels appears not to be affected by mutation of *Tgfbr2* (Choudhary et al., 2006).

In contrast to the neural crest-derived aorticopulmonary septum, the myocardium of the cardiac outflow tract originates from a distinct region of the pharyngeal mesoderm, known as the anterior or secondary heartfield. This recently identified region is thought to harbour

myocardial precursor cells located anteriorly and dorsally to the cardiac tube in mice (Buckingham et al., 2005; Kelly and Buckingham, 2002). Targeted deletion of *Tbx1* in the anterior heartfield results in a proliferation defect, while overexpression of *Tbx1* leads to upregulation of *Fgf8* and *Fgf10* (Hu et al., 2004; Xu et al., 2004). Thus, analogous to its role during endodermal expansion, Tbx1 might regulate proliferation of myocardial precursors in the anterior heartfield via FGF signaling. Neural crest cells are located adjacent to the anterior heartfield during outflow tract development (Kelly and Buckingham, 2002), hence raising the possibility of signaling crosstalk between neural crest cells and cell types derived from the mesodermal core of the pharyngeal arches.

7.4. Development of craniofacial and glandular pharyngeal derivatives

The formation of craniofacial derivatives from pharyngeal arches crucially relies on cephalic neural crest, also known as cranial neural crest. Cranial neural crest cells originate from the midbrain-hindbrain region anterior to the cardiac neural crest population. Similar to the cardiac neural crest, cranial neural crest cells undergo three distinct developmental phases: (i) migration into the pharyngeal system in a precise anteroposterior pattern (Kontges and Lumsden, 1996), (ii) proliferation, once the neural crest cells - now referred to as ectomesenchymal cells - have populated the arches, and (iii) differentiation into terminal structures including most of the craniofacial bones, cartilage, and connective tissue (Kontges and Lumsden, 1996).

Migrating cranial neural crest cells are thought to carry positional information from their origins alongside the midbrain and hindbrain, but are also proposed to respond to signaling cues from the surrounding pharyngeal epithelia and the mesodermal core (Clouthier and Schilling, 2004; Graham, 2003). Interestingly, the G-protein-coupled endothelin receptor type A (Ednra) is expressed in migrating cranial neural crest cells and ectomesenchymal cells, while the cognate ligand for Ednra, the signaling molecule endothelin 1 (Edn1) is expressed in the pharyngeal epithelia and the mesenchymal core (Clouthier and Schilling, 2004). Impaired endothelin signaling is associated with cartilage malformations, while migration and expression of migratory neural crest markers such as transcription factors AP-2 and distal-less homeobox 2 (Dlx2) are unaffected in *Edn1* and *Ednra* mutant mice. Endothelin signaling is

proposed to act around the time of cranial neural crest arrival in the pharyngeal system and is required for correct expression of transcription factors dHand and eHand, which are implicated in neural crest patterning within the pharyngeal arches (Clouthier et al., 2000; Thomas et al., 1998) (Figure 7.2).

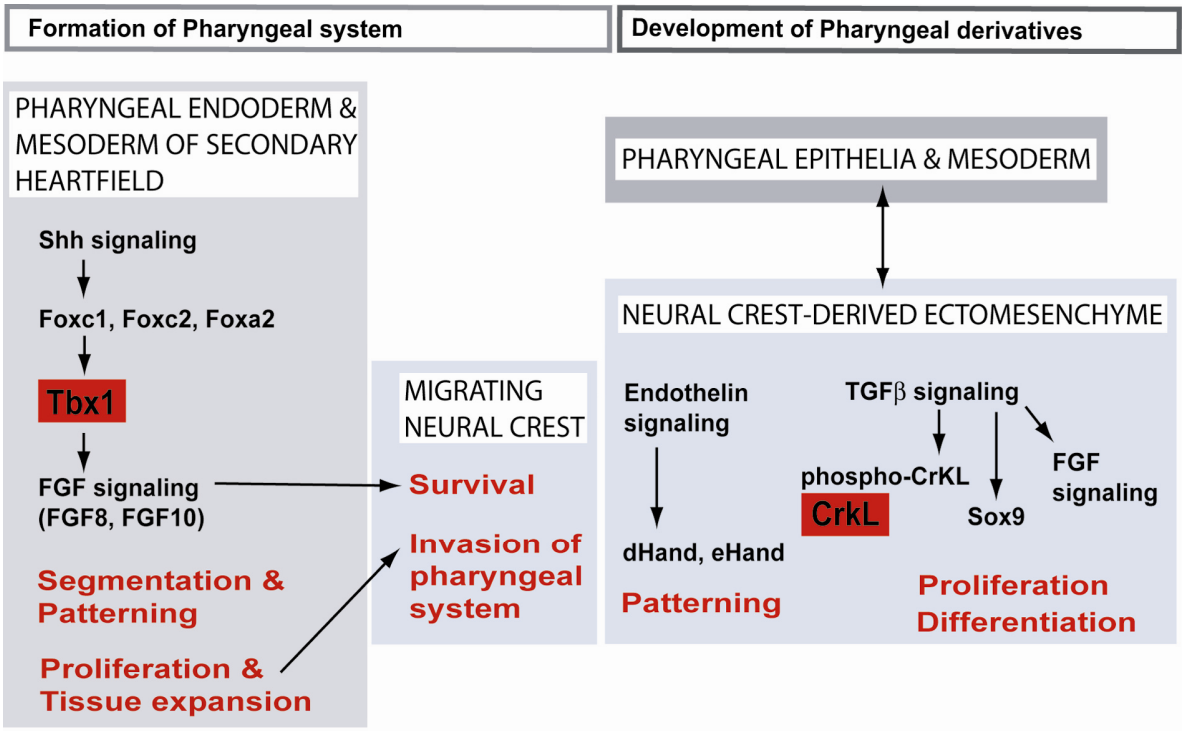

Figure 7.2. Hypothetical scheme of genetic factors and molecular interactions regulating pharyngeal system morphogenesis during two developmental phases in distinct tissues.
Tbx1 and potential modulators of this crucial transcription factor are implicated in the initial formation of pharyngeal arches and pouches, prior to neural crest migration. In contrast, endothelin, Crkl and TGFβ signal activity are likely required during postmigratory neural crest development. Moreover, interactions of the pharyngeal epithelia, core mesoderm and neural crest are thought to underlie the proper morphogenesis of pharyngeal derivatives, including craniofacial and glandular structures.

