The tetraspanin Tm4sf3 is localized to the ventral pancreas and regulates fusion of the dorsal and ventral pancreatic buds

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During embryogenesis, the pancreas develops from separate dorsal and ventral buds, which fuse to form the mature pancreas. Little is known about the functional differences between these two buds or the relative contribution of cells derived from each region to the pancreas after fusion. To follow the fate of dorsal or ventral bud derived cells in the pancreas after fusion, we produced chimeric Elas-GFP transgenic/wild-type embryos in which either dorsal or ventral pancreatic bud cells expressed GFP. We found that ventral pancreatic cells migrate extensively into the dorsal pancreas after fusion, whereas the converse does not occur. Moreover, we found that annular pancreatic tissue is composed exclusively of ventral pancreas-derived cells. To identify ventral pancreas-specific genes that may play a role in pancreatic bud fusion, we isolated individual dorsal and ventral pancreatic buds, prior to fusion, from NF38/39 Xenopus laevis tadpoles and compared their gene expression profiles (NF refers to the specific stage of Xenopus development). As a result of this screen, we have identified several new ventral pancreas-specific genes, all of which are expressed in the same location within the ventral pancreas at the junction where the two ventral pancreatic buds fuse. Morpholino-mediated knockdown of one of these ventral-specific genes, transmembrane 4 superfamily member 3 (tm4sf3), inhibited dorsal-ventral pancreatic bud fusion, as well as acinar cell differentiation. Conversely, overexpression of tm4sf3 promoted development of annular pancreas. Our results are the first to define molecular and behavioral differences between the dorsal and ventral pancreas, and suggest an unexpected role for the ventral pancreas in pancreatic bud fusion.

KEY WORDS: Xenopus, Pancreatic bud, Tm4sf3, Tetraspanin, Annular pancreas

INTRODUCTION

The pancreas is a single endodermal organ that embryologically is derived from three distinct primordia: one dorsal and two ventral (Kelly and Melton, 2000; Kumar and Melton, 2003; Slack, 1995). The dorsal pancreatic bud arises first from the dorsal side of the duodenum immediately below the notochord, whereas the two paired ventral pancreatic buds develop slightly later adjacent to the hepatic diverticulum (Tremblay and Zaret, 2005). In mammals, the smaller left ventral bud usually regresses (Lammert et al., 2001; Lewis, 1911; Odgers, 1930), whereas in chick and Xenopus the two ventral buds will become part of the mature organ (Kelly and Melton, 2000; Kim et al., 1997). Each bud gives rise to different regions of the mature organ: the dorsal pancreas, which contributes to the body, neck and tail; and the ventral pancreas, which contributes to the head and uncinate process (Delmas, 1939; Uchida et al., 1999). In Xenopus laevis, the dorsal anlage is first apparent at NF35/36 at the level of the pronephros, whereas the two ventral buds develop slightly later at NF37/38 adjacent to the hepatic cavity, where it merges with the gastroduodenal cavity (Chalmers and Slack, 1998; Kelly and Melton, 2000; Pearl et al., 2009). [We refer to stages of Xenopus development as NF followed by the stage number as defined by Nieuwkoop and Faber (Nieuwkoop and Faber, 1967).] Following formation of these three separate buds, subsequent morphogenesis results, first, in the fusion of the two ventral buds by NF38/39 and, second, in the fusion of the dorsal and ventral buds at NF40.

Both endocrine and exocrine cells are found dispersed throughout the adult pancreas, but, in Xenopus, initial differentiation of these cell types occurs in a spatially and temporally distinct manner (Pearl et al., 2009). Endocrine cells are specified and arise initially only from the dorsal pancreas (Horb and Slack, 2002; Kelly and Melton, 2000). Beta cells are first specified prior to fusion of the dorsal and ventral pancreas at NF32, whereas alpha and delta cells are detected only in the pancreas from NF44/45. By contrast, acinar cell markers are first detected only in the ventral pancreas shortly after fusion at NF40, with expression spreading rapidly into the dorsal pancreas by NF44 (Horb and Slack, 2002). After NF46, however, endocrine and exocrine cells are present throughout the entire pancreas, with no dorsal-ventral differences. By contrast, in adult mammals dorsal-ventral differences are seen in the endocrine composition of islets. The dorsal islets contain normal shaped islets rich in insulin and glucagon cells, but few PP cells (Bencosme and Liepa, 1955; Suda et al., 1981; Wittingen and Frey, 1974). The exact opposite is seen in the head and uncinate process: the islets are irregular in shape, being rich in PP cells and poor in insulin and glucagon cells (Uchida et al., 1999; Yi et al., 2004). Specification of the dorsal pancreas is not essential for normal development. In humans, dorsal pancreas agenesis has been reported as an uncommon congenital defect that, in most cases, is not diagnosed until the individual develops other symptoms later.
in life, such as diabetes mellitus or pancreatitis (Gilinsky et al., 1985; Gunz et al., 1976; Gurson et al., 1970; Klein et al., 1994; Lechner and Read, 1966; Shah et al., 1987; Wang et al., 1990; Wildling et al., 1993). On the other hand, there are no reports of ventral pancreas agenesis. Two congenital defects, however, have been attributed to improper development of the ventral pancreatic bud: pancreas divisum and annular pancreas (Cano et al., 2007). Pancreas divisum is a relatively common pancreatic congenital anomaly, with a prevalence of 5-10% (Agha and Williams, 1987). It occurs when the ventral and dorsal ducts do not fuse, resulting in the persistence of the dorsal accessory pancreatic duct (Klein and Affronti, 2004; Quest and Lombard, 2000). Annular pancreas, on the other hand, is a rare congenital defect that occurs when the ventral pancreas forms a complete ring around the duodenum, causing an obstruction of the duodenum (Jimenez et al., 2004; Ladd and Madura, 2001). Several hypotheses have been proposed to explain the development of annular pancreas (Baldwin, 1910; Kamisawa et al., 2001; Lecco, 1910). These hypotheses attribute the embryological origin of annular pancreas to defective ventral pancreatic development, but this has not yet been proven. Although the genetic basis for this defect is not known, 42% of Indian hedgehog (Ihh) mutant mice developed annular pancreas (Hebrok et al., 2000). However, it is unclear how loss of Ihh contributes to the development of this condition.

