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The *Gata5* target, *TGIF2*, defines the pancreatic region by modulating BMP signals within the endoderm

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Mechanisms underlying regional specification of distinct organ precursors within the endoderm, including the liver and pancreas, are still poorly understood. This is particularly true for stages between endoderm formation and the initiation of organogenesis. In this report, we have investigated these intermediate steps downstream of the early endodermal factor *Gata5*, which progressively lead to the induction of pancreatic fate. We have identified *TGIF2* as a novel *Gata5* target and demonstrate its function in the establishment of the pancreatic region within dorsal endoderm in *Xenopus*. *TGIF2* acts primarily by restricting BMP signaling in the endoderm to allow pancreatic formation. Consistently, we found that blocking BMP signaling by independent means also perturbs the establishment of pancreatic identity in the endoderm. Previous findings demonstrated a crucial role for BMP signaling in determining dorsal/ventral fates in ectoderm and mesoderm. Our results now extend this trend to the endoderm and identify *TGIF2* as the molecular link between dorsoventral patterning of the endoderm and pancreatic specification.

KEY WORDS: BMP, Gata5, TGIF2, Endoderm, Pancreas, *Xenopus*, Mouse

INTRODUCTION

The endodermal germ layer is fated to form the digestive and respiratory systems as well as associated organs, such as the liver and pancreas (Tam et al., 2003). Over the past few years extensive investigation has contributed to the elucidation of the molecular mechanisms underlying initial endoderm formation (Dickinson et al., 2006; Ober et al., 2003; Sinner et al., 2006; Tam et al., 2003; Taverner et al., 2005). However, we still know relatively little about subsequent endodermal patterning and how different domains of the endoderm progressively become specialized to generate distinct organ primordia. In this study, we have focused on the mechanisms underlying the establishment of the pancreatic domain within the endoderm.

Increasing evidence suggests that regionalization of the endoderm in vertebrates occurs at relative early developmental stages, beginning with a broad patterning within the endoderm as it first emerges during gastrulation (Lewis and Tam, 2006; Wells and Melton, 2000; Zorn et al., 1999). In the mouse embryo, the definitive endoderm might acquire positional identity already as it exits the primitive streak, such that cells recruited earlier will form the foregut and those recruited later will contribute to the posterior gut (Lawson and Pedersen, 1987; Lewis and Tam, 2006). In frog and zebrafish, gene expression and fate mapping analysis has unveiled the existence of a significant patterning along both the anteroposterior and dorsoventral axes within the endoderm long before organogenesis (Chalmers and Slack, 2000; Costa et al., 2003; Warga and Nusslein-Volhard, 1999). For instance, in *Xenopus* embryos dorsal endoderm explants, once dissected from early gastrula stage and cultured alone, express pancreatic markers, while ventral explants do not (Kelly and Melton, 2000). This suggests that as early as gastrula stage the endoderm is broadly regionalized, and prospective pancreatic endoderm forms in the quarter of the embryo around the dorsal blastopore lip. Fate map analysis of the *Xenopus* endoderm at neurula stage has shown that the pancreatic primordia emerges from two distinct regions of endoderm (dorsal and most anterior/ventral endoderm) (Chalmers and Slack, 2000). This is in line with the pancreatic specification map of the gastrula embryo, as both regions originate from dorsal endoderm and, through gastrulation movements, acquire different positions at neurula stage (Keller, 1975).

The molecular basis of this early endodermal patterning is clearly less well understood than those of the ectoderm and mesoderm, but several observations suggest that the molecular signals regulating early events of regionalization are shared among the three germ layers (Harland and Gerhart, 1997; Henry et al., 1996; Sasai et al., 1996; Zorn et al., 1999). For instance, TGFβ signaling influences anterior specification and patterning in both mesoderm and endoderm (Henry et al., 1996; Zorn et al., 1999). Furthermore, extracellular BMP antagonists, released from the dorsal mesoderm, specify dorsal fates within the ectoderm (neural tissue) and the mesoderm (notochord and somites) (Harland and Gerhart, 1997), and may also promote endoderm of dorsal character (Chen et al., 2004; Sasai et al., 1996). However, the direct role of BMP signaling in dorsal/ventral patterning of the endoderm has not been established. More importantly, how this early patterning of the endoderm leads to the establishment of specific organ domains, such as the pancreas, has yet to be molecularly characterized.