Further development of neural crest-derived ectomesenchymal structures into bone and cartilage again critically depends on signaling cues provided by the environment. One signaling cascade crucially involved in this process is canonical TGFβ signaling. Importantly, mutations in the genes encoding the receptors *TGFBR1* and *TGFBR2* have been linked to common cardiovascular and skeletal phenotypes in humans (Loeys et al., 2005). In mice,

inactivation of genes encoding the ligands *Tgfb2* and *Tgfb3* results in severe craniofacial malformations, and also mutation of downstream TGFβ signal mediators Smad2 and Smad3 interferes with normal craniofacial development (Dunker and Krieglstein, 2002; Liu et al., 2004). Interestingly, expression of transcription factor eHand is not affected in *Tgfbr2* mutant mice (Wurdak et al., 2005), suggesting that TGFβ signaling is required later than endothelin signaling, and is important for differentiation rather than neural crest migration or patterning. Consistently, several studies using two different conditional *Tgfbr2* mouse strains report normal neural crest migration at around E10.5 (Choudhary et al., 2006; Ito et al., 2003; Ittner et al., 2005; Wurdak et al., 2005). These were performed with mice harbouring either a deleted "floxed" exon2 of *Tgfbr2* (*Tgfbr2*$^{\Delta ex2}$) (Choudhary et al., 2006; Ito et al., 2003), or mice harbouring a deleted "floxed" exon4 of *Tgfbr2* (*Tgfbr2*$^{\Delta ex4}$) (Ittner et al., 2005; Wurdak et al., 2005). However, though *Wnt1-Cre*-mediated recombination was used, *Tgfbr2*$^{\Delta ex2}$-mutant mice show some relevant phenotypic differences compared to *Tgfbr2*$^{\Delta ex4}$-mutants for presently unknown reasons. *Tgfbr2*$^{\Delta ex2}$-mutant mice are viable to full term and have craniofacial and cardiac defects, but lack glandular anomalies (Choudhary et al., 2006). In contrast, *Tgfbr2*$^{\Delta ex4}$-mutant embryos die shortly before birth and display a complete DiGeorge syndrome-like phenotype with craniofacial, cardiac, thymus and parathyroid anomalies (Wurdak et al., 2005). In addition, these embryos have severe eye defects reminiscent of Axenfeld-Rieger`s anomaly (Ittner et al., 2005). Importantly, Tgfbr2 protein is absent in NCSCs of *Tgfbr2*$^{\Delta ex4}$-mutant embryos, resulting in inactivation of canonical TGFβ signal propagation in neural crest-derived pharyngeal, as well as eye structures (Ittner et al., 2005; Wurdak et al., 2005). Intriguingly, TGFβ signal-deficient cells within the anterior eye and pharyngeal apparatus fail to express key transcription factors required for proper differentiation, thus indicating an important role for TGFβ signaling in the context-dependent regulation of progenitor cell fate decision. In the anterior eye, TGFβ released from the lens is required for expression of paired-like homeodomain transcription factor 2 (Pitx2) and Foxc1 in the neural crest-derived cornea (Ittner et al., 2005). In the pharyngeal arches TGFβ signaling is necessary for normal expression of Sox9 in neural crest-derived ectomesenchymal cells (Wurdak et al., 2005). Sox9 is a transcription factor crucially involved in the specification of osteochondroprogenitor cells from the neural crest, and neural crest-specific ablation of Sox9 results in severe craniofacial defects (Mori-Akiyama et al., 2003). In

association with downregulation of Sox9, TGFβ signal-deficient ectomesenchymal cells fail to phosphorylate Crkl. Phosphorylated Crkl protein is concomitantly undetectable in cardiac neural crest cells populating the outflow tract, thus raising the possibility that Crkl mediates TGFβ-dependent non neural fate acquisition of ectomesenchymal and cardiac neural crest cells (Wurdak et al., 2005). Moreover, both *Crkl* and *Tgfbr2*$^{\Delta ex4}$-mutant embryos develop DiGeorge syndrome, strengthening the association between TGFβ signaling and Crkl modulation. However, taking into account, that Crkl mutants have a milder DiGeorge syndrome-like phenotype compared to *Tgfbr2*$^{\Delta ex4}$-mutants, it is conceivable that TGFβ signaling also acts independently from Crkl in neural crest derivatives, for example during palatogenesis (Dudas et al., 2004).

Increasing evidence from zebrafish studies suggests that signals from the pharyngeal endoderm such as FGF signaling pattern bones and cartilages of the pharyngeal arches and locally promote survival of skeletogenic ectomesenchymal cells (Crump et al., 2004a; Crump et al., 2004b; David et al., 2002). In addition to the implication of FGF signaling in pharyngeal pouch formation, there is ample evidence that FGF ligands and receptors have essential functions during cartilage and calvaria development. *Fgf10*-null mice display a cleft secondary palate (Alappat et al., 2005), and conditional inactivation of FGF receptor *Fgfr2* results in decreased osteoblast proliferation and bone formation (Yu et al., 2003). Interestingly, neural crest-specific inactivation of *Tgfbr2* has recently been associated with reduced proliferation and downregulation of *Fgf2* and *Fgfr2* in frontal bone primordia, suggesting that FGF may act as a local downstream mediator of TGFβ signaling during calvaria development (Sasaki et al., 2006).

7.5. Conclusion

Increasing evidence discussed in this review suggests that two major DiGeorge syndrome candidate genes *Tbx1* and *Crkl*, both of which are present in the del22q11 region can be modulated by signaling events implicated in normal pharyngeal system development (Figure 7.2). Distinct molecular and genetic networks are emerging that might crucially regulate two

distinct phases of pharyngeal apparatus formation, and hence also crucially contribute to the pathophysiology of DiGeorge syndrome in a spatial and temporal-specific manner:
During the initial phase of pharyngeal system formation, mutation of *Tbx1* and/or mutation of factors putatively modulating Tbx1 lead to disturbed segmentation and patterning of pharyngeal structures. Consequently, the development of pharyngeal pouch-derived organs such as thymus and parathyroid glands is profoundly impaired. Moreover, cranial and cardiac neural crest cells are unable to properly invade and complete the pharyngeal apparatus, leading secondarily to neural crest defects. Accordingly, penetrance and severity of the resulting DiGeorge syndrome phenotypic spectrum primarily depends on the degree of initial arch and pouch patterning defects. In contrast, upon homozygous depletion of *Crkl* or *Tgfbr2* neural crest cells populate the pharyngeal system, but they do not correctly form non-neural derivatives. Consequently, *Crkl* and $Tgfbr2^{\Delta ex4}$-mutant mice develop DiGeorge syndrome, likely due to impaired non-neural cell fate acquisition of ectomesenchymal and cardiac neural crest cells. Importantly, TGFβ signaling is sufficient and required for Crkl phosphorylation in neural crest cells. Thus, TGFβ signal modulation in neural crest differentiation may play a crucial role in the etiology of DiGeorge syndrome.

