Lack of TCF2/vHNF1 in mice leads to pancreas agenesis

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Heterozygous mutations in the human POU-homeobox *TCF2* (*vHNF1*, *HNF1β*) gene are associated with maturity-onset diabetes of the young, type 5, and abnormal urogenital tract development. Recently, pancreas atrophies have been reported in several maturity-onset diabetes of the young type 5 patients, suggesting that *TCF2* is required not only for adult pancreas function but also for its normal development. *Tcf2*-deficient mice die before gastrulation because of defective visceral endoderm formation. To investigate the role of this factor in pancreas development, we rescued this early lethality by tetraploid aggregation. We show that *TCF2* has an essential function in the first steps of pancreas development, correlated with its expression domain that demarcates the entire pancreatic buds from the earliest stages. Lack of *TCF2* results in pancreas agenesis by embryonic day 13.5. At earlier stages, only a dorsal bud rudiment forms transiently and expresses the transcription factors *Ipfl1* and *Hlxb9* but lacks the key transcription factor involved in the acquisition of a pancreatic fate, *Ptf1a*, as well as all endocrine precursor cells. Regional specification of the gut also is perturbed in *Tcf2*^{-/-} embryos as manifested by ectopic expression of *Shh* and lack of *Ihh* and *Ipfl1* in the posterior stomach and duodenum. Our results highlight the requirement of *Tcf2* for ensuring both accurate expression of key regulator molecules in the stomach–duodenal epithelium and proper acquisition of the pancreatic fate. This study provides further insights into early molecular events controlling pancreas development and may contribute to the development of cell-replacement strategies for diabetes.

diabetes MODY5 | homeodomain transcription factor | pancreas development | gut regionalization | tetraploid aggregation

In mammals, the pancreas emerges as ventral and dorsal evaginations from the foregut–midgut junction that subsequently fused to form a complex organ. The signaling molecule Sonic Hedgehog (SHH) demarcates a molecular boundary between the prepancreatic endoderm and adjacent stomach and duodenal anlagen and exerts an inhibitory action on pancreas development (1–3). Genetic studies in mice have identified a hierarchical regulatory network involved in pancreas morphogenesis, with significant and sequential differences between ventral and dorsal pancreas. In the mouse, the dorsal bud appears at embryonic day 9.5 (E9.5) concomitantly with the first differentiated glucagon-producing cells. The homeobox gene *Ipfl1* (*Pdx1*) is expressed before and during this budding, and all pancreatic cell types derive from IPF1⁺ progenitors (4, 5). However, in *Ipfl1*-deficient mice, pancreas development is arrested after budding (6, 7), implying that other factors promote pancreas specification. Recently, the transcription factor *Ptf1a* (*P48*) has been shown to be essential for the acquisition of a pancreatic fate by undifferentiated ventral foregut endoderm, being required for the specification of the ventral pancreas and robust outgrowth of the dorsal bud. In its absence, ventral pancreas progenitors differentiate into duodenal cells by default (8). By contrast, the homeobox gene *Hlxb9* is required only dorsally, for specifying the gut epithelium to a pancreatic fate (9, 10). A key regulator of endocrine development is the basic

helix–loop–helix protein Neurogenin3 (Ngn3), which is absolutely required to promote islet cell development (11). The *Isl1* gene, which encodes a LIM-homeodomain protein, performs two functions in the developing pancreas. It is initially required in the dorsal mesenchyme for proper exocrine differentiation and later in the pancreatic epithelium for islet survival (12). Downstream of them, other transcription factors are essential for proper pancreatic endocrine differentiation such as *Nkx2.2* and *Pax6* (13–15). However, the initial stages of pancreatic development occur early in mammalian embryogenesis, and molecular mechanisms governing these first steps remain to be elucidated.

In humans, mutations in the POU-homeobox *TCF2* gene are associated with the human disease maturity-onset diabetes of the young type 5, a form of dominantly inherited type II diabetes mellitus characterized by pancreatic beta cell dysfunction at the age of 25 years or younger, nondiabetic early onset renal disease, liver dysfunction, and abnormal urogenital tract development (16–18). In addition to these phenotypes, variable levels of pancreas atrophies have recently been associated with different *TCF2* mutations (19, 20). Remarkably, we have recently identified a severe pancreas hypoplasia in two fetuses carrying previously undescribed mutations in the *TCF2* gene (A. L. Delezoide, C.H., and S.C., unpublished results). These data, together with the observation that *vHnf1* (*Tcf2*)-mutant embryos show underdevelopment of the pancreas in zebrafish (21), strongly suggest a critical function of *Tcf2* in pancreas development. However, the molecular bases of these pancreatic phenotypes are poorly understood, as are the *Tcf2* target genes involved. In mice, the precise implication of *Tcf2* during early organogenesis remains essentially unknown, because *Tcf2*-deficient embryos die before gastrulation due to defective visceral endoderm formation (22, 23).