Indeed, at present, there is a significant gap in our knowledge of endodermal players acting in the window of time between the early stages of endoderm formation and the expression of the pancreatic marker *Pdx1* (also known as *Xlhbox8* in *Xenopus*) (Gamer and Wright, 1995; Jonsson et al., 1994; Kelly and Melton, 2000; Offield et al., 1996), which is induced just before organogenesis. A molecular understanding of this period of time will explain how the endoderm is progressively patterned to generate the presumptive pancreatic tissue.

Among the different transcription factors implicated in early embryonic endoderm formation, the Gata-type zinc-finger transcriptional activators play a prominent role (Holtzinger and Evans, 2005; Reiter et al., 2001; Tam et al., 2003; Weber et al., 2000; Zhao et al., 2005). Expression of *Gata4*, *Gata5* and *Gata6* has been described in endodermal cells of the embryo from early gastrula stage onwards,

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becoming restricted to anterior endodermal derivatives, such as the prospective liver and pancreas, as development proceeds in all vertebrates species so far analyzed (Tam et al., 2003; Weber et al., 2000). Although a functional redundancy during endoderm specification seems to exist among different Gata factors (Afouda et al., 2005; Holtzinger and Evans, 2005; Zhao et al., 2005), loss of *gata5* in the *faust* zebrafish mutant is sufficient to cause significant loss of early endoderm and, specifically, of anterior endodermal derivatives, such as the liver and the pancreas (Reiter et al., 2001). In addition, gain-of-function experiments in *Xenopus* demonstrate that *Gata5* is a potent inducer of endodermal fate and, specifically, of regionally restricted endodermal markers, such as *Pdx1* (Afouda et al., 2005; Weber et al., 2000), at tailbud stage. The cascade of molecular events downstream of *Gata5* in the endoderm leading to *Pdx1* induction is yet to be defined.

To begin to dissect this cascade we used a microarray approach to identify genes that operate between *Gata5* and *Pdx1* in *Xenopus* embryos. Overall, we identified 141 genes with expression that changes in response to *Gata5*. In this report, we present a subset of *Gata5* putative targets that are expressed in anterior endodermal derivatives, and provide an in-depth analysis of one of these targets, the TGFβ-induced factor 2 [also known as TG-interacting factor 2 (TGIF2)] (Imoto et al., 2000; Melhuish et al., 2001). We show that *TGIF2* defines the pancreatic region by modulating the TGFβ pathway in the endoderm, highlighting the prominent role of BMP inhibition in the regionalization of the pancreatic domain within the endoderm. Importantly, our study uncovers a crucial intermediate step in pancreas formation, in which *TGIF2* acts as a molecular connector between dorsoventral patterning of the endoderm and specification of pancreatic fate, linking *Gata5* to *Pdx1* induction.

MATERIALS AND METHODS

Embryo manipulations

Microinjections and dissections were performed as described (Spagnoli and Brivanlou, 2006). For the morpholino antisense oligonucleotides used in this study, see Fig. S3A in the supplementary material. The sequences of the morpholino antisense oligonucleotides against *Xenopus* chordin have been published (Oelgeschlager et al., 2003). The oligonucleotides were purchased from GeneTools LLC.

Microarray and plasmid construction

DNA microarrays were prepared as described (Altmann et al., 2001; Munoz-Sanjuan et al., 2002). Sample preparation and hybridization was performed as described previously (Munoz-Sanjuan et al., 2002) and below. The hybridization and scan of slides were performed at the Genomics Core Facility of the Rockefeller University. Array images files were gridded and analyzed using GenePix Pro image software and the data uploaded into GeneTraffic software. Sequences of the clones identified in the array were assembled using Sequencher 4.2.2 and blasted against public databases. The coding region of *Xenopus* Gata5 (kind gift of Todd Evans, AECOM, NY) was subcloned into pCS2++ by PCR. The plasmids Gata5-GR and Gata6-GR were generously provided by Roger Patient (University of Nottingham, UK). The majority of the clones regulated in the array contained full-length genes, including the clone 8B1/*xTGIF2*. The flag-tagged *xTGIF2* was generated by PCR and ligated into pCS2++. Full-length *mTgif2* (BC053438) was purchased from Open Biosystems and used for the rescue experiments. DN-*Alk3* corresponds to truncated *Xenopus* BMPRI (1-744 nt) (Suzuki et al., 1995), and CA-*Alk3* was a gift from Bill Smith (UC Santa Barbara, CA). The RNAs for all these constructs were synthesized on *Asc*I-linearized templates, with SP6 RNA polymerase using the mMessage mMachine Kit (Ambion).