Overall, targeted gene mutation and the use of animal models have provided fundamental new insights into the pathogenesis of DiGeorge syndrome. However, elucidating temporal and spatial patterns of action of factors in the disease etiology require further study. Hence, the use of timed and tissue-specific gene mutation combined with cell type-specific *in vivo* fate mapping conceivably is the most promising approach to strengthen the notion of signal modulation and intracellular crosstalk as key mechanisms facilitating the orchestration of pharyngeal structure morphogenesis.

8. OUTLOOK & CONCLUSIONS, PART 1-3

8.1. Proposal for isolation and characterization of a neural crest-derived, TGFβ-dependent postmigratory non neural progenitor cell

In part 1 and 2 of my thesis I have shown that neural crest-specific mutation of *Tgfbr2* in mice leads to phenotypic malformations reminiscent of clinical features typical for DiGeorge syndrome and Axenfeld-Rieger`s eye anomaly. Importantly, we showed that the developmental anomalies of *Tgfbr2*-mutant embryos do not result from defective migration of neural crest cells, but rather are caused by impaired non-neural cell fate decisions of neural crest cells. Neural crest cells properly populate structures of the eye and the pharyngeal system but fail to acquire distinct non-neural cell fates, which are closely associated with the expression of transcription factors specifying distinct differentiation programs. In particular, we could show that TGFβ signaling derived from the lens is required for the expression of transcription factors Pitx2 and Foxc1, which are critically involved in the proper differentiation and survival of corneal keratocytes and corneal endothelial cells (Ittner et al., 2005). Moreover, TGFβ signaling is necessary for normal expression of the chondrogenesis-associated transcription factor Sox9 in the first and second pharyngeal arches, and for a smooth muscle-like fate in the forming septum of the cardiac outflow tract.

Overall, our results allow different interpretations in terms of the TGFβ mode of action during neural crest development. First, TGFβ signaling might directly induce differentiation and lineage specification of postmigratory neural crest cells. Alternatively, TGFβ signaling might promote the exit from a NCSC-like state, thus allowing a more mature progenitor cell to respond to different, region-specific differentiation cues (Figure 8.1). Several observations speak in favour of the latter hypothesis. The finding that loss of TGFβ signaling affects transcription factor-dependent cell fate decisions in several different neural crest-derived cell types and structures stresses the important role of the cellular context in TGFβ signal interpretation (Ittner et al., 2005; Wurdak et al., 2005). Moreover, *Tgfbr2*-deficient neuroepithelial stem cells of the midbrain show a differentiation potential similar to control cells in culture, and are not biased towards a specific neural cell lineage at the expense of other lineages (Wurdak et al., manuscript submitted). Finally, the drastically increased self

renewal capacity of TGFβ-deficient midbrain neural stem cells compared to control cells, together with cell cycle modulation and tissue expansion of mutant dorsal midbrains strongly indicates that loss of TGFβ signaling causes persistence of neuroepithelial cells in a stem cell-like state over time (Wurdak et al., manuscript submitted). Though neural crest development is different from that of CNS development, it is possible that TGFβ signaling has a comparable function in both tissues. We hypothesize that TGFβ signaling may act on postmigratory neural crest-derived stem or progenitor cells present in the pharyngeal system that are common to non-neural cell lineages including smooth muscle cells, keratocytes and chondrocytes (Figure 8.1).

In contrast to naïve migratory NCSCs, postmigratory control and mutant neural crest cells populating the pharyngeal system start to express Sox9 but have already lost Sox10 expression (Wurdak et al., 2005). They still express p75 (Wurdak, Ittner, John and Sommer, unpublished observation) which would allow the preparative isolation of putative neural crest-derived non neural progenitor cells from the dissected pharyngeal apparatus by FACS. Although p75 has been used to enrich neural crest-derived neural progenitors (Morrison et al., 1999), the specificity of p75 for neural crest cells in the pharyngeal system is presently unclear. The pharyngeal system is a heterogeneous and complex structure that is composed of cells derived from ectoderm, endoderm, mesoderm and the neural crest. Therefore, I propose to isolate pharyngeal neural crest cells by combining *in vivo* fate mapping with preparative FACS using a fluorescent β-galactosidase substrate (Fluorescein Digalactoside, Molecular Probes). This should result in a homogenous population of neural crest-derived cells that can be used in different experimental setups, including comparative gene expression analysis. Importantly, the cells can either be incubated in adhesive culture or cultured in suspension using the neurosphere system (Reynolds and Rietze, 2005). The latter culture method can be used to address the self-renewal capacity of the putative neural crest progenitors and their differentiation potential. Analogous to the experiments described for midbrain neural stem cells in part 3 of this thesis, the behaviour of *Tgfbr2*-deficient pharyngeal neural crest progenitors should be compared to corresponding control cells, which might be cultured in the presence or absence of TGFβ ligands. These experiments should establish whether pharyngeal neural crest progenitor cells that are positive for p75 and Sox9 have *in vitro* characteristics of

multipotent, self-renewing stem cells or whether they represent lineage-restricted progenitor cells. Moreover, the experiments would reveal the effect of TGFβ signaling on the self-renewing activity and multipotency of the putative neural crest-derived progenitor cells over time. Experimental data based on cell cycle FACS analysis and immunostaining for the cell cycle regulatory protein p27 demonstrate that TGFβ signaling can modulate cell cycle control and cell cycle time of midbrain neuroepithelial cells (Wurdak et al., manuscript submitted). Hence, analogous experiments might be performed using the neural crest-derived progenitor population enriched from the pharyngeal system.

Another key question is whether and how TGFβ signaling is involved in the maturation of a Sox9-positive, p75-positive, and Sox10-negative pharyngeal non-neural neural crest progenitor derived from a p75-positive and Sox10-positive NCSC. In addition to the neurosphere culture system described above, the manipulation of neural tube explant cultures is a promising approach to address this issue. The *in vitro* generation of homogenous NCSC explants is well established in our laboratory (Hari et al., 2002; Kleber et al., 2005; Lee et al., 2004). The cells can be treated with different concentrations of TGFβ ligands in the presence or absence of other growth factors that are crucially involved in neural crest development (Taneyhill and Bronner-Fraser, 2005), for example diverse FGF receptor ligands. Additionally, different cell densities should be tested, as low cell density conditions are associated with a smooth muscle-like fate of neural crest cells upon treatment with growth factors of the TGFβ family (Hagedorn et al., 2000; Hagedorn et al., 1999; Kleber et al., 2005). In contrast, high density and long term culture conditions in combination with FGF factors are permissive for chondrogenesis (Ido and Ito, 2006; McGonnell and Graham, 2002; Kléber, Wurdak, John and Sommer, unpublished observations). In summary, these experiments might reveal a dosage-dependent, as well as time-dependent effect of TGFβ signaling on the formation of a non-neural progenitor cell which is able to generate both smooth muscle cells and chondrocytes. Indeed, preliminary data suggest that TGFβ applied in low concentrations for approximately 5 hours leads to upregulation of Sox9 and concomitant loss of Sox10 expression in neural crest explant cells (John, unpublished observation). In agreement with this finding, a recent publication describes the activation of Sox9 via Smad3, a central mediator of TGFβ signal transduction (Furumatsu et al., 2005). Furthermore, communities of

TGFβ-treated neural crest explant cells seem to undergo chondrogenesis in the presence of FGF, while they adopt a smooth muscle fate in the prolonged presence of TGFβ1 (John, unpublished observation). However, downstream effectors of TGFβ signaling specifying non-neural cell fates remain to be elucidated on the molecular level.