To follow the fate of dorsal and ventral pancreatic bud-derived cells, we took advantage of the embryological benefits of Xenopus (Blitz et al., 2006; Pearl and Horb, 2008) and created chimeric transgenic Elas-GFP/wild-type embryos. We found that ventral pancreatic cells migrate extensively into the dorsal pancreas after fusion of the two buds during normal development, whereas the dorsal pancreas-derived cells do not. In addition, we also found that annular pancreatic tissue is populated exclusively by cells derived from the ventral pancreas. To uncover molecular genetic differences between the dorsal and ventral pancreatic buds that might explain the behavior of the ventral pancreatic bud, we isolated individual dorsal and ventral pancreatic buds prior to their fusion and compared their gene expression profiles using microarrays. As a result of this comparison, we identified several new dorsal and ventral-specific genes. Here, we present the functional analysis of one ventral specific gene, transmembrane 4 superfamily member 3 (tm4sf3). Using antisense morpholino knockdown techniques, we examined its role in early pancreas development and found that it was involved in the regulation of pancreatic bud fusion and acinar cell development. By contrast, overexpression of tm4sf3 was sufficient to promote development of annular pancreas. These results are the first to identify distinct behavioral and molecular differences between the dorsal and ventral pancreas.

MATERIALS AND METHODS

Embryological dissections

For the Elas-GFP transgenic/wild-type transplantation experiments, we fertilized eggs from Xenopus F2 Elas-GFP females with sperm from transgenic males, and simultaneously fertilized eggs from wild-type females with sperm from wild-type males. The embryos were grown at different temperatures overnight. Once the embryos reached NF19-20, we removed the vitelline envelopes and transplanted dorsal halves onto host embryos, from which we had removed an equivalently sized dorsal piece. The chimeric embryos were then placed in Noble agar to immobilize them, coverslips were placed on top of them to prevent the transplanted piece from moving and left to heal overnight. At NF44/45, we isolated whole guts from anesthetized tadpoles and photographed with the Leica DFC480 digital camera mounted onto a Leica MZ-16FA microscope. Individual liver/pancreas tissue samples were subsequently isolated from whole guts. The same method was followed to create chimeric embryos in which the entire endoderm was labeled, and to target tm4sf3 overexpression or knockdown to dorsal or ventral endoderm.

Microarray analysis

Individual dorsal and ventral pancreatic buds were isolated at NF38-39 from numerous different fertilizations over the course of 3 months. Tissue samples were frozen until sufficient numbers were isolated and total RNA was then isolated using Trizol. Each pancreatic bud contained between 1 and 5 ng of total RNA, and we collected between 1000-3000 individual buds for each sample, two dorsal replicates and two ventral replicates. RNA analysis, cDNA preparation and hybridization to the Genechip Xenopus Genome Array were performed by the UCI DNA & Protein MicroArray Facility (University of California, Irvine), a shared resource, affiliated with the Chao Family Comprehensive Cancer Center, an NCI-designated Comprehensive Cancer Center (http://dnaf.biochem.uci.edu/).

The results were evaluated using the Affymetrix Expression Console and MAS5 algorithm. As we had only two replicates for each sample (too small a number to use methods based on analysis of variance or integral methods such as RMA or PLIER), we employed the method of consecutive sampling and coincidence test (Guibault et al., 2006; Novak et al., 2006a; Novak et al., 2006b; Novak et al., 2002). In two-array comparisons, the genes are ordered according to mean signal intensity and grouped in bins containing n consecutive genes (in the present case, n=25). The standard deviation is then calculated for each bin and the standard deviation function in linear approximation is determined by regression. Subsequently, specific probability intervals are evaluated so that the distance of corresponding points at upper and lower boundaries measured in standard deviations is invariant. The genes above and below a given interval are identified for both pairs of replicates and genes common to both sets are listed. Only those candidate genes above (ventral) or below (dorsal) 0.95 probability interval in three or all four comparisons are listed in Tables S1 and S2 in the supplementary material. The data discussed in this publication have been deposited in NCBI’s Gene Expression Omnibus (Edgar et al., 2002), and are accessible through GEO Series accession number GSE13603 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE13603).