In situ hybridization analysis

Whole-mount in situ hybridizations were performed according to Harland (Harland, 1991). In situ hybridization on cryostat sections was done as in Schaeren-Wiemers and Gerfin-Moser (Schaeren-Wiemers and Gerfin-Moser, 1993). In situ probes from array clones in pCS2++ were prepared as follow: linearized with *Sal*I and transcribed using T3 polymerase. Two different *xTGIF2* in situ probes were used: one full-length (*Sal*I/T3), and a short one produced by PCR including the sequence outside the homeodomain and part of the 3′UTR (from nt 568-898). Other in situ probes were prepared as follow: for *Pdx1*/*Xlhbox8* (PCR cloning into pGEMT, *Nco*I/SP6); Hex-KSII (*Bam*HI/T7).

RT-PCR analysis

RT-PCR was performed as described (Spagnoli and Brivanlou, 2006). Ornithine decarboxylase (ODC) was used as loading control. Twenty-one PCR cycles were performed for ODC and Endodermin primers, and 25 cycles for the other primers. Real-time PCR reactions were carried out using the SYBR Green Master Mix (Roche) on Light Cycler Roche. Succinate dehydrogenase (SDHA) was used as reference gene for mouse samples. According to the Light Cycler Roche instruction manual, the standard number of real-time PCR cycles (45 cycles) was performed.

IP and western blot analysis

Immunoprecipitation was performed as previously described in Yeo and Whitman (Yeo and Whitman, 2001) with mouse anti-FLAG M2 beads (Sigma) or anti-Smad1 monoclonal antibody (Santa Cruz Biotechnology) or anti-Smad2 goat polyclonal antibody (Santa Cruz Biotechnology). Antibodies used in western blots were: anti-Flag M2 monoclonal antibody 1:20,000 (Sigma); 1:1000 of a Smad1 polyclonal antibody (Upstate); 1:1000 of a Smad2 monoclonal antibody (BD); 1:10,000 of a α-tubulin monoclonal antibody (Sigma).

Cell culture and shRNA transfection

BTC6 cells were purchased from ATCC and cultured in DMEM containing 15% FBS. Short hairpin RNAs for *mTgif2* knockdown and the non-silencing shRNA mir control were purchased from Open Biosystems and transfected into BTC6 cells using the Fluorescent Arrest-In transfection reagent (Open Biosystems). C2C12 cells purchased from ATCC were maintained in DMEM supplemented with 15% FBS. Human recombinant BMP4 for stimulation of C2C12 mouse cells was purchased from R&D. Cells were transfected 24 hours after seeding using the Lipofectamine Plus reagent (Invitrogen). After 48 hours, lysates were prepared, and the luciferase activity was determined with the Dual Luciferase Assay System (Promega). Relative luciferase activities were normalized by the co-expressed *Renilla* luciferase activities. All luciferase assays were repeated at least three times and performed in triplicate each time.

RESULTS

Identification of *Gata5* transcriptional targets regulated in a window of time between gastrulation and organogenesis

In order to gain insight into regional specification of the endoderm and characterize mediators of the *Gata5*-induction of *Pdx1* between gastrula stage and before organogenesis, we performed a microarray analysis on *Xenopus* embryonic explants that were injected with *Gata5* mRNA. In our experimental strategy, transcriptional changes in *Gata5*-injected ectodermal explants versus naïve ectodermal explants, which are fated to give rise to epidermis and not to endodermal derivatives, were analyzed at tailbud stage using competitive hybridization on a *Xenopus laevis* cDNA microarray (Altmann et al., 2001; Munoz-Sanjuan et al., 2002) (Fig. 1). As positive control for *Gata5* activity, an aliquot of the RNA sample used for the microarray was assayed for induction of known targets of *Gata5*, such as the general endodermal marker, *Endodermin* (Sasai et al., 1996), and the pancreatic regional marker, *Pdx1*, by RT-PCR (Fig. 1A). In agreement with previous observations (Weber et al., 2000), *Gata5* ectopic expression specifically induced a subset of regionally restricted markers, the expression of which is confined to the anterior endoderm, but not to mid-posterior endoderm, such as *IFABP* (Henry et al., 1996) (Fig. 1A).