Interestingly, a recent publication suggests that TGFβ can mediate FGF signaling during frontal bone development (Sasaki et al., 2006). Moreover, inactivation of the DiGeorge candidate gene *Crkl* in mice is proposed to disrupt FGF8 signaling (Moon et al., 2006). As illustrated in part1, our findings indicate that TGFβ signal modulation in neural crest-derived cells might be involved in the pathogenesis of DiGeorge syndrome. We showed that TGFβ signaling is both required and sufficient to phosphorylate and activate Crkl in a Src kinase-dependent manner. Thus, the question arises as to whether TGFβ-dependent generation of non neural lineages is dependent on Crkl/Src activity and/or modification of FGF signaling. The expression of FGF ligands and receptors could be studied in the pharyngeal system of control and *Tgfbr2*-mutant mice by in situ hybridization analysis and immunohistochemistry. In addition, FGF receptor modulation could be investigated using antibodies specific for phosphorylation sites that are crucial for FGF receptor activity (Mohammadi et al., 1996).

Crkl function has not been addressed so far on the cellular level in Crkl-mutant mice. Crkl-deficient NCSCs could be isolated from these mutants and further characterized using neural tube explant cultures or heterogeneous pharyngeal arch cultures. Alternatively, a Crkl knockdown could be achieved in wild type neural crest cells using RNAi technology. Introduction of siRNA or dsRNA into cultured neural crest cells could be done by lipofectamine-based transfection or retroviral/lentiviral infection. Moreover, we have recently established a microinjection system, allowing the manipulation of single neural crest cells (Niederer, Wurdak and Sommer, unpublished data). The fate of Crkl-deficient neural crest cells compared to control cells could be monitored by immunostaining for appropriate lineage markers (Sox9, collagen type 2, collagen type 1 for chondrogenic cells and SMαA for smooth muscle-like cells). As an alternative to cultured mouse neural crest cells, the role of Crkl in neural crest development could be analyzed *in vivo* in chick embryos using RNAi and electroporation (Pekarik et al., 2003).

The above experimental suggestions focus on neural crest explant cells or on postmigratory neural crest cells from the pharyngeal system. In principle, they could also be applied to neural crest cells of the anterior eye, giving rise to corneal stroma and endothelium. However, manipulation and enrichment of this small cell population is extremely difficult in the mouse embryo, and thus in my opinion not feasible for a comprehensive study. However, corneal endothelial precursor cells can be isolated from adult human cornea using the neurosphere system (Uchida et al., 2005; Yokoo et al., 2005). We aim to use and establish an analogous experimental approach, allowing the enrichment, *in vitro* propagation and characterization of human corneal endothelial precursors of potential neural crest origin. To this end, we have started a collaboration with the group of Prof. Michael Thiel at the Eye Clinic of the University Hospital Zurich. A long term goal of this collaboration is to expand and differentiate human corneal endothelial precursors *in vitro* in order to achieve *de novo* tissue formation. Subsequently, newly generated endothelium might be used for transplantation into patients suffering from corneal endothelial diseases such as corneal edema.

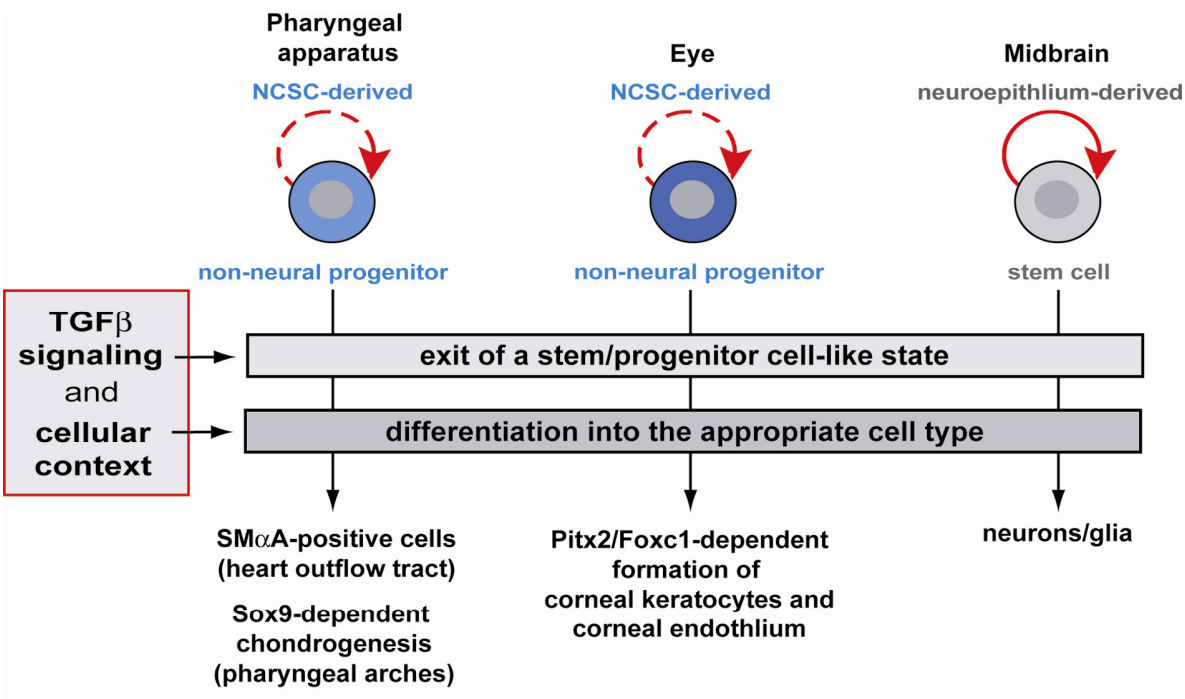

Figure 8.1. Hypothetical scheme of context-dependent interpretation of TGFβ signaling
Neural stem and progenitor cells derived from the neural crest or the midbrain neuroepithelium have to encounter TGFβ signaling during development. Consequently,TGFβ signaling promotes the exit of a stem/progenitor cell-like state, allowing cells to respond to environmental cues in a time and region-specific manner. Thus, differentiation of neural stem and progenitor cells into different appropriate cell types critically depends on the cellular context.

