Roles of HMG-box Transcription Factors in the Pancreas Development of the Mouse (*mus musculus*, Linnaeus, 1758)

Dissertation

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Summary

The mammalian pancreas is comprised of several cell populations, the exocrine cells, which are organized into acini, the endocrine cells, which form the islets of Langerhans, as well as the ductal cells, endothelial cells, and neurons. A key function of the endocrine pancreas is the control of blood glucose homeostasis. Loss or defects of the insulin-producing ß-cells in the pancreas lead to the pathological condition *diabetes mellitus*. One possible therapy for *diabetes mellitus* is the development of a culture system to generate replacement ß-cells *in vitro*. However, to develop such replacement therapy, we first need to identify the factors, which control ß-cell differentiation. It has been shown that several classes of tissue restricted transcription factors have crucial functions in the pancreatic endocrine cell differentiation. HMG box proteins are a class of transcription factors, whose function has not yet been explored in the pancreas. The overall goal of this research project was to analyze the expression of HMG-domain transcription factors in the developing mouse pancreas, and to study their function in pancreas development.

The HMG box class contains two transcription factor gene families, the *Sox* and *Tcf/Lef* transcription factors. In mammals, the *Sox* family of HMG box transcription factors is comprised of twenty members, which are classified into nine groups on the basis of sequence similarity and genomic organization. Sox transcription factors have been shown to control the development of numerous tissues and cell types during embryogenesis. However, little is known about their expression and function in the pancreas. One goal of this research project was to characterize the expression and function of Sox genes in the mouse pancreas. Expression of thirteen different Sox genes, which belong to groups C, D, E, F, G and H, was found in the developing pancreas or in adult endocrine islets. Subsequently, the expression patterns of seven Sox genes (*Sox4, Sox11, Sox5, Sox13, Sox8, Sox9, Sox10*) were analyzed in detail by *in situ* hybridization at several stages of pancreas development. Sox transcription factors were detected in pancreas from as early as E9.5 to adulthood. In the pancreatic epithelium, different Sox genes were often expressed in overlapping domains, suggesting that there may be functional redundancy. To study the function of Sox genes in pancreas development, two *Sox* mutant mouse strains, *Sox8* and *Sox10* mutant mice, were analyzed for defects in overall pancreatic morphology and in the expression of different cell lineage markers. Neither homozygous *Sox8* nor *Sox10* mutant mice displayed any defects in

pancreatic endocrine or exocrine differentiation, suggesting that both *Sox8* and *Sox10* are dispensable for endocrine and exocrine pancreas development. However, in *Sox10*-/- mice, Schwann cells, which are the islet-sheathing glial cells in the pancreas, were completely absent from the neonatal pancreas. Since endocrine or exocrine development was not affected in *Sox10*-/- mice, this finding suggests that pancreatic Schwann cells are not required for endocrine and exocrine differentiation. In summary, this novel information on the expression of Sox transcription factors in the embryonic and adult mouse pancreas will be the necessary basis for studying *Sox* gene functions in the pancreas.

In the second part of this research project, the role of TCF/LEF transcription factors in murine pancreas development was explored. TCF/LEF proteins are downstream effectors of the canonical Wnt signaling pathway. The canonical Wnt signaling pathway controls cell differentiation in numerous tissues during embryogenesis and has also been implicated in the control of stem cell maintenance in regenerating tissues, such as the hematopoietic cell lineage, skin and intestine. Stimulation of the Wnt signaling pathway results in the nuclear translocation of ß-catenin, which forms a complex with TCF/LEF proteins to activate Wnt target genes in the nucleus. To date, it is still unclear whether the pancreas contains true stem cells and if so, whether Wnt signals control their maintenance. As a first step to identify a possible role of Wnt signaling in the pancreas, the expression of *Tcf/Lef* genes as well as of other components of the canonical Wnt signaling pathway was studied during pancreatic development. It was found that all four *Tcf/Lef* genes, as well as genes coding for the Wnt ligands, the Frizzled receptors and other key factors of the canonical Wnt pathway were expressed in the developing pancreas from the earliest stages through adulthood.

Next, to study if Wnt signaling is active in the pancreas, pancreata of two independent Wnt reporter mouse lines, in which formation of an active TCF/LEF/ß-catenin complex leads to the expression of ß-galactosidase, were analyzed by enzymatic ß-galactosidase staining. The analysis showed that the canonical Wnt pathway is active from early formation of the pancreatic anlage until birth. However, no activity was detected in the adult pancreas. To address if the canonical Wnt signaling controls pancreas development, transgenic mice were generated, in which the canonical Wnt cascade was either blocked, or ectopically activated in early pancreatic progenitors. To block Wnt signaling, a dominant negative form of TCF4 (dnTCF4) was expressed under control of an early pancreas specific promoter, while a constitutively active form of TCF4 (caTCF4) was used to stimulate Wnt signaling. Late stage

embryos of neither one of the two transgenic strains displayed detectable pancreatic defects. Since the promoter which was used to drive the transgene, only targets a small population of cells at later developmental stages, the lack of transgene expression in appropriate cell populations could account for the absence of a phenotype. To overcome this problem, a bigenic *Cre-loxP* based system was employed, which through matings with different *Cre*-recombinase expressing mouse lines, allows for expression of a dnTCF4 or caTCF4 in various cell populations of the pancreas. In the mice expression of dnTCF4 or caTCF4 is controlled by the ubiquitous *ROSA26* locus, but expression is prevented by a STOP cassette. Only removal of this STOP cassette by *Cre* recombinase leads to heritable and stable expression of the TCF4 transgene. Thus far, double transgenic $Rosa26^{dnTcf4}$*:ins-*$cre^{+/tg}$ mice, in which canonical Wnt signaling is inhibited in mature ß-cells, have been generated.

The results of this study show that HMG box transcription factors of both the Sox and Tcf/Lef families are expressed during murine pancreas development. Moreover, detection of TCF/LEF/ß-catenin-mediated transcription in Wnt reporter mouse lines demonstrates that pancreatic progenitor cells receive canonical Wnt signals. The generation of a flexible, bigenic *Cre-loxP* based transgenic system will allow us to now study the role of Wnt signaling in pancreatic development, and adult ß-cell function.

I Introduction

The pancreas is an endocrine and exocrine organ, which plays an important role in the nutrient metabolism. The most abundant endocrine cell type is ß-cells, which produce the hormone of the glucose homeostasis, insulin. Loss or dysfunction of the ß-cells are the frequent causes of the pathologic condition diabetes mellitus. One of the promising approaches to the treatment of diabetes mellitus is the generation of replacement ß-cells. However, in order to be able to generate functional ß-cells *in vitro*, it is necessary to better understand the mechanisms and key factors, which regulate ß-cell differentiation during embryonic development. In the process of mammalian embryonic development, pancreatic multipotent progenitor cells give rise to the endocrine as well as exocrine cells of the pancreas. The processes of organ morphogenesis, progenitor cell expansion and differentiation are tightly controlled by a variety of factors, many of which are transcription factors (Edlund, 2002; Gu et al., 2004; Sander and German, 1997). However, our understanding of ß-cell differentiation is still incomplete. The functions of many transcription factors known to be crucial for the morphogenesis of other organs has not yet been analyzed in the developing pancreas. One key class of developmentally important factors, which has not been studied in the pancreas, is the HMG box family of transcription factors. In the present study, the roles of the HMG box transcription factors in mouse pancreas development are explored. First, regulation of gene expression by transcription factors will be introduced. Then, already known roles of two key subclasses of HMG box transcription factors in the mammalian development will be reviewed. Finally, an overview of the embryonic development of the mouse pancreas will be given.

1.1 Transcription Factors

1.1.1 Eukaryotic Gene, Regulation of Transcription

Metazoan organisms consist of a variety of highly specialized cell types. This cell type diversity is created during embryonic development by the temporally and spatially coordinated synthesis of specific proteins. In the cell, the production of protein amounts is controlled at different levels, such as chromatin remodeling, mRNA splicing and stability, mRNA translation, as well as the rate of protein degradation (Lewin, 2000). The research presented here focuses on the gene regulation at the transcriptional level.

Tight regulation of transcription is achieved by two different means. First, cis-acting DNA sequences determine the specificity of binding by regulatory factors; second, protein factors are recruited to the cis-DNA elements and form larger protein complexes to exert their transcription regulatory functions (Alberts et al., 1994). Promoters, enhancers and silencers comprise the cis-acting DNA sequences group. Promoters of the eukaryotic protein-coding genes mostly lie upstream of the transcription startpoint, vary in their sequence and show modular design, i.e. contain characteristic short sequence elements, which serve as recognition motifs for specific transcription factors (Lewin, 2000). Some of these sequence elements, as well as the factors, which recognize them, are common to a variety of different promoters; others are characteristic only to some genes (Alberts et al., 1994). In eukaryotic cells, protein-coding genes are transcribed by RNA Polymerase II protein complex (Alberts et al., 1994). The large number of factors which act in conjunction with RNA Polymerase II, can be divided into three classes:

- First, the ubiquitous factors required for the initiation of RNA synthesis at all promoters and determination of the initiation site. Together with RNA Polymerase II they constitute the basal transcription apparatus (Lewin, 2000).
- Second, DNA-binding factors that increase the efficiency of initiation. These factors are ubiquitous, bind upstream of the transcription startpoint, and their activity is not regulated (Lewin, 2000).
- Third, transcription factors with regulatory roles. They differ from the second group in that they are synthesized or activated at specific time points in specific cell types and tissues, and therefore control transcription spatially and temporally (Lewin, 2000). Proteins belonging to the third group are of high interest to the field of developmental biology, because they control cell differentiation (Alberts et al., 1994; Lewin, 2000). In this study, these factors will be referred to as “transcription factors”.

1.1.2 Transcription Factors

Analyses of transcription factors have revealed that many of them contain characteristic motifs, which are responsible for either binding to DNA, or to other proteins (Alberts et al., 1994). In general, these motifs are relatively short and comprise only a part of a transcription factor. Based on the similarities between the characteristic motifs, transcription factors can be classified into several groups. Within each multiprotein family, the members often display

closely related or essentially identical DNA binding properties, but are distinct outside their DNA-binding domain (Kadonaga, 2004). The HMG (High Mobility Group) box transcription factor class belongs to the superclass of ß-Scaffold Factors with Minor Groove Contacts.

1.2 Structure, Expression and Known Developmental Roles of the HMG Box Transcription Factors in Mammals

All factors of the HMG box class bear a single or multiple characteristic HMG domains. The 79 amino acid long HMG domain forms an L-shaped module composed of three helices that bind to the minor groove of the DNA, which leads to sharp bend of 80° to 135° in the DNA helix (Ferrari et al., 1992). Therefore, HMG box proteins are believed to act as architectural factors by bringing DNA-bound proteins into close proximity, and facilitate the assembly of larger protein complexes (Ohe et al., 2002; Travers, 2000; Wilson and Koopman, 2002). In mammals, the class of HMG box proteins consists of four different families: Sox, TCF/LEF, UBF, and HMG2-related family. Two different types of HMG box factors are distinguished: first, chromosomal HMG box proteins with two or more HMG domains that bind DNA with moderate affinity and little or no sequence specificity (UBF and HMG2-related families). The chromosomal HMG box proteins are purely architectural factors; they bend, distort, or modify the DNA structure bound by histones (Agresti and Bianchi, 2003; Soullier et al., 1999). The second group comprises Sox and TCF/LEF factors. Their characteristic is a single HMG domain, which binds to a specific recognition sequence in the promoters of target genes. (Bustin, 2001; Laudet et al., 1993; Soullier et al., 1999). As Sox and TCF/LEF proteins regulate cell-specific gene transcription, in the present study we focused on the roles of these two gene families, *Sox* and *Tcf/Lef*.

1.2.1 SOX Transcription Factors

Sox transcription factors are found in all metazoan species. In mouse and human, there are 20 different Sox transcription factors (Schepers et al., 2002; Soullier et al., 1999). Via their HMG-domain, all Sox factors recognize the same conserved motif in the DNA, 5'-(A/T)(A/T)CAA(A/T)G-3' (Connor et al., 1994; Harley et al., 1994). Outside the HMG box, Sox proteins are poorly conserved. All Sox proteins possess a variety of different domains through which they bind to other cofactor proteins, homo- or heterodimerize, or activate transcription (Wegner, 1999). The HMG box itself not only binds DNA, but also participates in protein-protein interactions (Wilson and Koopman, 2002).

Group	Gene	Major Known (or Suggested) Functions
A	*Sry*	Testis determination, (pre-mRNA splicing)
B1	*Sox1*	Lens development, (neural determination)
	Sox2	Neural induction, (lens induction, pluripotency)
	Sox3	(Neural determination, lens induction), *candicate gene for Borjeson-Forssman-Lehmann syndrome*
B2	*Sox14*	(Interneuron specification, limb development)
	Sox21	(CNS patterning)
C	*Sox4*	Heart, lymphocyte, thymocyte development
	Sox11	(Neuronal and glial maturation)
	*Sox12**	(Development of many tissues)
D	*Sox5*	Chondrogenesis
	Sox6	Chondrogenesis, (pre-mRNA splicing)
	Sox13	(Development of arterial walls)
E	*Sox8*	(Development of many tissues)
	Sox9	Chondrogenesis, sex determination, (pre-mRNA splicing), *Campomelic dysplasia*
	Sox10	Neural crest specification*, Waardenburg-Hirschsprung syndrome*
F	*Sox7*	(Development of vascular and many other tissues)
	Sox17	Endoderm specification, role in Wnt signaling cascade
	Sox18	Vascular and hair follicle development
G	*Sox15***	(Myogenesis)
H	*Sox30*	(Male germ cell maturation)

Table 1.1. Mouse and human *Sox* genes. The classification of Sox members into groups, and their functions as inferred from mouse null or mutant phenotypes and human mutations, are indicated. Functions in parentheses are deduced from *in vitro* experiments, expression patterns or other studies. Known human pathologies caused by mutations in a *SOX* gene are italicized. Human orthologs of the mouse *Sox* genes previously bore following names: (*),*SOX22*; (**), *SOX20*. Adapted and updated from Schepers et al., 2002.

The first successfully cloned and characterized *Sox* gene was the *SRY* gene, which encodes the testis-determining factor, located on the human and mouse Y chromosome (Clepet et al., 1993). This gene gave the Sox family its name (SRY-related HMG box). The other 19 human *SOX* genes were identified by the homology (amino acid identity of more than 50%) of their HMG boxes, in relation to that of the *SRY* gene. Sox transcription factors are classified into nine groups, designated A-H, according to the similarity of their HMG box domains (Schepers et al., 2002). In the Table 1.1, all mouse and human *Sox* genes are listed, and their known or deduced functions are indicated. Mutations in some of the *SOX* genes have been implicated in the pathogenesis of several human congenital syndromes; in those cases names of the syndromes are given in italics (Table 1.1). Numerous studies have demonstrated pivotal roles for *Sox* genes in the development of many tissues and cell types in mammals (reviewed in (Prior and Walter, 1996).

1.2.1.1 Expression and Functions of Sox Transcription Factors in Mammalian Embryonic Development

In mammals, Sox transcription factor genes are expressed from the earliest stages of development until adulthood, and the majority of *Sox* genes are expressed during the embryonic development (Pevny et al., 1998; Prior and Walter, 1996; Wegner, 1999). Many *Sox* genes are expressed in more than one tissue. For example, *Sox*9 is expressed in developing chondrocytes (Wright et al., 1995) and in Sertoli cells of the developing testis (Kent et al., 1996; Morais da Silva et al., 1996). The opposite is also true; many tissues and cell types express more than one *Sox* member at the same time point. For example, *Sox8* and *Sox9* are expressed together in kidney and testes (Kent et al., 1996; Sock et al., 2001; Wright et al., 1995), while *Sox5*, *Sox6* and *Sox9* are co-expressed in all chondrogenic sites of mouse embryos (Lefebvre et al., 1998). Sox genes have also been shown to play crucial roles in the development and function of many tissues (see Table 1.1). Loss and gain of function experiments in mice demonstrated roles in sex determination (*Sry*, *Sox*9), early embryogenesis (*Sox*2), neurogenesis (*Sox*2, *Sox*3, *Sox*1 and *Sox*11), chondrogenesis (*Sox*5, *Sox*6 and *Sox*9), neural crest cell development (*Sox*9 and *Sox*10), lens development (*Sox*2 and *Sox*1) and heart development (*Sox*4) (Cheung and Briscoe, 2003; Foster et al., 1994; Kent et al., 1996; Koopman et al., 1991; Kuhlbrodt et al., 1998a; Nishiguchi et al., 1998; Pevny et al., 1998; Schilham et al., 1996). The continued expression of *Sox* genes in late embryonic development and adulthood suggests that these factors may play a role in far more

developmental processes than previously thought. However, the early lethality of complete loss-of-function alleles often precludes the analysis of subsequent developmental processes. For example, *Sox4*-deficient mice die at E14 due to circulatory failure, precluding an analysis of the function for *Sox4* in the brain, gonads, lung, heart and thymus during mouse development (Schilham et al., 1996).

1.2.2 TCF/LEF transcription factor family

The *Drosophila* and *Caenorhabditis elegans* genomes contain each only one *Tcf* gene, called *pangolin* and *Pop-1*, respectively. In mammals, four genes of this family have been found, *Tcf-1, Lef-1, Tcf-3* and *Tcf-4.* The founding members of the family that gave it its name, TCF-1 and LEF-1, were originally identified in screens for transcription factors specific for T-lymphocytes. All four factors contain a single HMG box. Remarkably, TCF/LEF proteins possess no transactivation domains, and therefore cannot activate transcription of target genes alone in reporter assays. They all bind to the consensus DNA sequence 5'-AGATCAAAGGG-3' (Giese et al., 1991; van Beest et al., 2000).

Tcf/*Lef* genes are expressed in numerous tissues during mammalian development, as well as in adults, often in overlapping domains. The earliest embryonic expression of a *Tcf/Lef* gene in mice is that of *Tcf-3*, which is expressed between E (embryonic day) 6.5 and E10 ubiquitously, and subsequently downregulated (Korinek et al., 1998b; Merrill et al., 2004). Up until E10.5 of mouse development, limb buds, neural crest, nasal process and pharingeal arches express both *Tcf-1* and *Lef-1* (Oosterwegel et al., 1993). In the mid-gestation, *Tcf-1* and *Lef-1* are expressed in the lung, tooth buds, thymus and urogenital system, while *Tcf-4* expression is detected in di- and mesencephalon, as well as in the gut epithelium (Korinek et al., 1998b). In adult mice, *Tcf-1* is expressed in T-lymphocytes, whereas *Lef-1* is found in B-lymphocytes (Oosterwegel et al., 1993). The other two TCF/LEF members mark stem cells in the adults. *Tcf-3* is expressed in stem cells of the skin (DasGupta and Fuchs, 1999), while *Tcf-4* expression is maintained in the stem cell compartment of the intestinal epithelium (Korinek et al., 1998a).

1.2.2.1 Binding Partners of Tcf/Lef Factors

Through the interaction with different proteins, TCF/LEF factors can function as transcriptional activators or repressors (Brantjes et al., 2002). The first protein found to interact with TCF/LEF factors was ß-catenin (Molenaar et al., 1996), an end-step effector of canonical Wnt signaling cascade (see below). All members of the TCF/LEF family can bind ß-catenin via the well-conserved N-terminal domain of 55 amino acids. TCF/LEF factors lack the transcription activation domain, whereas a transcriptional activator ß-catenin has no DNA-binding properties. Upon binding of ß-catenin to TCF/LEF factors, a bipartite transcription factor is formed, which is able to activate the transcription of target genes through modification of the local chromatin structure (Brannon et al., 1999; van Noort and Clevers, 2002). *In vitro* experiments also showed that all TCF/LEF proteins are able to bind to the transcriptional corepressors of the *Grg* family (mammalian orthologs of Drosophila *groucho*) (Brantjes et al., 2001; Waltzer and Bienz, 1998). Grg proteins interact with HDAC (histone deacetylase) and thus repress gene transcription through chromatin condensation (Chen et al., 1999). Therefore, in the absence of ß-catenin, TCF/LEF transcription factors act as repressors through the interaction with the Grg co-factor proteins. Upon activation of the Wnt signaling pathway ß-catenin enters the nucleus and displaces Grg proteins, thereby leading to the activation of Wnt target gene transcription.

1.3 The Wnt Signaling Pathway

Wnt factors are secreted glycosylated proteins, which are highly conserved in the evolution. Wnts act in an autocrine and paracrine fashion *via* at least four signaling cascades. Wnt signaling plays important roles during animal development. In mice and humans, 19 different Wnt genes have been identified. All Wnt proteins signal through cellular surface receptors of the seven-transmembrane family of Frizzled (Fzd) proteins, which comprises 10 members in mouse and human. There is evidence that some Wnt ligands bind preferentially to specific Frizzled receptors to activate one of the intercellular signaling cascades (Huelsken and Birchmeier, 2001; Kuhl et al., 2000; Sheldahl et al., 1999). The four intracellular cascades, through which Wnt proteins signal are: (1) the ß-catenin pathway, called canonical Wnt pathway; (2) the Wnt/Ca^{2+} pathway, which involves activation of phospholipase C and protein kinase C; (3) the pathway regulating spindle orientation and asymmetric cell division; (4) the planar cell polarity pathway which activates jun N-terminal kinase and leads to the cytoskeletal rearrangements (Bienz, 2001; Huelsken and Birchmeier, 2001; Kuhl et al., 2000).

The present study focused on the canonical, TCF/LEF-mediated, Wnt signaling pathway (Fig.1.1).

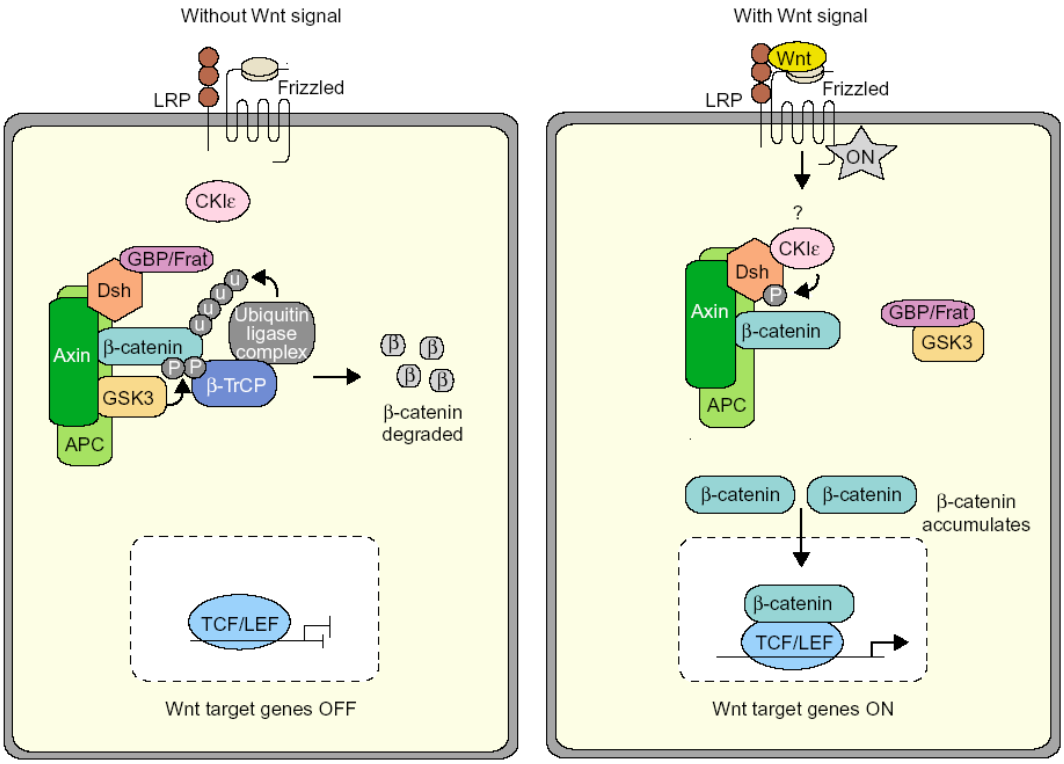

Figure 1.1. Canonical Wnt signaling cascade, 2-step scheme. When no Wnt ligand is presented to a cell, ß-catenin is targeted for degradation via phosphorylation by a large multiprotein complex containing Axin, APC, and GSK3. Target genes are repressed by a TCF/LEF transcription factor. Binding of a Wnt ligand to a receptor Frizzled and a co-receptor LRP inhibits the APC/GSK3 complex through an unknown mechanism. After ß-catenin is accumulated in the cytosol, it enters the nucleus, binds to a TCF/LEF transcription factor and activates target gene transcription. Adapted from: Miller, 2002.

In the canonical Wnt pathway, target gene transcription depends on the level and localization of ß-catenin in the cell. In the absence of a Wnt ligand, ß-catenin is targeted for degradation through specific phosphorylation on its N-terminus, ubiquitinated in a large protein complex (Fig. 1.1, left), and subsequently degraded by the proteosome. In the absence of nuclear ß-catenin, TCF/LEF factors repress the transcription of target genes. Upon binding of a Wnt ligand to a transmembrane receptor of the Fzd family and a low-density lipoprotein receptor-related protein (LRP) co-receptor (Fig. 1.2, right), the ß-catenin degradation machinery is inhibited (Behrens et al., 1998). This in turn leads to the accumulation of ß-catenin in the

cytosol and allows it to enter the nucleus. Nuclear ß-catenin forms a complex with TCF/LEF members and activates target gene transcription, most likely through chromatin modification (Huelsken and Birchmeier, 2001). A variety of extra- and intracellular proteins, which function in the canonical Wnt signaling cascade, have been identified. Soluble extracellular inhibitory factors, such as Dickkopf (DKK), Cerberus and Frizzled-related proteins (Fzb) inhibit Wnt binding to Fzd receptors and therefore block Wnt signal transduction. Intracellularly, different proteins inhibit Wnt signaling by regulating the ß-catenin stability. Adenomatous polyposis coli (APC) protein, Axin and glycogen synthase kinase 3 (GSK3) act within a multiprotein complex to mediate ß-catenin phosphorylation and subsequent degradation (Behrens et al., 1998; Cong and Varmus, 2004; Powell et al., 1992). Axin2/conductin, a protein related to Axin, is another inhibitor of the canonical Wnt pathway (Behrens et al., 1998). Interestingly, Wise, a factor recently discovered in Xenopus, functions in a context-dependent manner and exerts either activator or repressor functions in the Wnt signaling cascade (Itasaki et al., 2003). Thus, a canonical Wnt signal is modulated by the target cells through a variety of different factors.

1.3.1 Functions of the Wnt Signaling Pathway

Through the analysis of loss-of-function mutations of individual Wnt genes, early developmental functions of the Wnt signaling, such as axis formation, as well as late developmental functions, such as morphogenesis and homeostasis of many tissues, have been demonstrated (Ishikawa et al., 2003; Liu et al., 1999; Parr and McMahon, 1995; Popperl et al., 1997). However, as noted previously, Wnt ligands signal through the different intracellular cascades. As the canonical Wnt signaling is TCF/LEF-mediated, the roles of the canonical Wnt pathway are best illustrated by the *Tcf/Lef* loss-of-function mutations. The observation that *Lef-1*-deficient mice exhibit defects in mammary gland formation, and lack body hair and whiskers, indicated that the canonical Wnt signaling is required for the development of tissues which rely on the epithelial-mesenchymal interactions (van Genderen et al., 1994). Notably, *Tcf-1* and *Lef-1* deficient mice exhibit a more severe phenotype than caused by a loss of a single gene, which includes defects in placenta formation, presence of additional neural tubes and perturbed formation of apical ectodermal ridge of the limb bud, and reproduces a *Wnt3a* loss-of-function phenotype (Galceran et al., 1999). TCF-3 differs from the other three TCF/LEF factors in that it acts as a Wnt gene target repressor. This is illustrated by the

observation that duplicated axial mesoderm structures in the *Tcf-3* deficient embryos (Merrill et al., 2004) rather resemble the null mutation phenotypes of *Axin* or *APC*, two well-characterized inhibitors of Wnt signaling (Ishikawa et al., 2003; Zeng et al., 1997). Taken together, this data provides evidence that TCF/LEF transcription factors exert most, if not all of their functions within the canonical Wnt signaling cascade. In addition, inappropriate activation of the canonical Wnt signaling cascade has been implicated in tumorigenesis in mice and humans. Various mutations in genes coding for ß-catenin, APC, and Axin, which lead to the sustained stabilization of ß-catenin, can induce tumor formation in many cell types (Miller et al., 1999; Morin et al., 1997; Polakis, 2000; Powell et al., 1992; Satoh et al., 2000).

1.3.2 Roles of the Canonical Wnt Signaling Pathway in Stem Cell Maintenance

One of the remarkable roles of the TCF/LEF-mediated Wnt signaling cascade is the control of stem cell self-renewal and differentiation in regenerative tissues, such as adipose tissue, hematopoietic cells, skin and intestine (Alonso and Fuchs, 2003; Reya et al., 2003; Ross et al., 2000; Sancho et al., 2003). While most of the differentiated cell types possess the ability to divide symmetrically to generate two identical daughter cells, multipotent stem cells are able to undergo asymmetrical divisions, yielding a committed progenitor daughter and a stem cell daughter (Fuchs et al., 2004). Regulating the balance between symmetric and asymmetric stem cell divisions becomes critical in maintaining proper stem cell numbers and in meeting the demand for differentiated cells in the regeneration of the tissue. The role of the TCF/LEF-mediated Wnt signaling for the stem cell proliferation and differentiation is very well studied in the intestinal epithelium. In the intestine, four different lineages arise from a common source of pluripotent stem cells, which is located in the intestinal crypts. Several lines of evidence support the paradigm that the maintenance of these stem cells is contingent upon Tcf/Lef/ß-catenin mediated Wnt signaling. First, gene disruption has revealed that TCF-4 is required to establish the proliferative progenitors of the prospective crypts in the embryonic intestine (Korinek et al., 1998a). Second, transgenic expression of the secreted Wnt inhibitor Dickkopf-1 (Dkk1) in the intestine results in reduction of the proliferative compartment and absence of nuclear ß-catenin in adult intestinal crypts (Pinto et al., 2003). Finally, progenitors in adult intestinal crypts exhibit expression of the same genetic program that is turned on by activating mutations of the Wnt pathway; this genetic program is turned off in *Tcf-4*-deficient and in Dkk1 transgenic mice (van de Wetering et al., 2002). Remarkably, similar genetic programs control intestinal and pancreatic cell differentiation (Jenny et al., 2002; Jensen et al.,

2000). It remains to be determined whether the stem cell maintenance in the pancreas is regulated by the canonical Wnt signaling pathway.