Plasmids, RT-PCR and whole-mount in situ hybridization

All clones were isolated by PCR from NF42 whole-gut cDNA, and confirmed by sequencing. For all constructs, primers were designed based on full-length sequence information obtained from GenBank, and the PCR products cloned into pCRII (Invitrogen). The GenBank Accession Number for tm4sf3 is NM_001087390. RT-PCR was performed on isolated dorsal and ventral pancreatic buds and normalized to EF1-α. Whole-mount in situ hybridization with single probes were performed as described using BM Purple (Horb et al., 2003). Complete information for each clone is available upon request.

Antisense morpholino and mRNA injections

Antisense morpholino oligonucleotides were designed by Gene Tools. The antisense morpholinos were designed either to the translation start or in the 5'UTR. Morpholinos were injected into the dorsal vegetal blastomeres at the eight-cell stage. For functional analysis, we selected only those samples where the morpholino targeted the entire anterior part of the gut. Targeting to the stomach, liver and pancreas was confirmed by monitoring fluorescence from labeled oligonucleotides, only after isolation of whole guts from injected embryos. Those samples where only half the stomach or half the pancreas was targeted were not selected. The sequences of the antisense morpholinos used are: tm4sf3 5'GATGCGAAGAGCCTTCTTGA-3'; tm4sf3 start, 5'CACCTTGCAACCACG-CCATTTGG-3'. For mRNA injections, tm4sf3 was cloned into CS2+ and mRNA made using the Ambion mMessage machine kit. tm4sf3 mRNA was injected along with gfp mRNA and targeting confirmed by examining fluorescence. Each of the mRNA and morpholino injection experiments was performed at least three times using different batches of embryos.
RESULTS

Ventral pancreas derived cells migrate extensively after fusion of the dorsal and ventral pancreas

To determine the spatial distribution of dorsal and ventral pancreatic bud cells in the pancreas after fusion, we monitored the fate of cells derived from each bud by selectively labeling either the dorsal or ventral pancreas. This was accomplished by producing chimeric wild-type/F2 Elas-GFP transgenic embryos in which either the dorsal or ventral pancreatic bud was derived from a transgenic donor embryo and thus GFP+ (Jarikji et al., 2007). The Elas-GFP transgene directs expression throughout the entire pancreas at early tadpole stages (Beck and Slack, 1999). Hence, when the ventral pancreatic bud is derived from an Elas-GFP transgenic embryo, the ventral pancreatic cells would be GFP+, whereas dorsal pancreatic cells would be unlabeled. After fusion has occurred, the fate of ventral pancreatic bud cells can be determined by examining the location of GFP+ cells in the pancreas; the same can be done when the dorsal pancreatic bud is derived from an Elas-GFP transgenic embryo.

To produce these chimeric embryos, we transplanted part of the dorsal half of a wild-type or Elas-GFP transgenic NF20 embryo, which included the archenteron roof from where the dorsal pancreas is derived (Chalmers and Slack, 2000), onto the ventral half of a transgenic or wild-type host (Fig. 1A,H). At this stage, the dorsal and ventral endoderm is separated by the archenteron, thus defining the boundary between the dorsal and ventral endoderm, and allowing for consistent dissections. These chimeric embryos were named either DtgVwt or VtgDwt to indicate whether the dorsal (D) or ventral (V) half of the embryo was derived from a transgenic Elas-GFP embryo (tg) or a wild-type embryo (wt). The chimeras were then cultured until tadpole stage NF42-44 at which time we isolated the whole guts and examined the fate of GFP+ cells in the pancreas.

When the ventral pancreas was derived from an Elas-GFP transgenic embryo (VtgDwt), we did not find a smooth transition between labeled and unlabeled cells in 70% of the recombinants (n=20) (Fig. 1B,C). Instead, ventral-derived GFP+ cells were found intermingled with dorsal unlabeled cells in VtgDwt chimeras (Fig. 1E,F). By contrast, when the dorsal pancreas was derived from an Elas-GFP transgenic embryo (DtgVwt), no dorsal GFP+ cells were found within the ventral region of the pancreas in any of the recombinants (n=22) (Fig. 1I,J). Unlabeled ventral-derived cells, however, were found within the labeled dorsal pancreas in DtgVwt chimeras (Fig. 1L,M). In both sets of chimeric embryos, ventral pancreas-derived cells were found within the dorsal pancreas, showing that ventral pancreas cells migrate more extensively than the dorsal pancreatic cells.

As the archenteron collapses after NF20 prior to gut formation, an alternative interpretation to our results is that ventral endoderm cells migrate into the dorsal pancreatic endoderm when the archenteron collapses, prior to pancreatic bud formation. Previous lineage tracing data in Xenopus showed that dorsal and ventral endoderm cells do intercalate within the intestine; however, they did not examine the relative fate of cells within the pancreas (Chalmers...
Fig. 2. Ventral endoderm cells do not mix with the prepancreatic dorsal endoderm after the archenteron closes. (A) Fluorescent image of a VtgDwt chimeric embryo at NF32 showing GFP expression throughout the entire tadpole, but lacking in the dorsal region. (B) Isolated endoderm from NF35/36 VtgDwt chimeric tadpole showing GFP fluorescence throughout the ventral endoderm, but lacking in the dorsal-most region of the endoderm. (C) Double in situ hybridization for ptf1a (red) and GFP (purple) of endoderm shown in B. No gfp mRNA is detectable in the ptf1a expression domain. (D) Fluorescent image of a DgVwt chimeric embryo at NF32 showing GFP expression only in the dorsal part of the tadpole. (E) Isolated endoderm from NF35/36 tadpole showing GFP fluorescence only in the dorsal layer of the endoderm. (F) In situ hybridization for ptf1a shown in G. The ptf1a expression domain is located in the dorsal layer of the endoderm. dp, dorsal pancreas.