This analysis revealed 141 genes that were upregulated or downregulated following overexpression of *Gata5* in ectodermal explants by a factor of two or more. Table S1 provides a list of names and sequence identities of the modulated genes (see Table S1 in the supplementary material). These *Gata5* putative targets were submitted to three independent tests for priority, focusing initially on genes that were upregulated by *Gata5* (Fig. 1B). The first criterion was a validation of the results of the microarray by RT-PCR analysis on independent mRNA populations extracted from uninjected and *Gata5*-injected ectodermal explants at the same developmental stage (tailbud). RT-PCR analysis confirmed that more than 80% of the array clones analyzed were valid (see Fig. S1 in the supplementary material). The second criterion was based on the establishment of the temporal hierarchy of *Gata5* target activation, by using a hormone-inducible version of *Gata5*, referred to as Gata5-GR (Afouda et al., 2005). This allowed a classification of the time of induction of a subset of these targets between neurulation and tailbud stage (see Fig. S2 in the supplementary material; data not shown). Finally, the third criterion was to identify putative *Gata5* target genes that were co-expressed with *Gata5* (Weber et al., 2000) in endodermal derivatives by a whole-mount in situ hybridization approach (Fig. 2). *Gata5* targets that underwent RT-PCR confirmation, were temporally expressed in a window of time between *Gata5* and *Pdx1* expression and showed an expression pattern similar to that of *Gata5* were chosen for further analysis, with a special emphasis on genes that did not show any homology with sequences available in public databases (Fig. 1B and see Table S1 in the supplementary material).

A number of the clones analyzed by in situ hybridization showed expression in the endoderm from gastrula stage onward (Fig. 2A,B,F). For instance, *Frizzled 7* [(*Fz7*); clone 10B11] (Djiane et al., 2000; Sumanas et al., 2000) transcript was detected in dorsal/vegetal cells at the early gastrula stage and in anterior endodermal derivatives, such as the prospective hepatic and ventral pancreatic regions, at late stages (Fig. 2A). This staining clearly overlaps with the domains of expression of *Gata5* at similar developmental stages (Weber et al., 2000). *Hepatoma derived growth factor* [(*HDGF*); Clone 7C5] (Lepourcelet et al., 2005) showed a weak expression in endodermal cells during gastrulation (Fig. 2B), whereas *Xenopus* TGIF2 [(xTGIF2); clone 8B1] expression was enriched in dorsal and ventral/anterior endodermal cells at gastrula and neurula stages, respectively (Fig. 2F-J). At tadpole stage, its endodermal expression is confined to the pancreatic-duodenal region (Fig. 2K).

Some of the array clones were not detected in the endoderm at early embryonic stages, but as development proceeded their transcripts became abundant in endodermal derivatives, including the EST BG410109 (clone 7G9) in the liver, the EST BG410148 (clone 8C11) in the hepatic and duodenum region, and the EST BC094159 (clone 7B7) in the pancreas of the coiled gut (Fig. 2C,D,E). The fact that many of these putative *Gata5* targets showed an expression in endodermal territories starting at tailbud stage is consistent with the described temporal regulation that they undergo upon *Gata5* expression (see Figs S1 and S2 in the supplementary material). Finally, most of the array clones analyzed also showed sites of expression outside of the endoderm and often in the nervous system at various embryonic stages, suggesting that they may play additional roles during embryogenesis.