1.4 Mouse Pancreas Development and Functions

The pancreas consists of two functionally different parts: the exocrine and the endocrine (Fig. 1.2). The adult exocrine pancreas comprises the majority of the organ (95-99% of the cell mass) and includes secretory epithelial cells organized in structural units termed acini. A system of digestive ducts connects the acini and delivers the digestive enzymes (lipases, proteases and amylases) into the digestive tract. The endocrine pancreas consists of clusters of four types of endocrine cells, which are organized into the so-called islets of Langerhans and embedded in the exocrine tissue. To reach their target organs and perform their functions the endocrine hormones are secreted by the islet cells into the blood stream. Each of the endocrine cell types is characterized by the secretion of a unique hormone. Insulin-producing ß-cells account for the majority of islet cells and form the core of islets. The other three cell types are found in the periphery of islets: α-cells produce glucagon, δ-cells secrete somatostatin, and PP-cells produce pancreatic polypeptide (Fig. 1.2, B). There are several non-endocrine and non-exocrine cell types, which are found in the pancreas. First, the pancreas contains endothelial cells which belong to the network of blood vessels. Second, the pancreas contains neural cell bodies and nerve fibers, which innervate islets and acini to regulate the secretion of hormones and digestive enzymes (Persson-Sjogren et al., 2001; Salvioli et al., 2002). Finally, Schwann cells of the peripheral glia envelop the endocrine islets of Langerhans (Sunami et al., 2001; Teitelman et al., 1998; Ushiki and Watanabe, 1997).

Diabetes mellitus is a heterogeneous group of disorders, which are characterized by high glucose levels in the blood. Pancreatic ß-cells are central to the pathophysiology of diabetes. Type 1 diabetes is an autoimmune disease, in which destruction of ß-cells leads to an insulin deficiency (Bell and Polonsky, 2001). Type 2 diabetes is a complex condition characterized by variable degrees of ß-cell-dysfunction and insufficient production of insulin. (St-Onge et al., 1999). The only long-term treatment strategy for diabetes is replacement of the ß-cell mass (Chaudhari et al., 2001). Discovering stem cells that possess the capacity to differentiate to the ß-cell type will offer the possibility to generate replacement ß-cells *in vitro*. However,

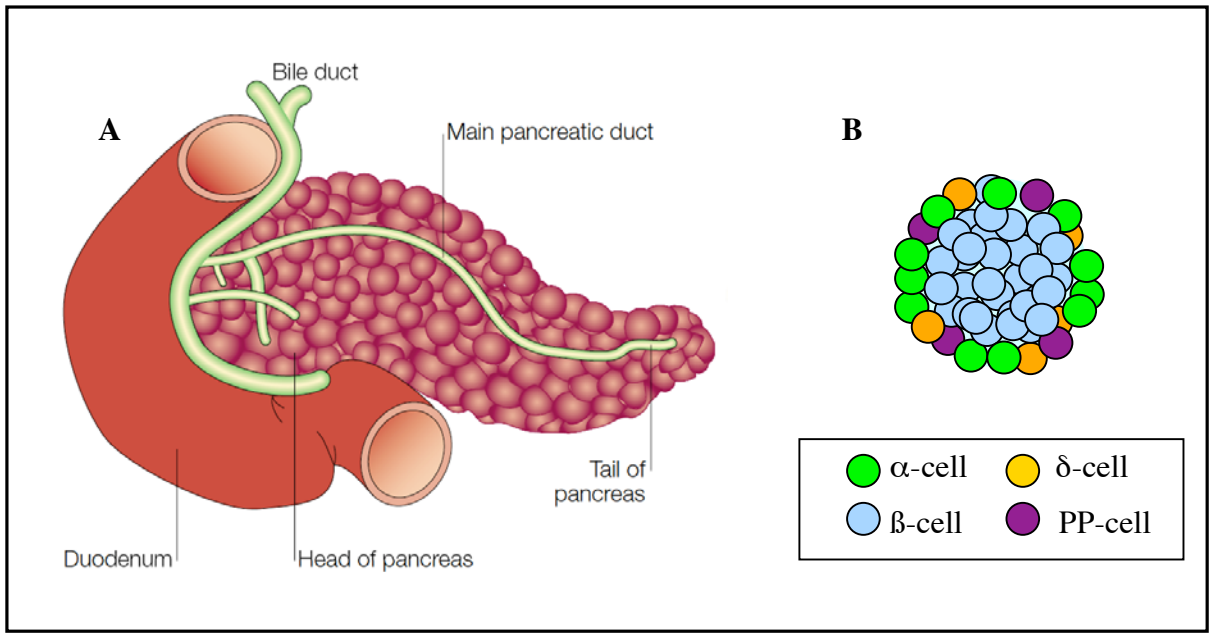

Figure 1.2. Schematic representation of adult mammalian pancreas. (A), Organ morphology. In humans, the pancreas is usually anatomically divided into the head, neck, body and tail; in mice this shape is not so strongly defined. Purple spheres depict exocrine acini. (B) Schematic representation of the endocrine islet of Langerhans. The core of the islet is made by α-cells (green), whereas on the periphery ß-cells (blue), δ-cells (orange) and PP-cells (violet) can be found. Adapted from (Edlund, 2002).

in order to produce these replacement cells, it is important to know how ß-cells are formed during pancreas development, and to be able to reproduce the signals promoting ß-cell differentiation. Second, it is necessary to identify the stem cell population in the pancreas and to study the mechanisms controlling the maintenance and differentiation of these cells.

1.4.1 Embryonic Development of the Mouse Pancreas

As demonstrated by numerous gene ablation studies in mice, the combination of cell autonomous and secreted factors, as well as inductive and permissive signals is essential for the embryonic development of the mammalian pancreas (Sander and German, 1997). To introduce the molecular mechanisms governing the development of the mouse pancreas, three major stages will be reviewed: specification of the pancreatic domains, outgrowth of the pancreatic primordium, and finally, endocrine and exocrine differentiation.

1.4.1.1 Specification of the pancreatic domains and early development

The mouse pancreas derives from two patches of endodermal epithelium that bud dorsally and ventrally from the gut epithelium, between the stomach and duodenum, beginning at approximately E9 (Fig. 1.3). Prior to and during budding, the pancreatic primordium expresses the homeodomain protein pancreatic and duodenal homeobox protein-1 (PDX-1) (Ohlsson et al., 1993). Through lineage tracing studies, it has been demonstrated that PDX1+ progenitors give rise to all pancreatic cell types (Gu et al., 2002). In the *Pdx-1* homozygous mutant mice the proliferation, branching and differentiation of the pancreatic epithelium is arrested after budding (Ahlgren et al., 1996; Offield et al., 1996). In this, the fact that the pancreatic buds form in the absence of *Pdx-1* indicates that other factors promote pancreas specification. Some of such signaling molecules have been identified. In Zebrafish, inhibition of retinoic acid (RA), bone morphogenetic protein (BMP), or hedgehog (Hh) signaling disrupts pancreas development. Conversely, excess activity of these pathways results in expanded or ectopic pancreas development (diIorio et al., 2002; Roy et al., 2001; Stafford and Prince, 2002; Tiso et al., 2002).

In mice, several transcription factors expressed in the pancreatic epithelium have been implicated in the specification of endoderm towards the pancreatic fate. During development, *Hlxb9* is expressed in both pancreatic buds, and in the absence of *Hlxb9* the dorsal bud fails to form (Li et al., 1999). In the dorsal prepancreatic epithelium, *Hlxb9* is required for the initiation of *Pdx-1* expression, which suggests that pancreatic progenitors require *Hlxb9* to engage into the pancreatic differentiation program. However, since direct lineage tracing of *Hlxb9*-deficient cells has not yet been performed, it remains unclear if progenitors adopt an alternate fate in the absence of *Hlxb9*. The conversion from a pancreatic to a duodenal phenotype has been demonstrated for the Ptf1a/p48-deficient cells (Kawaguchi et al., 2002). Together, this data suggests that extrinsic factors regulate anterior-posterior patterning of the endoderm in order to establish the position of the pancreas; this positional information is translated into the expression of transcription factors that specify pancreatic fate, and promote *Pdx1* expression and epithelial budding.

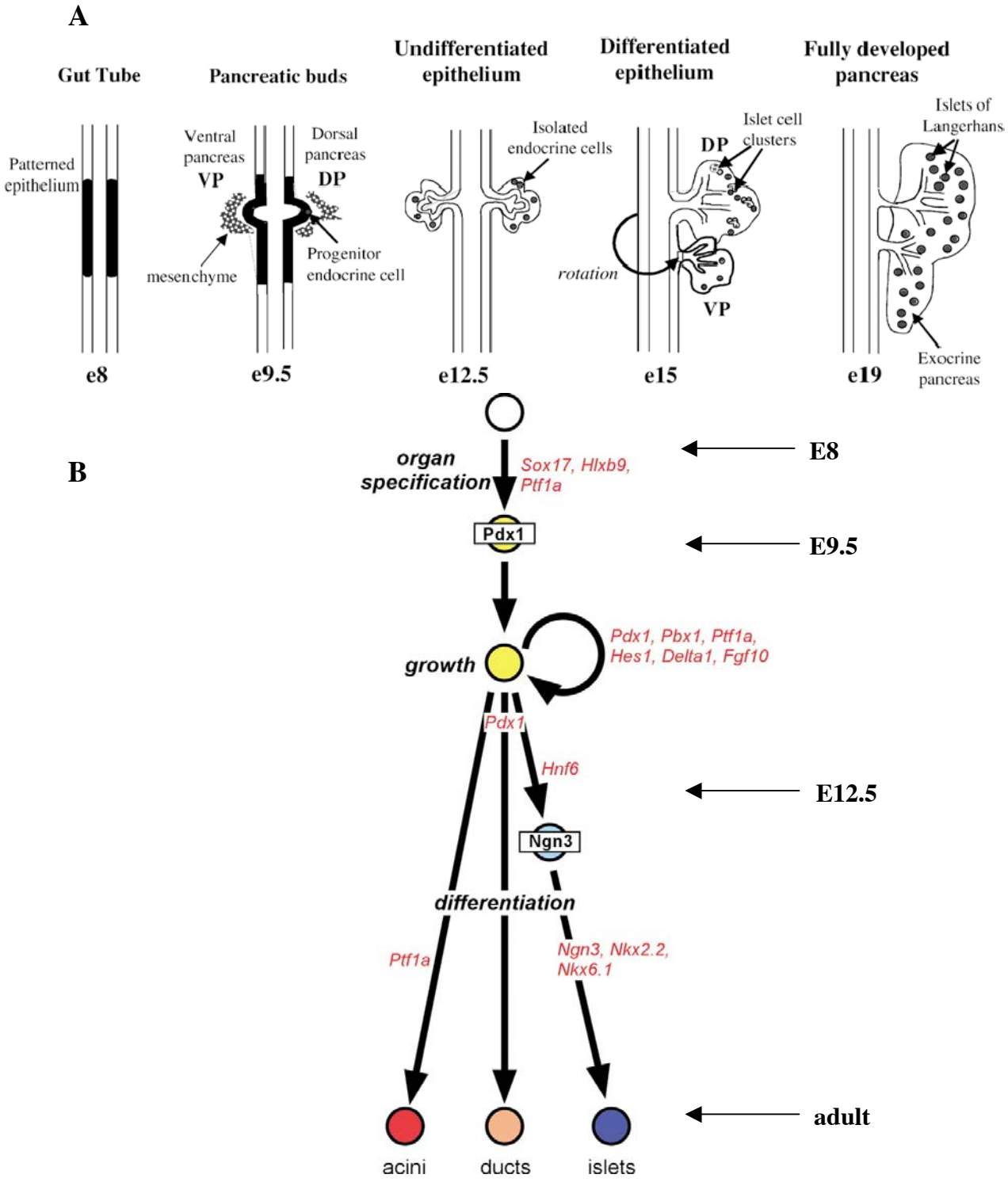

Figure 1.3. Mouse pancreas development. (A), schematic pancreas morphogenesis in embryo. (B) steps of pancreatic cell differentiation. (A) At E8, the endoderm is pre-patterned through by the signals from the neighboring cells. Between E9 and E10 two epithelial buds form, dorsal (DP) and ventral (VP). Outgrowth and branching are observed between E10 and E13.5. Around E13 during the second transition, endocrine and exocrine differentiation begins. Ventral and dorsal pancreata fuse at E15-E16 to form a single organ. At birth (E19) islets of Langerhans are mostly formed with their characteristic organization. (B), some of the identified factors controlling different steps of the pancreas development (details in the text). Modified from Kemp et al. 2003, and Murtaugh and Melton, 2003.

1.4.1.2 Growth of the Pancreatic Primordium

Epithelial budding of the pancreatic primordium is followed by rapid growth and branching. The growing epithelium is surrounded by mesenchymal cells, and epithelial-mesenchymal interactions are known to regulate the development of the mammalian pancreas (Golosow and Grobstein, 1962). In explant culture experiments, Golosow and Grobstein showed that pancreatic buds from E11 mouse embryos grew and branched in culture only if mesenchyme was left attached to the epithelium, or was cultured with the epithelium. Their results indicated that the signals from the mesenchyme seemed to be permissive rather than inductive. A number of mesenchymal factors are now known to be required for the growth-promoting effect of the mesenchyme (Murtaugh and Melton, 2003). One of them, a transcription factor ISL1, is required for the development of the dorsal pancreatic mesenchyme, where it is expressed during bud formation. In *Isl1* homozygous mouse mutants, not only this mesenchyme is absent, but also *Pdx-1* expression in the adjacent dorsal epithelium is reduced (Ahlgren et al., 1997). An identical phenotype is seen in mice lacking N-cadherin, which is also expressed and functionally required in the dorsal pancreatic mesenchyme (Esni et al., 2001). Another gene involved in epithelial-mesenchymal interactions is *Pbx1*, which is expressed in pancreatic epithelium as well as surrounding mesenchyme (Kim et al., 2002). In mice with a homozygous deletion of the homeodomain gene *Pbx1*, a severe hypoplasia of the dorsal pancreas and lack of acinar development are observed (Kim et al., 2002). Exocrine differentiation was rescued by recombination of *Isl1*- or *Pbx*1-deficient epithelium with wild-type mesenchyme, indicating that both ISL1 and PBX1 are required in the mesenchyme (Ahlgren et al., 1997; Kim et al., 2002). Recently, the fibroblast growth factor-10 (FGF10) has been identified as a pancreatic mesenchymal factor promoting proliferation and expansion of the pancreatic progenitors. Mice lacking *Fgf10*, as well as mice ectopically expressing a dominant-negative form of the fibroblast growth factor receptor-2b (FGFR2b), which binds FGF10, exhibit hypoplasia of dorsal and ventral pancreata (Bhushan et al., 2001; Celli et al., 1998; Ohuchi et al., 2000), which is less dramatic than the hypoplasia seen in the *Isl1*- or *Pbx1*-deficient dorsal pancreata. This evidence points to the existence of additional mesenchymal factors. Indeed, other mesenchymal signals may include members of the epithelial growth factor (EGF) family, as EGF-receptor homozygous mutant mice display moderately decreased pancreatic growth (Miettinen et al., 2000), and EGF has been shown to promote epithelial proliferation *in vitro* (Cras-Meneur et al., 2001). Yet there might be other, unidentified mesenchymal factors, which could play a critical role in

pancreatic progenitor proliferation. A likely candidate gene family is Wnt factors. For example, in kidney, another organ regulated by the epithelial-mesenchymal interactions, WNT2b acts as an early mesenchymal signal, controlling morphogenesis of the epithelial tissue; in this organ the canonical Wnt pathway may directly regulate ureter branching (Lin et al., 2001). The potential roles of the canonical Wnt signaling in the epithelial-mesenchymal interactions and in the outgrowth of the pancreatic epithelium still remain to be identified.

1.4.1.3 Specification and Differentiation of Endocrine and Exocrine Cell Types

In mice, first endocrine cells could be found in the dorsal pancreatic bud as early as E9 (Pictet and Rutter, 1972; Spooner et al., 1970). These early endocrine cells often co-express insulin and glucagon. However, as lineage-tracing analyses have shown, these early hormone-producing cells never give rise to the mature endocrine cells (Herrera, 2000). Subsequently, during the so-called secondary transition, starting around E13, exocrine differentiation begins, and endocrine cells undergo true differentiation in exponentially increasing numbers, with ß-cells predominating (Herrera et al., 1991; Pictet and Rutter, 1972). Differentiated endocrine cells aggregate to form islets of Langerhans, and the process of differentiation and islet formation continues postnatally (Kaung, 1994). However, little is known about the differentiation of pancreatic ducts, partly due to the lack of definitive markers for the ductal cells (Gu et al., 2002). Numerous transcription factors have been shown to control endocrine and exocrine differentiation (Edlund, 2002); here, a few key factors will be named.

During epithelial bud outgrowth, *Pdx-1* expression changes from pan-epithelial to high-level in ß-cells and low-level in the undifferentiated precursors (Guz et al., 1995). Conditional inactivation of the *Pdx-1* gene after bud formation prevents both acinar and endocrine differentiation (Holland et al., 2002), suggesting the roles of PDX-1 in regulating the pool of the multipotent progenitors cells in the pancreas. Another key regulator of the endocrine development is the basic helix-loop-helix protein Neurogenin3 (NGN3), which marks scattered cells of the pancreatic epithelium. *Ngn3* is expressed transiently in the pancreatic endocrine progenitor cells, and subsequently downregulated during differentiation (Schwitzgebel et al., 2000). NGN3 promotes endocrine cell fate, as in the absence of *Ngn3* no endocrine cells are formed (Gradwohl et al., 2000). In addition, *Ngn3* was sufficient to induce endocrine cell differentiation when misexpressed in the gut epithelium (Grapin-Botton et al., 2001). Furthermore, when *Ngn3* was misexpressed broadly in the whole pancreatic epithelium

under control of the *Pdx-1* promoter, the whole organ was converted to the endocrine fate (Schwitzgebel et al., 2000). The mechanisms for establishing the *Ngn3* expression only in select epithelial cells of the pancreas may be similar to that in the nervous system. In the developing nervous system, the expression of two related *Ngn* genes, *Ngn1* and *Ngn2*, is controlled by the process called lateral inhibition, which is mediated by Delta-Notch signaling (Adam et al., 1998). In lateral inhibition, a pool of initially equivalent cells is divided into the NGN-positive neuronal precursors surrounded by non-neuronal cells. Inhibition of the Notch signaling pathway removes the spatial restriction on the neuronal fate (Lewis, 1998). Indeed, mice lacking the Notch ligand *Delta1* or the Notch target gene *Hes1* exhibited widespread endocrine differentiation, at the expense of the exocrine progenitors (Apelqvist et al., 1999; Jensen et al., 2000), reproducing the phenotype of the broad Ngn3 overexpression. Thus, similar to the nervous system, Delta-Notch signaling controls the choice between differentiated endocrine and progenitor cell fates in the developing pancreas.

A number of transcription factors have been identified, which are required for the differentiation of the insulin-producing ß-cells (Sander and German, 1997). Two of the NK-homeodomain genes, *Nkx6.1* and *Nkx2.2* act downstream of *Ngn3*, or in parallel to *Ngn3*, because *Ngn3* expression is normal in mice which lack *Nkx2.2* or *Nkx6.1* (Schwitzgebel et al., 2000). In the *Nkx2.2* homozygous mutant mice, ß-cells are arrested before completing differentiation (Sussel et al., 1998). Mice lacking the *Nkx6.1* gene do not undergo the secondary transition, and the number of insulin-producing cells is drastically reduced (Sander et al., 2000). Thus far, the roles of Sox family of transcription factors in pancreas development have been poorly understood, and expression of only two Sox genes, *Sox9* and *Sox13,* have been detected so far in the developing and adult mammalian pancreas, Expression of *SOX13* was detected in the adult human islets, and autoantibodies to SOX13 occurred at high frequencies in human patients with autoimmune type 1 diabetes (Fida et al., 2001; Kasimiotis et al., 2000; Park et al., 2003) However, linkage and association studies of single nucleotide polymorphisms within *SOX13* have demonstrated that the *SOX13* gene itself is unlikely to contribute to type 1 diabetes susceptibility (Argentaro et al., 2001). *SOX9* expression has been detected in the human (Piper et al., 2002), and the Xenopus pancreas (Lee and Saint-Jeannet, 2003). In individuals who died from severe Campomelic dysplasia (CD), a sporadic autosomal dominant syndrome, characterized by skeletal malformations and associated with *SOX9* haploinsufficiency, pancreatic phenotypes have been described (Piper et al., 2002). In CD individuals, pancreatic epithelial cells were more loosely packed within

the mesenchymal stroma and islets were less morphologically distinctive than in control tissue (Piper et al., 2002). From these observations, it was suggested that SOX9 could play a role in human pancreas development. Importantly, the observed defects in the pancreas of the CD individuals resulted from the *SOX9* heterozygous mutations, but the consequence of a complete absence of the *SOX9* for the pancreas development, as well as its function, are not yet known.

When we started the present research, the roles of Wnt signaling in the mammalian pancreas development had not been previously analyzed. Recently, two studies were published that suggested that the Wnt cascade functions in the development and homeostasis of the mouse pancreas. In the study by Heller et al. (2002), patterns of expression of Wnts and Fzds in the embryonic pancreas were analyzed. In addition, the mouse *Pdx-1* gene promoter was used to target the expression of different Wnt members to the early pancreatic progenitor cells (Heller et al., 2002). By the sensitive radioactive multiplex reverse transcriptase polymerase chain reaction (RT-PCR), *Wnt1, Wnt2b, Wnt5a, Wnt7b,* and *Wnt11* mRNAs could be detected in the developing pancreas, mostly in the mesenchymal cells. Genes for all Frizzled receptors, *Fzd2* to *Fzd9*, and co-receptors *LRP5* and *LRP6,* were found to be expressed in the embryonic pancreas, with *Fzd2* being the most abundant. Transgenic mice ectopically expressing cDNAs of *Wnt2, Wnt4, Wnt6,* and *Wnt7a* under *Pdx-1* promoter in the early pancreatic epithelial cells did not exhibit any obvious phenotypes in pancreas when analyzed at birth (Heller et al., 2002). However, overexpression of *Wnt1* resulted in arrested development of pancreas and spleen. When *Wnt5a* expression was driven by the *Pdx-1* promoter, several structures derived from the proximal foregut showed reduction in size, including the pancreas, spleen, and stomach. Few remaining insulin cells lacked expression of the ß-cell marker *Isl1,* and no pancreatic ducts could be found (Heller et al., 2002). Taken together, these results suggested that Wnt signaling participates in epithelial-mesenchymal interactions, and may at least be involved in specifying the region identity in the anterior foregut. However, the apparent disadvantage of this approach was the use of the soluble Wnt factors, which could affect the neighboring tissues and result in broad foregut patterning defects. Secondly, it was not possible to determine whether observed pancreatic phenotypes were mediated by the canonical, TCF/LEF/ß-catenin mediated Wnt pathway. Other pieces of evidence suggested that Wnt signaling regulates the function of adult islets of Langerhans (Fujino et al., 2003). Mice lacking the gene coding for the Wnt co-receptor *LRP5* exhibited impaired glucose tolerance. Islets of the LRP5-mutant mice showed reduction in the levels of intracellular ATP

and Ca^{2+} in response to glucose, which resulted in the decreased glucose-induced insulin secretion (Fujino et al., 2003). The role of Wnt signaling in the normal glucose response of ß-cells could be further supported by the fact that the exposure of the cultured wild-type islets to WNT3a and WNT5a proteins stimulated glucose-induced insulin secretion, which could be blocked by adding the antagonist of Wnt signaling, Frizzled-related protein-1 (sFRP-1) to the culture medium (Fujino et al., 2003). Again, this study did not address the question whether the roles of *LRP5* in the insulin secretion were mediated by the canonical Wnt signaling.

1.5 Rationale and Hypothesis

The HMG box class of proteins comprises two families of cell-specific transcription factors, Sox and TCF/LEF. Both Sox and TCF/LEF proteins are expressed in a temporally and spatially controlled manner during mammalian development and in the adulthood. These factors are known to regulate the development of numerous mammalian tissues. To date, the role of Sox and TCF/LEF factors in the development of the mammalian pancreas is poorly understood. The overall goal of this research project was to analyze the expression of HMG-domain transcription factors in the developing mouse pancreas, and to study their function in pancreas development.

To elucidate the role of Sox transcription factors in pancreas development, first, an expression analysis was performed. To identify, which *Sox* genes are expressed in the developing mouse pancreas, RT-PCR on RNA extracted from the pancreas at different embryonic stages, as well as and *in situ* RNA hybridization on pancreatic sections were employed. Subsequently, two mouse lines deficient for select pancreatically expressed *Sox* genes were analyzed for defects in pancreatic development.

As down-stream effectors of the Wnt signaling pathway, TCF/LEF proteins mediate stem cell renewal in many regenerating tissues, such as skin or intestine. We wanted to test the hypothesis that TCF/LEF-mediated Wnt signaling is also involved in the maintenance of the stem or progenitor cells in the mouse pancreas. However, despite recent advances in the identification of factors involved in pancreas development and ß-cell function, it is still unclear whether the pancreas contains stem cells, and if so, which factors control their maintenance and differentiation. Similarly, the differentiation programs directing the

pancreatic progenitors to a ß-cell fate in mouse pancreas development are yet not fully understood. The identification of such factors would facilitate the development of a culture system for the in vitro generation of replacement ß-cells.

To test if canonical Wnt signaling mediates stem cell maintenance in the mammalian pancreas, it was first studied whether the canonical Wnt signaling cascade is active in the developing mouse pancreas. To answer this question, expression of TCF/LEF transcription factors, as well as other key factors of the canonical Wnt signaling pathway, was studied at different stages of mouse pancreas development. In addition, to identify sites of active TCF/LEF/ß-catenin-mediated transcription in the embryonic and adult pancreas, two independent Wnt reporter mouse lines were analyzed. The subsequent goal was to elucidate the potential roles of Wnt signaling in the maintenance and differentiation of pancreatic progenitor cells, as well as in the adult endocrine islet function. To study the effects of either blocking or activating Wnt signaling in the pancreas, mouse lines were generated which express dominant negative or constitutively active forms of TCF-4.

II Results

2.1 Analysis of the Roles of Sox Transcription Factors in Mouse Pancreas Development

2.1.1 Expression of *Sox* genes during Mouse Pancreas Development

2.1.1.1 Sox Gene Expression Analysis by RT-PCR

In mouse and human, there are 20 different members of transcription factors belonging to the Sox family (Schepers et al., 2002; Soullier et al., 1999). In order to narrow the search, I performed a degenerate RT-PCR (reverse transcriptase polymerase chain reaction) at three key stages of pancreas development. These were E (embryonic day) 12.5, the time point at which pancreatic epithelium starts to branch, E15.5, when extensive endocrine and exocrine differentiation takes place, and at six months after birth, when all four endocrine cell types have differentiated and formed mature islets of Langerhans. As a template for the RT-PCR, I generated cDNA from the total RNA extracted either from whole pancreata, or adult islets of Langerhans as described in the Methods section. A set of degenerate primers was used (Roose et al., 1998; Stock et al., 1996), which are complementary to the highly conserved Sox HMG box sequence, and therefore amplify non-specifically HMG box sequences of all Sox transcripts present in the cDNA mix. The amplified products were subcloned into the pGEM-T Easy vector (Promega) and transformed into *E.Coli*. Fifty clones from each age were randomly picked, plasmid DNA was extracted (see Methods), the HMG box inserts were sequenced and aligned to the HMG boxes of known Sox proteins. Results of the sequencing are summarized in the Table 2.1.

Random sequencing of independent cDNA clones revealed expression of eight different Sox genes in the pancreas belonging to the groups C, D, and E. Within group C, cDNA clones coding for Sox4 were most frequently isolated, but clones for *Sox11* and *Sox12* were also found in the embryonic pancreas. Among group D members, we found expression of *Sox5* and *Sox13*. Within group E, *Sox9* was predominantly isolated from embryonic pancreas, but I also identified transcripts for *Sox8* and *Sox10* during pancreas development. From 50 different clones sequenced from adult islets, we identified transcripts for *Sox4, Sox9*, and *Sox13*. This data shows the expression of at least eight different members of the Sox gene family in the embryonic pancreas, and three members in adult islet cells.

Group	Gene	E12.5	E15.5	Adult islet
C	*Sox4*	26	12	74
	Sox11	12	24	-
	Sox12	2	-	-
D	*Sox5*	10	4	-
	Sox13	6	2	6
E	*Sox8*	4	4	-
	Sox9	36	52	20
	Sox10	4	2	-

Table 2.1. Degenerate RT-PCR for Sox genes in mouse pancreas. RT-PCR was performed using degenerate oligonucleotides described in Appendix with RNA purified from isolated embryonic mouse pancreata at the indicated ages or mouse adult islets. The values show the frequencies of each gene as a percentage of a total of 50 sequenced subclones per age.

Group	Gene	E12.5	E15.5	E18.5
A	*Sry*	-	-	-
B1	*Sox1*	-	-	-
	Sox2	-	-	-
	Sox3	-	-	-
F	*Sox7*	+	+	+
	Sox17	+	+	+
	Sox18	+	+	+
G	*Sox15*	-	+	-
H	*Sox30*	+	+	+

Table 2.2. RT-PCR with gene-specific primers for group A, B, F, G, and H Sox genes in mouse pancreas. RT-PCR was performed using gene-specific primers as described in the Methods Section with RNA purified from isolated embryonic mouse pancreata at the indicated ages. (+) means that the gene transcript was detected, and (-) means that no PCR product could be detected, despite amplification from a control RNA.

Although a number of other researchers have successfully used this degenerate approach to discover Sox genes expressed in a variety of different tissues (Roose et al., 1998; Stock et al., 1996), it should be noted that the degenerate RT-PCR approach may not cover all Sox genes in the mouse pancreas, and that the relative number of sequences does not necessarily reflect the abundance of a particular Sox transcript at a given stage. Given the potential bias of the degenerate primers, I next used gene-specific primers to directly test if members of groups A, B, F, G or H are found in the developing pancreas. Although *Sry, Sox1, Sox2*, and *Sox3* were not detected, transcripts for *Sox7, Sox15, Sox17, Sox18*, and *Sox30* were amplified from the embryonic pancreas (Table 2.2), showing that numerous Sox genes from almost all groups are expressed in the embryonic pancreas.

2.1.1.2 Sox Gene *in situ* Expression Analysis on Pancreatic Tissue Sections

I next analyzed the expression of *Sox4, Sox11, Sox5, Sox 13, Sox9*, on tissue sections throughout the development of the mouse pancreas by *in situ* hybridization, and *Sox8* and *Sox10* by enzymatic X-gal staining. The genes are ordered below by the group they belong to.

From group C (*Sox4 and Sox11*), between E9.5 and E10.5, Sox11 was detected in the mesenchyme surrounding both pancreatic buds and in a few cells of the pancreatic epithelium (Fig.2.1). At E12.5, Sox11 was exclusively found in a subset of the epithelial cells. Sox4-positive cells were first observed at E12.5, showing wide epithelial expression in the pancreas. At E14.5, both *Sox11* and *Sox4* were broadly expressed in the epithelium. At the end of gestation, around E18.5, endocrine cells have begun to cluster into islets of Langerhans, and islets can be morphologically distinguished from exocrine acinar cells and pancreatic ducts. At E18.5, Sox11 and Sox4 were detected all throughout the forming endocrine islets. Both factors were coexpressed with glucagon, and their wide expression in the center of the islets suggests expression in insulin-producing cells. Transcripts of *Sox4* were also found in a subset of exocrine acinar cells.