and Slack, 2000). Therefore, to determine whether ventral endoderm cells migrated into the dorsal pancreatic endoderm during early tail bud stages when the archenteron collapses, we performed the same recombination experiments above, but instead of just labeling the pancreas the entire dorsal or ventral endoderm was labeled. We injected GFP mRNA into the vegetal pole of wild-type embryos, thus labeling the entire endoderm, and created chimeric embryos using GFP-injected and noninjected wild-type embryos (Fig. 2A,D). At NF35/36, we dissected out the entire endoderm and examined them for expression of ptf1a and gfp to determine whether ventral endoderm cells were present in the dorsal pancreatic bud. The dorsal-derived archenteron roof endoderm cells are a thin layer of cells and contribute only a small amount to the entire endoderm; they can be clearly seen when either the dorsal or ventral endoderm is labeled (Fig. 2B,E). When the ventral endoderm was labeled with GFP, we did not find any GFP+ cells within the dorsal pancreatic bud, as marked by ptf1a expression (Fig. 2B,C; n=10). By contrast, when the dorsal endoderm was labeled, GFP expression co-localized with ptf1a expression (Fig. 2E,F; n=6). In agreement with these results, when we dissected pancreata from chimeric Elas-GFP/wt embryos at NF40 (immediately after fusion), we did not detect intermingling of GFP+ and GFP- cells (data not shown). In conclusion, these results agree with our whole-mount in situ data, we isolated dorsal and ventral pancreatic anlagen from NF38/39 (2.5 days) and compared their gene expression profiles (Fig. 3A). In addition to the above data, our previous results also suggested that the ventral pancreas would be enriched with exocrine-specific genes, whereas the dorsal pancreatic bud would be enriched with endocrine-specific genes (Horb and Slack, 2002; Kelly and Melton, 2000). At early stages, just after fusion of the pancreatic buds, expression of several endocrine-specific markers (neuroD, pax6 and insulin) was localized to the dorsal region of the pancreas (Fig. 3B-F). By contrast, we found that Nkx2.2, another endocrine transcription factor, was expressed in both dorsal and ventral regions of the pancreas (Fig. 3F). These known endocrine-specific genes provided us with positive controls for the dorsal pancreatic bud.

Isolation of dorsal and ventral pancreatic anlagen and their genetic differences

Based on the above results, we hypothesized that genes involved in regulating dorsal-ventral bud fusion and migration would be localized to the ventral pancreas. Therefore, to identify ventral pancreas-specific genes, we isolated dorsal and ventral pancreatic buds prior to their fusion at NF38/39 (2.5 days) and compared their gene expression profiles (Fig. 3A). In addition to the above data, our previous results also suggested that the ventral pancreas would be enriched with exocrine-specific genes, whereas the dorsal pancreatic bud would be enriched with endocrine-specific genes (Horb and Slack, 2002; Kelly and Melton, 2000). At early stages, just after fusion of the pancreatic buds, expression of several endocrine-specific markers (neuroD, pax6 and insulin) was localized to the dorsal region of the pancreas (Fig. 3B-F). By contrast, we found that Nkx2.2, another endocrine transcription factor, was expressed in both dorsal and ventral regions of the pancreas (Fig. 3F). These known endocrine-specific genes provided us with positive controls for the dorsal pancreatic bud.

To determine whether our dissections of dorsal and ventral pancreatic buds were accurate, we examined whether differential expression of these endocrine-specific markers was evident in our isolated buds. In agreement with our whole-mount in situ data, we found pax6, neuroD, insulin and hhexb9 to be enriched in the dorsal pancreatic bud, whereas nkh2.2 was present in both dorsal and ventral pancreatic buds (Fig. 3G). These results confirmed that our embryological dissections were accurate and that molecular genetic
differences were faithfully maintained and detected in these isolated buds. We therefore used these samples to screen the *Xenopus* Affymetrix Genechip. Four different samples, two dorsal and two ventral, were reverse transcribed, labeled and hybridized to the *Xenopus* Affymetrix Genechip. Results were analyzed using the Affymetrix Expression Console and MAS5 algorithm, and because we only had two replicates, we employed the method of consecutive sampling and coincidence test (Guilbault et al., 2006; Novak et al., 2006a; Novak et al., 2006b; Novak et al., 2002). The results of this analysis yielded 158 genes as being ventral enriched and 68 genes as being dorsal enriched (see Tables S1 and S2 in the supplementary material).