8.2. The role of TGFβ signaling in neural stem cell maintenance in the developing and adult CNS

In part 3 of my thesis I have demonstrated that inactivation of TGFβ signaling in the midbrain/hindbrain region results in a drastic increase of midbrain size due to strong overproliferation and expansion of the neural stem cell number in the caudal midbrain neuroepithelium. Intriguingly, stem cell expansion in mutants is accompanied by an increase in canonical Wnt signaling *in vivo* and in cell culture, indicating that TGFβ signaling normally suppresses Wnt signaling. Hence, the question arises as to whether and how the balance between TGFβ and Wnt signaling controls stem cell maintenance vs. differentiation also in other areas of the CNS, including the developing forebrain and spinal cord, and neurogenic niches of the adult brain such as the subventricular zone. Interestingly, preliminary experiments using immunohistochemistry show that *Tgfbr2* is expressed not only in the developing midbrain but also throughout the CNS neuroepithelium, including the developing forebrain (Wurdak and Sommer, unpublished data). Moreover, wild type neuroepithelial cells isolated from the developing forebrain and treated with TGFβ1 display impaired self-renewal capacity in neurosphere culture (Wurdak and Sommer, unpublished data). Therefore, it is likely that TGFβ signaling is involved in stem cell regulation in CNS areas other than the midbrain. Alternatively, the role of TGFβ in regulating the number of neural stem cells might be specific to the midbrain.

To elucidate the *in vivo* function of TGFβ signaling in the developing forebrain a *Cre/loxP* based, forebrain-specific ablation of *Tgfbr2* could be performed. A breeding strategy comparable to that used for the conditional inactivation of *Tgfbr2* as described in this thesis can be used. Mice carrying the floxed *Tgfbr2* allele could be crossed with *Emx-Cre* mice expressing Cre recombinase specifically in the forebrain neuroepithelium (Iwasato et al., 2004). Expression of progenitor markers, for example Sox2, Hes1 and 5, and nestin as well as differentiation markers such as doublecortin and neuroD can be analyzed at different stages, as previously performed to study midbrain development (Wurdak et al., manuscript submitted). Furthermore, the establishment of cortical layers could be assessed in these mutants. Apoptosis and proliferation can be measured both *in vivo* and upon isolation of neuroepithelial cells in culture. BrdU incorporation assays and cell cycle FACS analysis could

be used to reveal whether cell cycle length and/or cell cycle exit is affected in mutant stem and progenitor cells (Wurdak et al., manuscript submitted). Furthermore, changes in stem cell number and self-renewing activity could be assessed by serial passaging of cells in neurosphere cultures, as described earlier for the analysis of midbrain neural stem cells.

It is possible that TGFβ signaling regulates the maintenance of neural stem cell pools in the CNS in general. Because *Cre* expression in the above mentioned *Wnt1-Cre* and *Emx-Cre* mice is restricted to the dorsal neural tube and the developing midbrain, and the forebrain, respectively, one could also use *Brn4-Cre* mice, facilitating Cre-mediated recombination throughout the developing neural tube with the exception of the forebrain (Ahn et al., 2001). However, though phosphorylated Smad2 protein could be detected at least at some embryonic stages in the neural tube (de Sousa Lopes et al., 2003), the expression of TGFβ receptors and ligands during neural tube development remains to be investigated in detail. Depending on the outcome of this expression analysis, inactivation of TGFβ signaling and, as a possible consequence, alterations of Wnt signaling in these mice, might be of particular interest with respect to the developing spinal cord. Interestingly, recent data from our laboratory suggest that Wnt signaling is modulated by BMP in the dorsal but not the ventral spinal cord, resulting in differential activities of Wnt along the dorsoventral axis of the developing spinal cord (Ille et al., manuscript in preparation). Thus, TGFβ signaling might elicit varying functions in the spinal cord, depending on the spatiotemporal signal distribution. The phenotypic analysis of mice lacking *Tgfbr2* throughout the neural tube might be carried out both *in vivo* and in cell culture, as described above for mutant midbrain and forebrain neuroepithelial cells.

As illustrated in part 3, TGFβ signal inactivation in the midbrain is associated with enhanced canonical Wnt/β-catenin signaling. To investigate whether such a signaling crosstalk occurs throughout the CNS neuroepithelium, one could study the spatiotemporal expression of Wnt ligands and activated, nuclear β-catenin in mice in which the *Tgfbr2* gene has been deleted either by *Emx-Cre* or *Brn4-Cre* activity. In addition to conventional immunohistochemistry, the TOPGAL mouse line (DasGupta and Fuchs, 1999), reporting activation of canonical β-catenin signaling *in vivo* could be crossed into the *Tgfbr2* mutant mouse line. Should TGFβ

signal inactivation in the forebrain, the spinal cord, and other brain areas also result in stem and progenitor cell expansion, I would expect this phenotype to be associated with Wnt signal activation as in the midbrain. The *in vivo* analysis should be combined with the analysis of cultured wild type neuroepithelial cells that can be isolated from the forebrain, the midbrain, and the spinal cord. The cells could either be treated with Wnt alone or with Wnt in combination with TGFβ (Wurdak et al., manuscript submitted, Ille et al., manuscript in preparation). This experiment should reveal whether TGFβ can functionally counteract Wnt signaling and whether this effect is brain-area specific.

Neural stem cells have not only been identified in the developing nervous system but also in the adult brain (Alvarez-Buylla et al., 2001; Temple, 2001). Thus, it will be important to elucidate whether mechanisms controlling stem cell fates during development also play a role in the adult organism. To address whether TGFβ and Wnt signaling control maintenance and/or differentiation of adult neural stem cells, the expression of TGFβ and Wnt signaling components in the subventricular zone and the hippocampus of adult mouse brains should be analyzed. Moreover, a neurosphere assay should be performed, as described for the midbrain neural stem cells (Wurdak et al., manuscript submitted). Should these experiments point to a possible involvement of TGFβ and/or Wnt signaling in stem cell behaviour, a recently established system (Hack et al., 2005) could be applied to conditionally delete *Tgfbr2* or to conditionally activate β-catenin signaling in the adult subventricular zone. Activation of β-catenin can be achieved by specific deletion of exon 3 of β-*catenin*, which leads to intracellular stabilization of β-catenin (Lee et al., 2004). To achieve local *Cre*-mediated gene deletion in dividing cells, a retroviral vector expressing Cre recombinase and GFP can be injected into the subependymal zone of adult mice carrying floxed alleles of the genes of interest (Hack et al., 2005). Proliferation and fate decisions of mutant adult neural stem cells, and their capacity to migrate through the rostral migratory stream (RMS), could be assessed exactly as reported by Hack et al., 2005. These experiments might reveal whether factors and factor combinations crucial for stem cell development in the embryo are also critically involved in the control of adult stem cells of neurogenic niches.