In this study, we rescued this early lethality by tetraploid aggregation, by using *Tcf2*^{-/-} embryonic stem (ES) cells. We observed in these rescued *Tcf2*-null embryos an absence of the ventral pancreatic bud and an extremely reduced and transient dorsal bud that leads to pancreas agenesis by E13.5. Our results uncover the requirement of *Tcf2* for the specification of the ventral pancreas and for proper morphogenesis and differentiation of the dorsal pancreas. They further suggest that *Tcf2* also is required for both accurate regionalization of the primitive gut through Hedgehog (Hh) signaling and proper acquisition of the pancreatic fate by regulating *Ptf1a* expression, thus placing this transcription factor at one of the highest positions in the genetic network that controls pancreas development.

Materials and Methods

Diploid and Tetraploid Chimera. Because chimeric embryos generated with our previously isolated *Tcf2*^{-/-} and *Tcf2*^{+/-} ES cells

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Abbreviations: En, embryonic day n; ES, embryonic stem; Hh, hedgehog; vHnf1, variant Hepatocyte nuclear factor 1; Shh, Sonic Hedgehog; Ihh, Indian Hedgehog; Ngn3, Neurogenin3; Isl1, Islet-1; Hnf6, Hepatocyte nuclear factor 6.

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Fig. 2. Early defective pancreas development in *Tcf2*^{-/-} embryos. (A–H) Overall morphology in whole-mount β -gal-stained *Tcf2* heterozygous and homozygous mutant embryos between E8.5 and E12.5. *Tcf2*^{-/-} embryos exhibit, in addition to severe liver hypoplasia and abnormal ureteric branching (L. Lokmane, M. Pares-Fessy, C.H., and S.C., unpublished data), defective pancreas development. β -gal activity is more intensely observed in the neural tube (nt) of E8.5 *Tcf2*^{-/-} than *Tcf2*^{+/-} embryos (A and B), as well as in mesonephric ducts (md) and primitive gut (g) at E9.5 (C and D), probably as a result of the presence of two copies of the *LaclZ* gene in mutant embryos and/or a negative autoregulation of *Tcf2*. The protruding dorsal pancreatic bud externally detected in heterozygous embryos between E9.5 and E12.5 is not observed in homozygous mutant embryos (black arrowhead, C–H). (I–X) Pancreatic bud morphogenesis in sagittal sections of control and *Tcf2* homozygous mutant embryos between E9.5 and E13.5. (I–K) IPF1 immunostainings of sagittal sections of whole-mount β -gal-stained embryos reveal ventral and dorsal pancreatic buds in *Tcf2*^{+/-} embryos, whereas the ventral pancreatic bud is totally absent and only a very reduced dorsal bud is observed in *Tcf2*^{-/-} embryos at E9.5. vp, ventral pancreas; dp, dorsal pancreas. (L–T) At later stages, the dorsal pancreatic bud (white arrowhead), the duodenum (d), and the posterior stomach are stained by IPF1 in control embryos, but only a remnant pancreatic bud is stained in *Tcf2*-deficient embryos, which is abnormally close to the posterior stomachal epithelium. Whereas the pancreatic bud exhibits an important growth in control embryos particularly from E12.5, the remnant pancreatic bud in *Tcf2*^{-/-} embryos regresses by E12.5 and is not further detected at E13.5 (pancreas agenesis) (U–X). (K, N, Q, and T) β -gal staining of the remnant pancreatic bud in sagittal sections of *Tcf2* homozygous mutant embryos. β -gal and IPF1-stained sections are counterstained by safranin. β -gal⁺ mutant cells are detected in the rudiment of the pancreatic bud in *Tcf2*^{-/-} embryos coexpressing IPF1 and display a broader expression domain including a thickness of the stomachal epithelium between E10.5 and E11.5. Note that the magnification in K, N, Q, and T is higher than in the corresponding IPF1-stained section in J, M, P, and S. (U–X) Stomachal epithelium morphology in E13.5 *Tcf2* control and homozygous mutant embryos. Trichromic staining of the IPF1-stained sagittal sections. Arrows indicate the posterior stomachal epithelium, which is surrounded by the stomachal mesenchyme. Whereas the normal posterior stomach exhibits a columnar vacuolized epithelium, the posterior stomach of *Tcf2*^{-/-} embryos appears squamous and nonvacuolized, as is normally the anterior stomach. In M, P, T, and X, an asterisk indicates thickening of the gastric epithelium.