In agreement with our hypothesis, the microarray data revealed dorsal enrichment of several endocrine differentiation markers [insulin, prohormone convertase 2 (pcsk2-A), secretogranin III (scg3-A), carboxypeptidase E and 7B2 pituitary protein] and pancreatic transcription factors (neuroD, pax6 and hlx9) (see Table S1 in the supplementary material). In fact, insulin II, insulin I and pcsk2-A were three of the most highly enriched dorsal genes, with 38-, 37- and 13-fold greater expression in the dorsal bud, respectively (see Table S1 in the supplementary material). As a first step to validate the microarray data, we examined the expression of several dorsal-enriched genes in isolated dorsal and ventral pancreatic buds by RT-PCR. We chose 12 of the most highly enriched genes (>2.5-fold) and found all to be highly enriched in the dorsal pancreas, which validates the microarray data (Fig. 4).

To define more accurately their localization within the pancreas, we examined the spatial expression of 12 dorsal-enriched genes at NF40 in isolated liver/pancreas tissue samples by whole-mount in situ hybridization. We did not use NF38/39 pancreatic buds (the stage when the buds were isolated for the microarray) because the individual pancreatic buds are much too small to process for whole-mount in situ analysis, and are very difficult to isolate in large numbers. NF40 is the earliest stage after fusion of the dorsal and ventral pancreatic buds when the tissue samples are easier to isolate, and still maintain their dorsal-ventral differences, as was seen for insulin, neurod and pax6 (Fig. 3C-E). In addition to these three, we confirmed dorsal-specific localization for six other genes identified in the microarray: hlx9, pcsk2-A, scg3-A, frzb-1, brunol1 and insm1 (Fig. 4C-E and data not shown). The expression pattern for eight of these genes was very similar, showing punctate domains of expression within the dorsal pancreas. Only frzb-1 showed a different pattern of expression, being localized to the dorsal pancreatic mesoderm (data not shown). Overall, most of the top 20 dorsal-enriched genes are known endocrine-specific genes, whereas the majority of the remaining 48 genes are either unknown ESTs or are known genes that have not been studied in pancreas development.

Although many more genes were identified as enriched in the ventral pancreas (see Table S2 in the supplementary material), much less is known about the molecular genetics of ventral pancreas development. As with the dorsal subset, we initially selected a subset of genes with greater than twofold enrichment and confirmed their differential expression in isolated dorsal and ventral pancreatic buds by RT-PCR (Fig. 4B). Those selected were transmembrane 4 superfamily 3 (tm4sf3), transmembrane 4 superfamily member 4 (tm4sf4), inter-alpha trypsin inhibitor heavy chain 2 (itih2), ephrin B1, Ets2 repressor factor (erf), anterior gradient 2 (agr2) and Xl.1424. Ventral bud-specific expression was confirmed for five out of these seven genes — tm4sf3, tm4sf4, itih2, agr2 and Xl.1424 (Fig. 4B) — whereas ephrin B1 and erf were equally expressed in the dorsal and ventral bud fractions.

Tm4sf3 is required for acinar cell differentiation, dorsal-ventral pancreatic bud fusion and stomach development

One of the first ventral-enriched genes we chose to study was transmembrane 4 superfamily member 3 (tm4sf3), which was enriched 10-fold in the ventral bud fraction. Tm4sf3 belongs to the tetraspanin family of proteins, which are cell-surface proteins that span the membrane four times, and are present in many different organisms (Hemler, 2005; Zoller, 2009). There are 33 vertebrate tetraspanins that are implicated in regulating cell migration and fusion, although their exact functions are not fully characterized (Berditchevski, 2001; Hemler, 2005; Lazo, 2007; Levy and Shoham, 2005a; Levy and Shoham, 2005b). *TM4SF3* was originally identified as the cDNA for the human tumor-associated antigen CO-029 (D6.1 in rat) expressed in gastric, colon rectal and pancreatic
carcinomas (Szala et al., 1990). A previous microarray screen in mice identified Tm4sf3 as being expressed in Pdx1-eGFP<sup>+</sup> cells, although it was only expressed in the duodenum (Gu et al., 2004). The role of tm4sf3 during embryonic development, however, has not been investigated.

To determine where tm4sf3 was expressed in the whole embryo, we examined the developmental expression of Xenopus tm4sf3 by whole-mount in situ hybridization. We did not detect expression of tm4sf3 during gastrula, neurula or tail bud stages. Beginning at tadpole stage 40, tm4sf3 expression was found throughout the stomach/duodenum (Fig. 5A), in agreement with the previous data in mice. As the gut continued to develop, expression of tm4sf3 increased in the stomach/duodenum, and was also found enriched in the bile duct (Fig. 5B). As much of the pancreas is obscured by the stomach and duodenum at these stages, we examined its expression in the pancreas in isolated liver/pancreas samples. We found abundant expression of tm4sf3 in the ventral pancreas at NF40, with low-level expression also in the bile duct (Fig. 5C). As mentioned above, the expression in the ventral pancreas was localized to the junction where the two ventral pancreatic buds fuse. By NF44 the expression of tm4sf3 in the pancreas decreased, whereas its expression in the developing bile duct increased (Fig. 5D).