Within group D, Sox13 was first expressed in a subset of epithelial cells at E10.5, and broadly detected in the epithelium at E12.5 (Fig. 2.2). The first Sox5 expressing epithelial cells were observed at E12.5; at E14.5 both Sox5 and Sox13 were widely expressed in the pancreatic epithelium. At E18.5, Sox13 and Sox5 mRNAs were no longer detected by *in situ* hybridization, but could be amplified by RT-PCR from whole pancreas RNA with gene-specific primers (Fig. 2.2, F). This data could either suggest that their expression levels

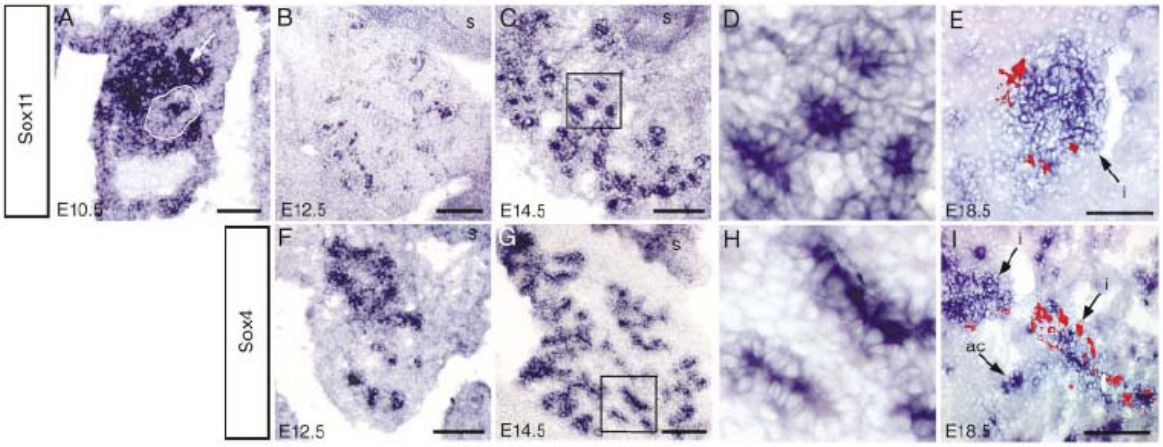

Figure 2.1. Expression of *Sox11* and *Sox4* in the developing mouse pancreas. *In situ* hybridization for *Sox11 (*A-E) and *Sox4* (F-I) on cryosections through the pancreas at the indicated ages. (A) The dorsal pancreatic epithelium is circled, and *Sox11* expression in the pancreatic mesenchyme indicated by an arrow. In (C) and (G) the boxes represent the region that is shown in (D) and (H), respectively, in higher magnification. (E, I) To visualize the outer boundary of the islets, sections were immunostained with an anti-glucagon antibody (red) after *in situ* hybridization. The fluorescence images for glucagon and the light microscopy images were overlaid in Photoshop. The arrows indicate islet cells (i) and exocrine acinar cells (ac). Abbreviations: s, stomach. Scale bars, 100 µm.

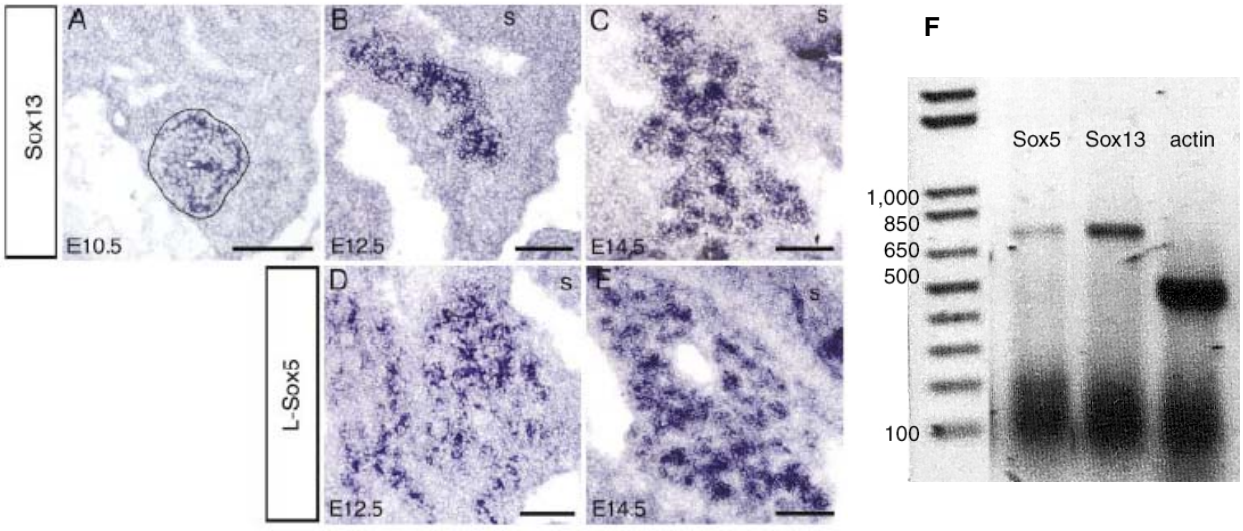

Figure 2.2. Expression of *Sox13* and *Sox5* in the developing mouse pancreas. *In situ* hybridization for *Sox13 (*A-C) and *Sox5* (D, E) on cryosections through the pancreas at the indicated ages. (F), Electrophoresed RT-PCR products on mRNA from E18.5 pancreas, with primers specific for *Sox5*, *Sox13*, or control gene *gamma-actin* as indicated. (A) The dorsal pancreatic epithelium is circled. (F), a molecular weight ladder is on the left. Abbreviations: s, stomach. Scale bars, 100 µm.

decrease in late embryogenesis, or alternatively that the increased levels of RNases in the perinatal pancreas impair the detection of the transcripts by *in situ* hybridization.

Among group E members, *Sox9* was expressed in the epithelium of both pancreatic buds at E9.5 (Fig. 2.3). Between 10.5 and E12.5, *Sox9* continued to be strongly expressed in the epithelium. By E14.5, a majority of pancreatic epithelial cells were still Sox9-positive. In late gestation, *Sox9* expression became more restricted and was found in the islets of Langerhans, in a subset of ductal epithelial cells, and in few exocrine acinar cells (Fig. 2.3).

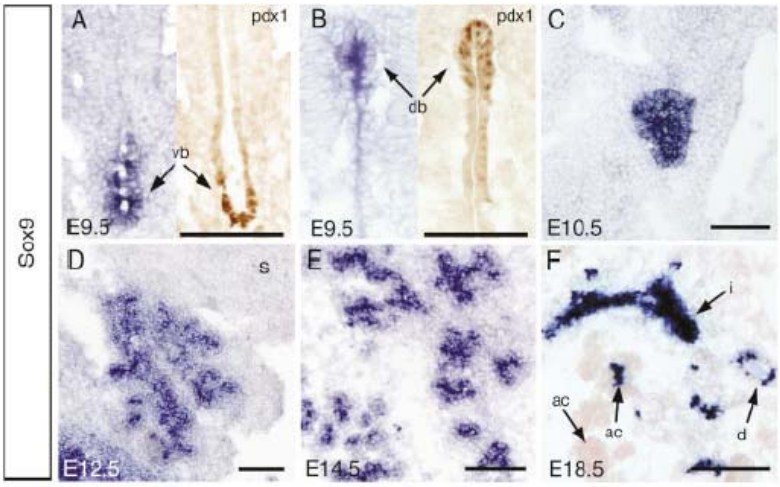

Figure 2.3. Expression of *Sox9* in the developing mouse pancreas. *In situ* hybridization for *Sox9 (*A-F) on cryosections through the pancreas at the indicated ages. Expression of *Sox9* in the ventral (vb) (A) and dorsal (db) pancreatic buds (B). To visualize the pancreatic epithelium, the right half of each image shows an immunostaining for Pdx1 on an adjacent section. (C, D) *Sox9* expression marks the entire dorsal pancreatic epithelium. (F) The arrows indicate *Sox9* signal in islet cells (i), exocrine acinar cells (ac), and ductal cells (d). Note that the majority of acinar cells do not express *Sox9*. Abbreviations: s, stomach. Scale bars, 100 µm.

Expression of *Sox8* and *Sox10* from the group E was studied by enzymatic X-gal staining, using $Sox8^{+/LacZ}$ and Sox $10^{+/LacZ}$ mice heterozygous for a gene replacement of the coding sequence with ß-galactosidase (Britsch et al., 2001; Sock et al., 2001). I found Sox8 and Sox10 in scattered cells at the epithelial/mesenchymal boundary at E10.5 (Fig. 2.4, A, F). At E12.5, individual Sox8- and Sox10-positive cells were dispersed between mesenchyme and epithelium and did not express glucagon (B, G). A very similar pattern of *Sox8* expression was observed in the developing chick pancreas (Bell et al., 2000). In late embryogenesis,

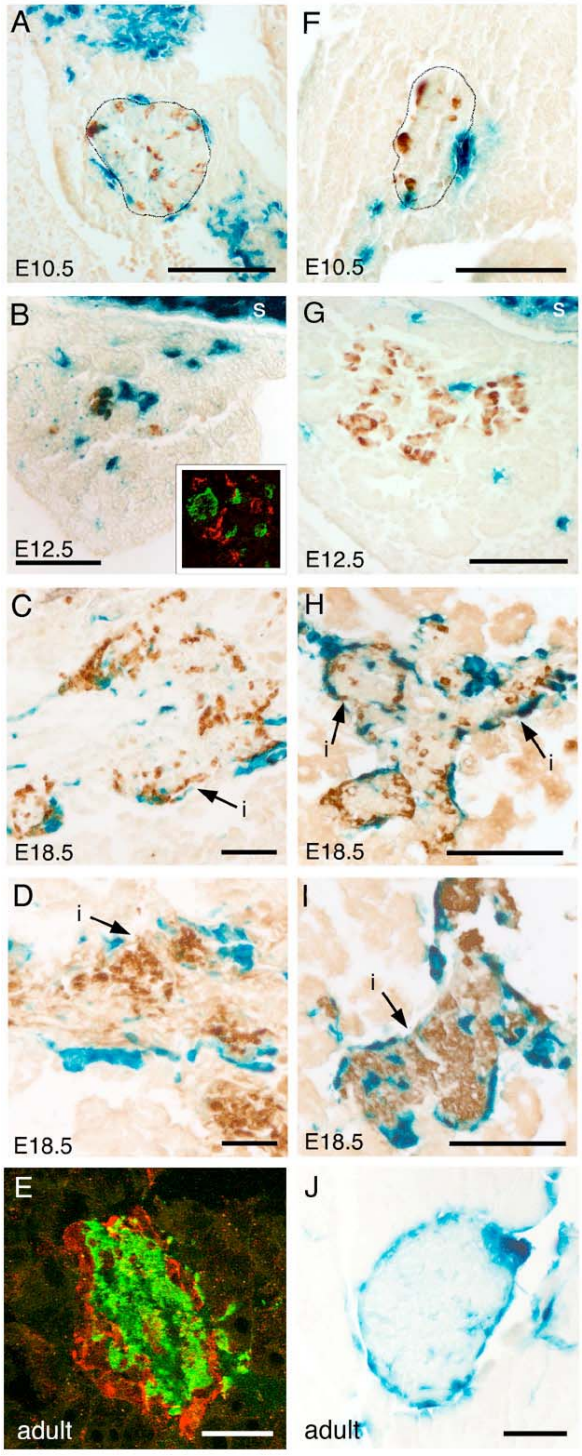

Figure 2.4. Expression of *Sox8* and *Sox10* in the developing and adult mouse pancreas. ß-galactosidase staining for *Sox8* (blue in A-D) in Sox8 $^{+/LacZ}$ embryos, or *Sox10* (blue in F-J) in Sox10 $^{+/LacZ}$ embryos on paraffin sections through the pancreas at the indicated ages. Subsequent immunodetection of glucagon (brown in A-C and F-H) or insulin (brown in D, I) was performed. (A, F) The dorsal pancreatic epithelium is circled. (B) The inset shows a confocal image of a double-immunofluorescence with anti-ß-galactosidase (red) and anti-glucagon (green) antibodies at E12.5. (E), confocal image of a double-immunofluorescence with anti-ß-galactosidase (red) and anti-insulin (green) antibodies (C, D, H, I). Arrows indicate hormone-positive pancreatic islets (i). Abbreviations: s, stomach. Scale bars, 100 µm.

Sox8- and Sox10-expressing cells were found at the periphery of forming islets (C, D and H, I). Immunohistochemistry with anti-glucagon, anti-insulin, anti-somatostatin and anti-PP antibodies on X-gal stained tissue sections revealed that these Sox8- and Sox10-positive cells did not express any of the four hormones (data not shown). The peri-islet expression of *Sox8* and *Sox10* was maintained in the adult pancreas (E, J).

2.1.2 Analysis of Sox Null Mutants for Their Pancreatic Phenotype

Sox8-deficient mice are fertile and appear grossly normal except for a slight weight reduction (Sock et al., 2001). In contrast, mice homozygous for Sox10 deletion have impaired peripheral glia formation and die at birth, due to effects unrelated to pancreas development (Britsch et al., 2001).

To investigate possible roles for Sox8 and Sox10 in pancreas development, I analyzed pancreatic sections from E18.5 $Sox10^{LacZ/LacZ}$ homozygous mutant embryos, because of perinatal lethality of Sox10-deficient mice, as well as from eight-month-old $Sox8^{LacZ/LacZ}$ mutant mice. Pancreata from $Sox8^{LacZ/LacZ}$, as well as $Sox10^{LacZ/LacZ}$ mutants did not exhibit any histological abnormalities (hematoxylin-eosin staining in Fig.2.5, A-D), and showed normal expression of the endocrine hormones (Fig.2.5, E-H) and amylase (data not shown). Additionally, to rule out the possibility that the insulin and glucagon protein levels in the Sox8-deficient pancreas are altered, I performed a radiommuno-assay (RIA, Linco), as described in Methods Section. Both insulin and glucagon levels in the pancreas were within the normal ranges as compared to those of the wild-type control littermates (Table 2.3). Together this data suggests that endocrine and exocrine differentiation is not contingent upon Sox8 or Sox10 function.

Hormone concentration	**+/+**	**$Sox8^{LacZ/LacZ}$**
Glucagon (ng/mg protein)	425,0 +/- 55,0 (n=2)	420 +/-32,15 (n=3)
Insulin (μg/ mg protein)	13,45 +/- 1,75 (n=2)	15,67 +/-1,36 (n=3)

Table 2.3: Quantification of insulin and glucagon hormone concentrations in Sox8-deficient mice. Concentrations of hormones were determined in pancreata of P2 (post-natal day 2) Sox8-deficient mice and their wild-type littermates, by the radioimmuno assay (individual pancreata were analyzed; average values +/- standard error of the mean).

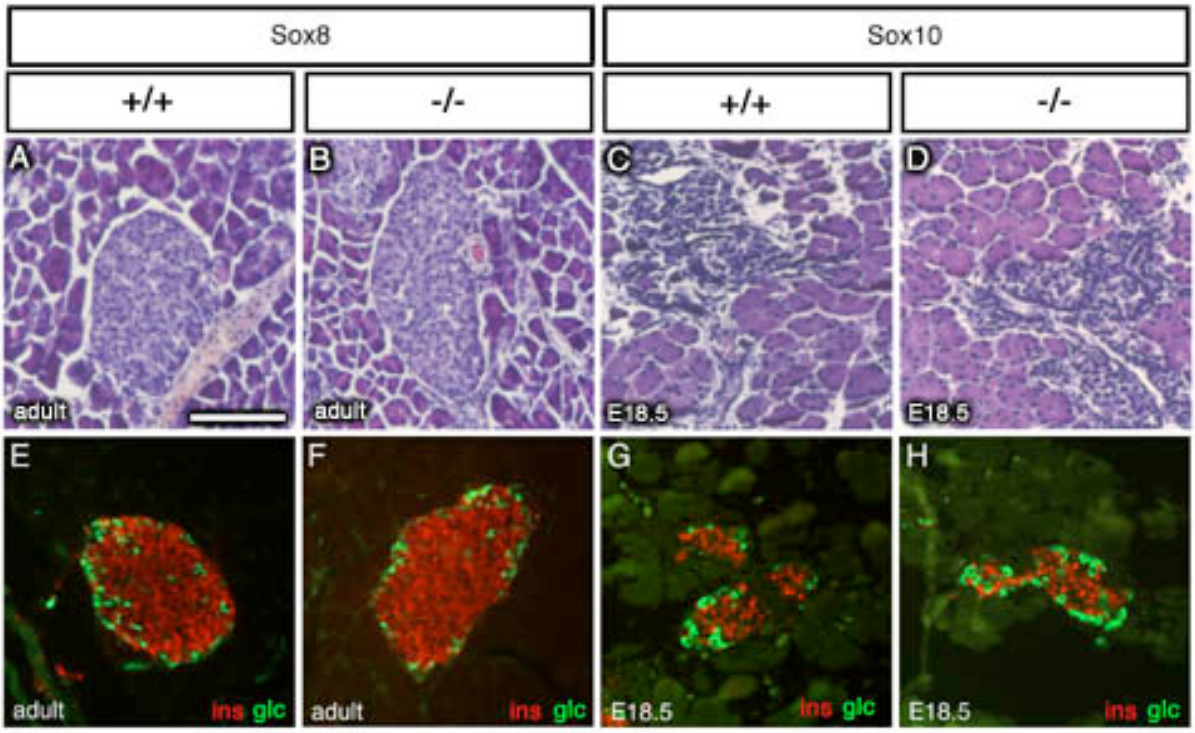

Figure 2.5. Expression of islet hormones in *Sox8*- and *Sox10*-deficient mice. (A-D) Hematoxylin-Eosin staining of pancreatic sections from adult wild type (+/+) and *Sox8* mutant mice (-/-), as well as from wild type (+/+) and *Sox10* mutant (-/-) embryos at E18.5. (E-H) Immunofluorescence staining with anti-insulin (ins) (shown in red) and anti-glucagon (glc) (shown in green) antibodies. Five independent pancreata were analyzed from both wild type and mutant mice.

In the peripheral nervous system, Sox10 and Sox8 are expressed in glial cells, and Sox10 has been shown to be requisite for the development of these cells (Britsch et al., 2001; Sock et al., 2001). Because pancreatic islets are enveloped in a sheath of glial cells (Teitelman et al., 1998), it is possible that the Sox10-positive cells in the pancreas are indeed glial cells. Our finding that no ß-galactosidase-positive cells could be detected in pancreas of Sox10-mutant embryos (Fig. 2.6) further suggests that these cells require Sox10 for their development.

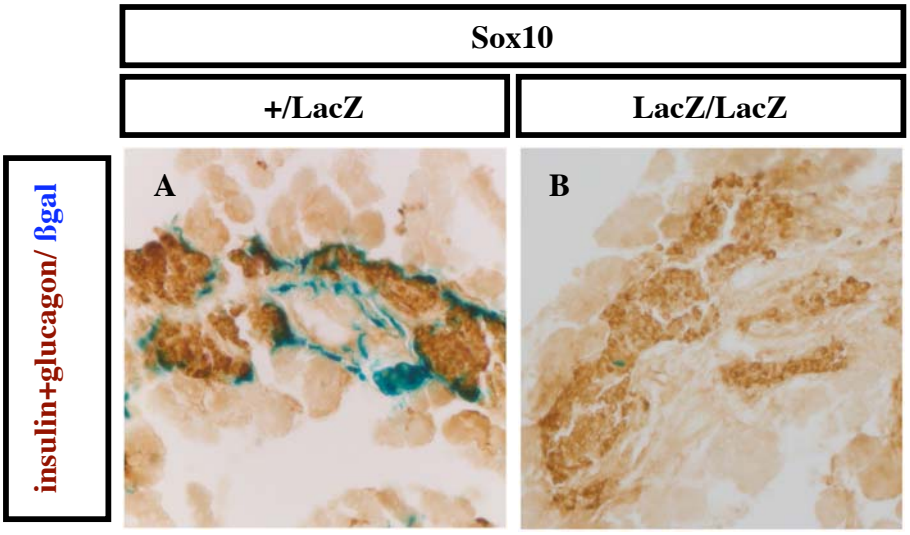

Figure 2.6. Loss of ß-galactosidase expression in Sox10$^{LacZ/LacZ}$ mutants suggests Schwann cell loss. Combined immunohistochemical staining for insulin and glucagon (brown) to visualize the islets, and in situ enzymatic ß-galactosidase staining for Sox10 on E18.5 paraffin-embedded pancreatic sections. (A), islet of a heterozygous Sox10$^{+/LacZ}$ mouse, used as control; (B), islet of a mutant Sox10$^{LacZ/LacZ}$ littermate. Note the absence of ß-galactosidase-positive cells on the periphery of the islet.

2.2 Analysis of the Roles of TCF/LEF Transcription Factors in Pancreas Development

2.2.1 Expression of *Tcf/Lef* genes during mouse pancreas development

2.1.1.1 RT-PCR Expression Analysis of *Tcf/Lef* Genes.

The expression and functions in the developing pancreas of the members of the other HMG box transcription factor family, TCF/LEF factors, have previously not been studied. In order to determine their expression pattern, I first employed a highly sensitive reverse transcriptase polymerase chain reaction (RT-PCR) technique with gene-specific primers to detect transcripts of all four *Tcf/Lef* genes at different stages of pancreas development (Table 2.4). For the detection of alternatively spliced mRNAs of *Tcf-1, Lef-1,* and *Tcf-4* (Kobielak et al., 2001; Van de Wetering et al., 1996) Primers were designed in such a way that they anneal to the DNA sequence common to different splice variants. I synthesized cDNA from the total RNA extracted from whole pancreata at embryonic ages E12.5, E15.5, and E18.5, as well as RNA extracted from the adult endocrine islets (for islet isolation, RNA extraction and cDNA synthesis see Methods). The RT-PCR analysis showed that one member of the family, *Tcf-3*,

was detectable at all stages of development, whereas the others were found either until E15.5 (*Lef-1, Tcf-4*), or after E15.5 (*Tcf-1*). Thus, expression of all *Tcf/Lef* genes in the developing pancreatic tissue was detected by RT-PCR.

Gene	E12.5	E15.5	E18.5	Adult islet
Tcf1	-	-	+	+
Lef1	+	+	-	-
Tcf3	+	+	+	+
Tcf4	+	+	-	-

Table 2.4. RT-PCR analysis of *Tcf/Lef* gene expression in developing mouse pancreas and adult endocrine islets. (+) means that the gene transcript was detected, and (-) means that no PCR product could be detected, despite amplification from a control RNA.

2.1.1.2 LEF/TCF Gene Expression Analysis by *in situ* Hybridization on Pancreatic Sections

In order to obtain information about tissue distribution of TCF/LEF transcription factors in the pancreas, I performed *in situ* RNA hybridization with RNA probes specific for all four *Tcf/Lef* genes (information in the Methods Section) at three different stages (E10.5, E12.5, and E14.5). The results are briefly summarized in Table 2.5 and illustrated in the Figures 2.7.

Gene	E10.5	E12.5	E15.5
Tcf1	+(*)	-	-
Lef1	-	-	-
Tcf3	?	?	?
Tcf4	+	+	-

Table 2.5. *In situ* hybridization analysis of Tcf/Lef members in the developing pancreas. (+) means that the mRNA was detected, and (-) means that no mRNA could be detected, despite signal from the positive control tissue. (*), expression observed in the mesenchyme surrounding the pancreatic epithelial bud. (?), not detected, but no positive control exists at these ages.

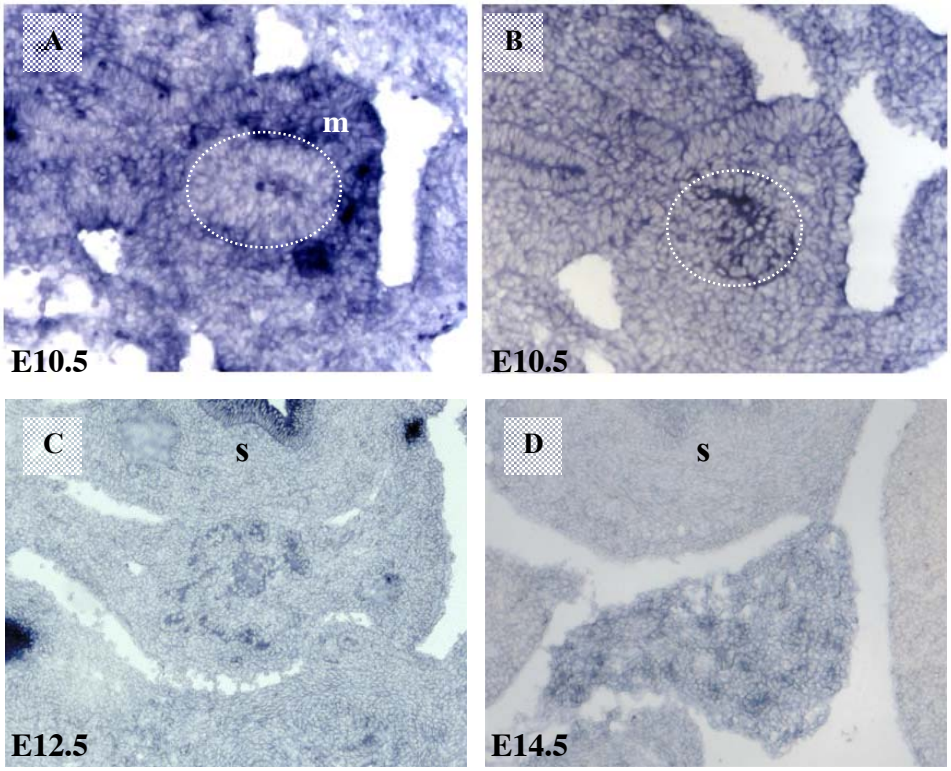

Figure 2.7. Expression of *Tcf1* and *Tcf4* in the developing pancreas. *In situ* RNA hybridization for *Tcf1* and *Tcf4* on cryosections through the developing mouse pancreas at the indicated ages. (A), E10.5, Tcf1. Expression is detected in the pancreatic mesenchyme (B), E10.5, Tcf4. Pancreatic epithelial bud at E10.5 is circled. (C), E12.5, Tcf4. (D), E14.5, Tcf4. Abbreviation: m, mesenchyme; s, stomach.

In concordance with the RT-PCR results, *Tcf-1* was not found either at E12.5, or E15.5, whereas it appeared in the mesenchyme immediately adjacent to the early developing pancreatic bud at E10.5 (Fig. 2.7, A). However, in contrast to the RT-PCR results, pancreatic *Lef1* was not detected by *in situ* hybridization analysis either at E10.5, E12.5, or at E14.5, despite specific signal observation in control tissues known to express *Lef-1*. This discrepancy could be explained by differences in the sensitivity of the two detection methods. While low levels or even trace amounts of mRNA transcript could be detected by the RT-PCR, *in situ* hybridization technique has a higher threshold for transcript detection. Thus, it is most likely that *Lef-1* is expressed in the pancreas, but at low levels. The same could hold true for *Tcf-3*, which despite its amplification by RT-PCR, could not be detected in pancreas at any analyzed stage by *in situ* hybridization. *Tcf-4* was first detected by *in situ* hybridization at E10.5 in the pancreatic epithelial bud (Fig. 2.7, B) and continued to be widely expressed in the pancreatic epithelium at E12.5 and E14.5 (Fig. 2.7, C and D).

2.2.2 Expression of Wnt Pathway Components during Mouse Pancreas Development

Thus, expression of all four members of Tcf/Lef family was detected in the developing pancreas. Since Tcf/Lef transcription factors are the downstream effectors of the canonical Wnt signaling pathway, I next tested if other components of Wnt signaling pathway are also expressed in the mouse pancreas. For this purpose, I employed gene-specific RT-PCR approach as well as RNA *in situ* hybridization techniques. The results of these analyses are summarized in the Table 2.6.

GENE	E10.5	E12.5	E14-15	E18	adult
Wnt2b	-	RT-PCR	-	RT-PCR	RT-PCR
Wnt5a	-	in situ	-	n.t	-
Fz2	in situ	in situ	in situ	n.t	n.t
Fz4	-	in situ	-	n.t	-
Axin2	X-gal	X-gal	X-gal	X-gal	X-gal
Tcf1	Mesenchyme.In situ	-	-	RT-PCR	RT-PCR
Lef1	-	RT-PCR	RT-PCR	-	-
Tcf3	-	RT-PCR	RT-PCR	RT-PCR	RT-PCR
Tcf4	In situ	in situ/RT-PCR	in situ/RT-PCR	-	-

Table 2.6. Summary of expression of different key components of the Wnt signaling cascade in the mouse pancreas. Green shading indicates that the transcript was detected at this stage, with a remark by which technique (RT-PCR or *in situ*); red shading means that by neither RT-PCR nor *in situ* hybridization the gene was detected. N.t., not tested. X-gal, enzymatic ß-galactosidase staining.

In summary, Wnt ligands (Wnt5a and Wnt2b, Fig. 2.8, A), the transmembrane receptors Frizzled (Fzd2, Fzd7, and Fzd4, Fig. 2.8, B-F; data not shown), intercellular adaptor protein Dvl1 were detected by RT-PCR in the developing pancreas and in the adult endocrine islets. In addition, X-gal staining of pancreata from heterozygous *Axin2*$^{+/LacZ}$ embryos revealed expression of *Axin2*, a negative intercellular Wnt cascade regulator, in pancreatic tissue.

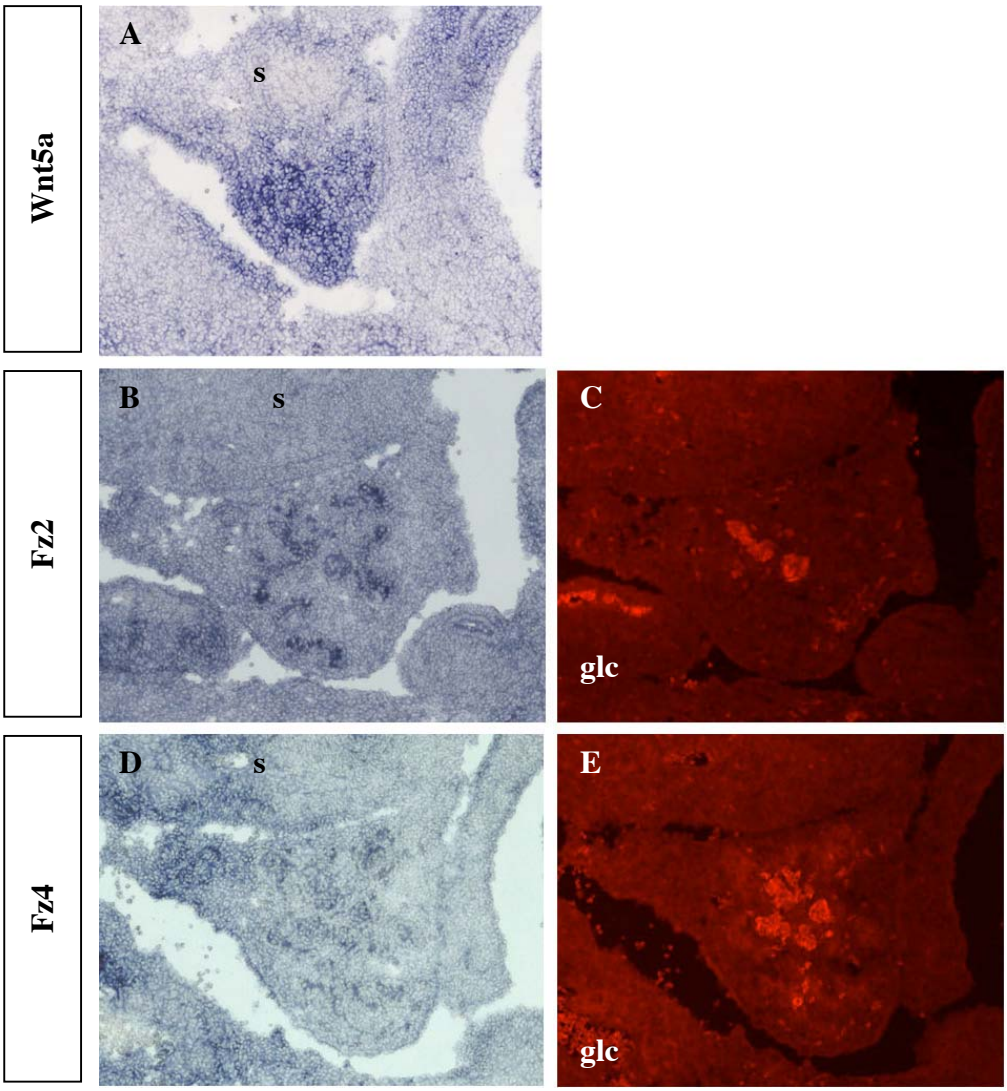

Figure 2.8. Expression of Wnt5a, Fzd2, and Fzd4 in the developing pancreas. *In situ* RNA hybridization for Wnt5a (A), Fzd2 (B) and Fzd4 (D) on cryosections through the mouse pancreas at E12.5. (A), Wnt5a transcript is found in both pancreatic epithelium and mesenchyme. (B), (D), Fzd2 and Fzd4 are broadly expressed in pancreatic epithelium. (C), (E). To visualize the early glucagon-producing cells after *in situ* hybridization, sections were immunostained with a fluorescent anti-glucagon antibody (red). Both Fzd2 and Fzd4 are expressed in early glucagon cells as well as in other cells of the pancreatic epithelium. S, stomach.