Interestingly, a number of *Gata5* targets among the identified sets are genes known to influence the TGFβ signaling pathway, such as, for example, *Coco* (clone 57H9) (Bell et al., 2003), *TAB3* (clone 57G10) (Munoz-Sanjuan et al., 2002) and *TGIF2* (Imoto et al., 2000; Melhuish et al., 2001). While the TGFβ signaling

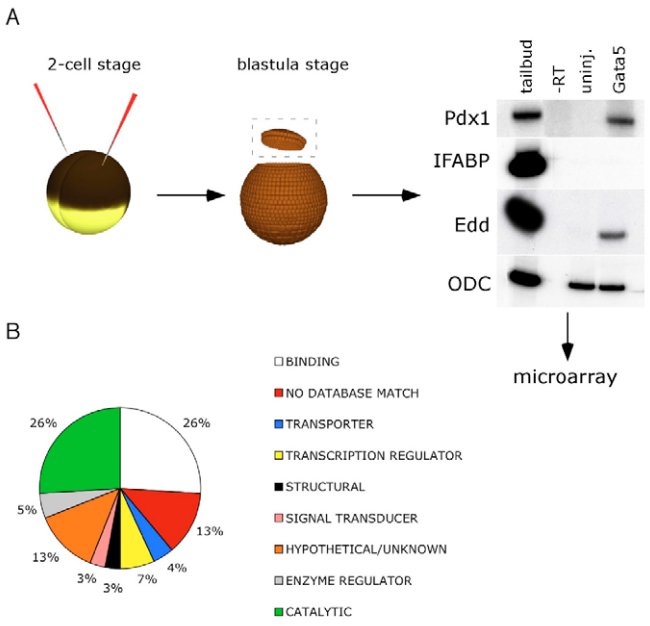

Fig. 1. Schematic of experimental strategy. (**A**) *Xenopus* embryos were injected into both animal blastomeres at the two-cell stage with *Gata5* (500 pg) mRNA. Ectodermal explants were isolated at stage 9 from both *Gata5*-injected and uninjected embryos and cultured until stage 28 (tailbud). Transcriptional differences were analyzed on 5000-clone gastrula-stage cDNA microarray (Munoz-Sanjuan et al., 2002). An aliquot of each RNA sample was assayed for expression of the indicated *Gata5* targets by RT-PCR. ODC was used as loading control. (**B**) Pie chart of the classification of the genes upregulated by *Gata5* based on the GO molecular function categories. The majority of the upregulated clones in the *Gata5* array fall into four main categories: (1) catalytic activity (26%); (2) binding activity (26%), including the large group of nucleic-acid-binding as well as protein-binding factors; (3) hypothetical/unknown function (13%), including full-length sequences conserved in the mouse and human databases the function of which is unknown; (4) no database match (13%), including a number of clones with no hits in database searches, which might be either genes unique to the frog or partial cDNAs. Edd, *endodermin*; –RT, minus reverse transcriptase; ODC, *ornithine decarboxylase*.

pathway has been described to influence endoderm development (Henry et al., 1996; Zorn et al., 1999), *Coco*, *TAB3* or *TGIF2* have not previously been associated with endoderm formation or patterning, suggesting novel mechanisms of control of the pathway in the endoderm. Among these three factors, *TGIF2* is the only one that showed an endodermal expression from gastrula stage onwards (Fig. 2F) (Bell et al., 2003; Munoz-Sanjuan et al., 2002). We therefore decided to undertake an extensive characterization of the *Gata5* target, *TGIF2*, in the context of early endodermal patterning.

TGIF2, a target of *Gata5*, is a modifying endodermal factor that promotes pancreatic fate

TGIF2, which we identified as a novel target of *Gata5*, encodes a homeodomain protein that belongs to the TALE (three-amino-acid loop extension) superfamily of homeodomain proteins (Imoto et al., 2000; Melhuish et al., 2001). No embryological or endoderm-specific function has been assigned to this protein.