To address the function of tm4sf3 in early pancreas development, we designed an antisense morpholino to the 5′ UTR to inhibit its translation (see Materials and methods). As a control, we used a second morpholino designed to the translation start site of tm4sf3 that did not affect translation. These tm4sf3 morpholinos were injected at the eight-cell stage into the two dorsal vegetal blastomeres to target the anterior endoderm. Injection of 20 ng of the tm4sf3-utr morpholino resulted in a phenotype of a small ventral pancreas, a normal dorsal pancreas and liver; the stomach was also smaller and abnormal. The dorsal and ventral pancreatic buds had not fused and remained separate (Fig. 6). This phenotype was seen in 86% of injected tadpoles (n=197). Expression of ptf1a in the non-fused dorsal and ventral pancreatic buds was normal at NF41 (Fig. 6A,B). Expression of the liver marker hex was also normal, though in almost every case the liver was fused with the intestine (Fig. 6C,D). By contrast, we found reduced expression of the stomach/duodenum marker frp5 (Fig. 6E,F). Targeting of the morpholino to only the dorsal pancreas did not affect fusion of the dorsal and ventral pancreatic buds (see below). Serial histological sections confirmed the lack of fusion between the dorsal and ventral pancreatic buds (Fig. 6G-V). Three-dimensional reconstruction of the whole gut demonstrates clearly that, in contrast to control, the dorsal and ventral pancreatic buds remained separate (Fig. 6W,X).

To determine which pancreatic cell types were affected by loss of tm4sf3, we performed whole-mount in situ hybridization on whole guts for different pancreas markers. We were unable to detect expression of the late acinar differentiation marker elastase (Fig. 6J). As tm4sf3 is not expressed in the dorsal pancreas, we were surprised to find that expression of elastase was inhibited in the unfused dorsal pancreatic bud. To rule out the possibility that loss of late acinar differentiation markers was due to nonspecific results from tm4sf3 morpholino expression in the dorsal pancreatic bud, we specifically targeted knockdown of Tm4sf3 in the dorsal pancreas by creating chimeric tm4sf3 morpholino/wild-type embryos. Dorsal halves of NF20 embryos injected with tm4sf3 morpholino were transplanted onto ventral halves of wild-type embryos and grown to NF44, at which time we isolated the whole guts. Unlike the previous morpholino injection results (that were targeted to the entire anterior endoderm) fusion of the dorsal and ventral pancreatic buds and expression of elastase were normal (data not shown).

We next determined whether initial differentiation of exocrine pancreatic cells was normal in Tm4sf3 knockdown embryos by examining whether expression of the early exocrine differentiation marker, pancreatic protein disulphide isomerase (Xpdi), was affected by loss of tm4sf3. In contrast to elastase, we found normal expression of Xpdi in the unfused dorsal and ventral pancreatic buds of tm4sf3 morphants (Fig. 6K,L). We believe the reason for these differences in effects on exocrine differentiation markers is due to the fact that Xpdi is expressed in both dorsal and ventral pancreatic buds before they have fused at NF39, earlier than elastase (Afelik et al., 2004). It is possible that dorsal pancreas expression of late acinar differentiation markers is dependent on cell-cell interactions that initiate in the ventral pancreas, and knockdown of Tm4sf3 in the ventral pancreas disturbs this initial expression in the ventral pancreas.

In contrast to the effects on exocrine differentiation, we found normal expression of the endocrine marker insulin in the dorsal pancreas (Fig. 6M,N). However, in 45% of cases ectopic insulin expression was also detected in the ventral pancreatic bud (Fig. 6N). At this early stage, insulin expression is normally detected only in the dorsal pancreas, and is not expressed in the ventral part of the pancreas until NF45-46 (Horb and Slack, 2002). With regards to the other endocrine cell types, we found reduced expression of somatostatin and glucagon in the stomach and duodenum (Fig. 6M-P). Expression of these two endocrine markers is not detected in the pancreas until after NF44 (Pearl et al., 2009). In agreement with its spatial distribution, these results demonstrated that Tm4sf3 was necessary for pancreatic acinar cell differentiation and stomach/duodenal endocrine cell differentiation, but not for specification of endocrine beta cells. In addition to its effects on cell fate, we also found that knockdown of Tm4sf3 affected the proliferation of endodermal cells. In tm4sf3 morphants, there was a 51% decrease in endodermal phospho histone H3 cells at NF40 (data not shown).

To confirm that the knockdown phenotype is directly related to the loss of Tm4sf3, we attempted to rescue the morpholino-induced phenotype by co-injecting tm4sf3 mRNA lacking the 5′ UTR along...
Dorsal-ventral pancreatic bud fusion

**Fig. 6. Tm4sf3 is required for acinar and stomach/duodenum development.** (A,B) Expression of the general pancreas marker Ptf1a is normal in tm4sf3 morphants (n=7), but also reveals that the dorsal (dp) and ventral (vp) pancreatic buds have not fused. The liver position (L) has changed compared with normal and is present below the pancreatic buds in the region of the stomach/duodenum. (C,D) Expression of the liver marker Hex was normal (n=18). P, pancreas. (E,F) Expression of the stomach/duodenum marker frp5 was almost completely abolished (40/44). (G,H) Schematic highlighting the phenotype seen in Tm4sf3 knockdown embryos. The pancreas normally grows behind the duodenum, as illustrated by the darker shading in the wild-type gut. In tm4sf3 morphants, the dorsal and ventral pancreatic buds do not fuse. (I,J) The acinar cell marker elastase was substantially reduced or completely abolished in Tm4sf3 knockdown embryos (19/27 absent, 8/27 reduced). (K,L) Expression of the early acinar differentiation marker XPDPip was normal in the dorsal and ventral pancreatic buds in tm4sf3 morphants (n=42). (M,N) Insulin expression was normal in the dorsal pancreatic buds in a little over half the cases (16/29). Interestingly, ectopic insulin expression was found in the ventral pancreatic bud in 45% of our samples (13/29). (O,P) No expression of glucagon or somatostatin was detected in the stomach/duodenum (n=8). At this stage, neither is yet expressed in the pancreas. (Q-V) Representative serial sections from an individual isolated NF42 whole gut that were previously stained for ptf1a expression. (W,X) Three-dimensional reconstruction of the samples based on all serial sections. Pancreas is blue, liver is pink, intestine is yellow and the gall bladder is green.