Area measurements of proliferative vs. differentiation zone in the *Tgfbr2*-mutant midbrain indicate that neuroepithelial growth in these mutants is exclusively due to lateral tissue expansion (Wurdak et al., manuscript submitted). Lateral expansion is achieved when symmetrical divisions of self-renewing neural stem cells at the apical surface of the ventricular zone are favoured over asymmetric neurogenic divisions (Gotz and Sommer, 2005). To investigate whether TGFβ signaling affects symmetric vs. asymmetric cell divisions, it would be a promising approach to establish an adhesive clonogenic culture system (Capela and Temple, 2006; Sun et al., 2005). This would allow us to study the behaviour of neuroepithelial cells from control and *Tgfbr2*-mutant developing forebrain, midbrain and spinal cord, as well as of neural stem cells from the adult brain. Briefly, single neuroepithelial cells could be virtually mapped and their fate could be followed using video time lapse microscopy and immunocytochemistry. In my opinion, neural stem cell populations should first be enriched based on their surface marker expression, before using them in adhesive culture. Promising candidate markers are prominin1 and LeX, which have already been successfully used to isolate stem cell-like populations by FACS (Capela and Temple, 2002; Capela and Temple, 2006; Lee et al., 2005). Should symmetric divisions be promoted upon TGFβ signal inactivation, single mutant stem cells will divide to produce clones predominantly consisting of stem cells, while asymmetric neurogenic divisions, generating for instance doublecortin-positive neuronal cells should be impaired (Sun et al., 2005). Treatment of neural stem cells with TGFβ should conversely decrease symmetric divisions and increase the generation of differentiated progeny by asymmetric cell divisions or by lack of self-renewal (Figure 3.2c).

The transition from uncommitted self-renewal to lineage commitment and differentiation is thought to be associated with lengthening of the cell cycle time in neural stem cells (Calegari et al., 2005), and/or the decision of stem/progenitor cells to exit the cell cycle (Durand et al., 1998; Tarui et al., 2005, Siegenthaler and Miller 2005). Several signaling events including TGFβ signaling have been reported upstream of factors such as p27, regulating cell cycle decisions (Castelo-Branco et al., 2003; Go et al., 2000; Mishra et al., 2005). Thus, cell cycle control is very likely crucially involved in the different mechanisms of stem cell self-renewal, and in the switch from self-renewal to differentiation (Figure 3.2). However, it will be an

important goal of future studies to investigate how the combined action of TGFβ signaling and other signaling pathways regulate the cell cycle of different stem/progenitor cell types. Moreover, the molecular interactions between signaling pathway components and components of the cell cycle machinery remain to be elucidated.

9. MATERIALS AND METHODS

9.1. Generation of mutant embryos and *in vivo* fate mapping

Animals homozygous for the floxed allele of exon4 of *Tgfbr2* (Leveen et al., 2002), encoding the transmembrane domain and essential parts of the intracellular domain of the TGFβRII protein, were used for time-matings with *Wnt1-Cre* mice heterozygous for the *Tgfbr2* floxed allele. The morning of the day when vaginal plugs could be found was designated as E0. Genotyping was performed as previously described (Leveen et al., 2002). The penetrance of the overall mutant phenotype described in this study was 100% in *Tgfbr2*-mutant animals. Littermates carrying a wild-type allele or lacking *Wnt1-Cre* displayed no overt phenotype and were used as controls. All animal experiments where performed on the C57BL/6 background.

The *Rosa26* reporter (*Rosa26R*) mouse strain expresses β-galactosidase upon Cre-mediated recombination (Soriano, 1999). To define distinct NC contribution to embryonic structures during development *Rosa26R* mice were crossed with *Wnt1*-Cre transgenics (Hari et al., 2002). At least three whole embryos per different stage were stained with the β-galactosidase substrate 5-bromo-4-chloro-3-indolyl-β-D-galactopyranosid (X-gal; Sigma) and subsequently fixed in 4% (v/v) paraformaldehyde over night at 4 °C. Subsequently, embryos were paraffin embedded, sectioned at 7 μm, and dewaxed for mounting with DFX (Fluka). Some sections were counterstained with eosin (Fluka). In addition, *LacZ*-reporter gene expression was detected on cryosections (Hari et al., 2002).

9.2. Histology, Immunohistochemistry and TUNEL staining

Embryonic tissue was fixed in 4% (v/v) paraformaldehyde. Fixation period durations were depending on the embryonic stage and size of organs and tissue (For example, fixation of a E10.5 embryo was for approximately 4 hours at room temperature). Subsequently, paraffin embedding and sectioning (5 μM sections unless specifically stated otherwise) was done according to standard and partially automated techniques (for a detailed protocol of the embedding procedure see http://baygenomics.ucsf.edu/protocols/Embedding). Sections were deparrafinized and Hematoxylin-Eosin staining was performed according to a standard

protocol. Alternatively, deparrafinized and rehydrated sections were used for immunostaining. Antigen retrieval was achieved by wet heat treatment of the tissue sections in 1M Citrate Buffer, pH 6 for 10 minutes at 95°C. Subsequent blocking was done for 30 minutes at RT in PBS containing 10% (v/v) FCS and 0.5% (v/v) TritonX-100. The blocking solution was also used for all washing steps. The following tissue/materials, staining procedures, primary antibodies, secondary antibodies and detection methods were used in part 1-3 of this thesis:

Part 1:

Whole mount E10.5 embryos were X-Gal-stained for at least 5 hours at room temperature. Subsequently, the embryos were paraffin embedded and sectioned. Paraffin sections (7 μm) were stained with goat anti-PTH antibodies using the Vectastain method (VectorLab). Specificity of the antibody was tested by saturation with PTH(1-34). Anti-smooth muscle α-actin (Clone 1A4; Sigma) and anti-TGFβRII-antibodies (C-16; Santa Cruz Biotechnology Inc.) were applied (1:100). Visualization was done using Alexa546-linked secondary antibodies (Abcam Inc.) or by the Vectastain method using NovaRed as a substrate (VectorLab), and phospho-Smad2 and phospho-Crkl (1:100, Cell Signaling Technology, Inc.) by Metal Enhanced DAB (Pierce). Sections were observed using an Eclipse E600 microscope (Nikon) with a CCD camera (Kappa).