2.2.3 Detection of Cells that Receive a Wnt Signal during Mouse Pancreas Development

A previous study has shown that Wnt ligands, Frizzled receptors, and other modifiers of Wnt signaling activity are expressed in the developing pancreas (Heller et al., 2002). However, the question remains open, which cells receive the Wnt signal in the developing pancreas *in vivo*. There are several ways to visualize cells that transduce a Wnt signal *(see* http://www.stanford.edu/~rnusse/assays/reporter.html).

First, two independent, but based on the same principle, transgenic mouse lines have been generated, *TOP-Gal* by dasGupta and Fuchs (DasGupta and Fuchs, 1999) and *BAT-Gal* by Maretto et al. (Maretto et al., 2003), that respond to Wnt signals in intact cells by inducing expression of the ß-galactosidase gene. Both reporter lines carry the same transgenic DNA construct, which is composed of a multimerized TCF/LEF binding site regulating expression of the ß-galactosidase gene from *E.Coli*. Thus, in these transgenic mice ß-galactosidase is produced in cells which express any of the TCF/LEF transcription factors, and also have a nuclear localized ß-catenin as a result of an active Wnt signal. The coding sequence for ß-galactosidase includes a nuclear localization signal (NLS) to retain ß-galactosidase protein in the nucleus. This facilitates the detection of ß-galactosidase expression either by specific anti-ß-galactosidase antibodies, or by enzymatic *in situ* staining with the ß-galactosidase substrate X-gal.

The second approach to visualize sites of active Wnt signaling in the pancreas is based on the experimental evidence that *Axin2* expression is regulated by the canonical Wnt/ß-catenin signaling. It should be noted that there are 2 vertebrate *Axin* genes, and both proteins, AXIN and AXIN2 act as negative regulators of the Wnt signaling cascade. *Axin* is constitutively expressed, but expression of *Axin2* (also called *Conductin*) is induced by active canonical Wnt signaling (Jho et al., 2002; Lustig et al., 2002; Yan et al., 2001). To facilitate the visualization of *Axin2* expression, a transgenic mouse line was generated (Jho et al., 2002), in which green fluorescent protein (GFP) is expressed under control of the *Axin2* gene promoter. The pattern of GFP expression in the developing mouse embryo fully reproduced the well-known pattern of endogenous canonical Wnt signaling (Jho et al., 2002), thus validating *Axin2* expression as a marker for the active TCF/LEF-mediated Wnt signaling pathway.

First, to study Wnt signaling activity in the developing pancreas, I analyzed pancreata of heterozygous *Axin2*$^{+/LacZ}$ mice (unpublished) in collaboration with the laboratory of Dr. Walter Birchmeier at the Max-Delbrück Center in Berlin, who provided us with *Axin2*$^{+/LacZ}$ mice. This laboratory generated the mouse strain with *Axin2* coding sequence replaced by the ß-galactosidase gene carrying an NLS. Thus, ß-galactosidase expression is regulated by the *Axin2* promoter, and in heterozygous *Axin2*$^{+/LacZ}$ mice the pattern of ß-galactosidase expression reproduces endogenous *Axin2* expression pattern (Dr. Markus Morkel, personal communication). Second, I analyzed ß-galactosidase expression at different stages of the developing pancreata of *BAT-Gal*$^{+/tg}$ mice (Maretto et al., 2003) in collaboration with the laboratory of Dr. Stefano Piccolo (University of Padua).

I performed enzymatic staining with X-gal as a substrate for the ß-galactosidase in *Axin2*$^{+/LacZ}$ mice at different embryonic stages of pancreas development (E10.5-E18.5) and in the adult pancreas. In my analysis of embryonic pancreata from *Axin2*$^{+/LacZ}$ mice I found expression of *Axin2* at E10.5 in most cells of the pancreatic epithelial bud (Fig 2.9, A), which was visualized by subsequent immunohistochemical staining with antibodies against glucagon. At E13.5 I found *Axin2* expression at high levels in the pancreatic mesenchyme and at lower levels in scattered cells in the pancreatic epithelium (Fig. 2.9, B). Before birth *Axin2*-positive cells were found in some insulin and glucagon cells of the islets (Fig. 2.9, C and D), in pancreatic embryonic ducts (Fig. 2.9, C), as well as in cells of unknown identity scattered throughout the pancreas. No *Axin2*-positive cells were found in the pancreas of adult mice. These results indicate that Wnt signaling is likely to be active throughout the development of the pancreatic epithelium.

To confirm the data on cells receiving Wnt signal in the developing pancreas which I obtained by analyzing *Axin2* expression pattern, I additionally analyzed ß-galactosidase expression at different stages of the developing pancreata of *BAT-Gal*$^{+/tg}$ mice (Maretto et al., 2003). This work was done in collaboration with Dr. Stefano Piccolo (University of Padua, Italy), who kindly provided us with this mouse strain. I performed enzymatic staining with X-gal as a substrate for the ß-galactosidase in *BAT-Gal*$^{+/tg}$ mice at different embryonic stages of pancreas development (E10.5-E18.5) and in the adult pancreas. The expression pattern of *BAT-Gal* in this study is highly similar to that of *Axin2* in the developing pancreas, although overall there are less positive cells, which are also more scattered in appearance (Fig. 2.10). In summary, at E10.5 a subset of cells of the pancreatic epithelium are *BAT-Gal* positive (A). At E12.5 *BAT-Gal* expressing cells are found mostly in pancreatic mesenchyme, but also in scattered cells in

the epithelium, as well as in some early glucagon-positive cells (B). At E14.5, ß-galactosidase-positive cells are found in all pancreatic epithelium and in some glucagon cells (C). Before birth *BAT-Gal* expression marks some islet cells, duct cells (D, longitudinal section plane) as well as cells of unknown identity scattered throughout some, but not all, pancreatic areas analyzed. Six weeks after birth no *BAT-Gal* positive cells were detected in the pancreas of *BAT-Gal*$^{+/tg}$ mice. Taken together, expression patterns of *Axin2* and *BAT-Gal* confirm that the pancreatic epithelium receives a TCF/LEF-mediated Wnt signal throughout its development.

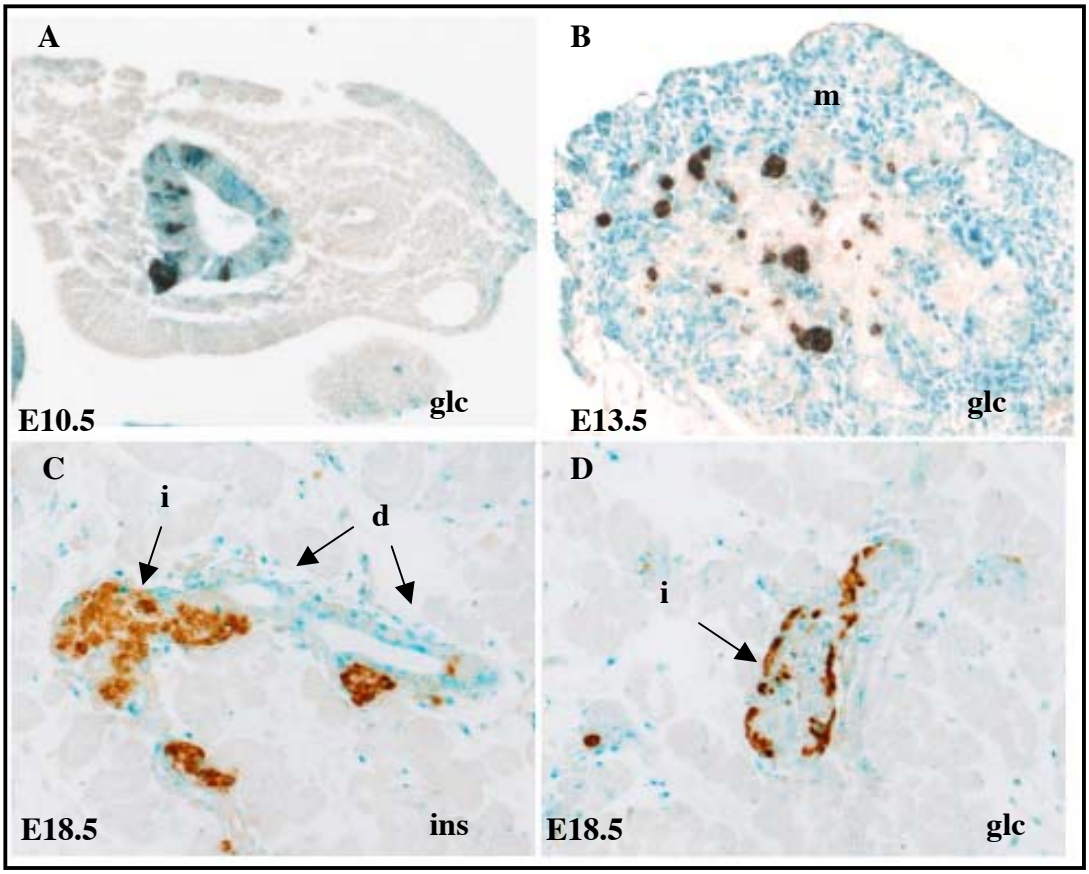

Figure 2.9. *Axin2* expression in the developing mouse pancreas. Expression of ß-galactosidase in the *Axin2*$^{+/LacZ}$ mouse pancreas at different stages of development, as visualized by the enzymatic X-gal staining of the tissue, yielding *Axin2* expression pattern. Paraffin-embedded sections through the X-gal stained pancreatic tissue were subsequently immunohistochemically stained with antibodies to glucagon (A; B, D) or insulin (C). Ages as indicated. (A), *Axin2* is expressed in most of the pancreatic epithelial cells at E10.5 as marked by antibodies against glucagon. (B), Most of the pancreas mesenchyme (m) is *Axin2*-positive at E12.5, whereas scattered *Axin2*-expressing cells are also found in the pancreatic epithelium. (C, D), at E18.5 *Axin2* is expressed in islets of Langerhans (i), in some scattered cells of unknown identity throughout pancreatic tissue, as well as in embryonic ducts (d). Abbreviations: d, duct; i, islet; m, mesenchyme.

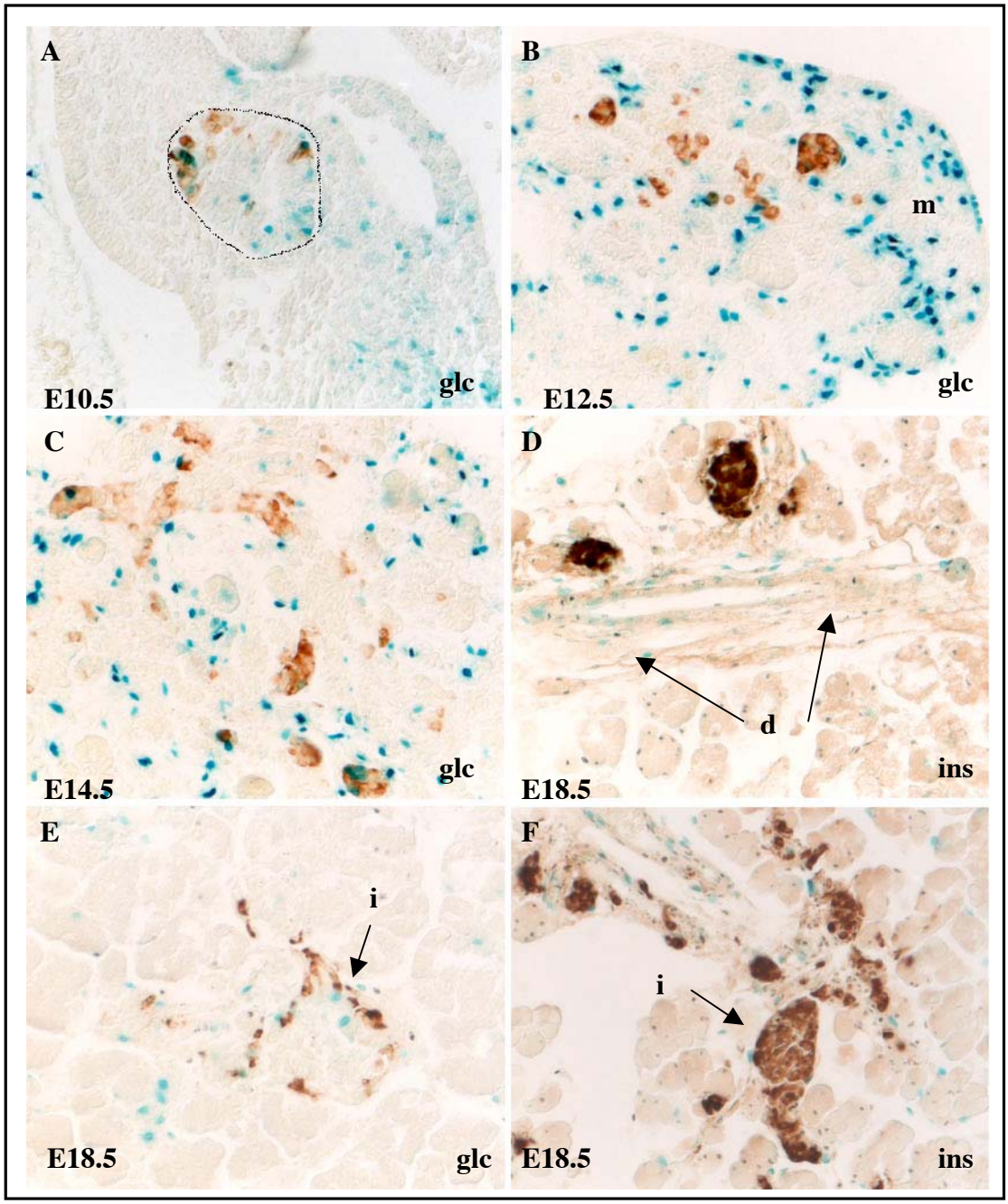

Figure 2.10. *BAT-Gal* expression in the developing mouse pancreas. Expression of ß-galactosidase in the *BAT-Gal*$^{+/tg}$ mouse pancreata at different stages of development, as visualized by the enzymatic X-gal staining of the tissue. Paraffin-embedded sections through the X-gal stained pancreatic tissue were subsequently immunohistochemically stained with antibodies to glucagon (A – E) or insulin (F). Ages as indicated. In (A), pancreatic epithelium is circled. (A), *BAT-Gal*-positive cells are found in some pancreatic epithelial cells at E10.5 as marked by antibodies against glucagon. (B), at E12.5 pancreas mesenchyme (m) is *BAT-Gal*-positive, whereas scattered *BAT-Gal*-expressing cells are also found in the pancreatic epithelium. (C), *BAT-Gal* is expressed in scattered cells throughout the pancreas at E14.5. (D), at E18.5 *BAT-Gal* is found in ductal cells (here a longitudinal section plane). (E, F), *BAT-Gal* marks some cells of islets of Langerhans (i), as well as some scattered cells of unknown identity throughout pancreatic tissue. Abbreviations: d, duct; i, islet; m, mesenchyme.

2.2.4 Creation of Modified Forms of TCF4

One possible approach to analyze the roles of TCF/LEF factors in pancreas development is generation and analysis of mice deficient for one of the *Tcf/Lef* genes. Mouse lines lacking individual *Tcf/Lef* genes have been generated and analyzed (Korinek et al., 1998a; Merrill et al., 2004; van Genderen et al., 1994; Verbeek et al., 1995). All four *Tcf/Lef* deficient lines exhibit mutant phenotypes resulting from aberrant Wnt signal transduction (reviewed in the Introduction). However, so far in none of the *Tcf/Lef* mouse lines lacking individual *Tcf/Lef* genes any obvious defects in pancreas development have been reported. Therefore, I next sought to investigate the function of TCF/LEF-mediated gene transcription in pancreas development and function of islets of Langerhans. Because TCF/LEF proteins exert their biological functions as the most downstream effectors of the canonical Wnt cascade, a decision was made to interfere with canonical Wnt signaling cascade in the developing pancreas. There are different possibilities to affect Wnt pathway in a living system at different levels of the cascade (for reference, see http://www.stanford.edu/~rnusse/assays/inhib.htm).

The final decision was made upon the pancreas-specific overexpression of modified forms of the TCF4 protein, resulting in either activation, or inhibition of canonical Wnt signaling, due to following reasons. First, modified TCF proteins act in a cell-autonomous manner; and therefore, neighboring cells will not be affected by the ectopic expression of a modified TCF4 transcription factor. Second, dominant-negative and constitutively active modified forms of TCF/LEF proteins have been generated and successfully tested *in vitro* and *in vivo* (Aoki et al., 1999; Molenaar et al., 1996; van de Wetering et al., 2002; Wang et al., 2002). Finally, TCF-4 was chosen because of its endogenous expression in the early developing pancreas and during pancreas development. I aimed to employ two complementary approaches to interfere with canonical Wnt signaling cascade in the developing pancreas. Dominant-negative TCF-4 protein was successfully used in mouse transgenic models, where it inhibited Wnt signaling pathway (van de Wetering et al., 2002; Wang et al., 2002). A constitutively active fusion protein form between LEF-1 and ß-catenin has been tested *in vitro* and in cell culture, and was shown to activate specific LEF-1-target genes (Aoki et al., 1999). In this study, the structure of the dominant-negative form of TCF-4 was reproduced, and a constitutively active TCF4-ß-catenin fusion protein was constructed similar in design to the described LEF-1-ß-catenin fusion protein. The modified forms of TCF4 (dnTCF4 and caTCF4) that I generated are shown schematically in the Figure 2.11.

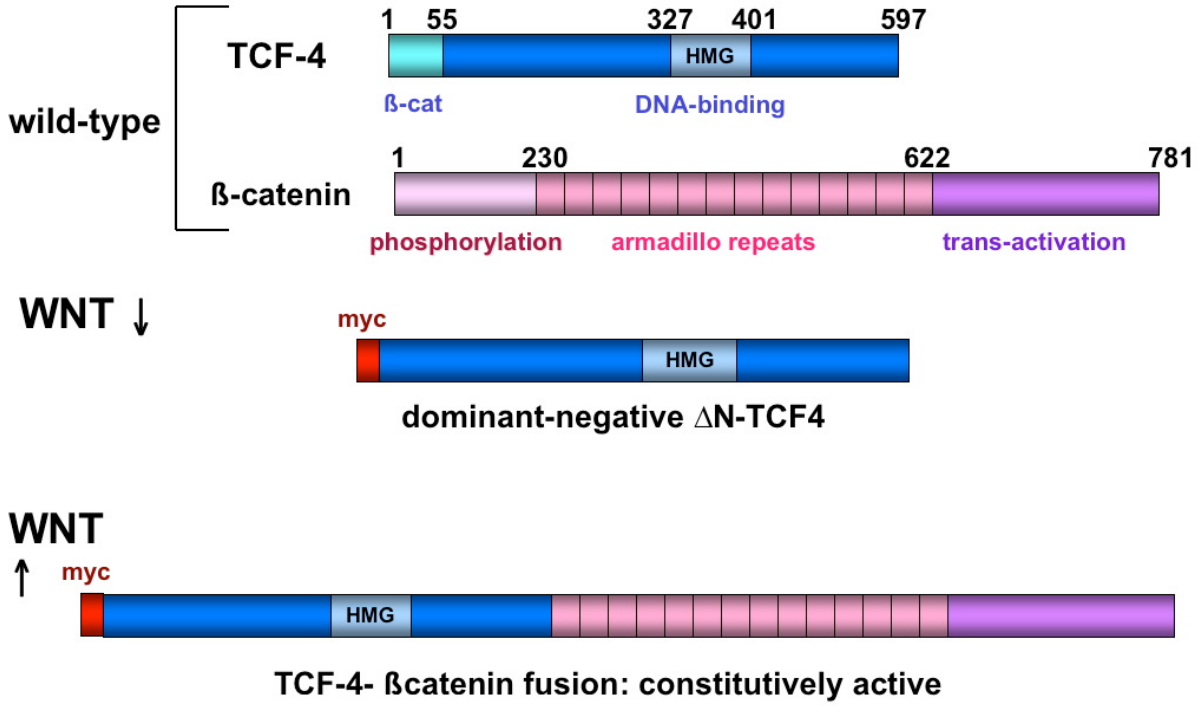

Figure 2.11. Wild-type and modified forms of TCF4 transcription factor aimed to interfere with endogenous Wnt signaling. In the wild-type TCF4 protein, first 55 amino acids comprise the ß-catenin interaction domain. Amino acids 327 to 401 form the HMG box domain interacting with DNA of target genes. In the dominant-negative form of TCF4 (termed dnTCF4), 31 N-terminal amino acids are deleted, thus abolishing the capacity of TCF4 to bind to ß-catenin to activate the transcription. This leads to repression of Wnt signaling (Wnt↓). Wild-type ß-catenin protein can be divided into the 81 amino acids long N-terminal domain responsible for degradation through phosphorylation, the armadillo repeats domain (amino acids 230-622) for binding to Tcf/Lef and other cofactors proteins, and the C-terminal trans-activation domain for activation of target gene transcription. In the constitutively active TCF4 form (termed caTCF4) a fusion protein is made between a dominant-negative TCF4 and a ß-catenin molecule lacking 81 N-terminal amino acids necessary for its degradation. Thus, a bipartite TCF-ß-catenin transcription factor is reconstructed, which leads to ectopic activation of Wnt signaling cascade (Wnt↑). Both modified TCF4 forms, dnTCF4 and caTCF4 bear a myc epitope on their N-termini for the detection in cells.

The dominant-negative form of TCF4 (dnTCF4) lacks 31 N-terminal amino acids necessary for binding to ß-catenin. Thus, dnTCF4 expression leads to occupying Tcf/Lef binding sites on the promoter of target genes, but no transcriptional activation by dnTCF4 can occur

without interaction with ß-catenin. Therefore, Wnt signaling is inhibited in cells expressing dnTCF4. In the constitutively active TCF4 form (caTCF4), dnTCF protein is fused to ß-catenin which lacks N-terminal 81 amino acids (comprising the ß-catenin degradation domain). Thus caTCF4 factor possesses both DNA-binding and transcription activation properties, and escapes ß-catenin degradation mechanism by APC/GSK3ß/Axin complex. Therefore, Wnt signaling is activated in cells expressing caTCF4.

I created cDNAs for dnTCF4 and caTCF4 factors by modification of cDNAs of ß-catenin and TCF-4. For this purpose, PCR (polymerase chain reaction) was employed to delete fragments of cDNAs, or to fuse cDNAs to other sequences. Plasmid containing ß-catenin coding sequence was a kind gift from Dr. M. Watermann (University of California, Irvine), and the plasmid containing TCF-4 cDNA was a kind gift from Dr. A. Hecht (Max-Planck Institut für Immunbiologie, Freiburg). Both dnTCF4 and caTCF4 cDNAs were fused on their 5'-end to the sequence consisting of three elements: (1) the SDK oligonucleotide (combined Shine-Dalgarno sequence and a Kozak sequence), for efficient initiation of mRNA translation on ribosomes; (2) an ATG transcription start codon, and (3) a myc epitope sequence to facilitate the detection of the modified TCF-4 protein forms in cells (sequences in Appendix). In the dominant-negative dnTCF4 cDNA, I deleted the sequence coding for 31 N-terminal amino acids (ß-catenin interaction domain) (Fig. 2.11). In the caTCF4 cDNA, I fused the TCF-4 coding sequence lacking 31 N-terminal amino acids to the sequence coding for the amino acids 82-721 or ß-catenin protein (Fig. 2.11).

The objective was to express the dnTCF4 and caTCF4 factors in developing mouse pancreas. First possibility was to use promoters of genes developmentally expressed in pancreas to drive expression of modified TCF4 forms in a transgenic mouse model. The most suitable promoter for this purpose is the promoter of the *pdx-1* (pancreatic and duodenal homeobox gene 1). *Pdx-1* gene is first expressed at 10-12 somites stage in mice, in the dorsal and ventral walls of the primitive foregut at the positions where pancreas will later form (Jonsson et al., 1994). Subsequently *pdx-1* is highly expressed in pancreatic progenitors at E8-E10 and later in differentiating ß-cells, as well as weakly in exocrine cells (Jonsson et al., 1994; Offield et al., 1996). However, if pancreas development is perturbed in mice expressing modified TCF4 proteins under control of the *pdx-1* promoter, no stable transgenic line can be established. To circumvent this problem, a second approach was used. In this experimental setup, a bigenic *Cre-loxP* system is employed to temporally and spatially control the expression of dnTCF4 and caTCF4 in mouse pancreas. The *Cre-loxP* bigenic system relies on the *in vivo*

recombination activity of the P1 bacteriophage *Cre* recombinase. It is a 38 kD protein that catalyzes the recombination between two of its 34 bp-long recognition sites, called *loxP* sites (Hamilton and Abremski, 1984). A random occurrence of a specific 34 bp sequence requires a 10^{18} bp length of DNA, whereas the entire mammalian genome is less than 10^{10} bp long, thus making *Cre*-mediated *loxP*-excision highly specific. In the bigenic approach modified TCF4 cDNA is placed into the genome in a silent, transcriptionally inactive state. In cells expressing phage *Cre*-recombinase an *in vivo* genomic recombination between two *loxP* sites occurs, activates the expression of the modified TCF4 construct. Not only these cells, but also their progeny will express modified TCF4 protein forms. Numerous transgenic mouse lines have been created that express *Cre* recombinase in specific cell types under control of tissue-specific promoters (Nagy, 2000). In my research, I pursued both directions, (a) transient transgenic expression of dnTCF4 and caTCF4 in all pancreatic precursor cells under control of the *Pdx-1* gene promoter, and (b) stable and heritable expression of dnTCF4 and caTCF4 either in whole pancreas, or in mature ß-cells using the bigenic *Cre-loxP* based system.

2.2.5 Testing the Modified Forms of TCF4 for Their Functionality *in vitro*

To test if the modified TCF4 protein forms are functional *in vitro*, we collaborated with the laboratory of Dr. Marian Waterman at the University of California in Irvine, an expert in the field of Tcf/Lef transcription. We used a cell based transfection assay to test if dnTCF4 or caTCF4 indeed inhibit or enhance Tcf/Lef-mediated transcriptional activation. In this assay, a CMV (cytomegalovirus) promoter-driven expression construct for dnTCF4 or caTCF4 was co-transfected with the TOPFLASH reporter plasmid.

In the TOPFLASH plasmid three consensus binding sites for LEF/TCF factors upstream of the c-fos minimal promoter drive expression of a luciferase reporter gene. The TOPFLASH plasmid is often used as a standard Wnt-responsive reporter plasmid (van de Wetering et al., 1997). The activity of luciferase in the cell serves as a reporter for the activity of the canonical Wnt signaling pathway. Luciferase expression increases if cells are co-transfected with Tcf/Lef and ß-catenin. In this assay luciferase activity can easily quantified.

I subcloned both modified cDNAs constructs (dnTCF4 and caTCF4) including the 5'-SDK sequence, as well as the wild-type full-lenth TCF4 and beta-catenin, into the pcDNA3 mammalian expression vector (see Methods). All subsequent experiments were carried out by

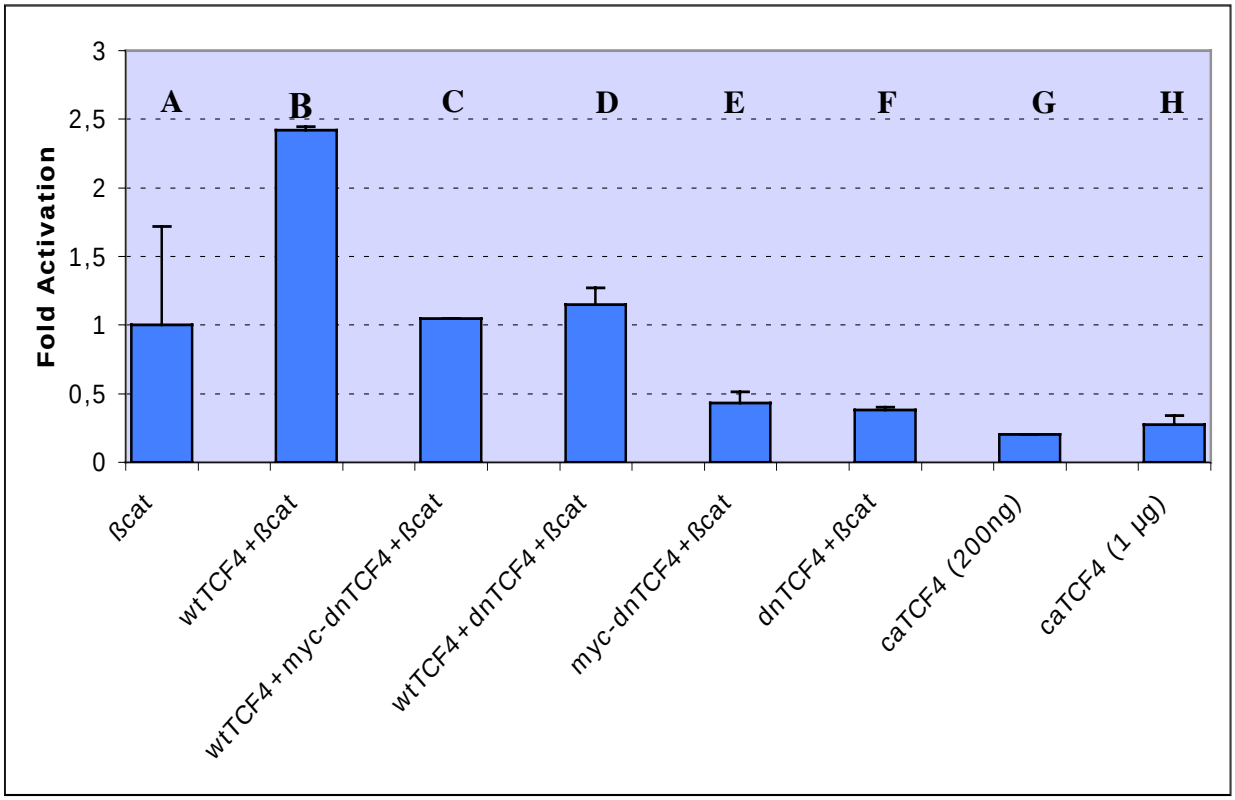

Figure 2.12. dnTCF4 blocks Tcf/Lef-mediated transcription *in vitro*. COS cells were transiently transfected with TOPFLASH luciferase reporter construct (0.4 µg), expression vectors for the indicated proteins (0.4 µg), and ß-galactosidase expression construct (0.1 µg). Cells were harvested 18-20 h after transfection, and ß-galactosidase activity was determined to normalize luciferase activity for each point (each point was performed in duplicate). The –fold induction was calculated relative to the luciferase reporter plasmid co-transfected with ß-catenin alone (A). Results are shown as mean+SEM (standard error of the mean). (A), ß-catenin, reference luciferase activation value. (B), wild-type TCF4 with ß-catenin activate the reporter approx. 2.5 fold. (C, D) co-transfection of dnTCF4 with wild-type TCF4 and ß-catenin abrogated luciferase activation, regardless of myc epitope presence on the dnTCF4 protein (in D, dnTCF4 lacks N-terminal myc epitope). (E, F). co-transfection of dnTCF4 with ß-catenin represses luciferase activity approx. 2-fold, regardless of myc epitope presence on the dnTCF4 protein (in F, dnTCF4 lacks N-terminal myc epitope). (G), caTCF4 is not able to activate the reporter gene; (H) increased amounts of caTCF4 show the same effect as in (G).