With 20 ng of the morpholino. At NF42, we examined the resultant tadpoles for morphological rescue of dorsal-ventral pancreatic bud fusion and acinar differentiation. In control embryos, the dorsal and ventral pancreatic buds have fused and elastase expression is detected throughout the entire pancreas (Fig. 7A). As previously demonstrated, the dorsal and ventral pancreatic buds did not fuse and no expression of elastase was detected in 84% (n=58) of tm4sf3 morphants (Fig. 7B). When 1.8 ng of tm4sf3 mRNA (lacking the 5′UTR) was co-injected with 20 ng of tm4sf3 morpholino, expression of elastase was restored and the dorsal and ventral pancreatic buds fused in 53% of injected embryos (Fig. 7C, n=112). Forty-two percent of injected embryos still showed the knockdown phenotype, whereas 5% developed annular pancreas, indicative of an overexpression phenotype (see below). At mRNA doses greater than 1.8 ng, we observed less of a rescue to normal morphology and instead saw increased development of annular pancreas (data not shown). These results demonstrate that the morpholino-induced phenotype is specifically due to the loss of Tm4sf3.

**Tm4sf3 promotes annular pancreas formation**

We next examined whether tm4sf3 was sufficient to promote ectopic migration and fusion of the dorsal and ventral pancreas by overexpressing tm4sf3 mRNA. Following the same procedure as with the morpholino, tm4sf3 mRNA was injected into the two dorsal vegetal blastomeres at the eight-cell stage. At doses lower than 1 ng, we did not observe any effects, whereas at 1.6 ng we observed a phenotype of annular pancreas in 79% of injected embryos (n=140). Within the ectopic pancreas, we detected abundant expression of acinar differentiation markers (Fig. 8A,B), but no endocrine marker expression (Fig. 8C,D). Development of other parts of the anterior endoderm was normal, and we did not observe any ectopic development of stomach or duodenal tissue (Fig. 8E,F). No change in expression of the early pancreas markers ptf1a and pdx1 was observed at NF35 in tm4sf3 injected tadpoles (data not shown). To determine whether the induction of annular pancreas by tm4sf3 was due to increased proliferation, we stained injected embryos for phospho histone H3, but did not find any significant increase in proliferation within the annular pancreas (data not shown). Development of annular pancreas was confirmed in histological sections (Fig. 8C,F,J), and can be clearly seen in the three-dimensional reconstruction (Fig. 8L).

The fact that the only phenotype observed upon tm4sf3 overexpression was development of annular pancreas suggested that tm4sf3 overexpression was effective only in the ventral pancreas. To determine whether this was indeed the case, we targeted tm4sf3 overexpression to the dorsal pancreas by creating chimeric tm4sf3 mRNA/wild-type embryos. As outlined above with the tm4sf3 morpholino, we transplanted dorsal halves of NF20 embryos overexpressing tm4sf3 mRNA onto ventral halves of wild-type embryos. In these embryos, however, we did not observe development of annular pancreas (data not shown).

One of the ways in which tetraspanins have been shown to affect cell migration is by regulating integrin signaling through ligand-induced internalization (Berditchevski and Odintsova, 2007), and TM4SF3 has been shown to interact with integrins that affect cell motility and metastasis in colon, liver and pancreatic cancer cells (Claas et al., 1998; Claas et al., 2005; Gesierich et al., 2005; Herlevsen et al., 2003). The promotion of cell migration by another metastasis-associated tetraspanin, CD151, was found to be dependent on its ability to regulate integrin trafficking, as mutation of the C-terminal endocytosis/sorting motif in CD151 abolished its ability to promote cell migration (Liu et al., 2007). Similar tyrosine-based sorting motifs (YXXΦ) have been identified within the C terminus of 12 other tetraspanins, including TM4SF3 (Berditchevski
and Odintsova, 2007). To determine whether Tm4sf3 function is dependent on its interaction and internalization of integrins, we created a mutant Tm4sf3 without the last seven amino acids YCQIGKK (Tm4sf3Δc), and examined whether it was still able to promote formation of annular pancreatic buds. We found that overexpression of tm4sf3Δc induced the development of annular pancreas (n=106, data not shown). However, the frequency with which tm4sf3Δc induced annular pancreas was slightly lower than that observed for tm4sf3 injections carried out at the same time: 51% (54/106) when compared with 71% for tm4sf3 (32/45). If integrin signaling were involved in tm4sf3 promotion of ectopic migration and fusion of the pancreas, then we would not have expected to find development of annular pancreas in tm4sf3Δc-injected embryos. These results demonstrate that tm4sf3 promotion of annular pancreas formation is not dependent on its interaction with integrins.