Standard protocols were used for bone and cartilage staining with alizarin S red and alcian blue. Briefly, the calvaria were dissected by removing skin and brain tissue completely. After fixation in 95% (v/v) ethanol for 24 hours at room temperature, alcian blue solution (stains acid mucopolysaccharides; cartilage) was applied for 3 days, followed by incubation with 95% (v/v) ethanol with 2% KOH for 24 hours and incubation with alizarin S red solution (indicator of calcium phosphate; bone) for 24 hours at room temperature. Stained calvaria were kept in clearing solution with 1:1 glycerol, 95% (v/v) ethanol. Frontal sections of the E18 palate were stained by standard van Kossa's method.

TUNEL assays on paraffin-embedded tissue sections were performed following the producer's guidelines (Roche Diagnostics). Briefly, after fixation with 4% (v/v) paraformaldehyde for 15 minutes at room temperature, the sections were treated with 3% (v/v) H_2O_2 in PBS for 10 minutes at room temperature, followed by treatment with proteinase K (50 μg/mL) for 10 minutes at room temperature. Subsequently, blocking was done in

blocking buffer containing 1% (v/v) TritonX-100 , 10% FCS, 0.1% (w/v) BSA in PBS, followed by equilibration in TDT buffer (30 mM Tris-HCL, pH 7.2 140 mM sodium cacodylate, 1 mM cobalt chloride) and incubation in TUNEL reaction mix (0.2U/µL TDT enzyme, 6nM biotin-16-dUTP, 6 nM dATP) for 90 minutes at 37°C. The staining was visualized using Cy3-conjugated streptavidin (1 : 200 dilution, Jackson ImmunoResearch Laboratories) and fluorescent microscopy.
Note that each marker was analyzed on at least 3 embryos per stage.

Part 2:

Eyes of at least three embryos per stage were stained with primary antibodies to TGFβ1, TGFβ2, and TGFβ3 (1:100, Santa Cruz), Tgfbr2 (also termed TGFβRII in part 1 and part 3, 1:100 Santa Cruz), pSmad2 (1:100, Cell Signaling), Ki-67 (1:100, LabVision), Brn3A (Fedtsova and Turner, 1995), Pax6 (1:100, Chemicon), neurofilament (1:200, Chemicon), Foxc1 (1:100, Santa Cruz), Pitx2 (1:100, Hjalt et al., 2000), and GFAP (1:200, Sigma). For visualization the ABC elite Kit (Vector) with Metal enhanced DAB (Pierce) or AP substrate kit I (Vector) as substrates was used. TUNEL assays were performed following the producer's guidelines (Roche Diagnostics) as described above. Sections were observed using an Eclipse E600 microscope (Nikon) with a CCD camera (Kappa). Standard protocols were used for tissue processing of semi-thin sections and subsequent toluidine blue staining (Wenzel et al., 2000). The Van Gieson's staining procedure was used to visualize collagen formation in the cornea. All sections were observed using an Eclipse E600 microscope (Nikon) with a CCD camera (Kappa).

Part 3:

Midsagittal and parasagittal brain sections of at least three embryos per stage were stained with polyclonal primary antibodies to TGFβRII (1:100, Santa Cruz), pSmad2 (1:100, Cell signaling), KI67 (1:100, LabVision) and DCX (1:500, Santa Cruz). For visualization the ABC elite Kit (Vector) with Metal enhanced DAB (Pierce), or AP substrate kit was used. Sections were observed using an Eclipse E600 microscope (Nikon) with a CCD camera (Kappa). DCX-positive and DCX-negative areas were determined using the PicEd Cora software version 8.08 (Jomesa). At least two different sections from 3 different embryos were

analyzed. Anti-β-catenin staining was performed using a monoclonal antibody (1:300, Sigma) and visualized by an Alexa546-linked secondary antibody in combination with standard DAPI DNA-labeling. Sections were analyzed using the confocal system TCS SP2 (Leica) and microscope (DMIRBE, Leica) equipped with a 40X / NA 1.25 oil immersion objective. Digital images were obtained using Leica confocal software 2.61.

9.3. In situ hybridization

Non radioactive in situ hybridization with digoxigenin-labeled riboprobes was performed on cryosections as described (Hari et al., 2002; Lee et al., 2004). Antisense riboprobes (Sommer laboratory) were to sox9, sox10, dhand, ehand, tbx1, tgfβ1-3, pax3, crabp1, dct (also termed trp2), wnt1, sox2, otx2. NBT/BCIP were used to visualize hybridization signals. Sections were analyzed using an Eclipse E600 microscope (Nikon) with a CCD camera (Kappa).

9.4. Neurosphere culture

Neurosphere cultures were prepared as previously described (Reynolds and Weiss, 1996) in presence or absence of 0.5 ng/mL TGFβ1 (R&D) as indicated. E12.5 or E15 dorsal midbrains were dissected and mechanically triturated with a fire-polished glass pipette. For primary neurosphere cultures, cells were resuspended at 20.000 cells/mL in DMEM/F12 medium (Gibco), supplemented with B-27 (Gibco) and 20 ng/mL FGF (R&D), as well as 20 ng/mL EGF (R&D). After five days of growth, primary cultures were dissociated and secondary neurospheres were grown at clonal density (1’000 cells/mL). Subsequently, serial passaging of neurospheres was done in seven day intervals, again at clonal density. The neurospheres of each passage were observed using a Zeiss Axiovert microscope and the sphere diameters were determined using an Axiocam camera and Axiovision Release 4.3 software. Only spheres with a diameter >50μM were counted. The cell number per dissociated sphere was analyzed using a standard Neubauer counting chamber. In order to induce differentiation, secondary neurospheres were plated onto coverslips pre-coated with poly-L-lysine (Sigma) in DMEM/F12 supplemented with B-27. After five days, neurospheres were fixed in 4% formaldehyde and immunostained with primary antibodies against GFAP (1:300, DAKO) for

astrocytes, β3-tubulin (1:300, Sigma) for neurons, and O4 (1:100, Chemicon) for oligodendrocytes.

9.5. Neuroepithelial cell culture

Dorsal midbrain cells were isolated from embryonic rat brains at E14 (equivalent to mouse E12.5) and cultured in non-differentiating conditions using neuroepithelial (NEP) basal medium (Kalyani et al., 1997) with 25 ng/mL FGF and 10% chicken embryo extract for 2 days. Three independent experiments were performed and the NEP cell population was used (I) for clonal analysis in order to address Wnt-mediated proliferation of single NEP cells ± TGFβ1, and (II) for mass culture in order to address neuronal and glial differentiation ± TGFβ1.