In order to begin our functional analysis of *TGIF2* in the context of endoderm patterning, we tested its ability to change the character of endodermal cells using vegetal pole (prospective endoderm

Fig. 2. Whole-mount in situ hybridization of selected array clones. (**A**) *Xenopus* embryos at early gastrula (st. 10), tailbud (st. 30) stage and dissected whole gut (st. 43) were hybridized with antisense probe for *Fz7*/clone 10B11. Arrowheads indicate expression in the endoderm; the dotted line depicts the dorsal blastopore lip. Staining in endodermal derivatives, such as the hepatic and pancreatic regions, is indicated by a bracket in the middle panel. (**B**) Embryos at early gastrula (st. 10), tadpole (st. 35) stage and dissected whole gut (st. 43) were hybridized with *HDGF*/clone 7C5. Arrowheads point to expression in the endoderm; and the bracket in the middle panel indicates endodermal derivatives, such as the hepatic and pancreatic regions. (**C**) Embryos at gastrula (st. 10.5), tadpole (st. 37) stage and dissected whole gut (st. 42) were hybridized with clone 7G9 [EST similar to hypothetical protein (BG410109)] indicating expression in the ectoderm and hepatic rudiment (bracket in the middle panel). (**D**) Embryos at gastrula (st. 10), tadpole (st. 36) stage and dissected whole gut (st. 42) were hybridized with clone 8C11 [EST similar to hypothetical protein KIAA0592 (BG410148)] showing expression in the dorsal ectoderm, brain, pronephros, liver and duodenum. (**E**) Embryos at gastrula (st. 10), early tailbud (st. 27) stage and dissected whole gut (st. 42) were hybridized with clone 7B7 [EST similar to hypothetical protein (BC094159)] indicating expression in the ectoderm, brain, branchial arches and pancreas. (**F-J**) In situ hybridizations on serial sections of gastrula and neurula stage embryos using *Xenopus TGIF2* (*xTGIF2*) and *Gata5* probes show expression of both genes in a subset of endodermal cells. *xTGIF2* is also strongly expressed in the ectoderm and mesoderm. (F,F′) In situ hybridization on section of stage 10 embryo using antisense probe for *TGIF2*/clone 8B1 (* indicates the dorsal side). Red dashed box outlines the *TGIF2*-positive endodermal region, which is magnified in F′. Arrowheads point to endodermal cells. (G) *Gata5* in situ hybridization on section of stage 10 embryo. Arrowheads point to endodermal cells. (H,H′) In situ hybridization on section of stage 10.5 embryo shows enriched staining in dorsal endodermal cells (* indicates the dorsal side). Red dashed box outlines the *TGIF2*-positive endodermal region, which is magnified in H′. Arrowheads point to endodermal cells. (I,I′) In situ hybridization on neurula stage whole (I) and sectioned (I′) embryos shows *TGIF2* staining in anterior endodermal cells (red dashed box in I′). (J) *Gata5* in situ hybridization on section of stage 17 embryo. (**K**) Dissected whole gut (st. 43) stained with *TGIF2* probe shows expression in the pancreatic rudiment. a, anterior; d, duodenum; lv, liver; pa, pancreas; p, posterior.

tissue) explants, as embryological assay. Fig. 3A shows that expression of *xTGIF2* strongly induced the expression of *Pdx1* in ventral vegetal cells that are normally devoid of pancreatic markers. Conversely, the expression of the hepatic marker, *Hex* (also known as *Hhex* – Mouse Genome Informatics) (Zorn et al., 1999; Zorn and Mason, 2001), appeared to be specifically downregulated in both ventral and dorsal vegetal cells injected with *xTGIF2* (Fig. 3A). Interestingly, the transcription factor *Foxa2*, a marker of anterior endoderm and hepatic bud (Zorn and Mason, 2001), was slightly induced by *xTGIF2* on the ventral vegetal side (Fig. 3A). This reflects the fact that *Foxa2* is more widely distributed than *Hex* in the anterior endoderm of tadpole embryos, for instance being expressed also in the pancreas (Zorn and Mason, 2001). Finally, the level of the posterior gut marker, *IFABP*, remained unchanged (Fig. 3A). These results clearly indicated that *xTGIF2* has a modifier activity within the endoderm, changing the character of ventral to dorsal.