Dr. Waterman's laboratory at the University of California in Irvine. Cells were transiently transfected with either TOPFLASH luciferase reporter construct alone, expression plasmids containing wild-type TCF4 and ß-catenin as well as caTCF4 and dnTCF4, and ß-galactosidase expression construct. Activity of ß-galactosidase was determined to normalize luciferase activity for each point. The results are summarized in the Figure 2.12:

Wild-type full length TCF4 activated the luciferase gene approximately 2.5-fold when co-transfected with ß-catenin (Fig. 2.12, B) Upon additional co-transfection of dnTCF4, luciferase reporter activation was abolished (C, D). When dnTCF4 was co-transfected with ß-catenin, this reduced the luciferase activation level approximately 2-fold (E, F). The myc-tagged form of dnTCF4 exhibited the same ability to reduce TCF4/ß-catenin mediated transcription as dnTCF4 bearing no myc sequence (Fig. 2.12, compare C to D and E to F), showing that addition of N-terminal myc epitope did not change the property of the dnTCF protein. Constitutively active form (caTCF4) did not activate the luciferase reporter gene in this assay (G, H). There are two possible explanations for that. First, caTCF4 protein could be not functional in this cellular context. Second, caTCF4 protein might have reduced stability in COS cells, being degraded before exhibiting its transcription activation potential. However, *in vitro* transfection assays might not fully reflect the behavior of protein in the natural context. In the developing mouse tissues, the physiological amounts of transcription factors that are necessary to regulate target genes are much lower than those used in cell transfections. Therefore, I decided to use both dnTCF4 and caTCF4 factors bearing the N-terminal myc tag in order to interfere with canonical Wnt signaling cascade in the developing pancreas.

2.2.6 Generation of *pdx-dnTCF*$^{+/tg}$ and *pdx-caTCF*$^{+/tg}$ Transgenic Mice

2.1.1.1 Design of the Constructs Used for Generation of *pdx-dnTCF*$^{+/tg}$ and *pdx-caTCF*$^{+/tg}$ Transgenic Mice

To express dnTCF4 or caTCF4 in the developing mouse pancreas, I generated constructs in which the promoter of the mouse *Pdx-1* gene drives expression of dnTCF4 or caTCF4. To visualize transgene-expressing cells in the pancreatic tissue, ß-galactosidase bearing a nuclear localization signal was also expressed under control of the *Pdx-1* promoter, using a bicistronic vector with the single polyadenilation site on the 3'-end (Fig. 2.13).

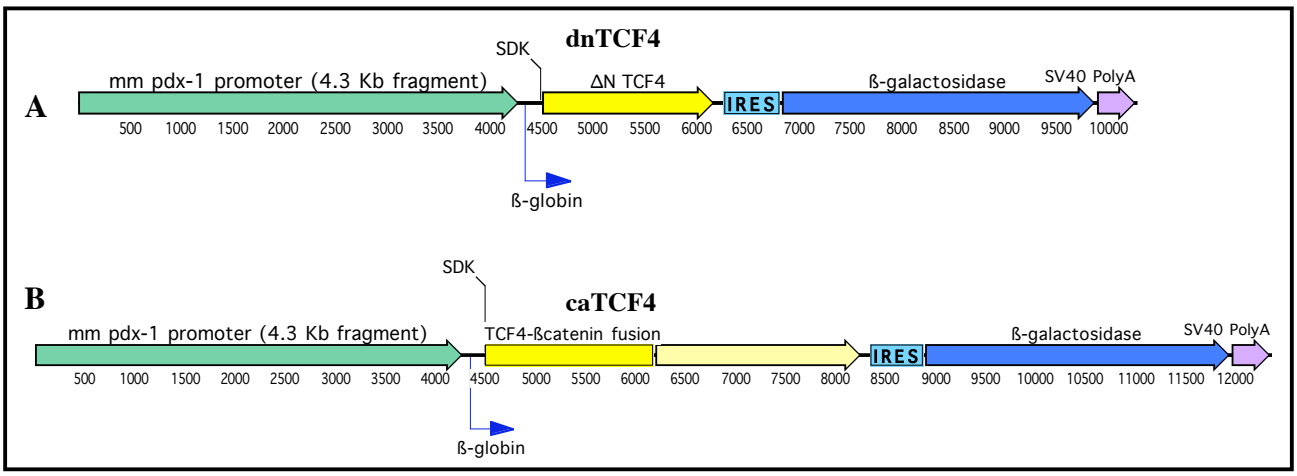

Figure 2.13. Schematic representation of linearized vectors used for generation of *pdx-dnTCF*$^{+/tg}$ and *pdx-caTCF*$^{+/tg}$ transgenic mice. A, *pdx1-dnTCF-LacZ* construct; B, *pdx1-caTCF-LacZ* construct. Mouse *Pdx-1* gene promoter drives bicistronic expression of dnTCF4 (A) or caTCF4 (B) coding sequences, followed by the ß-galactosidase gene. Abbreviations: IRES, internal ribosome entry site; SDK, Shine-Dalgarno Kozak sequence.

2.1.1.2 Generation of *pdx-dnTCF*$^{+/tg}$ and *pdx-caTCF*$^{+/tg}$ Transgenic Mice.

The pronuclear injections were performed by the transgenic mouse facility of the Center for Molecular Neurobiology in Hamburg. Both *pdx1-dnTCF4-LacZ* and constructs were microinjected into pronucleus stage mouse embryos and implanted into pseudopregnant C57Bl/6J females. Fifty-three embryos were injected with the *pdx1-dnTCF4-LacZ* construct and implanted into 5 foster mothers, and 62 embryos injected with the *pdx1-caTCF4-LacZ* construct were transferred into 6 foster mothers. It should be noted that integration of the DNA sequence into the genome in transgenic animals is a random process; therefore, each transgenic embryo was unique and needed to be analyzed independently.

Just before birth (E18.5) mice were sacrificed, embryonic pancreata were taken out and cryo-embedded (see Methods). From the *pdx1-dnTCF4-LacZ* injections, 34 embryos were recovered and assigned numbers *dn1* to *dn34*. Fourty-four embryos were recovered from the *pdx1-caTCF4-LacZ* injections and given numbers *ca1* to *ca44*. From a small part of the embryo DNA was extracted and analyzed for the presence of the ß-galactosidase coding sequence by PCR. In the analysis, six embryos were found to carry the *pdx1-dnTCF4-LacZ*

construct (*dn21, dn23, dn26, dn27, dn30,* and *dn34*) and two embryos that carry the *pdx1-caTCF4-LacZ* construct (*ca1* and *ca6*).

2.1.1.3 Analysis of Pancreata of the *pdx-dnTCF*$^{+/tg}$ and *pdx-caTCF*$^{+/tg}$ mice for the Expression of the ß-galactosidase Gene.

All *pdx-dnTCF*$^{+/tg}$ and *pdx-caTCF*$^{+/tg}$ embryos were analyzed for the expression of ß-galactosidase in the pancreas (Fig. 2.14). Two *pdx-dnTCF*$^{+/tg}$ embryos (*dn21*, C and *dn34*, D) one *pdx-caTCF*$^{+/tg}$ embryo (*ca6*, B) were found to express ß-galactosidase strongly in the pancreas, while the other transgenic embryos carrying *pdx1-dnTCF4-LacZ* or *pdx1-caTCF4-LacZ* constructs exhibited only marginal expression of ß-galactosidase in the pancreas (as exemplified by *ca1* in Fig. 2.14, A). Very strong expression of the ß-galactosidase gene was observed in the nuclei of the exocrine acinar cells (magnified in the insert in Fig. 2.14, B).

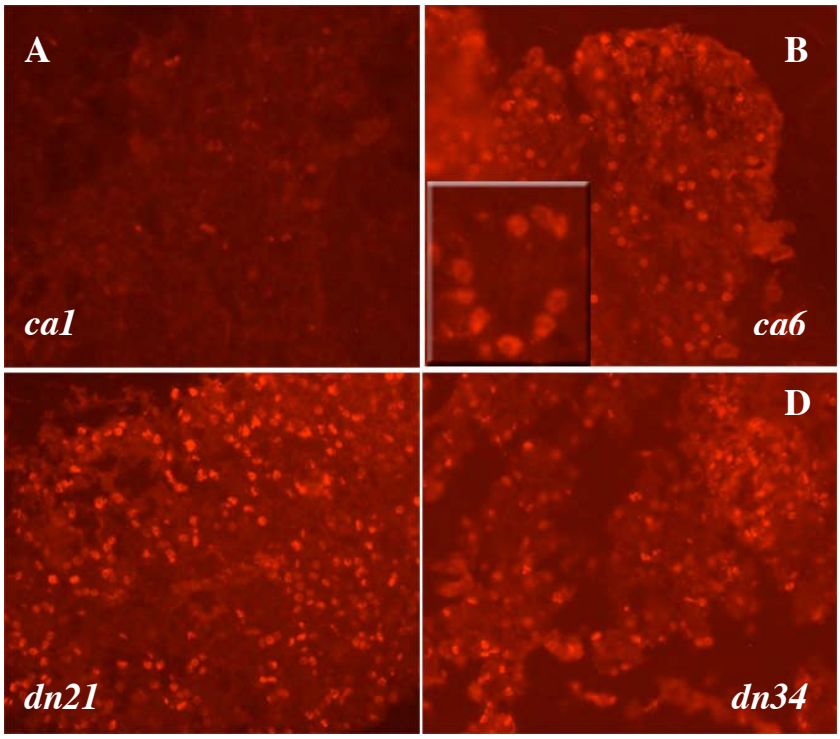

Fig. 2.14. Expression of ß-galactosidase in the pancreata of the *pdx-dnTCF*$^{+/tg}$ and *pdx-caTCF*$^{+/tg}$ transgenic mice. Immunohistochemical staining with antibodies against ß-galactosidase on E18.5 pancreas cryosections of the *pdx-dnTCF*$^{+/tg}$ and *pdx-caTCF*$^{+/tg}$ mice. Fluorescent staining is found in the cell nuclei because of the nuclear localization signal in the ß-galactosidase coding sequence. (A), *ca1* and (B), *ca6* are *pdx-caTCF*$^{+/tg}$ -transgenic mice, (C), *dn21* and (D), *dn34* are *pdx-dnTCF*$^{+/tg}$ -transgenic. In (B), a magnified single exocrine acinus with nuclear ß-galactosidase expression is shown in the insert.

2.1.1.4 Analysis of the *pdx-dnTCF*$^{+/tg}$ and *pdx-caTCF*$^{+/tg}$ Mice for the Expression of Pancreatic Markers.

Pancreata of *pdx-dnTCF*$^{+/tg}$ and *pdx-caTCF*$^{+/tg}$ mice expressing ß-galactosidase at high levels did not exhibit any morphological abnormalities, either in organization of exocrine acini, or in size or shape of forming endocrine islets (data not shown). To exclude that endocrine cell types have differentiation defects, pancreata of three "strong" expressors (*dn21*, *dn34*, and *ca6*) were analyzed for the expression of endocrine cell lineage markers, such as insulin, glucagon, somatostatin and pancreatic polypeptide, by immunohistochemical staining with antibodies against the respective hormones. In Figure 2.15, the results of the immunohistochemical analysis of the dn34 and ca6 embryos, as well as the wild-type control embryo, are shown.

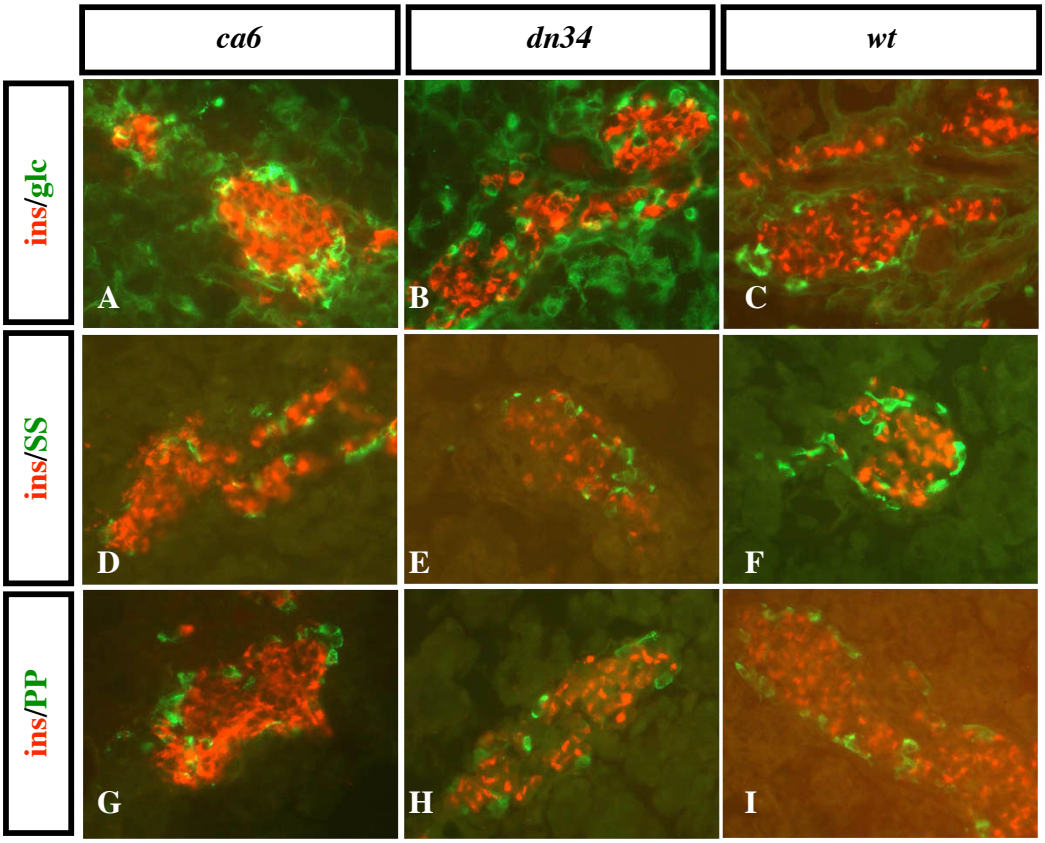

Fig. 2.15. Expression of endocrine markers in islets of *pdx-dnTCF*$^{+/tg}$ and *pdx-caTCF*$^{+/tg}$ E18.5 transgenic mice. Immunofluorescent staining with antibodies against four pancreatic hormones was performed on the cryosections through the pancreas of one *pdx-caTCF*$^{+/tg}$ embryo (*ca6* in A, D, G), one *pdx-dnTCF*$^{+/tg}$ embryo (*dn34* in B, E, H), and one wild-type control embryos (*dn32* in C, F, I) at E18.5. Double immunohistochemical staining was performed with antibodies against insulin and glucagon (A, B, C), insulin and somatostatin (D, E, F), and insulin and pancreatic polypeptide (G, H, I). Abbreviations: glc, glucagon; ins, insulin; PP, pancreatic polypeptide; SS, somatostatin.

No difference in the expression of endocrine cell markers could be seen between any of the transgenic pancreata and their wild-type littermate controls. Lack of a discernable phenotype in *pdx-caTCF*$^{+/tg}$ and *pdx-dnTCF*$^{+/tg}$ embryos could result from an inability to target the appropriate cell population with the *pdx-1* promoter, as *pdx-1* expression becomes restricted to ß- and δ-cells after E14.5. Alternatively, it is possible that the Tcf/Lef-ß-catenin mediated Wnt signaling is dispensable for embryonic pancreas development. However, the results of these experiments do not exclude that the canonical Wnt signaling is required for proper pancreas function postnatally.

2.2.7 Bigenic *Cre-LoxP* System for Expression of *dnTCF4* and *caTCF4* Factors in Mouse Pancreas

To inhibit or activate TCF/LEF activity in a constitutive and heritable fashion *in vivo*, I employed a bigenic *Cre-LoxP*-based system with a floxed dnTCF4 or caTCF4 transgene. In this system, cells only activate dnTCF4 or caTCF4, when exposed to *Cre* recombinase (Fig. 2.16). I targeted the cDNAs encoding for dnTCF4 or caTCF4 to the ubiquitously expressed Rosa26 genomic locus, preceded by a strong transcriptional STOP-sequence flanked by unidirectional *loxP* sites. In the absence of *Cre* recombinase, transcription of dnTCF4 or caTCF4 is blocked; upon expression of *Cre* recombinase, the STOP sequence is deleted, resulting in expression of dnTCF4 or caTCF4. Notably, the expression of *Cre* recombinase leads to a non-reversible removal of the STOP-sequence, resulting in heritable and constitutive dnTCF4 or caTCF4 expression in all progeny of cells that activated *Cre* recombinase. The myc epitope that I added to the N-termini of dnTCF4 and caTCF4, serves to visualize cells expressing modified forms of TCF-4 and to follow their fate *in vivo*. Importantly, the results of cell transfection experiments have shown that an N-terminal myc epitope does not change the biochemical properties of the modified TCF4 proteins.

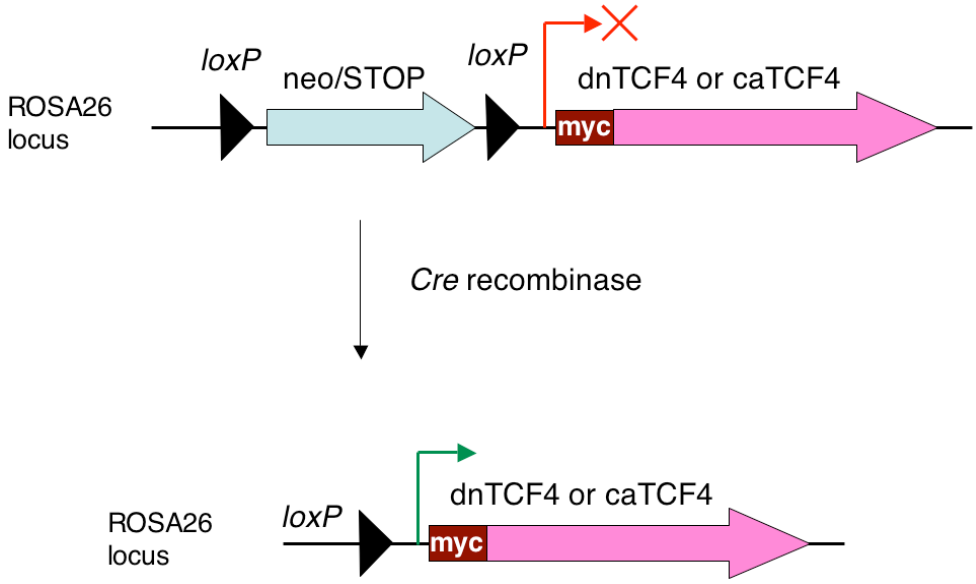

Figure. 2.16. Bigenic system for ectopic expression of modified TCF4 forms in the developing mouse pancreas. In ROSA26^{dnTCF4} and ROSA26^{caTCF4} mice, the neo/STOP cassette blocks transcription of dnTCF4 or caTCF4, respectively. The neo/STOP cassette is flanked by *loxP* sites (black triangles). Upon tissue-specific *Cre* recombination the neo/STOP cassette is excised, permitting stable expression of the dnTCF4 or caTCF4 protein in all progeny.

My modular transgenic system offers several advantages over classic transgenic approaches. First, in case of early postnatal lethality due to a pancreatic defect or expression of the transgene outside the pancreas, I can still analyze large numbers of offspring at various developmental stages from a single founder. Thus, this system eliminates the variations in expression levels or patterns seen as a result of different transgene integration sites. Second, with the use of appropriate *Cre*-expressing mice, transgene expression can be directed to different cell populations. To interfere with the canonical Wnt signaling either in all pancreatic cells, or in the differentiated ß-cells exclusively, I employed two different *Cre*-expressor mouse lines: first, a *pdx1-cre* line, expressing Cre-recombinase in whole early pancreatic epithelium under control of *Pdx-1* gene promoter, and an *insulin-cre* line, which expresses *Cre* recombinase in ß-cells under control of the insulin gene promoter. Both transgenic *Cre* lines have been described, demonstrated to direct *Cre*-mediated excision in appropriate cell populations, and successfully used (Herrera, 2000). Finally, the bigenic *Cre-loxP* system ensures constitutive and sustained expression of the transgene independently of the promoter activity driving the transgene.

To generate ROSA26^{dnTCF4} and ROSA26^{caTCF4} mice, I inserted modified TCF-4 forms into one allele of ROSA26 genomic locus through homologous recombination in embryonic stem (ES) cells. ROSA26 locus is transcriptionally active in virtually all cells of the mammalian organism (Friedrich and Soriano, 1991; Zambrowicz et al., 1997). To activate the expression of dnTCF4 or caTCF4, ROSA26^{dnTCF4} and ROSA26^{caTCF4} mice, respectively, must be crossed with transgenic mice expressing *Cre*-recombinase in pancreas. In the offspring, *in vivo* genomic DNA recombination takes place only in cells expressing *Cre* recombinase. All progeny of cells that undergo recombination will maintain their recombined status. Therefore, by choosing a respective *Cre*-expressing mouse strain I can control the pool of cell types affected. I decided to cross ROSA26^{dnTCF4} and ROSA26^{caTCF4} mice with *insulin-cre*$^{+/tg}$ and *pdx1-cre*$^{+/tg}$ transgenic mice to target two different cell populations in the pancreas. If an *insulin-cre*$^{+/tg}$ mouse is used for matings, only mature insulin-producing ß-cells will express modified forms of TCF4; and in case of *pdx1-cre*$^{+/tg}$ all pancreatic epithelium, including exocrine and endocrine cells, will express modified forms of TCF-4, because Pdx-1 promoter is active from the earliest steps of development of the pancreatic epithelium (Jonsson et al., 1994).

To be able to monitor *in vivo* if Tcf/Lef-ß-catenin-mediated Wnt signaling is affected, ROSA26^{dnTCF4} and ROSA26^{caTCF4} mice are bred into the *BAT-Gal*$^{+/tg}$ transgenic reporter background. As mentioned before, both modified proteins, dnTCF and caTCF, bear a myc epitope on their N-termini (red circle in Fig. 2.16) to allow visualization of their expression domains in the pancreas.

2.2.8 Generation of *ROSA26^{dnTCF}* and *ROSA26^{caTCF}* Mice

ROSA26^{dnTCF} and ROSA26^{caTCF} mice were generated through the standard technique of gene targeting (Capecchi, 2001).

2.2.8.1 Construction of the *pROSA-dnTCF4* and *pROSA-caTCF4* Targeting Vectors

For the insertion of the dnTCF4 or the caTCF4 into the genomic ROSA26 locus I used two targeting constructs, pROSA-dnTCF4 and pROSA-caTCF4, respectively, based on the

pROSA26-1 vector (Soriano, 1999) and pBigT vector (Srinivas et al., 2001). The sequences I used for the construction of the targeting vectors, and details of the targeting vector construction are summarized in the Methods Section.

2.2.8.2 Generation of *ROSA26^{dnTCF}* and *ROSA26^{caTCF}* mice

To generate ROSA26^{dnTCF4} and ROSA26^{caTCF4} mice, the targeting vectors were transfected into ES cells. Embryonic stem cells were expanded, and after undergoing positive and negative selection, analyzed for the correct integration of pROSA-dnTCF4 and pROSA-caTCF4 targeting constructs (see Methods). I analyzed 192 individual ES cell clones from each targeting construct for successful homologous recombination events as described in Methods section. From each construct, ten clones were shown to correctly integrate the targeting constructs. An example of a Southern Blot autoradiogram on genomic DNA from the wild-type and targeted clones is shown in Fig. 2.17, A.

Two positive ES cell clones from pROSA-dnTCF4 and three clones from pROSA-caTCF4 targeting construct were injected into blastocysts and transferred into pseudopregnant C57Bl/6J foster mice in the transgenic animal facility at the Center for Molecular Neurobiology in Hamburg. Chimeric offspring were born in both *ROSA26^{dnTCF}* and two of the three *ROSA26^{caTCF}* mouse lines. In one *ROSA26^{dnTCF}* line, the targeted allele was successfully transferred to the offspring (Fig. 2.17, B). So far no offspring carrying the targeted *ROSA26^{caTCF}* allele were produced by *ROSA26^{caTCF}* chimeric males.

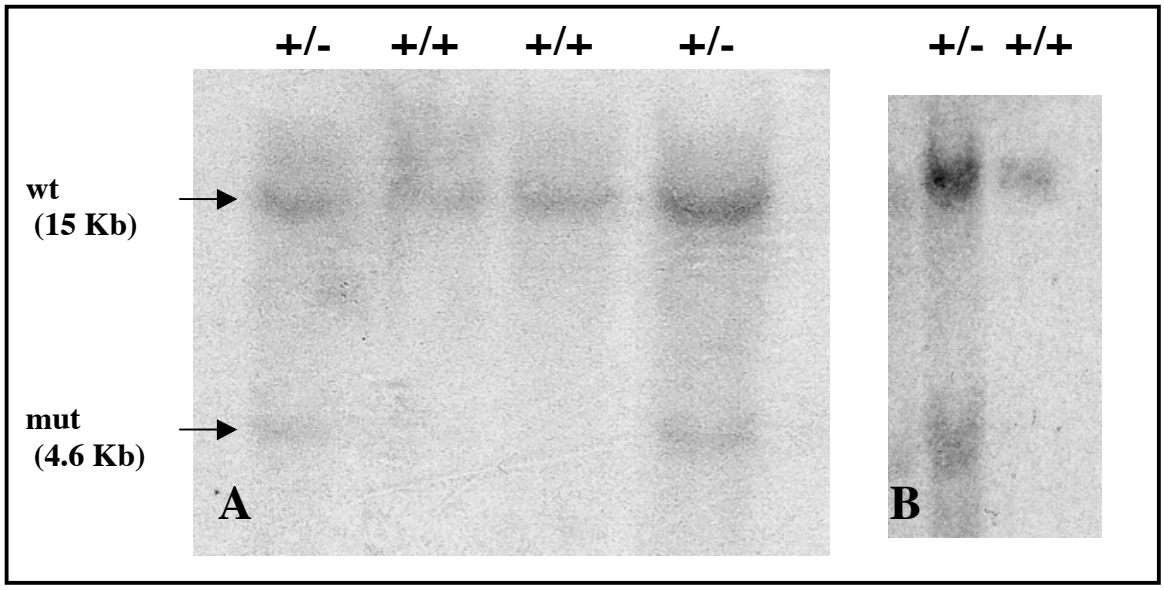

Figure 2.17. Identification of ES cell clones positive for the correct genomic integration of pROSA-dnTCF4 and pROSA-caTCF4 targeting constructs. (A), an example of Southern Blot autoradiogram on genomic DNA from ES cell clones. Wild-type 15 kb large band is present in all clones analyzed. However, in the first and the last lanes, an additional mutant 4.6 kb band is detected, resulting from one ROSA26 targeted allele. (B), Southern Blot autoradiogram of genomic DNA from two offspring of a chimeric *ROSA26*^{dnTCF} male. In the left lane, mutant band is detected, whereas in a wild-type littermate only the 15 kb wild-type band is present. In (A) and (B), genomic DNA was digested with *EcoRI* and hybridized with the probe A (Mao et al., 1999)(see Methods for details).

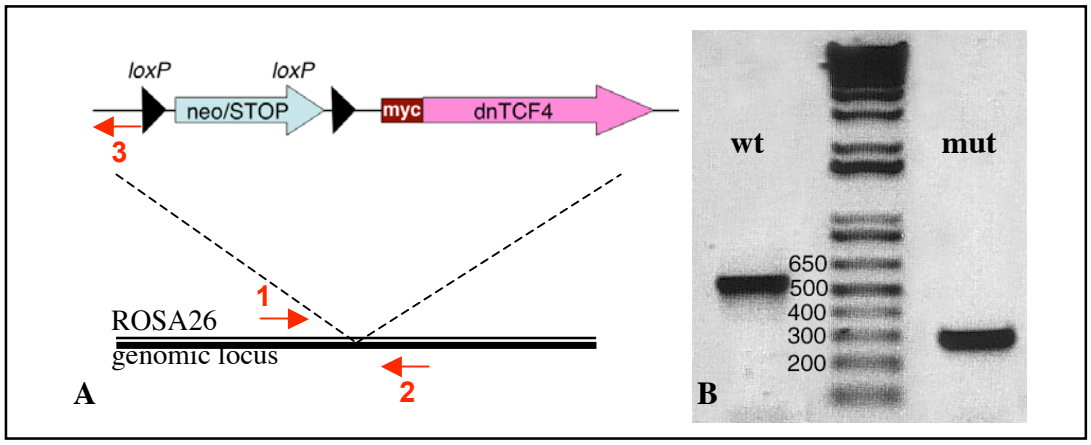

Figure 2.18. PCR-based genotyping of heterozygous ROSA26$^{dnTCF/+}$ mice. (A), Scheme of the PCR design. Primers 1 and 2 anneal to the wild-type *ROSA26* genomic sequence and yield a band of 550 bps in a PCR. In the targeted allele, primers 1 and 3 produce a band of 300 bps. (B), electrophoresed products of a PCR reaction on the DNA from a heterozygous ROSA26$^{dnTCF/+}$ mouse. Both reactions were performed on the same DNA sample. Primer sequences can be found in Appendix.

All subsequent genotyping of *ROSA26*^{caTCF} mice for the presence of the targeted allele was performed by the PCR technique (Fig. 2.18) as described elsewhere in the literature (Soriano, 1999). Heterozygous *ROSA26*$^{dnTCF/+}$ mice were crossed to the *BAT-Gal*$^{+/tg}$ mice to generate *ROSA26*$^{dnTCF/+}$*:BAT-Gal*$^{+/tg}$ mice. Transgenic *BAT-Gal* allele was detected by the LacZ-PCR method (all primer sequences in Appendix).