(Fig. 2 B, D, F, and H). Tetraploid chimeric embryos generated by aggregation of *Tcf2*-heterozygous ES cells were phenotypically normal at the stages examined here (data not shown).

Pancreatic bud formation in *Tcf2*^{-/-} and control embryos (see *Materials and Methods*) was analyzed from E9.5 to E13.5, by scoring the expression of *Ipfl1* (Fig. 2 *I, J, L, M, O, P, R, S, U*, and *V*). We verified that all *Ipfl1*⁺ cells in *Tcf2*^{-/-} embryos were mutant β -gal⁺ cells (Fig. 2 *K, N, Q*, and *T*). At E9.5, we observed a severely reduced dorsal pancreatic bud (an $\approx 60\%$ reduction) in *Tcf2* mutants, whereas the ventral bud was undetectable (Fig. 2 *D* and *J*). The dorsal pancreatic bud rudiment did not grow further, remaining close to the stomach lumen (Fig. 2 *M, P*, and *S*), in contrast to control embryos that displayed an important branching phase between E10.5 and E12.5 (Fig. 2 *L, O*, and *R*). The pancreatic bud in *Tcf2*^{-/-} embryos was extremely reduced

at E12.5 (Fig. 2S) and became absent at E13.5 (Fig. 2V). In accordance with these observations, by using the mitosis marker phosphorylated histone-H3 (26, 27), we found a lower proliferating rate in the mutant pancreatic bud from E9.5, whereas the number of proliferating cells in the surrounding mesenchyme was not affected (Fig. 3G). TUNEL experiments showed that cells of this remnant dorsal bud did not undergo apoptosis because the percentage of apoptotic cells of *Tcf2*^{-/-} vs. control pancreatic bud was not significantly different (Fig. 3H), suggesting that these cells were either eliminated by necrosis or recruited to another tissue.

We subsequently examined the expression of early pancreatic markers (28) by immunohistochemical (Fig. 4 A–J) and *in situ* hybridization (Fig. 4 K–L) analyses in E11.5 sagittal sections. We observed that the *Ipfl* dorsally reduced expression domain in

Fig. 3. Decreased cell proliferation in pancreas of *Tcf2*^{-/-} embryos. (A and B) The pancreatic bud in control and *Tcf2*-homozygous mutant embryos is defined by Ipf1 expression domain. (C and D) The mitosis marker antiphosphorylated histone H3 (P-H3) antibody detects proliferating cells in the pancreatic bud (circled by white dashed lines). (E and F) TUNEL experiments show cells in apoptosis in control and *Tcf2*-mutant pancreas (circled by white dashed lines). (G) The percentage of P-H3⁺ cells among IPF1⁺ cells of control and *Tcf2*^{-/-} pancreatic buds reveals an important cell proliferation decrease in *Tcf2*-mutant pancreas. (H) The percentage of TUNEL⁺ cells among IPF1⁺ cells reveals no significant difference between control and *Tcf2*^{-/-} pancreatic buds. A total of 13 sections from control (*n* = 5) and 7 sections from *Tcf2*^{-/-} (*n* = 4) of E9.5 and E10.5 embryos were evaluated.

mutant embryos also expressed the early pancreatic marker *Hlxb9* (Fig. 4 *B* and *D*). By contrast, both *Ipf1* and *Hlxb9* expression were not detected in the presumptive ventral pancreatic bud area. Remarkably, we found no expression of the key transcription factor *Ptf1a* (8) (Fig. 4*L*). Moreover, *Tcf2*^{-/-} mutants displayed a very reduced expression of *Hnf6* and no *Ngn3* expression in the remnant dorsal pancreatic bud, two factors required for endocrine fate acquisition (11, 29) (Fig. 4 *N* and *P*). Consistent with this absence of *Ngn3*, the earliest known marker of endocrine precursors (11), expressions of *Isl1*, *Pax6*, and glucagon were lost (Fig. 4 *F*, *H*, and *J*). *Isl1* remained, however, expressed in the mesenchyme (7) (Fig. 4*F*), a tissue where *Tcf2* was not expressed. Thus, endocrine precursors are totally absent in *Tcf2*^{-/-} pancreatic epithelium.

Taken together, our results show that *Tcf2* controls initial specification of the ventral pancreas and is required for proper proliferation and differentiation of the dorsal pancreas.