To determine whether *xTGIF2* has an inducer activity in addition to its modifier activity within the endoderm, we injected *xTGIF2* mRNA into the pluripotent prospective ectoderm (animal pole) of the embryos alone or simultaneously with mRNA encoding a general endodermal inducer, such as the transcription factor VegT (Xanthos et al., 2001). The ectodermal explants were cultured until late tailbud stage (stage 32) and examined for the expression of the pan-endodermal marker, *Endodermin*, and regionally-restricted endodermal markers, such as *Hex* and *Pdx1* by RT-PCR (Fig. 3B). Only when *xTGIF2* was presented together with *VegT* did we observe induction of the pancreatic marker *Pdx1*, which was accompanied by downregulation of *Hex* (Fig. 3B). These results suggest that *xTGIF2* is not an inducer of pancreatic endoderm, but rather a modifier of endodermal fate, confirming our findings in vegetal pole explants (Fig. 3A). Finally, we did not observe induction of posterior endodermal markers, such as *IFABP*, or of mesodermal and neural fates, as judged by the absence of expression of the mesodermal-specific marker *MyoD* and neural-specific marker *NCAM* (Fig. 3B). More specifically, we also investigated a potential effect of *xTGIF2* on mesodermal markers that are associated with lateral plate derivatives and have been reported previously to be present in vegetal pole explants (Horb and Slack, 2001). Fig. 3C shows that