Double heterozygous *ROSA26*^{caTCF}*:BAT-Gal*$^{+/tg}$ mice that I generated were viable, fertile and did not exhibit any visible abnormalities, as expected.

2.2.9 *In vivo Cre*-mediated Activation of *dnTCF4* Expression in the Pancreatic Islets

Double heterozygous *ROSA26*^{caTCF}*:BAT-Gal*$^{+/tg}$ female mice were crossed with *ins-cre*$^{+/tg}$ males, expressing *Cre* recombinase in ß-cells. Offspring were analyzed by PCR for the presence of *ROSA26*^{dnTCF4} mutant allele, *BAT-Gal* transgene, and the *ins-cre* transgene. At the age of 2 weeks, *ROSA26*^{dnTCF}*:ins-cre*$^{+/tg}$ mice did not differ from their wild-type littermates or otherwise exhibit any morphological or physiological abnormalities. *ROSA26caTCF:ins-cre+/tg* mice will be sacrificed at the age of 20 weeks and analyzed for their islet structure, islet marker expression, and by immunohistochemistry with antibodies against the myc epitope for the *in vivo* activation of dnTCF4 expression in pancreatic ß-cells.

In summary, I have demonstrated that numerous members of Sox transcription factor family are expressed in the developing and adult mouse pancreas. I have analyzed expression patterns of seven Sox factors in detail in the embryonic mouse pancreas and could show that Sox8 and Sox10 are dispensable for the embryonic development of the mouse pancreas. I have also shown that all four transcription factors of the Tcf/Lef family are expressed during mouse pancreas development. To improve the analysis of functions of canonical Wnt signaling cascade in mouse pancreas, I created two modified forms of the TCF4 transcription factor. I generated and analyzed transgenic *pdx-caTCF*$^{+/tg}$ and *pdx-dnTCF*$^{+/tg}$ mice, which expressed *dnTCF4* and *caTCF4*, respectively, in the early pancreatic epithelium. Furthermore, I developed a bigenic *Cre-loxP* system for expressing *dnTCF4* and *caTCF4* factors stably and inheritably under control of the pancreas-specific *Cre*-recombinase. *ROSA26*^{caTCF} mice bearing a dominant-negative form of TCF4 were successfully generated.

III Discussion

3.1 Co-expression of Sox Genes in Overlapping Domains of the Developing Pancreas

This study demonstrates that thirteen *Sox* genes from groups C, D, E, F, G, and H are expressed in the developing mouse pancreas. Detailed expression analysis of seven *Sox* factors by in situ RNA hybridization on pancreas sections at various stages of mouse development showed that the expression domains of different *Sox* factors often overlap. This suggests the possibility that different *Sox* genes could exhibit functional redundancy. Indeed, functional redundancy has been documented amongst members of other developmentally expressed transcription factor gene families, such as the *MyoD* family (Rudnicki and Jaenisch, 1995) and the *Hox* family (Maconochie et al., 1996). Within the *Sox* family, the basis for the redundancy is provided by high homology in the HMG-domain and DNA binding characteristics of the Sox proteins (Kamachi et al., 2000). Therefore, different Sox proteins could bind the same target sequence and potentially regulate transcription of the same target genes. Indeed, the exchange of HMG-domains between Sox factors does not lead to any detectable changes in the DNA-binding properties *in vitro*. This is supported by the finding that replacement of the HMG domain of Sox1 with the Sox9 HMG domain and vice versa, does not lead to any change in the ability of these proteins to activate the Sox1 target gene δ-*crystallin* e or the Sox9 target gene *Col2a1* (Kamachi et al., 1999). Further, the specificity of the regulation of the gene transcription by the Sox factors depends on the set of co-factors that Sox proteins bind to (Wilson and Koopman, 2002). It is known that the protein domains outside the HMG box are involved in such protein-protein interactions (Wegner, 1999). As proteins of the same *Sox* group exhibit high homology also outside the HMG-box domain, and therefore can interact with the same co-factors, it is likely that the common target genes are activated by the related Sox transcription factors. The potential for the functional redundancy has been supported by experimental evidence. Indeed, it has been shown that ectopic expression of a mouse *Sox2* transgene in flies rescues the defects in differentiation of the midline glia caused by a mutation in the *Drosophila Sox* gene *Dichaete* (Soriano and Russell, 1998). *In vivo*, functional redundancy between Sox factors of the same group has been demonstrated as well, as in the case of Sox5 and Sox6. In mouse development, *Sox5* and *Sox6* are expressed at the same chondrogenic sites. *Sox5* and *Sox6* single null mice display only mild skeletal abnormalities at birth, whereas *Sox5*/*Sox6* double null mice die before birth

with a severe chondrodysplasia (Smits et al., 2001). Functional redundancy has been further suggested for the group B proteins Sox1, Sox2, and Sox3 (Nishiguchi et al., 1998). Expression of all three Sox group B proteins strongly overlaps in the developing nervous system, and Sox2 and Sox3 function might explain surprisingly mild central nervous system defects in *Sox1* homozygous mutant mice (Nishiguchi et al., 1998).

Major cell differentiation in the pancreas begins at E13.5. At this stage, between E12.5 and E15.5, a number of Sox genes, including *Sox4, Sox11, Sox5, Sox13,* and *Sox9,* are broadly expressed in the pancreatic epithelium. These Sox genes could control pancreatic cell differentiation. In addition, islet expression of *Sox4, Sox11* and *Sox9* at E18.5, and of *Sox4* and *Sox9* in the adult suggests their potential role in endocrine cell function. Given the possible redundancy between different Sox factors, the analysis of single mutant mice for these Sox factors may prove not to be informative to study the role of these factors in pancreatic cell differentiation. It is possible that each single *Sox* mutant will display either minor or no defects in the pancreas. In this case, the analysis of Sox gene function in pancreatic cell differentiation will require the generation of double, triple or even quadruple gene inactivation. As an alternative to studying loss-of-function mouse models for each Sox gene separately, one could inhibit the function of all Sox genes by expression of a dominant-negative form of Sox transcription factors. However, to date such forms of Sox factors have not yet been described in the literature.

3.2 Unique Sox Expression Domains in the Developing Pancreas

The expression pattern of the different Sox genes in the developing pancreas allows to make some predictions about their potential function in the development of the organ. *Sox9, Sox11,* and *Sox13* genes were expressed in early pancreatic epithelium at E10.5, which implies a possible role in the control of early either pancreatic specification or growth of the pancreatic epithelium. At this stage the epithelium contains multipotent precursor cells that give rise to endocrine, exocrine, as well as duct cells (Gu et al., 2002). Notably, Sox9 labeled most epithelial cells already at E9.5, and in its onset preceded the expression of *Sox11* and *Sox13* in the pancreatic epithelium (Fig. 2.1, 2.2 and 2.3). At this stage, Sox9 is co-expressed with PDX-1, an early pancreatic progenitor marker (Jonsson et al., 1994). Similar expression patterns, as I observed for *Sox9*, have been described for *Nkx2.2* and *Nkx6.1*, which are crucial

determinants of endocrine cell differentiation (Sander et al., 2000; Sussel et al., 1998). Indeed, a recently published study implicated *SOX9* in human pancreas development (Piper et al., 2002). In this study, in humans with a haploinsufficiency for *SOX9*, pancreatic epithelial cells were more loosely packed within the mesenchymal stroma and islets were less morphologically distinctive than in control tissue. However, the neonatal lethality of mice lacking even a single allele of *Sox9* (Bi et al., 2001) precludes the direct analysis of a homozygous deletion of *Sox9* in the pancreas development. Instead, two alternative approaches to analyze the functions of *Sox9* in pancreas development are possible. First, embryonic stem (ES) cells with a homozygous mutation in the *Sox9* gene can be used to create aggregation chimeras. In this type of experiment, ES cells, labeled with an easily detectable marker (ß-galactosidase) contribute randomly to all host tissues when injected into the blastocysts (Hogan et al., 1994). Should *Sox9* be cell-autonomously required for the development of any pancreatic lineage, the *Sox9*-/- ES cells will fail to contribute to this particular lineage. The second approach to overcome early embryonic lethality is based on the bigenic *Cre-loxP* system, in which *Sox9* gene is flanked by two *loxP* sites, and is ablated only in cells expressing the *Cre* transgene. Different transgenic *Cre* deletor lines with specific expression in select pancreatic lineages have been generated (Gu et al., 2002; Herrera, 2000). Depending on the *Cre* deletor line used, it will be possible to separately study the roles of *Sox9* in early pancreatic progenitors (*pdx1-cre*), endocrine progenitors (*ngn3-cre*) and differentiated ß-cells (*ins-cre*).

In contrast to pan-epithelial pancreatic *Sox9* expression at E10.5, Sox13 and Sox11 marked only a subset of cells of the epithelial bud. Recently, gene expression profiling of approximately 60 individual epithelial cells in the pancreatic bud at E10.5 revealed 6 distinct expression profiles at this stage (Chiang and Melton, 2003). However, since *Sox9, Sox11* and *Sox13* were not tested for in this gene profiling analysis, it cannot be determined if these Sox factors are expressed in similar or distinct cell types of the early pancreatic epithelium. A possible way to study if these early *Sox*-expressing cell populations in the pancreatic epithelium are distinct or overlapping, would be to develop specific antibodies against these Sox factors and perform co-immunofluorescence analysis on pancreatic sections. A more rigorous test for addressing which cells arise from these Sox-positive progenitors consists of a direct lineage tracing experiment, as already performed for *Pdx-1*- and *Ngn3*-expressing progenitors (Gu et al., 2002). In these experiments, the *Pdx-1* or *Ngn3* promoters were used to drive the expression of the *Cre* recombinase in reporter mice, which express an easily

detectable marker human placental alkaline phospatase (HPAP) only after *Cre*-dependent excision of a transcriptional stop sequence. Thereby this procedure labeled irreversibly the progeny of PDX1+ or NGN3+ cells by HPAP expression. In similar experiments, transgenic Sox deletor mouse lines can be generated, which express *Cre* recombinase under control of the *Sox9*, *Sox11*, or *Sox13* gene promoters. First, it needs to be confirmed that in these deletor lines *Cre* expression reproduces the endogenous pattern of expression of the respective Sox gene. Then, upon crossing between the Sox deletor and the HPAP reporter line, the progeny of Sox9+, Sox11+, or Sox13+ cells will be marked by the HPAP expression. Co-staining for HPAP and the immunohistochemical markers for different pancreatic cell types at later stages will provide an answer on the fate of the Sox-expressing cells in the early pancreatic epithelial bud.

While many Sox factors have overlapping patterns of expression in the pancreatic epithelium, *Sox11* was the only Sox factor that was expressed in the pancreatic mesenchyme at E10.5. The importance of epithelial-mesenchymal interactions for the development of the pancreas has been demonstrated more than thirty years ago (Golosow and Grobstein, 1962) in tissue recombination experiments. To evaluate the role of *Sox11* for the pancreatic epithelial outgrowth or branching morphogenesis, mice with a homozygous deletion of *Sox11* need to be generated and analyzed for any pancreogenesis defects. To determine if the mesenchymal expression of *Sox11* plays essential roles in the development of the mouse pancreas, similar *ex vivo* tissue recombination experiments (Crisera et al., 2000; Miralles et al., 1999; Percival and Slack, 1999) could be performed between wild type mesenchyme and Sox11-deficient epithelium. In these assays, the ability of the wild-type pancreatic mesenchyme to rescue the morphogenesis of the Sox11 mutant pancreatic epithelium, could be evaluated. In complementary experiments, by combining Sox11-deficient mesoderm with the wild-type epithelium, an ability of mesenchymally-expressed *Sox11* to promote growth of the epithelium *in vitro* could be directly tested.

3.3 Schwann Cells are Dispensable for Pancreas Differentiation

Several observations suggest that *Sox8* and *Sox10*-expressing cells in the developing pancreas are a specific type of peripheral glial cells, the Schwann cells. Glial cells are abundant in the central nervous system (CNS), where they perform functions such as providing physical

support to neurons, separation and insulation of neurons, regulation of neurotransmitter levels and mediating embryonic brain development (Stevens, 2003). Schwann cells is the type of glia found outside the CNS. Schwann cells are also found in the pancreas, where they envelop the islets (Sunami et al., 2001; Teitelman et al., 1998; Ushiki and Watanabe, 1997). In the peripheral nervous system, *Sox10* and *Sox8* are expressed in glial cells (Kuhlbrodt et al., 1998b; Sock et al., 2001), and *Sox10* has been shown to be required for the development of those cells (Britsch et al., 2001). The scattered pattern of *Sox8* and *Sox10* expression in early pancreas development, as well as the peri-islet localization of *Sox8* and *Sox10* with the beginning of islet formation, suggest that Sox8 and Sox10-positive cells in the pancreas are Schwann cells. In *Sox10* homozygous mutant mice, Schwann cell precursors arrest their differentiation and undergo cell death (Britsch et al., 2001). The finding that no ß-galactosidase-positive cells could be detected in pancreas of $Sox10^{-/-}$ mutant embryos in late gestation (Fig. 2.6) indicates that these cells require *Sox10* for their development, and therefore are likely to be Schwann cells. Glial cell identity is additionally suggested by the fact that neither *Sox8*, nor *Sox10* are co-expressed with any pancreatic endocrine or exocrine cell markers. To unambiguously elucidate the cell identity of Sox8- and Sox10-positive cells in the mouse pancreas, co-immunostaining against Sox8 and Sox10, combined with cellular markers for Schwann cells, such as an intermediate filament glial fibrillary acidic protein (GFAP) or S-100ß, needs to be performed. Alternatively, as Schwann cells can be well recognized in transmission electron microscopy, the electron micrographs of the pancreata immunolabelled for Sox8 or Sox10, could be analyzed to confirm the Schwann cell identity of the Sox8- and Sox10-positive cells in the pancreas.

Expression of endocrine markers and islet size or morphology in $Sox8^{-/-}$ adult and $Sox10^{-/-}$ E18.5 mutant pancreata did not differ from their wild-type littermates (Fig. 2.5, Table 2.3). The observation that pancreatic endocrine and exocrine differentiation were not perturbed in Sox10 mutant mice suggests that Schwann cells are not required for the embryonic pancreas morphogenesis and endocrine cell differentiation. To date, very little is known about the exact roles of Schwann cells in the mammalian pancreas. Schwann cells may be involved in endocrine regeneration in the pancreas, as previous studies have shown that pancreatic Schwann cells become activated after islet injury and begin to express increased levels of nerve growth factor (NGF) and the neurotrophin receptor p75 (Teitelman et al., 1998). Functional significance of glial cells in the adult endocrine pancreas function still remains to

be determined. Unfortunately, the perinatal lethality of $Sox10^{-/-}$ mutants hinders the fulfillment of these studies.

3.4 Inhibition or Activation of the Canonical Wnt Signaling Pathway by Expression of Dominant Negative or Constitutively Active Forms of Tcf4 in Mouse Pancreas

The analysis of the second family of the HMG-box transcription factors, TCF/LEF proteins, has shown that all four TCF/LEF factors are expressed in the developing mouse pancreas (Tables 2.4 and 2.5, Figure 2.7). Because TCF/LEF proteins transduce Wnt signals in the nucleus in a complex with ß-catenin, it was hypothesized that the canonical, TCF/LEF-mediated, Wnt signaling pathway regulates pancreas development and islet cell function.

One of the key roles of the canonical Wnt pathway in tissue differentiation is the regulation of progenitor and stem cell maintenance, Such role for Wnt signaling has been demonstrated in the progenitors in the central nervous system (Chenn and Walsh, 2002; Megason and McMahon, 2002; Zechner et al., 2003) and neural crest cells (Lee et al., 2004), as well as in stem cells of the skin (Alonso and Fuchs, 2003) and intestine (Sancho et al., 2003). In contrast to actively regenerating epithelial tissues, such as skin or intestine, it is still unclear if the pancreas contains a stem cell population that is capable of producing ß-cells in the adulthood. After birth, the size and cell numbers in the mammalian pancreas increase. During early postnatal growth of the pancreas, proliferation is observed within all three types of differentiated pancreatic cells, the ductal, acinar and islet cells, as detected by the radioactive autoradiographic label retaining assay (Magami et al., 2002). This observation indicates that part or all of postnatal pancreas growth can result from the replication of existing mature cells. While this study approached quiescent condition of the adult pancreas, another study focused on the regeneration of ß-cells in the neonatal pancreas after their specific ablation by a chemical compound streptozotocin. In this model, ß-cell kinetic analysis suggests that ß-cell mitotic activity is insufficient to explain the observed rapid increase in ß-cell numbers following the injury (Wang et al., 1995). Although this substantial ß-cell regeneration ability of the neonatal pancreas may suggest the presence of stem cells or multipotent progenitor cells in the immediately postnatal pancreas, their presence had not been clearly demonstrated. Discovering a stem cell compartment within the mammalian pancreas, and identification of factors that could ensure stem cell maintenance in the pancreas, would facilitate the

development of a culture system to generate ß-cells for subsequent transplantation *in vivo*. I attempted to evaluate the possibility that Wnt signaling controls the stem cell compartment in the pancreas.

To begin to understand a possible role for Wnt signaling in the developing pancreas, the expression of key components of the canonical Wnt pathway during pancreas development was analyzed. First, expression of all four *Tcf/Lef* genes was detected during mouse pancreas development and in adult (Table 2.4, Fig. 2.7). In addition, expression of the soluble ligands Wnt2b and Wnt5a, and the Wnt receptors Fzd2 and Fzd4 was detected in the embryonic pancreas by RNA *in situ* hybridization (Table 2.6, Fig. 2.8). This data suggests that an active Wnt signal may be received by the pancreatic epithelium. However, some Wnt ligands and Frizzled receptors may activate non-canonical, TCF/LEF-independent pathways (Kuhl et al., 2000). Especially Wnt5a and Fzd2 have been implicated in the non-canonical Wnt pathway through the stimulation of the protein kinase C (Sheldahl et al., 1999). Further support for a role of Wnt5a in non-canonical Wnt signaling comes from the observation that Wnt5a inhibits the canonical Wnt pathway in mouse limb development (Topol et al., 2003). Despite the finding that many key components of the Wnt signaling pathway have been found in the pancreas, it did not indicate whether, and in which cells, the TCF/LEF-mediated Wnt pathway is activated.

To answer the question whether the canonical Wnt pathway is active in the pancreas, and to identify the sites of active Wnt signaling in the mouse pancreas, expression of Wnt signaling markers in two independent Wnt reporter mouse lines was studied. One Wnt reporter line is based on the expression of the *Axin2* gene. The analysis of *Axin2* expression in mice has shown that *Axin2* expression is exclusive to tissues, where the canonical Wnt pathway is active (Jho et al., 2002; Lustig et al., 2002). To study which cells in the pancreas express *Axin2*, mice heterozygous for a gene replacement of the *Axin2* coding region with the ß-galactosidase gene ($Axin2^{+/LacZ}$ mice) were analyzed. As a second Wnt reporter, pancreata from transgenic BAT-$Gal^{+/tg}$ mice, which express the ß-galactosidase reporter gene under control of tandem repeats of TCF/LEF binding sites (Maretto et al., 2003), were studied. In these mice, cells which receive a TCF/ß-catenin-mediated Wnt signal are marked by the production of ß-galactosidase. The analysis of $Axin2^{+/LacZ}$ mice revealed *Axin2* expression at E10.5 in the pancreatic epithelium. At E12.5, expression of *Axin2* was detected at a high level in the pancreatic mesenchyme and in a subset of epithelial cells. Before birth, Axin2-positive

cells were found in forming endocrine islets and pancreatic duct-like structures (Fig. 2.9). The pattern of ß-galactosidase expression in BAT-Gal$^{+/tg}$ and Axin2$^{+/LacZ}$ mice was very similar, but not identical (Fig.2.10). In BAT-Gal$^{+/tg}$ mice, less cells were labeled throughout pancreas development than in Axin2$^{+/LacZ}$ mice, probably reflecting the differences in the sensitivity of these two different Wnt reporter lines. The ductal expression of both canonical Wnt signaling markers is of particular interest with respect to regulation of the endocrine progenitors or stem cells by the TCF/ß-catenin-mediated Wnt pathway, because some embryonic duct-like structures were shown to contain endocrine progenitors that later coalesce to form mature islets (Gu et al., 2002).

Surprisingly, *Tcf-4* was expressed in the pancreatic epithelium at E12.5 and E14.5 in much broader domains than the BAT-Gal and Axin2 reporters (Fig. 2.7 and 2.10). This suggests that not every TCF-4-positive cell in the pancreas transduces the ß-catenin-mediated Wnt signal. There are at least two possible explanations for this observation. First, it is known that all TCF/LEF proteins are able to bind to several different transcriptional co-repressors, among them all five members of the transcriptional corepressor family *Grg* (mammalian orthologs of Drosophila *groucho*) (Brantjes et al., 2001; Levanon et al., 1998). In addition to interacting with the Grg corepressors, the presence of two consensus motifs for interaction with the CtBP suggests that TCF/LEF proteins interact with additional co-repressors. Therefore, in the absence of ß-catenin, TCF/LEF proteins may act as transcriptional repressors in complex with Grg proteins or CtBP, and be converted into transcriptional activators when corepressors are competed away by the nuclear ß-catenin (Brantjes et al., 2002). As Grg proteins are abundantly expressed in the pancreatic epithelium (Dr. Shelley Nelson, personal communication), it is likely that TCF/LEF proteins have two functional properties in the pancreatic epithelium. The observation that TCF-3 can inhibit canonical Wnt signaling may further complicate the evaluation of functions fulfilled by TCF-4. Low levels of *Tcf-3* mRNA which were detected in the developing pancreas by RT-PCR, but not by *in situ* hybridization (Table 2.6) could be sufficient to perform its biological functions. In transgenic overexpression experiments in skin it was shown that at least in that cellular context, TCF-3-mediated regulation of the target gene expression is ß-catenin-independent, in contrast to the LEF-1-regulated genes (Merrill et al., 2001). Second, the duplicated axial mesoderm structures seen in the *Tcf-3* deficient embryos resemble the null mutation phenotypes of Axin or APC, two well-characterized inhibitors of Wnt signaling (Ishikawa et al., 2003; Zeng et al., 1997), thus indicating that TCF-3 can inhibit ß-catenin-mediated Wnt signaling *in vivo*.

No Axin2- or BAT-Gal-positive cells could be detected in the adult pancreas by enzymatic in situ X-gal staining for the ß-galactosidase activity. This result was surprising, especially since Wnt signaling has been suggested to regulate insulin secretion in islets of Langerhans (Fujino et al., 2003). One possible explanation for the lack of ß-galactosidase staining in adult islets of BAT-Gal$^{+/tg}$ mice is that the BAT-Gal reporter sensitivity is insufficient to reveal all sites of active Wnt signaling. The BAT-Gal reporter may fail to detect low, yet biologically relevant, TCF/ß-catenin-mediated transcriptional activation. This interpretation is supported by the finding that BAT-Gal$^{+/tg}$ mice do not express ß-galactosidase in intestinal crypts (Maretto et al., 2003), despite the demonstrated role of TCF-4-mediated Wnt signaling for the homeostasis of the stem cell compartment in the intestinal crypts (Korinek et al., 1998a; Pinto et al., 2003). Another possible reason for the lack of the TCF/LEF-mediated Wnt signaling activity in the adult islets is that a Wnt signal may be transduced through a non-canonical pathway. Indeed, insulin secretion by the adult islets was stimulated by WNT5a, a Wnt member implicated in ß-catenin-independent pathways (Fujino et al., 2003; Veeman et al., 2003).

The finding that several *Tcf/Lef* genes were expressed in the developing pancreas (Table 2.6) suggested possible functional redundancy between TCF/LEF factors in the pancreas. To analyze the role of the TCF/LEF-mediated Wnt signaling, we blocked all TCF/LEF-mediated Wnt signaling by expressing a dominant-negative (dnTCF4) or constitutively active (caTCF4) form of TCF-4 protein in different cell populations of the pancreas. Dominant negative forms of the TCF/LEF proteins have previously been used to inhibit Wnt signaling *in vitro* as well as *in vivo*. Deletion of the N-terminal 31 amino acids of TCF/LEF proteins eliminates the interaction domain for ß-catenin without impairing DNA binding, and therefore prevents activation of Wnt target genes by TCF/LEFs. (Molenaar et al., 1996; van de Wetering et al., 1997; van de Wetering et al., 2002). Importantly, dnTCF4 has been previously used to block TCF/LEF-mediated transcriptional activation in transgenic mice (Wang et al., 2002), and an analogous dominant-negative LEF-1 protein has been successfully used in transgenic mouse models as well (Niemann et al., 2002). Indeed, mice expressing a dominant-negative form of LEF-1 display more severe brain abnormalities than *Lef-1*-deficient mice, likely resulting from inhibition of other TCF/LEF factors (Galceran et al., 2000). Previously, to constitutively activate the transcription of Wnt target genes, dnLEF1was directly fused to ß-catenin. This construct was able to activate the TOPFlLASH reporter independently of ß-catenin and had

similar cell transforming activity as activation of the canonical Wnt pathway (Aoki et al., 1999).

Since TCF-4 was the predominant TCF/LEF factor in the pancreatic epithelium, analogous to the constructs described above dnTCF4 and caTCF4 constructs were generated for expression in the pancreas of mice. The addition of an N-terminal myc epitope allows to visualize cells expressing modified TCF-4 proteins and to follow their fate *in vivo*. Importantly, previous studies have shown that an N-terminal myc-tag does not change the biochemical properties of dominant-negative TCF proteins *in vivo* (Niemann et al., 2002). In collaboration with Dr. Marian Waterman's lab (University of California, Irvine) this was confirmed for the newly generated TCF-4 constructs (Fig. 2.12). These experiments also showed that only dnTCF4 efficiently inhibited TCF/ß-catenin mediated transcriptional activation (Fig. 2.12), while caTCF4 failed to activate the transcription of the reporter gene. Two possible reasons could account for this inability of caTCF4 to activate the TOPFLASH reporter in the absence of ß-catenin. First, COS cells, in which the transfection experiments were made, could differ from the pancreatic epithelial cells in the expression of essential co-factors, such as for example chromatin remodeling factors. Second, the direct fusion of ß-catenin to dnTCF4 could result in the production of an unstable protein, which could be quickly degraded and therefore not able to exert its transactivation potential *in vitro*. It is also possible that physiological amounts of transcription factors that are necessary to regulate target genes in the developing mouse tissues are much lower than those needed to transactivate a reporter gene in cell transfections. Should the caTCF4 construct finally fail to activate Wnt pathway *in vivo*, we will construct another constitutively active form of TCF-4, in which dnTCF4 is directly fused to the heterologous activation domain of the *herpes simplex* virus protein VP16 domain. A previous study has shown that a fusion protein between LEF-1 and VP16 activated the TOPFLASH reporter gene as efficiently as a fusion form between LEF-1 and ß-catenin (Aoki et al., 1999). We will design a similar fusion protein between the dnTCF4 and the VP16 activation domain (ΔNTCF4-VP16). Another possibility to achieve ectopic activation of the canonical Wnt pathway in the pancreas is cell-specific expression of a stabilized form of ß-catenin. A modified allele of the ß-catenin has been described, in which two *loxP* sites flank exon3 of the ß-catenin gene (Harada et al., 1999). This exon of the ß-catenin gene encodes a protein stretch containing serine/threonine residues, responsible for phosphorylation and targeting ß-catenin protein for degradation (Hart et al., 1998). Upon expression of *Cre*-recombinase, stabilized ß-catenin is produced (ΔN-ßcatenin) that cannot be phosphorylated, and thus accumulates in

the cytoplasm, enters the nucleus and binds to TCF/LEF proteins to activate Wnt target genes (Harada et al., 1999). Therefore, by using mouse lines, which express *Cre*-recombinase in specific cell types of the pancreas, a similar effect to expressing a constitutively active dnTCF4-VP16 fusion protein is achieved. However, in order to activate Wnt target genes, the ΔN-ßcatenin modified protein must rely on the endogenous TCF/LEF proteins in the target cells. Therefore, in contrast to ΔNTCF4-VP16, ΔN-ßcatenin activates Wnt target genes only in cells, which express endogenous TCF/LEF proteins. Because the results of the present study have shown that majority of the pancreatic epithelium expresses TCF/LEF factors throughout the development (Fig. 2.7, Table 2.6), the effect of ΔN-ßcatenin and ΔNTCF4-VP16 on the canonical Wnt pathway are likely to be the same.

In a transient transgenic experiment, mice that expressed dnTCF4 and caTCF4 under control of *Pdx-1* gene promoter were generated. Two *pdx-dnTCF*$^{+/tg}$ and one *pdx-caTCF*$^{+/tg}$ transgenic embryo showed high levels of expression of the reporter gene, ß-galactosidase, in the pancreas, indicating that the modified TCF-4 forms were expressed in the pancreas. These transgenic mice were analyzed for their pancreatic phenotypes at E18.5. The analysis of pancreatic shape and organization of endocrine and exocrine tissue, as well as endocrine markers did not show any difference between *pdx-dnTCF*$^{+/tg}$ or *pdx-caTCF*$^{+/tg}$ transgenic pancreata and their wild-type littermate controls. Several reasons could explain lack of discernable pancreatic phenotypes in *pdx-caTCF*$^{+/tg}$ and *pdx-dnTCF*$^{+/tg}$ embryos. First, it could result from an inability to target the appropriate cell population with the *Pdx-1* promoter, as *Pdx-1* is broadly expressed only at E9.5-E11.5 in the pancreatic epithelium, but becomes restricted to differentiated ß- and δ-cells after E14.5. Alternatively, it is possible that TCF/LEF-ß-catenin mediated Wnt signaling is dispensable for embryonic pancreas development. Since embryos were harvested at E18.5, these experiments do not address the question whether canonical Wnt signaling is required for proper pancreas function after birth.

In contrast to the findings described here in the *pdx-dnTCF*$^{+/tg}$ or *pdx-caTCF*$^{+/tg}$ transgenic mice, a recent report showed that overexpression of *Wnt1* under control of the *Pdx-1* promoter resulted in complete splenic and pancreatic loss. In the same study, overexpression of Wnt5a lead to reduction of size of spleen, stomach and pancreas, as well as perturbed expression of pancreatic markers (Heller et al., 2002). The difference between the published results and the approach described in this study is that *pdx-dnTCF*$^{+/tg}$ or *pdx-caTCF*$^{+/tg}$ act in a cell-autonomous manner, whereas secreted Wnt proteins signal to surrounding cells. In both *pdx1-*

Wnt1 and *pdx1-Wnt5a* transgenic mice the whole foregut region was affected, indicating that Wnt ligands might have diffused beyond the PDX-1-positive domain. Therefore, Wnt proteins, when secreted by PDX-1-positive cells, signal to a broader endodermal domain than the pancreas epithelium alone, which may result in more generalized defects in foregut development. In this study, a mouse model was generated to study the effects of cell-autonomous inhibition of the Wnt signaling pathway.