Lack of TCF2 Perturbs Regionalization of the Primitive Gut. Because Hh signaling was shown to exert an inhibitory action on pancreatic development (1, 30), we further investigated whether the impairment of pancreatic development in *Tcf2*^{-/-} embryos could result from a modified expression of either Sonic hedgehog (*Shh*) or Indian hedgehog (*Ihh*). Whereas *Ihh* was expressed at E12 in the caudal epithelium of the stomach and duodenum in WT embryos (Fig. 4S), no *Ihh* transcripts were detected in *Tcf2*^{-/-} embryos (Fig. 4T). We propose that *Ihh* could be a direct target gene of *Tcf2*, because we found that *Ihh* is also absent in *Tcf2*^{-/-} embryoid bodies (31). By contrast, *Shh*, normally not expressed in the caudal stomach and in the pancreatic bud (Fig. 4Q), was highly ectopically expressed in the stomachal epithelium and duodenum with a rostrocaudal gradient of expression, but remained excluded from the mutant pancreatic bud (Fig. 4R). As expected, the Hh receptor Patched (*Ptc*) was intensively expressed along the *Shh* expression domain with the corresponding rostro-caudal expression gradient (Fig. 4V), suggesting that the Hh pathway is active. Interestingly, in E13.5 *Tcf2*^{-/-} embryos, the posterior stomach exhibited characteristics of the anterior stomach and appeared essentially squamous and non-vacuolized, instead of exhibiting a columnar epithelium with mucin-negative vacuoles as posterior stomach of control embryos (Fig. 2 W and X). A similar anteriorly directed transformation of the posterior stomach resulting from ectopic *Shh* expression also has been observed in mice carrying mutations of activin receptors ActRIIA and ActRIIB (32). However, *Tcf2*^{-/-} embryos present a more severe pancreatic phenotype than that of *ActRIIA*^{+/-} *ActRIIB*^{-/-} double-mutant embryos, suggesting an additional role of TCF2 in early pancreas development.

We also observed a thickening of the posterior stomach epithelium by E10.5 (Fig. 2 *M* and *P*). Subsequently, part of this multilayered stomachal epithelium appeared to be delaminated by E12.5 and remained as clusters of tissue in the lumen of a distended stomach (Fig. 2 *T* and *X*). This phenomenon might be caused by the loss of *Ihh* expression, because a multilayer epithelium is also observed in the colon of *Ihh*^{-/-} embryos (33).

These results show that loss of *Tcf2* function results in a perturbed anteroposterior regionalization of the primitive gut, through a deregulation of Hh signaling.

TCF2 Is a Critical Regulator in the Transcriptional Network That Governs Pancreas Morphogenesis. Our data strongly suggest a critical role for *Tcf2* in the orchestrated network of transcription factors and secretory molecules (34) controlling the expansion of endodermal progenitors and their differentiation into pancreatic primordia.

Remarkably, *Tcf2*-deficient embryos exhibit a very close phenotype to that caused by *Ptf1a* deficiency, in regard to the absence of a ventral pancreas and a reduced dorsal pancreas. Intriguingly, we identified a TCF1/2-DNA consensus-binding site (ATTAATGTTTAAC) in the *Ptf1a* promoter at -5,092 bp from the initiation site, within a domain highly conserved between mouse and human, which specifically binds TCF1/2 proteins (data not shown). This finding suggests that TCF2 could regulate *Ptf1a* expression directly. However, the phenotype of *Tcf2*^{-/-} embryos is more severe than that of *Ptf1a*^{-/-} embryos, because in the absence of *Ptf1a* expression the dorsal pancreas is maintained and relatively developed, with endocrine cells still present (8, 35), indicating that other regulatory factors may contribute to *Tcf2* mutant phenotype. In this context, *Ipfl* and *Ngn3* were previously identified as direct target genes of TCF2 (36, 37). Yet, *Ipfl* was still detectable in the *Tcf2*^{-/-} pancreatic bud rudiment (Fig. 2), implying that TCF2 is not required for *Ipfl* initial induction. By contrast, the expression of *Ngn3*, a gene whose expression was transiently reduced in *Hnf6*^{-/-} embryos (29, 38), was completely abolished in *Tcf2*^{-/-} embryos. Because *Hnf6* expression also was