**DISCUSSION**

Little information exists pertaining to the functional relevance of the embryological origin of the pancreas from separate dorsal and ventral pancreatic buds. In this study, we demonstrate that ventral, and not dorsal, pancreatic bud cells migrate extensively after fusion. We also find that annular pancreatic tissue is populated exclusively by ventral pancreas derived cells. By comparing isolated dorsal and ventral pancreatic buds we have identified molecular genetic differences between them, and our identification of ventral pancreas specific genes is the first demonstration of such a subset of genes. Of these, we characterized the function of Tm4sf3 and defined a new role for it in pancreatic bud morphogenesis. This is the first study to examine differences between the dorsal and ventral pancreatic buds and demonstrate distinct functions for each bud.

By selectively labeling either the dorsal or ventral pancreas, we determined the fate of ventral and dorsal pancreatic bud cells in normal development and found that cells from the ventral pancreatic bud migrate extensively into the dorsal bud after fusion, whereas dorsal pancreatic bud cells do not. By recombining dorsal and ventral halves of transgenic F2 Elas-GFP embryos with wild-type embryos, we were able to selectively label either the dorsal or ventral pancreatic bud. When the dorsal pancreas was derived from transgenic embryos, we found a sharp boundary between unlabeled ventral pancreatic bud cells and GFP+ dorsal pancreatic bud cells. By contrast, when the ventral pancreas was derived from Elas-GFP embryos, we found GFP expression throughout the dorsal pancreas after fusion. These results are the first to directly examine the final spatial location of dorsal or ventral bud cells in the pancreas after fusion of the two buds.

One congenital disorder associated with inappropriate development of the ventral pancreas is annular pancreas, which occurs when the pancreas completely encircles the duodenum, causing a partial obstruction of the duodenum. Several different theories have been put forth to explain this developmental anomaly, including Baldwin’s hypothesis and Lecco’s theory (Baldwin, 1910; Cano et al., 2007; Kamisawa et al., 2001; Lecco, 1910). Although the accepted notion is that the annular pancreas is derived from ventral pancreatic bud cells, this has not been proven. In our chimeric embryos, we identified several different cases of annular pancreas, allowing us to lineage trace the fate of cells that populated the annular pancreas. Our results demonstrate that it is indeed ventral pancreatic cells that populate the annular pancreas. The fact that we find GFP+ ventral pancreatic cells extending completely across the duodenum supports the notion that excessive migration of ventral pancreatic cells is responsible for the development of annular pancreas.

One the main phenotypes associated with the knockdown of Tm4sf3 was the lack of fusion of the dorsal and ventral pancreatic buds. Several reasons may explain this phenotype. The first explanation, which we favor, is that Tm4sf3 directly regulates the migration of ventral pancreatic bud cells. The fact that annular
pancreas develops in the gain-of-function phenotype supports this notion. An alternative explanation is that Tm4sf3 function is required only during fusion of the dorsal and ventral pancreatic buds, and not for migration. Although we cannot discount this possibility, we find it unlikely because if Tm4sf3 were simply involved in mediating fusion, then the buds should have migrated towards each other and simply not fused, but this is not what we observed. In conclusion, we believe that Tm4sf3 directly regulates the migration of ventral pancreatic bud cells.

Separate from the effects on pancreatic bud morphogenesis, the early appearance (stage 42) of endocrine β cells in the ventral pancreatic bud in tm4sf3 morphants has interesting implications. In normal development, insulin is not expressed in the ventral region of the pancreas until NF45/46. This differentiation of β cells in the ventral pancreatic bud 3 days earlier in Tm4sf3 knockdown tadpoles suggests that Tm4sf3 may also have an unexpected role in the repression of endocrine cell differentiation. Identification of the molecular differences between the ventral pancreatic buds in Tm4sf3 knockdown and control tadpoles will help to identify genes that need to be repressed to allow differentiation of β cells in the ventral pancreas.

Our study is the first to define defective ventral pancreas development with normal liver and dorsal pancreas development. Previous results identified a bi-potential precursor population for the ventral pancreas to be expressed in the pancreas from stage 18 up to stage 42, and the absence of expression in these cells was associated with the formation of a short third branch. In the current study, we analyzed the expression of a specific subset of genes in the ventral pancreas using Tm4sf3 knockdown embryos. Our findings suggest that Tm4sf3 may have an important role in the development of the ventral pancreas.

We also found that the expression of Tm4sf3 is not only associated with the differentiation of β cells, but also with the development of the dorsal pancreas. This is consistent with previous studies that have shown that Tm4sf3 is expressed in both the dorsal and ventral pancreas, and that its expression is required for the proper development of both glands.

In conclusion, our study provides new insights into the role of Tm4sf3 in pancreatic development, and highlights the importance of understanding the molecular mechanisms that regulate the development of both the dorsal and ventral pancreas. Further research is needed to elucidate the precise role of Tm4sf3 in the development of the pancreas, and to understand how this gene functions in the context of other genes and signaling pathways involved in pancreatic development.