(I) The cells were treated with BrdU for 1 day to allow retrospective identification of clones and labeled with PKH26 (Sigma) to allow prospective identification of clone founder cells. Cells were plated at a density of 50 cells per well in 12 well dishes (Nunclon) onto Wnt1-producing and control feeder layers, respectively (Kleber et al., 2005). Single adherent cells giving rise to clonal progeny were mapped and incubation was for 2 days in differentiating NEP medium (basal NEP medium plus 25 ng/mL FGF, 50 ng/mL NGF and 100 ng/mL EGF) in presence or absence of 5 ng/mL TGFβ1. Subsequently, cells were fixed in 4% formaldehyde and nuclei were labeled using the anti-BrdU labeling kit (Roche). The number of cells per clone, as well as the number of lost clones was determined. The fate of at least 200 clones per experiment was analyzed.

(II) 1000 NEP cells per well were plated on 24 well dishes (Nunclon) coated with Poly-D-lysine (Sigma), and Fibronectin (Sigma). Incubation was for 2 days in differentiating NEP medium with or without 5 ng/mL TGFβ1. Subsequently, the cells were immunostained using primary antibodies against β3-tubulin and GFAP. Secondary antibodies were AP-coupled (1:1000, Southern Biotechnology Associates). Detection was performed using a 4-Nitrophenyl phosphate solution (Fluka) at RT for 5 minutes. The OD was measured at 405 nm using a calibrated ELISA reader (Bio-Rad).

9.6. Culture experiments, Western blot analysis

Part 1

Primary neural tube explant cultures were done as described (Hari et al., 2002; Lee et al., 2004). Cells gained from neural crest explants were fixed after 20 hours with 2% formaldehyde for 10 minutes at room temperature before staining. Monc1 cells were cultured and treated with TGFβ1 (5 ng/ml, R&D) for 15 minutes at 37 ^{o}C as reported (Chen and Lechleider, 2004). For inhibition of Src kinases, PP1 or PP2 (Calbiochem) were added to the medium 40 min prior to TGFβ treatment. Cells were lysed in protein gel loading buffer containing 50 mM sodium orthovanadate (Sigma). Western blot analysis of Monc1 cell extracts was carried out as described (Koller et al., 2004). Primary antibodies were against Actin (Chemicon Inc.), phospho-Smad2 (S465/467; Cell Signaling Technology, Inc.), phospho-Src (Y416; Cell Signaling Technology, Inc.) and phospho-Crkl (Y207; Cell Signaling Technology, Inc.). Immunocytochemistry of Monc1 cells treated with TGFβ for 3 days was done as described (Lee et al., 2004).

Part 2

Rat embryonic fibroblasts (rat2; ATCC) were cultured in DMEM:F12 medium (Gibco) containing 10% fetal bovine serum (Sigma). Following 60 min incubation in DMEM:F12 medium containing 0.1% bovine serum albumin at 37°C, cells were treated with TGFβ (5 ng/mL) for 90 min at 37°C as described above. For short term tissue culture experiments, the eyes with periocular tissue were removed from nine embryos at E11 by microdissection. Left and right eyes were pooled separately and kept in DMEM:F12 medium containing 0.1% bovine serum albumine and antibiotics with and without TGFβ (5ng/mL), respectively, for 6 hours at 37°C. Western blot analysis of rat2 extracts and eye tissue extracts were carried out as described (Ittner et al., 2005). Primary antibodies were against Actin (Chemicon), pSmad2 (Cell Signaling), Foxc1 (Santa Cruz) and Pitx2 (Hjalt et al., 2000). Each experiment was done at least three times.

Part 3

Dorsal midbrains were removed from E12.5 or E15 embryos and the cell extracts were used for Western blot analysis (Wurdak et al., 2005). Primary antibodies were against actin (Chemicon) and dephosphorylated (activated) β-catenin (Upstate).

9.7. Cell cycle FACS analysis

Cells were isolated from different dorsal midbrains of E10.5 (n=3) and E13 (n=4) control and mutant embryos and mechanically triturated. The cell suspension was split 1:1 in order to use one half of cells for fixation with 70% (v/v) ethanol for 30 minutes at room temperature, and the other half for fixation with 4% (v/v) PFA in PBS, 0.01% (v/v) saponin for 10 minutes at room temperature, respectively. The fixative was washed away and cells were resuspended in FACS buffer (PBS containing 1% FCS, 5mM EDTA). The DNA content of ethanol-fixed cells was stained with propidium iodide (PI, final concentration 1mg/mL) for 10 minutes and used for FACS analysis. To stain proliferative cells, polyclonal anti-KI67 (1:100, Abcam) was added to the PFA-fixed cells in FACS buffer containing 0.01% (v/v) saponin. After 30 minutes at room temperature, the cells were washed, incubated with FITC-labelled goat anti-mouse Ig (1:300, Jackson) for 30 minutes at room temperature, and subsequently used for FACS analysis. The intensity of PI and KI67 staining was analyzed in a FACS calibur (BD Biosciences) in the fl3 channel.

9.8 Intracardial ink injection and assessment of ocular growth

Intracardial indian ink injection was carried out as reported (Jerome and Papaioannou, 2001). Briefly, a glass capillary was placed under visual control into the heart of E10.5 embryos or into the left ventricle of E18.5 embryos. Serial pictures were taken after ink injection.

Ocular growth was assessed using at least three *Tgfbr2*-mutant and control embryos per stage. Embryos were embedded and sectioned. Midorgan sagittal sections of both eyes were measured using an Eclipse E600 microscope (Nikon) equipped with a CCD-camera (Kappa) and the PicEd Cora software version 8.08 (JOMESA).

9.9 Statistical analysis

Results are shown as mean ± standard deviation of the mean (SD). Graphs and statistical analysis (two-tailed unpaired Student's t-test; calculation of SD) were done with Excel 2003 SP1 software (Microsoft) with Prism 4.01 (GraphPad Software).

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11. PUBLICATIONS

Wurdak H.*, Ittner L.M.*, Ille F., Falk S., Paratore C., Lang K.S., Leveen P., Suter U., Karlsson S., Born W., Sommer L. (2006) TGFβ signaling regulating midbrain size and maintenance of neuroepithelial stem cells. *shared first authorship
MANUSCRIPT SUBMITTED

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Ittner L.M.*, **Wurdak H***, Schwerdtfeger K., Kunz T., Leveen P., Hjalt T.A., Suter U., Karlsson S., Born W., Sommer L. (2005) Compound developmental eye disorders upon TGFβ signal inactivation in neural crest stem cells. J. Biol, 4, 11.1-11.16. *shared first authorship

Wurdak H.*, Ittner L.M.*, Lang K.S., Leveen P., Suter U., Fischer J.A., Karlsson S., Born W., Sommer L. (2005) Inactivation of TGFβ signaling in neural crest stem cells leads to multiple defects reminiscent of DiGeorge syndrome. Genes Dev, 19, 530-535. *shared first authorship

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13. CURRICULUM VITAE

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