Fig. 4. Impaired early pancreatic cell differentiation in *Tcf2*^{-/-} embryos. (A–J) Immunohistochemical analysis of E11.5 control and *Tcf2*^{-/-} dorsal pancreatic buds. Anti-Ipf1 (A and B), anti-Hlxb9 (C and D), anti-Isl1 (E and F), anti-Pax6 (G and H), and anti-glucagon (I and J) antibodies were used on sagittal sections of WT and *Tcf2*-mutant embryos. (A and B) Ipf1 was detected in the remnant dorsal pancreatic bud (marked by dashed lines), but not in the duodenum (circled by a white line), of *Tcf2*^{-/-} embryos. (C and D) Hlxb9 also is expressed in this bud. (E–J) None of the early endocrine pancreatic markers, Isl1, Pax6, and glucagon, were detected in the dorsal pancreatic epithelium of *Tcf2* mutants. (E and F) Isl1, although absent in pancreatic epithelial cell clusters, is still expressed in the mesenchyme. (K–V) *In situ* hybridization analysis of control and *Tcf2*^{-/-} E11.5 dorsal pancreatic buds. In contrast to *lpf1*, *Ptf1a* and *Ngn3* are not expressed in the remnant dorsal pancreatic bud of *Tcf2* mutants (K, L, O, and P), whereas *Hnf6* is severely reduced (M and N). Whereas *Ihh* expression is abolished in *Tcf2*^{-/-} mutants (S and T), *Shh* is highly expressed in the anterior stomach with an expanded expression domain in posterior stomach and duodenum (Q and R) and induced *Ptc* expression (U and V).

severely reduced in *Tcf2*^{-/-} dorsal pancreatic bud, activation of *Ngn3* may require the concurrent action of *Tcf2* and *Hnf6*. Recent studies have reported a transient reduction in TCF2 levels in the pancreatic duct cells in *Hnf6*-deficient embryos

from E13.5 to E15.5, leading to the suggestion that *Hnf6* is upstream of *Tcf2* (38). Nevertheless, the more severe pancreatic phenotype of *Tcf2*-mutant embryos, compared with that of *Hnf6*^{-/-} embryos (29), indicates that this transcriptional hi-

Fig. 5. Proposed model for *Tcf2* function in the development of the pancreas and gut endoderm. (A) Expression domains of the transcription factors *Tcf2*, *lpf1*, and *Ptf1a*, as well as the signaling molecules *Shh* and *Ihh*, in the fore-midgut area of *Tcf2*^{+/+} and *Tcf2*^{-/-} embryos. *Tcf2* deficiency leads to an extremely reduced pancreatic bud expressing *lpf1* but missing *Ptf1a* expression, associated with a perturbed gut regionalization reflected by an expansion of *Shh* expression domain and an absence of *Ihh* and *lpf1* expression in stomach and duodenum. +, expressed gene; -, nonexpressed gene. (B) Proposed model of the regulatory network that governs differentiation of pancreatic cells. The diagram illustrates the epistatic relations of genes required for pancreas differentiation, leading to endocrine and exocrine cells. *Tcf2* is required early in pancreas development, activating *Ptf1a*, and regulating the *lpf1*-*Shh* network, with *Shh* repression vs. *Ihh* activation. TCF2 also activates *Ngn3* in endocrine precursor cells, in agreement with the hypothesis that TCF2-positive cells are the precursors of NGN3 positive cells (38).

erarchy most likely is not involved at earlier stages of pancreas development.

Regulatory circuits also are involved in regional specification of the gut endoderm. One important aspect of this regionalization is the restricted expression of *Ipfl* and *Shh*, essential to permit pancreas development (12). Several studies have suggested that *Shh* and *Ipfl* mutually repress their expression through a regulatory loop in the gut endoderm (1, 21, 30). In correlation with this finding, we found in *Tcf2*^{-/-} embryos an expanded domain of *Shh* expression in the posterior stomach and duodenum, whereas the *Ipfl* expression domain remained restricted to the rudimentary dorsal pancreatic bud but was absent in posterior stomach and duodenum (Fig. 4). Thus, *Tcf2* appears to regulate regional specification of the gut endoderm through the *Shh-Ipfl* network.

Taken together, these findings allow us to propose a model highlighting the critical role played by *Tcf2* in the control of pancreas development, in relation with the regionalization of the primitive gut (Fig. 5). We propose that in the absence of TCF2, *Ptf1a* expression is not induced, leading to defective specification of the ventral pancreas and a reduced dorsal pancreas, which is subsequently not maintained because of an altered regionalization of the gut through deregulation of Hh signaling.

This study provides further insight into the early molecular events controlling pancreas development in mice and the function of the transcription factor TCF2 in this process. Our observation of pancreas hypoplasia in two fetuses carrying novel *TCF2* mutations (A. L. Delezoide, C.H. and S.C., unpublished data) suggests that decreased levels of TCF2 also perturb normal pancreas growth and function in humans. The role played by *Tcf2* in pancreas development thus appears to be conserved during evolution. Then, understanding how *Tcf2* together with other regulatory molecules direct early pancreas development in mice may help to elaborate cell-replacement strategies for diabetes mellitus.

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