To inhibit or activate canonical Wnt signaling in different cell populations of the pancreas, mouse lines were generated, in which dnTCF4 or caTCF4 constructs are expressed under control of the ubiquitous *ROSA26* locus upon cell-specific expression of *Cre*-recombinase (*ROSA26*^{dnTCF} and *ROSA26*^{caTCF} mice). The *ROSA26*^{caTCF} and *ROSA26*^{caTCF} mouse lines will be instrumentary in testing the following hypotheses about possible roles of the canonical Wnt signaling pathway in the mammalian pancreas. First, if in analogy to the role of Wnt signaling in the intestine and skin, the TCF/ß-catenin-mediated Wnt pathway controls the maintenance of the progenitor cell pool in the pancreas, we expect to see an arrest of whole pancreas development, upon expression of dnTCF4 in PDX1-positive early epithelial pancreatic progenitors. This expression is achieved by crossing *ROSA26*^{dnTCF} to *pdx1-cre*$^{+/tg}$ mice, which express *Cre*-recombinase in early pancreatic progenitors under control of the *Pdx-1* promoter. In contrast, expression of caTCF4 in PDX1+ cells might result in aberrant outgrowth of the pancreatic anlage or in perturbed pancreatic cell differentiation. Second, crossing *ROSA26*^{caTCF} and *ROSA26*^{caTCF} mice to mice which express *Cre*-recombinase in mature ß-cells under control of the insulin gene promoter, may reveal the role of canonical Wnt signaling in the ß-cell function and regulation of glucose-mediated secretion.

The results of the previous study by Fujino et al. (2003) suggested that Wnt signaling might be important for insulin secretion in the mature endocrine islets. In this study, mice lacking the gene coding for the Wnt co-receptor low-density lipoprotein receptor-related protein 5 (*Lrp5*) exhibit impaired glucose tolerance (Fujino et al., 2003). Islets of these mice show reduction in the levels of intracellular ATP and Ca^{2+} in response to glucose, which results in decreased glucose-induced insulin secretion (Fujino et al., 2003). A direct role for the regulation of the ß-cell insulin secretion was further supported by the fact that exposure of the cultured wild-type islets to WNT3a and WNT5a proteins stimulated glucose-induced insulin secretion, but not production, which could be blocked by adding the soluble antagonist of Wnt signaling, Frizzled-related protein-1 (sFRP-1) to the culture medium (Fujino et al., 2003). As

the LRP5 co-receptor binds to different Wnt ligands (Kato et al., 2002), and Wnt3a has been assigned roles in predominantly activating the canonical Wnt pathway (Galceran et al., 2001; Veeman et al., 2003), the results provided evidence that insulin secretion in ß-cells is contingent upon TCF/LEF-mediated Wnt signaling. To inactivate TCF/LEF-mediated Wnt signaling cell-autonomously in ß-cells, *ROSA26*^{dnTCF} were mated to the *ins-cre*$^{+/tg}$ mice. Thus far, four double heterozygous *ROSA26*^{dnTCF}*:ins-cre*$^{+/tg}$ offspring have been obtained, which are now 2 weeks old. At 2 weeks of age, the *ROSA26*^{dnTCF}*:ins-cre*$^{+/tg}$ offspring are indistinguishable from their wild-type littermates. Since *Cre* recombination may occur with a time-delay and ß-cell neogenesis is known to continue for as long as 3 weeks after birth (Sander and German, 1997), it is necessary to analyze *ROSA26*^{dnTCF}*:ins-cre*$^{+/tg}$ mice not younger than 3 weeks of age. In addition, glucose sensitivity by ß-cells is only acquired after birth (Hole et al., 1988), and the response to glucose reaches maturity after weaning (Otonkoski et al., 1988). Therefore, the possible impaired glucose sensing by *ROSA26*^{dnTCF}*:ins-cre*$^{+/tg}$ islets will be revealed at a later time point. In the study of *Lrp5*-deficient mice, impaired glucose tolerance was revealed at 6-8 months of age (Fujino et al., 2003). To evaluate pancreas endocrine functions in *ROSA26*^{dnTCF}*:ins-cre*$^{+/tg}$ mice with fully matured islets, intraperitoneal glucose tolerance tests will be performed at 6 months of age, to evaluate their ability to metabolize glucose (Heller et al., 2001). In addition, the structure and appearance of islets of Langerhans and expression of markers of mature ß-cells will be thoroughly examined. Possible abnormalities will further be investigated with careful tests of insulin secretion and measurement of insulin content in the pancreata of *ROSA26*^{caTCF}*:ins-cre*$^{+/tg}$ mice by radioimmunoassay (Methods).

To analyze the possible role of the canonical Wnt signaling in the maintenance of the pancreatic progenitor pool, *ROSA26*^{dnTCF}*:pdx1-cre*$^{+/tg}$ bigenic mice will be generated and evaluated for the alterations in the pancreas. Given the widespread role of Wnt signaling in progenitor cell maintenance and cell differentiation (Alonso and Fuchs, 2003; Reya et al., 2003; Sancho et al., 2003), we expect to reveal a similar function in the pancreas. Therefore, we predict to find a reduction in organ size and/or absence of specific lineages in *ROSA26*^{dnTCF}*:pdx1-cre*$^{+/tg}$ mice. Provided that *ROSA26*^{dnTCF}*:pdx1-cre*$^{+/tg}$ bigenic mice are viable, we will also explore the possibility that inhibited TCF/LEF/ß-catenin signaling results in the ablation of stem cells after birth. To test this possibility, we will study the capacity for endocrine and exocrine regeneration in a partial pancreatectomy model. Together, these experiments will determine the role of the canonical Wnt signaling cascade in progenitor or

stem cell maintenance in the embryonic and adult pancreas. Finally, to analyze the roles of the TCF/LEF/ß-catenin mediated Wnt signaling cascade in other tissues and cell types of the embryo, the bigenic transgenic system can be used to mate *ROSA26*^{dnTCF} and *ROSA26*^{caTCF} mice with other mouse lines that express *Cre*-recombinase in specific tissues.

IV Materials

4.1 Material Sources

All experiments were carried out using commercially available chemicals and molecular biology reagents (AppliChem, Ottoweg, Germany; Sigma, Aldrich Chemicals GmbH, Schnelldorf; Roche, Mannheim; Gibco-BRL Life Technologies, Karlsruhe; Roth, Karlsruhe; Clontech, Palo Alto, California; Amersham-Pharmacia, Amerscham Biosciences Europe GmbH, Freiburg; MBI Fermentas GmbH, St. Leon-Rot, Germany. Media and cell culture reagents were obtained from Gibco-BRL Life Technologies (Karlsruhe). Other materials purchased from particular companies are listed here or in the Methods.

4.2 List of Solutions and Media

Denhardt's (50X)	1g Ficoll 400; 1g Polyvenylpyrrolidine; 1g BSA; qs 100ml ddH_20
DEPC-H_2O	Diethylpyrocarbonate: diluted 1:1000 in ddH_20. Stir overnight before autoclaving.
LB-Medium	1 % Casein-Hydrolysate, 0,5 % Yeast Extract, 0,5 % NaCl, 0,1 % Glucose, pH7,0
PBS-Buffer	0,14 M NaCl, 2,7 mM KCl, 3,2 mM Na_2HP04, 1,5 mM KH_2P0_4 25 mM Tris-HCl, pH 8,0
PCI	Phenol:Chloroform:Isoamylalcohol (25:24:1)
PFA Fixative	4% Paraformaldehyde in PBS
SSC-Buffer (20x)	3 M NaCl, 0,3 M Sodium citrate pH7.0
TAE-Buffer (1X)	0,2 M Tris-Acetate, 10 mM EDTA pH8
1M Tris Buffer	121g Tris is dissolved in 800ml ddH_20. pH value is adjusted with HCl. Adjust volume to 1L with ddH_20.

4.3 Bacterial Strains Used

DHS5a	New England Biolabs
XL1-Blue	New England Biolabs

4.4 Basic Vectors Used for Cloning

pBluescript KS+/SK-	cloning vector (Stratagene)
pGEM-5Zf(+)	cloning vector (Promega)
pGEM-T Easy	T-A cloning vector (Promega)
pcDNA3	eukaryotic expression vector (Invitrogen)

4.5 ES Cell Line

For the generation of *ROSA26*^{dnTCF} and *ROSA26*^{caTCF} mouse lines, embryonic stem cell line E14.1 was used, which originates from a blastocyst of a male 129/Ola mouse (Kuhn et al., 1991).

4.6 Mouse Strains Used

The wild-type C57Bl/6J mice were bred at the UKE Hamburg. Sox8 (Sock et al., 2001) and Sox10 (Britsch et al., 2001) mutant mice were kindly provided by Dr. M. Wegner (Erlangen). Axin2 knock-out mice (not published) were kindly provided by Dr. W. Birchmeier (Berlin). Transgenic BAT-Gal reporter mice (Maretto et al., 2003) were kindly provided by Dr. S. Piccolo (Padua).

4.7 Probes Used for *in situ* Hybridization

Gene	Plasmid containing the cDNA, number in the Sander's Lab Plasmid Database	Generation of the in situ probe (antisense): enzyme to linearized the plasmid, RNA Polymerase	Creator of the plasmid
Sox4	222	HindIII, T7	C.Janiesch, Sander's Lab
Sox5	89	BamHI, T3	O.Lioubinski, Sander's Lab
Sox9	42	HindIII, T7	E.Sock, Erlangen
Sox11	91	BamHI, T3	O.Lioubinski, Sander's Lab
Sox13	92	BamHI, T3	O.Lioubinski, Sander's Lab
Lef-1	318	EcoRI, T3	Dr. R.Grosshedl's Lab, München
Tcf-1	316	XbaI, T7	Dr. R.Grosshedl's Lab, München
Tcf-3	317	BamHI, T7	Dr. R.Grosshedl's Lab, München
Tcf-4	249	EcoRI, T7	Dr. W.Birchmeier's Lab, Berlin
Wnt5a	13	BglII, SP6	Dr. A.McMahon's Lab, Harvard
Frizzled-2	322	XhoI, T3	Dr. Jeremy Nathans, Chevy Chase
Frizzled-4	323	EcoRI, T7	Dr. Jeremy Nathans, Chevy Chase

4.8 Antibodies Used

4.8.1 Primary Antibodies

Antibody (anti-)	Source	Species made in	Used Dilution
α-Amylase	Sigma	Rabbit	1:500
Glukagon	Linco	Guinea Pig	1:8000
Glukagon	Sigma	Mouse	1:8000
Insulin	Linco	Guinea Pig	1:8000
Ngn3	Gift from M. German, San Fransisco,	Rabbit	1:3000
Pancreatic Polypeptide	Dako	Rabbit	1:3000
Pdx1	Gift from H. Edlund, Umea, Sweden	Rabbit	1:3000
Somatostatin	Dako	Rabbit	1:4000

4.8.2 Secondary Antibodies

Antibody (anti-)	Source	Species made in	Used Dilution
Cy3-a-rabbit	Dianova	Goat IgG(H+L)	1:2000
Cy3-a-giunea pig	Dianova	Goat IgG(H+L)	1:2000
Cy3-a-mouse	Dianova	Goat IgG(H+L)	1:2000
Cy3-a-rabbit	Dianova	Goat Fab Fragment	1:50 -1:100
Alexa 488-a-mouse	Mobitech	Goat IgG(H+L)F(ab‘) 2 Fragment	1:2000
Alexa 488-a-rabbit	Mobitech	Goat IgG(H+L)F(ab‘) 2 Fragment	1:2000

4.9 Oligonucleotides

Oligonucleotides were purchased from Metabion AG (Martinsried, Germany). The list of oligonucleotides used in this work can be found in the Appendix.

V Methods

5.1 Isolation and Purification of Plasmid DNA

5.1.1 Analytical Scale Purification of DNA (Minipreps)

A miniculture of bacteria was started by picking a single bacterial colony from an agar plate and inoculating 3-4 ml of LB medium including an antibiotic, followed by incubation at 37°C overnight while shaking. 1.5 ml of the overnight-culture was transferred to Eppendorf reaction tubes and centrifuged 2 min. The pellet was resuspended in 350 μl STET (8% sucrose; 0.5% Triton X-100, 50 mM Tris-Hcl, pH 8.0; 50 mM EDTA, pH 8.0) and vortexed before adding 20 μl lysozyme (10 mg/ml), inverting tubes to mix and boiling for 2 min. After 5 min centrifugation at 14,000 rpm at RT (Eppendorf Tabletop Centrifuge, 5417), the pellet consisting of the cell debris was removed with a toothpick. DNA was extracted by the addition of 25μl of phenol followed by vortexing, and then 400 μl isopropanol was added and mixed. After centrifugation for 5 min at RT, pellets were rinsed with 70% EtOH before dissolving in 100 μl TE containing RNase A (20 μg/ml).

5.1.2 Large Scale Purification of DNA (Maxipreps)

To produce a large bacterial culture, minicultures were grown (see 5.1.1), 25-50 μl of which were used to inoculate 250 ml of LB containing 0.1 mg/ml ampicilin before incubation at 37°C O/N, shaking. Plasmids were purified using the QIAfilter Plasmid Maxiprep Kit (Qiagen) or the Concert High Purity Plasmid Maxiprep Kit (Gibco-BRL) according to the manufacturer's instructions.

5.1.3 Determination of DNA and RNA Concentration

The concentration of DNA and RNA in solution was spectrophotometrically determined by measuring the optical density (OD) using wavelengths of 260 nm and 280 nm (Ultrospec 3000, Pharmacia). An OD_{260} of 1 corresponds to approximately 50 μg/ml for double-stranded DNA, 40 μg/ml for single-stranded DNA and RNA, and 20 μg/ml for single-stranded oligonucleotides. The ratio between the readings at 260 nm and 280 nm (OD_{260}/OD_{280}) provides and estimate of the purity of the nucleic acid, with pure preparations of DNA and RNA having OD_{260}/OD_{280} values of 1.8 and 2.0, respectively. For quantitating the amount of DNA or RNA, 200 μl of a 1:50 or 1:100 dilution was assayed for absorbance. The

concentration was calculated by multiplying the OD_{260} by 0.005 or 0.04 (for DNA and RNA, respectively) and by the dilution to yield mg/ml.

5.1.4 Purification of DNA by Phenol Extraction and Ethanol Precipitation

To remove proteins from nucleic acid solutions, an equal volume of phenol/chlorophorm/isoamylalcohol (25:24:1) was added and thoroughly vortexed to form an emulsion. Separation of the organic and aqueous phases was accomplished by centrifugation (Eppendorf Table Centrifuge 5417) for 5 min at RT. The upper, aqueous phase was transferred to a fresh tube. The DNA/RNA was recovered by ethanol precipitation. One tenth of a volume of 3 M NaOAc, pH 5.2 and 2.5 volumes of 100% EtOH were added to the aqueous phase, mixed, and allowed to precipitate at –80°C for 10 min or at –20°C for 30 min, and centrifuged to form a DNA pellet. The pellet was then washed with 70% EtOH, dried in a vacuum and resuspended in ddH20 or TE buffer.

5.2 Production, Purification, and Cloning of DNA fragments

5.2.1 DNA Digests Using Restriction Enzymes

DNA digests using restriction enzymes were performed in the manufacturer-provided buffer. The reactions were set up using 1-2 U of enzyme per μg DNA and then incubated at 37°C (unless otherwise directed by the manufacturer) for 1-2 hours. The DNA fragments of choice were then gel-purified (see 5.4.2.1). Analytical digests were generally carried out with 1-2 μg DNA in a total volume of 20 μl, using the guideline: 1 U enzyme digests 1 μg DNA in 1 h.

5.2.2 Hybridization/Annealing of Synthetic Oligonucleotides

10 μg of each oligonucleotide (sense and anti-sense) were combined in a total volume of 50 μl of TE, 100 mM NaCl, thereby creating a stock concentration of 200 μg/ml. The reaction was heated to 85°C for 3 min before allowing to slowly cool to RT.

5.2.3 Amplification of DNA by Polymerase Chain Reaction (PCR)

PCR is used to amplify a segment of DNA that lies between two regions of known sequence. The template DNA is first denatured by heating in the presence of large molar excess of each of the two oligonucleotides and the four dNTPs. The reaction mixture is then cooled to a temperature that allows the oligonucleotide primers to anneal to their target sequences, after which the annealed primers are extended with DNA thermostable polymerase. The cycle of

deenaturation, annealing, and DNA synthesis is then repeated many times, with the products of one round serving as templates for the next, thereby leading to the exponential amplification of the desired DNA product.

To amplify target DNA, template DNA (1-20 ng plasmid DNA or 0.2-2 μg genomic DNA), 5 μl 10x PCR Buffer (100 mM Tris-Hcl, pH 8.4; 500 mM KCl; 0.8% NP-40), 2 μl 50 mM $MgCl_2$, ca 500 ng of each primer, 5 μl of 2 mM dNTP-Mix, 2.5 U Taq-Polymerase (Gibco-BRL) were combined in a 500-μl Eppendorf reaction tube and the total volume was raised to 50 μl with ddH2O. Using a PTC-2000 Peltier Thermal Cycler (MJ Research), the reaction was heated to 94°C for 30 sec and then cycled 30 times through the steps of denaturation (94°C, 30 sec), annealing at the temperature determined by the primers (30 sec) and amplification (72°C, 1.5 min). Generally, the annealing temperatures were determined based on the manufacturer-provided melting temperature. At the end the reaction was incubated at 72°C for 5 min before being cooled to 4°C.

5.2.4 Cloning DNA Fragments

5.2.4.1 DNA Extraction from Agarose Gels

Restriction enzyme-digested DNA fragments were separated in an agarose gel by electrophoresis, visualized with UV-light and excised from the gel with a razor blade. The DNA was purified through a QIAquick Gel-Purification Column (QIAGEN) according to the manufacturer's instructions.

5.2.4.2 Ligation of DNA Fragments and Vectors

Ligation of the insert DNA to the prepared vector DNA was catalyzed by the T4 DNA ligase. Approximately 50 ng vector DNA was combined with a 3- to 5-time molar excess of insert DNA (depending on the insert size), 1 μl of 10x T4 Ligase Buffer and 10 U of T4 DNA Ligase (Roche) in a total volume of 10 μl. The reaction was incubated O/N at 16°C before transforming 5 μl of the reaction into competent DH5α *E. coli* bacteria cells.

5.2.4.3 Cloning PCR Fragments

The amplification and isolation of DNA fragments is described in Sections 5.2.3 and 5.2.4.1, respectively. If the primers were designed to contain restriction enzyme sites, the fragments were digested with the appropriate enzymes (see 5.2.1) and gel purified before ligation (5.2.4.2). Alternatively, PCR-amplified fragments were first cloned into the pGEM T-Easy

Vector using the pGEM T-Easy Cloning kit (Promega) following manufacturer's instructions. Taq polymerase adds a single A to the 3' ends of PCR products. The linearized pGEM T-Easy vector has single, overhanging 3' T residues which allow efficient ligation with PCR products and reduced self-ligation of vector DNA. Following transformation and minipreps, this intermediate cloning plasmid could be digested and cloned into the plasmid of choice.

5.2.4.4 Producing Competent Bacteria

To prepare competent bacterial cells, only autoclaved, sterile solutions and equipment were used. A glycerol culture of the DH5α *E. coli* strain was streaked onto an agar plate and grown O/N at 37°C. Single colonies were then picked to start small (3-5 ml) cultures in LB medium containing 10 mM $MgCl_2$ was inoculated with 1 ml of the overnight culture and incubated on a shaker until the bacteria had grown to mid-log phase (when A595 attained 0.4-0.5). The bacteria were then pelleted at 5,000 rpm for 5 min at 4°C (Beckman J2-21M/E Centrifuge, JA10 rotor). After discarding the medium, the cell pellet was resuspended in 50 ml of ice-cool 100 mM $CaCl_2$, transferred to a 50-ml conical tube and incubated on ice for 30 min with occasional swirling. The cells were then centrifuged in the Tabletop centrifuge (Minifuge RF, Heraeus, Hannover, Germany) for 5-10 min at 5,000 rpm, 4°C. The pellet was resuspended in 10 ml ice-cold 100 mM $CaCl_2$, 15% glycerol and incubated on ice O/N. The cells were aliquoted the next day into sterile, chilled Eppendorf 1.5 ml tubes before freezing at –80°C.

5.2.4.5 Bacterial Transformation

For each transformation, 100 µl of DH5α were removed from storage at –80°C and thawed on ice. DNA (1-25 ng) was added and the mixture incubated on ice for 30 min. Immediately following a 90-sec heatshock at 42°C, the Eppendorf reaction tubes were briefly returned to ice before adding 1 ml of LB media and incubating the tubes, shaking, at 37°C for 30-60 min. The Eppendorf reaction tubes were centrifuged at 4,000 rpm for 2 min and then approximately 1 ml of the supernatant was removed. The cells were resuspended in the remaining LB media and plated onto LB-agar plates containing ampicilin, followed by O/N incubation at 37°C.

5.2.4.6 DNA Sequencing

DNA sequencing was performed by the Service Group at the Center for Molecular Neurobiology in Hamburg, led by Dr. Kullman and Fr. Däumingen. Samples were prepared by combining ca 800 ng DNA and 15 pmol sequencing primer in a total volume of 8 µl (qs with H_2O).

5.3 Identification of DNA Fragments via Hybridization

5.3.1 Random Primer Labeling of DNA

The Klenow fragment of E. coli DNA polymerase I is used to fill in the recessed 3' termini, such as those created by DNA digestion. Here, DNA fragments complementary to the DNA sequence to be detected were denatured and the single-stranded DNA was bound by random hexamer primers, allowing the Klenow enzyme to fill in the rest using the Rediprime II DNA Labelling System kit (Amersham Biosciences). Approximately 20 ng of digested, gel-purified plasmid DNA was brought to a total volume of 45 μl with TE, incubated 3-5 min at 95°C to denature the double-stranded DNA, and then placed directly on ice to prevent annealing of the now single-stranded DNA. DNA was added to the contents of the Rediprime II Labeling tubes as recommended by the manufacturer, and 5 μl α-^{32}P-dCTP were added. After mixing, the reaction was incubated at 37°C for 15 minutes, snap-cooled on ice for 5 minutes, and used for DNA hybriditzation (5.3.3).

5.3.2 Transfer of DNA from Agarose Gels to the Nitrocellulose Membrane (Southern Blotting)

Purified genomic DNA, which has been digested by restriction enzymes and separated by electrophoresis on a 0.8% agarose gel, was photographed next to a ruler and transferred to a nylon membrane by capillary blotting. Following electrophoresis, the gel was soaked in 0.25 M Hcl for 30 min, rinsed briefly with ddH2O, then denatured (1.5 M NaCl; 0.5 M NaOH) for 30 min. After a second rinse in ddH2O, the gel was then neutralized (0.5 M Trix-HCl, pH 7.0; 1.5 M NaCl) for 30 min, then for 15 min with fresh solution. Finally, the gel was soaked for 30 min in 20x SSC before being placed, upside-down, on a long piece of Whatman-3MM paper which had been soaked in 20x SSC. The paper was lying lengthwise in an agarose gel chamber with the ends resting in the buffer reservoirs filled with 20x SSC. A nylon membrane (Hybond N, Amersham; pre-treated by soaking for 5 min in ddH2O followed by 5 min in 20x SSC; cut to the same size as the gel) was then carefully laid upon the gel and bubbles were removed by rolling a pipette over the top of the membrane. Next, 3 pieces of 3MM paper (also cut to the same size as the gel) were laid on top of the nylon membrane, followed by the addition of a ca 10 cm high stack of paper towels (also cut to match the gel size). A weight was placed on top of the stack, and capillary blotting was allowed to take place O/N. The blot was then disassembled, the positions of the wells were marked with a pencil, and the nylon

membrane was allowed to air dry before UV cross-linking the DNA to the membrane, using 120,000 microjoules/cm^2 (UV Stratalinker 2400, Stratagene).

5.3.3 Probe Hybridization to Southern Blots

Analysis of EcoRI-digested DNA from ES cell clones transfected with pROSA26^{dnTCF4} and pROSA^{caTCF4} targeting vectors, and offspring from mating of chimeric ROSA26^{dnTCF4} and ROSA^{caTCF4} mice to the wild-type mice, was accomplished by Southern hybridization performed with a 5'-specific DNA probe A (Mao et al., 1999). The probe, derived from wild-type ROSA26 genomic locus, was ^{32}P-labeled by random priming (5.3.1). Southern blotting proceeded using ExpressHyb (Clontech) according to the manufacturer's instructions. Briefly, the Southern Blot membrane was placed in hybridization tubes and prehybridized in ExpressHyb solution for 30 min at 63°C. Radiolabeled probes were denatured at 95°C for 5 min, chilled quickly on ice, and then diluted in fresh ExpressHyb to a final concentration of 1-2 x 10^6 cpm/ml. The prehybridization solution was then replaced with the ExpressHyb containing the radiolabeled probe and incubated at 63°C for 2 hours. The blot was washed of the unbound probe according to the protocol before being wrapped airtight in plastic wrap and exposed to Kodak X-OMAT Blue film at –70°C O/N. Wild-type 15 kb large band was present in all DNA lanes, whereas an additional 4.6 kb band corresponding to the targeted locus was present only in DNA from clones which undergone homologous recombination (An example of a autoradiogram of a Southern Blot is shown in Fig. 2.17).

5.4 Gel Electrophoresis

5.4.1 Agarose Gel Electrophoresis

Depending upon the size of the DNA fragments to be analyzed, DNA was separated in 0.8-2.0% agarose gels (containing 0.5 μg/ml ethidium bromide). The 10x DNA loading dye (10% glycerol, 0.25% bromophenol blue, 0.25% xylene cyanol blue) was added to the samples before loading them onto the gel together with a 1-Kb DNA Ladder (Gibco-BRL) and electrophoresing at ca 100 V for 1-2 h. The separated DNA fragments were subsequently visualized and photographed using a UV-Gel Documentation System (Herolab, Wiesloch, Germany).

5.5 Cell Culture Methods

5.5.1 Mouse Embryonic Fibroblasts Cell Culture

Primary mouse embryonic fibroblasts ("feeder cells") were obtained from embryos from matings between weild-type mice and transgenic neomycin-resistant mice. These neomycin-resistant fibroblast compose the matrix, on which embryonic stem (ES) cells grow. Fibroblasts were mitomycin C - inactivated before co-culturing with ES cells to inhibit cell division. For that purpose, 100 μl of the mitomycin C solution (1 mg/ml mitomycin C in PBS, 5% DMSO, Sigma) were added to 10 ml of culture medium. After the incubation for 2 h at 37°C cells were rinsed twice with PBS and transferred to new plates. Inactivated fibroblasts could be maintained two to three weeks in cell culture.

5.5.2 Culture, Transfection and Selection of Embryonic Stem Cells

After rapid thawing of the liquid-nitrogen frozen stocks ES cells of a E14.1 line were resuspended in ES cell medium and cultured on top of the confluent layer of mouse embryonic fibroblasts in 10 cm culture dishes. Medium was exchanged each day until the cells reached confluency. For the transfection, 2 x 10^7 ES cells in 800 μl PBS were mixed in an electroporation cuvette (Gene-Pulser Cuvette 0.4 cm, Bio-Rad, München) with 20-25 μg of the linearized targeting vector ($ROSA26^{dnTCF}$ or $ROSA26^{caTCF4}$) and electroporated at 300 V, 1200 μF with a 2 ms impulse (electroporator by L. Fischer, Heidelberg). After electroporation, ES cells were resuspended and plated onto a 10 cm culture dish on top of the confluent layer of fibroblasts.

Starting from the second day after electroporation, positive selection for the homologously recombined ES cell clones was performed for 7 days, by addition of 400 μg/ml geneticin (= G418, Gibco) to the ES cell medium. Geneticin-resistant clones were isolated after washing with PBS and hand-picking the ES cell clones into separate wells of the 96 well plates (196 clones from each electroporated construct). Subsequently the ES clones were trypsinized with 25μl 0.05% Trypsin / 0.02% EDTA at 37°C, and transferred into a well of 96-well plates already containing a confluent layer of fibroblasts. Two days later ES cells were washed with PBS, trypsinized as described, diluted with fresh ES cell medium, and split into two halves. One half of ES cell suspension was transferred to a gelatinized well of a 96 well plate for DNA extraction, the other half to a well of a 96 well plate containing a layer of fibroblasts. In the plates containing fibroblasts, ES cell were cultured until large individual

colonies were obtained, and then frozen as following: ES cells were washed with PBS, trypsinized as indicated, and added an equal volume of ice-cold 2x freezing medium (60% FCS, 20% DMSO). The 96-well plates were wrapped in several layers of paper and frozen at –70°C for 3 days before transferring them to the liquid nitrogen. In the gelatinized plates ES cells were grown to confluency and digested to extract genomic DNA. ES clones undergone homologous recombination were identified by Southern Blot analysis, corresponding clones from the replica plates were thawed and transferred to the wells of 96 well plates on top of a layer of fibroblasts.

5.6 Genomic DNA Isolation

5.6.1 Isolation of Genomic DNA from ES Cells

To identify ES cell clones which undergone homologous recombination by Southern Blot analysis, genomic DNA was extracted in 96-well plates. Confluent ES cells growing on gelatinized 96-well plates were washed twice with PBS and incubated O/N in 50 µl of ES Lysis Buffer per well (10 mM Tris pH 7.5; 10 mM EDTA; 10 mM NaCl; 0.5% N-laurylsarcosine; 200 µg/ml Proteinase K) at 55°C. On the next day, 100 µl of ice-cold mixture EtOH / 0.25 M NaCl was added to each well and DNA was precipitated 30 min at room temperature. Supernatant was removed by turning the plates onto blotting paper. Wells were washed twice with 70% EtOH and air-dried 20 min. Subsequently genomic DNA was dissolved directly in 50 µl of restriction digest mix (1x optimal restriction buffer; 100 µg/ml BSA; 50 µg/ml RNase A; 10-15 U restriction enzyme) and incubated O/N at 37°C. Half of the restriction digest was loaded onto the agarose gel and analyzed by Southern Blotting as described.

5.6.2 Isolation of Genomic DNA from Tail Tips or Tissue Biopsies

Small pieces of embryonic tissue or tail tips of adult mice were lysed in 200 µl of Tail-Tip buffer (100 mM Tris pH 8,5; 200 mM NaCl; 5 mM EDTA; 0.2% SDS; 100 µg/ml Proteinase K) O/N at 55°C while shaking. After phenol/chloroform extraction (5.4.1) DNA was precipitated with EtOH and dissolved in ddH_2O.

5.7 RNA Extraction from Tissues and cDNA Synthesis

5.7.1 RNA Extraction from Pancreas or Whole Embryos

Tissue was dissected in ice-cold PBS, minced to achieve pieces of 1-10 mm^3 in size, and transferred immediately into the tubes containing RNAlater solution (Ambion). For isolation of RNA from tissues the RNeasy Kit and Qiashredder Kit (both from Qiagen) were used as recommended by the manufacturer. Briefly, tissue samples were thawed on ice, manually transferred from the RNAlater solution to 350 μl of RLT buffer mixed with 10 μl ß-mercaptoethanol, vortexed and transferred onto a Qiashredder Mini-column to homogenize tissue fragments. Lysate was eluted by centrifugation at 14,000 rpm for 2 min, mixed with the same volume of 70% EtOH, and loaded onto a RNeasy Mini-column. To bind RNA on the silica membrane, lysate was centrifuged 15 sec at 10,000 rpm, and the column was then washed with 350 μl RW1 buffer. DNase I digestion was carried out for 15 min directly on the membrane of the column (RNase free DNase I set, Qiagen). RNA was washed as recommended by the manufacturer and eluted from the column by 2 x 50 μl DEPC H2O by centrifugation at 10,000 rpm for 15 sec. RNA was used as a template for cDNA synthesis by in vitro transcription (5.7.2).

5.7.2 cDNA Synthesis by In Vitro Transcription

For the synthesis of the first-strand cDNA on the total RNA from the whole embryos, embryonic pancreata or the adult islets, a SuperScriptII (Invitrogen) reverse transcriptase kit was used as recommended by the manufacturer. Shortly, total RNA (10 ng–5 μg) was mixed with 25 ng of $Oligo(dT)_{12-18}$ primers, 4 μl of 5x First-Strand Buffer, 10 μM DTT, and 0.5 mM of each dNTP. 200 U of SuperScriptII reverse transcriptase were used in a synthesis at 42°C for 1 h. The reaction was stopped by heating at 70°C for 15 min. The cDNA was aliquoted and stored at –20°C.

5.8 Immunohistochemical methods

5.8.1 Tissue preparation / cryo

Mouse embryos were dissected and fixed in 4% paraformaldehyde in PBS (pH 7.4) at 4°C for 2 hours to O/N, depending on the size of the tissue, equilibrated with 30% sucrose in PBS, and mounted in O.C.T. embedding medium (TissueTek, Sakura) at –80°C. Embryos or

isolated pancreata were sectioned on a cryostat (2800 Frig-Cut E, Reichert-Jung) at 10 μm, transferred to glass slides and stored at –80°C until use.

5.8.2 Tissue preparation / paraffin

Mouse embryos were dissected and fixed in 4% paraformaldehyde in PBS (pH 7.4) at 4°C for 2 hours to O/N, depending on the size of the tissue. Then tissue was washed 2x PBS for 20 min, and lead through the row of ethanol solutions (50%, 70%, 2x 96%, and 3x 100%), for 45 min each to dehydrate. After 2x 1 hour washes in xylene tissue was transferred to the melted paraffin (HistoWax, Roth) at 65°C and paraffin was exchanged every day for 3 days. Tissues were embedded on a paraffin embedder (Leica EG1160). Sections were made on Leica Microtome (6 μm thick for non-stained tissue, 10 μm for X-gal stained tissue), stretched on top of the 42°C water bath, transferred to coated glass slides and air-dried for 12 hours at 37°C. For immunohistological staining sections were deparaffinized in the alcohol row (2x xylene, 2x 100% EtOH, 2x 96% EtOH, 2x 70% EtOH, 50% EtOH, H_2O), 2 min in each solution and proceeded as described.

5.8.3 In situ Enzymatic ß-Galactosidase Staining on Tissue

The enzymatic ß-galactosidase reaction with the substrate X-gal was performed on the E9-E10.5 whole embryos and isolated pancreata of the embryos older than E11. X-Gal (5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside) is a chromogenic substrate for ß-galactosidase, which hydrolyzes X-Gal forming an intense blue precipitate. This reaction was carried out on the embryos with the following genotypes: $Sox8^{+/LacZ}$, $Sox10^{+/LacZ}$, $Sox10^{LacZ/LacZ}$, $Axin2^{+/LacZ}$, and BAT-$Gal^{+/tg}$, and their wild-type littermate controls. Embryos were dissected in ice-cold PBS and fixed 15-45 min depending on the age in the fixing solution (1% formaldehyde; 0.2% gluteraldehyde; 0.002% NP-40; 1x PBS). After fixation the embryos were washed 2x 20 min in PBS and incubated for 12-16 hours at 30°C in the dark with the staining solution (1 mg/ml X-Gal; 5 mM K3Fe(CN)6; 5 mM K4Fe(CN)6; 2 mM MgCl2; 0.02% NP-40). The staining reaction was stopped by washing the tissues with PBS. Subsequently the tissues were dehydrated and embedded in paraffin (5.8.2).

5.8.4 Immunohistochemistry

For immunohistochemistry, sections were allowed to thaw, rehydrated in PBS and blocked unspecific binding sites with 5% normal goat serum (Sigma) in PBS at room temperature for one hour. Primary antibodies (see Materials) were diluted in 5% normal goat serum in PBS

and allowed to bind O/N at 4°C. For the fluorescence signal, the sections were then washed 3 times in PBS before incubation with the appropriate secondary fluorochrome-conjugated antibody for 1 hour at RT in the dark. Following three washes with PBS, sections were coverslipped using Vectashield Mounting Medium (Vector) and stored at 4°C in the dark. The sections were documented using a confocal microscope (Leica DM IRBE, Heidelberg), and True Confocal Scanner (Leica TCS SPII, Heidelberg). For the peroxidase signal on X-gal stained tissues, after incubation with the primary antibody sections were washed 3 times in PBS and incubated 1 hour at RT with the secondary, biotin-conjugated antibody. Subsequently the sections were washed 2x in PBS and incubated with the ABC solution (Vectastain ABC Kit, Vector Laboratories) for 1 hour to bind horseradish peroxidase on the biotin residues. Then sections were washed in PBS, transferred to 0.1 M Tris-HCl (pH 7.9) solution, and presented a DAB staining solution (Diaminobenzidine Tablet Set, Sigma) until brown precipitate formed. The staining reaction was stopped by placing slides in water. Sections were dehydrated and coverslipped with Histokitt mounting medium (Roth).

5.9 In situ Hybridization on Tissue Sections

5.9.1 In situ RNA Hybridization with Digoxygenin (DIG)-labeled RNA Probes

Plasmids containing fragments of cDNA from the genes to be detected, were linearized by restriction enzymes, and used as templates for T3, T7 and SP6-directed RNA synthesis, using the DIG RNA Labeling Mix and accompanying protocol (Roche), thereby generating RNA single-strand antisense probes. In situ hybridizations were performed essentially as described (Strahle et al., 1994). Briefly, mouse embryo cryosections were defrosted, and then hybridized O/N at 65°C in the presence of antisense RNA probes diluted 1:100 in hybridization buffer (0.2 M NaCl; 10 mM $NaPO_4$; 5 mM EDTA; 10 mM Tris-HCl, pH 7.5; 50% formamide; 10% dextran sulfate; 1 mg/ml tRNA (Gibco); 1x Denhardt's). They were then washed 4-5 times 30 min at 65°C in 1x SSC, 50% formamide, 0.1% Tween-20, followed by two 30 min washes at RT in MABT (100 mM maleic acid, pH 7.5; 150 mM NaCl; 0.1% Tween-20), then blocked 1 h at RT in MABT + 2% Blocking Reagent (Roche) + 20% heat-inactivated goat serum (Sigma). Alkaline-phosphatase-conjugated anti-DIG antibody (Roche) was diluted 1:2500 in blocking solution and incubated O/N at RT. Sections were then washed 5 x 20 min in MABT, then twice 10 min in AP Staining Buffer (100 mM NaCl; 50 mM $MgCl_2$; 100 mM Tris-HCl, pH 7.9; 0.1% Tween-20; 0.5 mg/ml levamisole). Sections were

incubated with AP staining buffer containing 3.5 µl each of NBT and BCIP/ ml for 1-3 days until the signal developed. Sections were then washed in ddH_2O, air-dried and mounted in Histokitt mounting medium (Roth).

5.10 Islet isolation

Islets were isolated from the 6-8 months old wild-type C57Bl/6J mice for the total RNA extraction. Pancreas was isolated in ice-cold PBS and the collagenase P solution (freshly prepared, 0.7 U/ml in Hanks' Balanced Salts (HBSS), pH 7.4) was injected into multiple sites. The inflated pancreas was incubated at 37°C for 15 min with the collagenase solution. Then 20 ml of ice-cold HBSS (138 mM NaCl; 5.6 mM Kcl; 1.2 mM $MgCl_2$; 5 mM HEPES; 3 mM glucose; 1 mM EGTA; 0.1% w/v BSA) were added to the pancreas, shaken vigorously by hand for 1 min, and divided into two 15 ml falcon tubes. The islets were let sediment on ice for 3 min, then HBSS was removed, and 20 ml of fresh HANKS solution were added to the sediment. The procedure was repeated twice. Finally, islets were resuspended in 10 ml of ice-cold HBSS, transferred to the culture dish and hand-picked in the dark field with a mechanic pipette. From one pancreas it was possible to obtain 50-100 islets.

5.11 Protein Analysis

5.11.1 Protein Extraction from Tissue

Individual pancreata of neonatal mice were isolated in ice-cold PBS and placed on ice in 50 µl protein extraction solution (80% EtOH; 18% H_2O; 2% HCl). Tissue was homogenized by ultrasound for 20 seconds and incubated O/N at 4°C. Large cell fragments were separated by subsequent centrifugation for 15 min at 14,000 rpm. The supernatant was transferred to fresh tubes and stored at –80°C for the following protein analysis. Part of the protein extract was analyzed for the total protein concentration by Bradford assay (5.11.2).

5.11.2 Total Protein Concentration Determination by Bradford Assay

Total protein concentration in protein extracts from pancreas was determined according to the Bradford method (Bradford, 1976) using BSA (bovine serum albumin) as a standard. 800 µl of PBS were combined with 10 µl of the protein-containing solution to be analyzed. To this, 200 µl of the Bio-Rad reagent (Bradford Assay Reagent, Bio-Rad) were added and the mixture was incubated at RT for at least 5 min before measuring the light absorbance at 595

nm (A_{595}). Comparison of this value with a BSA standard curve allowed calculation of the protein concentration.

5.11.3 Radioactive Hormone Concentration Determination in Total Pancreas Extracts.

Insulin and glucagon concentration in pancreas extracts from neonatal wild-type and Sox8-/- mice were determined by the competitive radioimmunoassay (RIA). For this purpose, Rat Insulin RIA Kit or Glucagon RIA Kit (both from Linco Research Inc.), respectively, were used. Both assays contain a specific antibody against insulin (or glucagon) and ^{125}I-labeled form of the same hormone, termed "tracer". For hormone concentration determination, tracer is incubated with the specific antibody. If unlabeled hormone contained in the pancreatic protein extract is added to this reaction, it competes with the iodine-labeled tracer. The amount of bound tracer is reverse proportional to the amount of the corresponding hormone in the extract, and can be quantified by measuring γ-radiation in the reaction precipitate. Exact procedure was performed as recommended by the manufacturer.

5.12 Gene-Modified Mice

5.12.1 Creation of the Modified Forms of TCF4

I created cDNAs coding two modified forms of TCF4 transcription factor (dnTCF4 and caTCF4), both based on the sequence of the wild-type TCF4. Dominant-negative form of TCF4 (dnTCF4) lacks 31 N-terminal amino acids necessary for binding to ß-catenin. In the constitutively active TCF4 form (caTCF4), dnTCF protein is fused to ß-catenin which lacks N-terminal 81 amino acids (comprising the ß-catenin degradation domain). I created cDNAs for dnTCF4 and caTCF4 factors by modification of cDNAs of ß-catenin and TCF4. I employed PCR (polymerase chain reaction) with specifically designed primers to delete fragments of cDNAs, and to fuse cDNAs to other sequences.

DNA sequences and vectors that I used for creation of modified TCF4 sequences and vectors are summarized in the Table 5.1. Plasmid containing ß-catenin coding sequence was a kind gift from Dr. M. Watermann (University of California, Irvine), and the plasmid containing TCF4 cDNA was a kind gift from Dr. A. Hecht (Max-Planck Institut für Immunbiologie, Freiburg). Both dnTCF4 and caTCF4 cDNAs were fused on their 5'-end to the sequence consisting of three elements: (1) the SDK oligonucleotide (combined Shine-Dalgarno

sequence and a Kozak sequence), for efficient initiation of mRNA translation on ribosomes; (2) an ATG transcription start codon, and (3) a myc epitope sequence to facilitate the detection of the modified TCF4 protein forms in cells (sequences in Appendix). This multi-purpose oligonucleotide linker was chemically synthesized by Metabion (Martinsried, Germany). PCR products were subcloned into the pGEM-T Easy Vector (Promega) for propagation in *E. coli*.

Sequence	**Origin of the Sequence / Reference**
myc-tag	Invitrogen
beta-catenin cDNA	Dr. M. Waterman (Prieve and Waterman, 1999).
TCF4E cDNA	Dr. A.Hecht (Hecht et al., 2000)
IRES-LacZ	SacI-NotI fragment; modified from (Theil et al., 1998).
LoxP-neo-*LoxP* -SV40PolyA-DTA	pBigT; S. Srinivas; (Srinivas et al., 2001) AscI-PacI fragment
ROSA26 genomic sequence	pRosa26-1; (Soriano, 1999)
pBluescript KS	Stratagene
pGEM-5Zf	Promega

Table 5.1. Origin of sequences used for the construction of the transgenic vectors pdx1-dnTCF and pdx1-caTCF, and targeting vectors pROSA26-dnTCF and pROSA26-caTCF.

5.12.2 Generation of of *pdx-dnTCF*$^{+/tg}$ and *pdx-caTCF*$^{+/tg}$ Transgenic Mice

To create vectors for producing the transgenic mouse lines I assembled all components of the plasmids pdx1-dnTCF and pdx1-caTCF in the vector pGEM-5Zf (Promega) by introducing an engineered multiple cloning site and inserting all necessary parts of the constructs using different restriction enzymes. The dnTCF or caTCF cDNA is immediately followed by a gene coding ß-galactosidase from E.coli (ß-galactosidase gene with a nuclear localization signal was a generous gift from Dr. Thomas Teil). as a bicystronic mRNA with a single poly-A tail on its 3'-terminus. Internal Ribosome Entry Site (IRES) sequence preceding LacZ gene allows the ribosomal machinery to initiate translation from a secondary site, within a single transcript. Upstream I placed the regulatory sequence controlling the spatial and temporal

expression of construct, the 4.3 Kb-XbaI-SalI promoter fragment of mouse Pdx-1 gene, which was a kind gift from Dr. Maureen Gannon. This promoter has been previously shown to faithfully reproduce the endogenous pattern of *Pdx-1* expression in transgenic mice when a ß-galactosidase reporter gene was placed under its transcriptional control (Stoffers et al., 1999).

The pronuclear injections to produce transgenic *pdx-dnTCF*$^{+/tg}$ and *pdx-caTCF*$^{+/tg}$ mice were performed in the transgenic mouse facility at the Center of Molecular Neurobiology in Hamburg. For this purpose, DNA for injection was released from the pdx1-dnTCF and pdx1-caTCF plasmids by restriction digest with PmeI, to get rid of any unwanted prokaryotic sequences, then electrophoresed on low percent agarose gel, excised and finally eluted in H_20. DNA concentration was adjusted to 1μg/μl as recommended by the transgenic facility. Constructs were microinjected into pronucleus-stage embryos and implanted into the pseudopregnant females.

5.12.3 Generation of ROSA26^{dnTCF4} and ROSA26^{caTCF4} mice

To generate ROSA26^{dnTCF4} and ROSA26^{caTCF4} mice, I inserted modified TCF4 forms into one allele of ROSA26 genomic locus through homologous recombination in embryonic stem (ES) cells. For the insertion of the dnTCF4 or the caTCF4 into the genomic ROSA26 locus I used two targeting constructs, pROSA-dnTCF4 and pROSA-caTCF4, respectively, based on the pROSA26-1 vector (Soriano, 1999) and pBigT vector (Srinivas et al., 2001) (sequences I used for the construction of the targeting vectors are summarized in Table 5.1). First, I introduced cDNAs of dnTCF4 or caTCF4 in the multiple cloning site in the pBigT vector, then PacI-AscI fragment was released and shuttled into the pROSA26-1 vector. The pROSA26-1 vector contains sequences of the mouse genomic ROSA26 locus, which provide molecular basis for homologous recombination in ES cells, as well as a Diphteria toxin alpha chain gene (DTA), which serves as a negative selection marker in ES cells. pBigT vector contains a neomycin resistance cassette (consisting of a neo gene and an active promoter of a phosphoglycerate kinase gene), a strong transcriptional stop sequence (tpA), flanked by two loxP sites, a splice acceptor site, a polylinker bearing multiple restriction enzyme recognition sequence for insertion of a desired cDNA, and a bovine growth hormone polyadenilation sequence (bGH) for mRNA stability (Fig. 5.1, A).

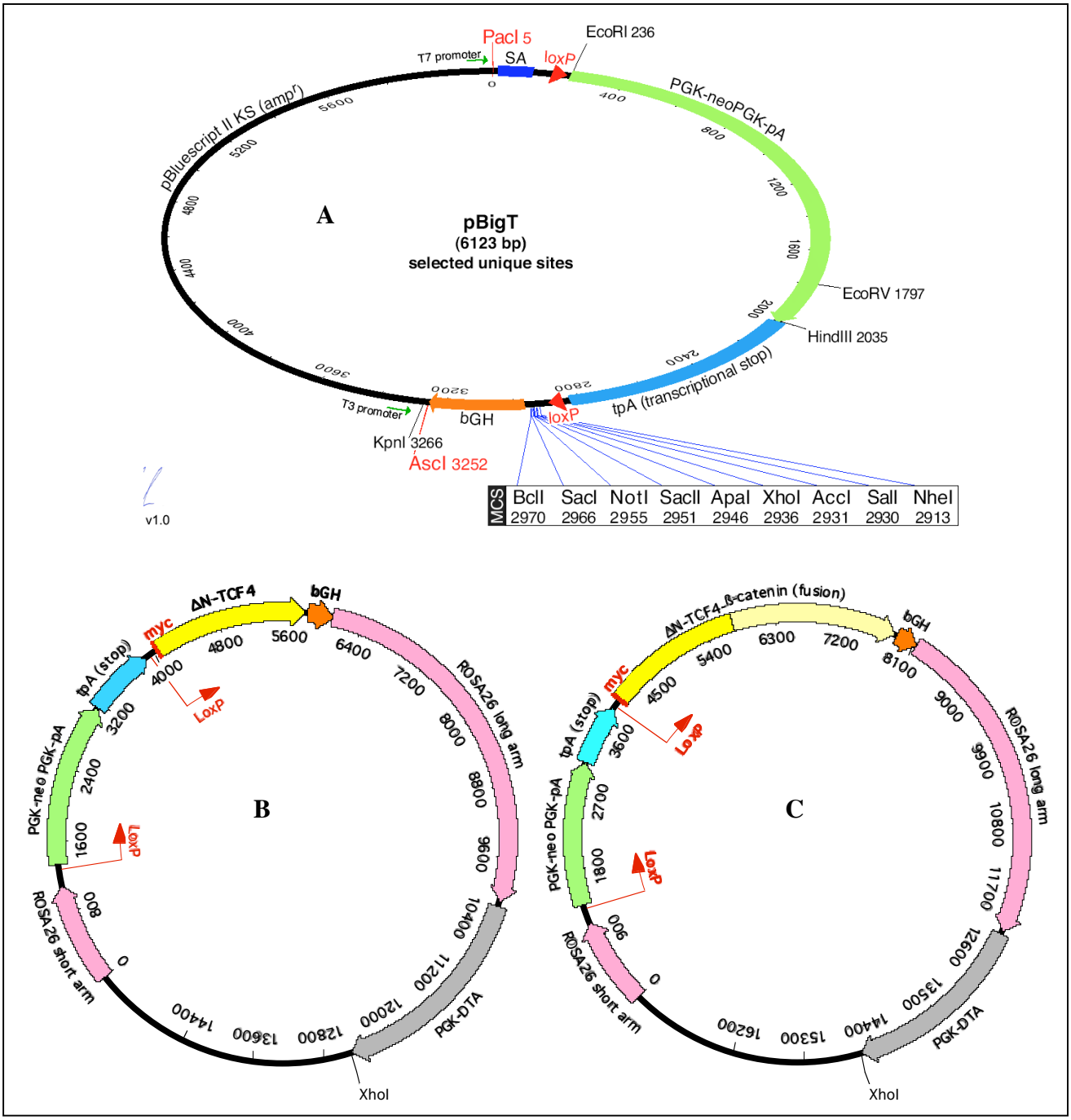

Figure 5.1. Targeting vectors pROSA-dnTCF4 and pROSA-caTCF4. (A), intermediate plasmid pBigT (Srinivas et al., 2001). (B), pROSA-dnTCF4 for overexpression of dominant-negative TCF4. (C), pROSA-caTCF4, for overexpression of constitutively active TCF4.

Assembled targeting vectors pROSA-dnTCF4 and pROSA-caTCF4 were linearized with PmeI and electroporated into the ES cells as described in 5.5.1. Subsequently clones were analyzed for the correct integration of the targeting vector into the genome by Southern blotting.

In case of ES cells electroporated with the pROSA-dnTCF4 construct, 134 DNA samples could be evaluated (i.e. in which the wild-type band was clearly visible). Out of these, in 10 ES cell clones an additional band of smaller size was observed, corresponding to the targeted ROSA26 allele. Out of ES cells transfected with the pROSA-caTCF4, 140 clones could be reliably evaluated, and in case of 10 DNA samples an additional mutant band was present (Fig. 2.18). Two positive ES cell clones from pROSA-dnTCF4 and three clones from pROSA-caTCF4 targeting construct were injected into blastocysts and transferred into pseudopregnant C57Bl/6J foster mice in the transgenic animal facility at the Center for Molecular Neurobiology in Hamburg. Chimeric offspring were born in both *ROSA26*^{dnTCF} and two of the three *ROSA26*^{caTCF} mouse lines. In one *ROSA26*^{dnTCF} line, the targeted allele was successfully transferred to the offspring (Fig. 2.17, B). So far no offspring carrying the targeted *ROSA26*^{caTCF} allele were produced by *ROSA26*^{caTCF} chimeric males.

5.13 Genotyping of Gene-Modified Mice

Mouse lines bearing a ß-galactosidase gene (Sox8$^{+/LacZ}$, Sox10$^{+/LacZ}$, Axin2$^{+/LacZ}$, BAT-Gal$^{+/tg}$, *pdx-dnTCF*$^{+/tg}$ and *pdx-caTCF*$^{+/tg}$) were genotyped for the presence of the ß-gal allele by the commonly used LacZ-PCR. Sox8$^{LacZ/LacZ}$ and Sox10$^{LacZ/LacZ}$ homozygous mutant mice were genotyped by the Sox8- or Sox10-specific PCR. *ROSA26*^{dnTCF} and *ROSA26*^{caTCF} mouse lines were initially genotyped by the Southern hybridization (5.3) and subsequently by the ROSA26-PCR. *Ins-cre*$^{+/tg}$ and *pdx1-cre*$^{+/tg}$ lines were genotyped for the presence of the *Cre* recombinase transgene by the *Cre*-PCR. All primer sequences can be found in the Appendix section.

5.14 Transient Transfection Assay

To determine the functionality in vitro of modified forms of TCF4 I created, we established collaboration with Dr. Marian Waterman's lab (University of California, Irvine). The cDNAs for dnTCF4 and caTCF4 (with and without a myc epitope) were subcloned into the pcDNA3

vector; all cell transfections were performed in the laboratory of Dr.Waterman. COS cells were transiently transfected with Effectene reagent according to the manufacturer's protocol (Qiagen). COS cells were plated at a density of 150,000 cells/well in six-well plates 20 h before transfection. The TOPFLASH luciferase reporter plasmid (0.4 μg) was co-transfected with ß-catenin (0.4 μg), full-length TCF4 (0.2 μg), dnTCF4 or caTCF4 (0.2 μg) and ß-galactosidase (0.1 μg) expression constructs. Cells were harvested 18-20 h after transfection, and ß-galactosidase activity was determined using the Galacton-Plus substrate (Applied Biosystems) to normalize luciferase activity for each point (each point was performed in duplicate). The -fold induction was calculated relative to the luciferase reporter plasmid alone.

VI Appendix

6.1 General Abbreviations

A, mA	ampere, miiliampere
ATP	adenosine triphosphate
BME	ß-mercaptoethanol
bp	base pairs
ca	approximately
cDNA	complementary DNA
Ci	curie
cm, mm	centimeter, millimeter
cpm	counts per minute
Cys	cystine
ddH_2O	double distilled water
DEPC	diethylpyrocarbonate
dNTPs	deoxyribonucleoside triphosphates
DMEM	Dublecco's modified Eagle medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EDTA	ethylenediaminetetra-acetate
EtOH	ethanol
FA	formaldehyde
FCS	fetal calf serum
g, mg, μg	gram, milligram, microgram
h, min, sec	hour, minute, second
HBSS	Hanks' Balanced Salts
HOAc	acetic acid
kb	kilobases
kDA	kilodaltons
L, ml, μl	liter, milliliter, microliter
M, mM, μM	molar, miilimolar, micromolar
MeOH	methanol
mRNA	messenger RNA
mut	mutant
NaOAc	sodium acetate
NP-40	Nonidet P-40
OD	optical density
O/N	overnight
32P	phosphorus-32
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
qs	bring to a total volume of
RNA	ribonucleic acid
rpm	rotations per minute
RT	room temperature
SDS	sodium dodecyl sulfate
Tris	Tris (hydroxymethyl) aminomethane

UTR	untranslated region
UV	ultraviolet
V	volts
X-gal	5-bromo-4-chloro-3-indolyl-ß-D-galactoside
wt	wild-type

6.2 Specialized Abbreviations

APC	Adenomatous polyposis coli
CD	campomelic dysplasia
CNS	central nervous system
CtBP	C-terminal binding protein
DKK	dickkopf
DTA	diphteria toxin A-chain
E	embryonic day
EGF	epithelial growth factor
ES	embryonic stem (cell)
FGF	fibroblast growth factor
Frzb	Frizzled-related soluble proteins
Fzd	Frizzled receptor
Grg	Groucho related genes
GSK3-ß	glycogen synthase kinase 3-ß
HDAC	histone deacetylase
HPAP	human placental alkaline phospatase
LEF	lymphoid enhancer factor
LRP	low-density lipoprotein receptor-related protein
NGN3	neurogenin3
NLS	nuclear localization signal
PDX-1	pancreatic and duodenal homeobox factor-1
PNS	peripheral nervous system
TCF	T-cell factor

6.3 List of Oligonucleotides Used

Name	**Sequence (5'-3')**	**Product size, bp**	**Purpose of Use**
Sox fw	AAG GCC GGA TCC ATG AA(CT) GC(ACT) TT(CT) AT(AGT) GT(TGCA) TGG	≈300	degenerate Sox RT-PCR
Sox rev	AAG GCC GGA TCC (TGCA)GG TCT T(AG)T A(CT) TT(AG) TA(AG) TC(TGCA) GG		degenerate Sox RT-PCR
Sry fw	GAG AGC ATG GAG GGC CAT	266	gene-specific RT-PCR
Sry rev	CCA CTC CTC TGT GAC ACT		gene-specific RT-PCR
Sox1 fw	TGC AGG AGG CAC AGC TGG CCT AC	280	gene-specific RT-PCR
Sox1 rev	TGC CGC CAC CGC CGA GTT CTG G		gene-specific RT-PCR
Sox2 fw	AAG TAC ACG CTT CCC GGA GGC TTG	411	gene-specific RT-PCR
Sox2 rev	AGT GGG AGG AAG AGG TAA CCA C		gene-specific RT-PCR
Sox3 fw	TCT CCG CCG CCC GCC ATC CGT TCG	174	gene-specific RT-PCR
Sox3 rev	CCG TTC CAT TGA CCG CAG TC		gene-specific RT-PCR

Sox5 fw	TGG AGA TTC TGA CGG AAG CG	682	gene-specific RT-PCR
Sox5 rev	CTT GTC CCG CAA TGT GGT T		gene-specific RT-PCR
Sox7 fw	ACC TTC AGG GGA CAA GAG TTC G	345	gene-specific RT-PCR
Sox7 rev	GTT TTT CTC AGG CAG CGT GTT C		gene-specific RT-PCR
Sox13 fw	CCC CAC AAC CAC TGA ACC TC	683	gene-specific RT-PCR
Sox13 rev	TGG ACG CCG TGT CCT CAT		gene-specific RT-PCR
Sox15 fw	TGG AGC GTC TGG GGG ACT TC	525	gene-specific RT-PCR
Sox15 rev	TGG GGA TAG GTA AGG GGA GAA AG		gene-specific RT-PCR
Sox17 fw	AAG GCG AGG TGG TGG CGA GTA G	488	gene-specific RT-PCR
Sox17 rev	CCT GGC AGT CCC GAT AGT GG		gene-specific RT-PCR
Sox18 fw	CGA ATC AGG GCG CTA TGG CTT TG	411	gene-specific RT-PCR
Sox18 rev	AGT GGG TAG CTC GCG GAA GG		gene-specific RT-PCR
Sox30 fw	CGG TTC TCC TTT CAT CAC CC	333	gene-specific RT-PCR
Sox30 rev	CCA AGG CTC CAA TGT CCA GA		gene-specific RT-PCR
LacZ fw	GCG TGT ACC ACA GCG GAT GGT TCG G	675	genotyping
LacZ rev	GTC AAT CCG CCG TTT GTT CCC ACG G		genotyping
Cre fw	CTA AAC ATG CTT CAT CGT CGG TCC G	200	genotyping
Cre rev	CGT AAC AGG GTG TTA TAA GCA ATC C		genotyping
Sox8 fw	GTC CTG CGT GGC AAC CTT GG	-	genotyping
Sox8 wt	GCC CAC ACC ATG AAG GCA TTC	430	genotyping
Sox8-mut	TAA AAA TGC GCT CAG GTC AA	617	genotyping
Sox10 fw	CGT TGG GCT CTT CAG GAG GAC	-	genotyping
Sox10 wt	GCT CTT GCT GGC ACC GTT GAC	400	genotyping
Sox10-mut	ACT GTC CTG GCC GTA ACC GAC	600	
Rosa fw	AAA GTC GCT CTG AGT TGT TAT	-	genotyping
Rosa rev wt	GGA GCG GGA GAA ATG GAT ATG	320	genotyping
Rosa rev mut	GCG AAG AGT TTG TCC TCA ACC	583	genotyping
TCF-1 fw	GTT CAC CCA CCC ATC CTT GAT GC	430	gene-specific RT-PCR
TCF-1 rev	GAT TCT TGA TGT TTT TCC CTT GAC		gene-specific RT-PCR
LEF-1 fw	CCA GAG AAC ACC CTG ATG AAG GA	390	gene-specific RT-PCR
LEF-1 rev	CAG GCT GAC CTT GCC AGC CAA T		gene-specific RT-PCR
TCF-3 fw	GTG CGA AAT CCC CAG TTA CGG TG	410	gene-specific RT-PCR
TCF-3 rev	CAA GGC TTC TCA GGG GGC AGG T		gene-specific RT-PCR
TCF-4 fw	CGA ATG TTT CCT AAA TCC TTG CCT	420	gene-specific RT-PCR
TCF-4 rev	CCA GAG CCC CAT TGG GAC AGA G		gene-specific RT-PCR
Wnt2b fw	TGG AGA GCA CTC TCA GAC TTC C	190	gene-specific RT-PCR
Wbt2b rev	GCC TTG TCC AAG ACA CAG TAG T		gene-specific RT-PCR
Wnt5a fw	CTT CCG GAA GGT GGG CGA TGC	205	gene-specific RT-PCR
Wnt5a rev	TTG CAC AGG CGT CCC TGC GTG		gene-specific RT-PCR
Fzd4 fw	GCT TGT GCT ATG TTG GGA ACC CAC	230	gene-specific RT-PCR
Fzd4 rev	ACA GGT TGC AGG AAC CGT		gene-specific RT-PCR
Fzd7 fw	GGC CAT CGA GGC CAA CTC GCA	150	gene-specific RT-PCR
Fzd7 rev	CGC AAT CGA TCC ACA CTA GAC		gene-specific RT-PCR

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