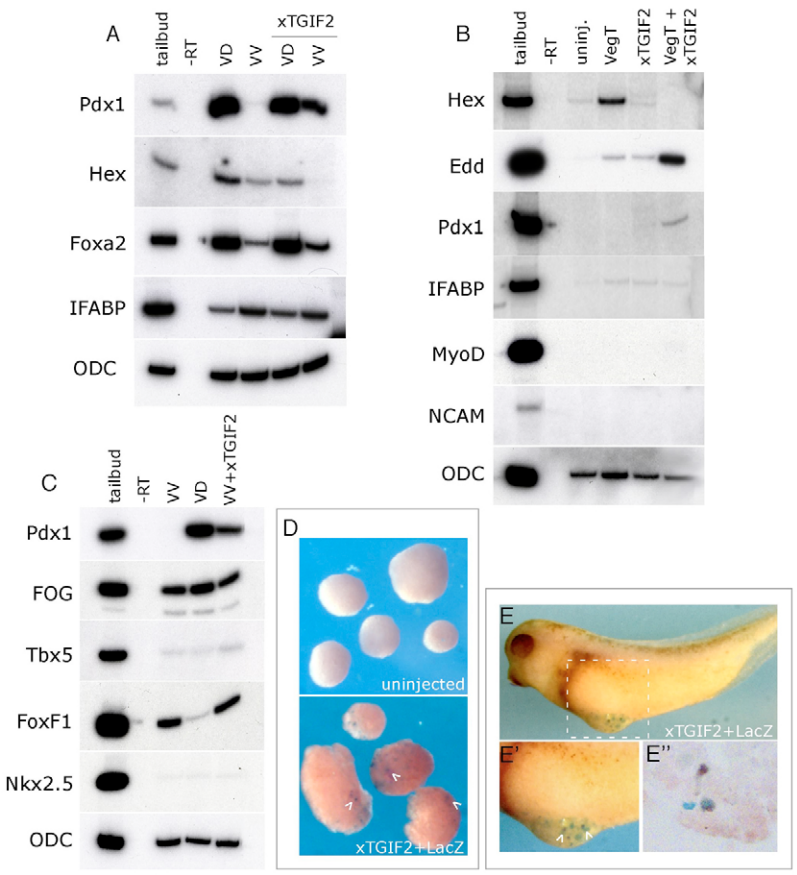

Fig. 3. *xTGIF2* behaves as a modifier of the endoderm. (**A**) *Xenopus* eight-cell stage embryos were injected into both ventral (VV) or dorsal vegetal (VD) blastomeres with *xTGIF2* (1 ng) mRNA. Vegetal explants were dissected at early gastrula stage (stage 10). Uninjected VV or VD pole halves were used as control for their regional differences in the expression of endodermal markers. All explants were collected at tailbud stage and assayed for expression of the indicated markers by RT-PCR analysis. (**B**) Animal caps injected with *VegT* (60 pg) mRNA and/or *xTGIF2* mRNA, as indicated. The explants were cultured until stage 30 (tailbud) and analyzed for expression of the indicated markers. (**C**) Eight-cell stage embryos were injected into ventral vegetal (VV) blastomeres with *xTGIF2* (1 ng) mRNA. Uninjected VV or VD pole halves were used as control. All explants assayed at tailbud stage for expression of the indicated mesodermal markers by RT-PCR analysis. (**D-E″**) Lineage-tracing analysis of endodermal cells injected with *xTGIF2* mRNA. (D) Ventral vegetal explants isolated from uninjected or *xTGIF2+lacZ*-co-injected embryos were stained for β-gal and assayed for *Pdx1* expression by in situ hybridization at tailbud stage. The β-gal staining (blue) and ectopic *Pdx1* (red) expression co-localize in endodermal explants (see arrowheads). (E) Whole-mount in situ hybridization analysis of *Pdx1* expression in *xTGIF2+lacZ*-co-injected embryos. White dashed outline demarcates the region of the embryo magnified in E′. Arrowheads indicate purple cells stained for β-gal (blue) and positive for *Pdx1* (red). (E″) Transverse section through the stained endodermal region shown in E′.

induction of *Pdx1* in ventral vegetal cells injected with *xTGIF2* was not accompanied by induction or modulation of gut-surrounding mesoderm markers, such as *FoxF1*, *FOG*, *Tbx5* and *Nkx2.5*.

Finally, to confirm that *xTGIF2* acts in the endoderm, we performed a lineage-tracer analysis by co-injecting *xTGIF2* and *lacZ* mRNAs in embryonic explants as well as in the whole embryo. In both cases, ectopic expression of *Pdx1* was observed in injected cells (β-Gal positive) in the endoderm (Fig. 3D-E″). Altogether, these results suggest that *xTGIF2* acts cell-autonomously in the endoderm to promote pancreatic fate.

TGIF2 is necessary for the establishment of the pancreatic domain within the endoderm

In order to address the in vivo function of xTGIF2 during the regionalization of the endoderm and, specifically, of the pancreatic region, we designed antisense morpholino oligonucleotides (referred to as TGIF2-Mo) targeting both *Xenopus laevis TGIF2* pseudoalleles found in the EST databases (see Fig. S3 in the supplementary material). To inhibit the translation of *xTGIF2* mRNA specifically within the territory where pancreas is formed, we injected the TGIF2-Mo into the dorsal vegetal blastomeres of eight-cell stage embryos. Injection of the TGIF2-Mo into this region of the embryo resulted in a clear drop of the level of *Pdx1* expression, as judged by RT-PCR analysis on embryonic explants (Fig. 4A). This reduction in pancreatic character of the dorsal vegetal half was accompanied by a slight increase of the hepatic marker *Hex*, whereas the expression of *IFABP* and gut-surrounding mesoderm markers (*FoxF1* and *FOG*) was unaffected (Fig. 4A). Real-time RT-PCR analysis on TGIF2-Mo-injected embryonic explants confirmed our results, showing a tenfold downregulation of *Pdx1* mRNA when TGIF2-depleted dorsal vegetal explants were compared with uninjected ones (Fig. 7A).

Similar observations were made by analyzing the expression pattern of *Pdx1* and *Hex* in antisense-injected embryos cultured to stage 35 by whole-mount in situ hybridization using specific probes (Fig. 4B,C). Importantly, the reduction of the *Pdx1* domain of expression in TGIF2-Mo-injected embryos was extended to both pancreatic buds and the intermediate duodenum region, indicating that xTGIF2 is crucial for both regions (Fig. 4B). In line with our RT-PCR analysis (Fig. 4A), the domain of expression of *Hex* was expanded in TGIF2-Mo-injected embryos (Fig. 4C). Notably, the loss of *Pdx1* expression in the prospective pancreatic region, due to the knockdown of endogenous xTGIF2 activity, could be rescued by the injection of mRNA encoding mouse TGIF2 (mTGIF2), which lacks the sequences targeted by TGIF2-Mo (Fig. 4B and Fig. 7A). Finally, at late tadpole stage, the pancreatic tissue was drastically reduced or absent in the gut of embryos depleted of TGIF2, as judged by the dramatic downregulation of *insulin* and *amylase* expression (Fig. 4D). Taken together, these results indicate that TGIF2 activity is required within the endoderm for proper establishment of the pancreatic region.

TGIF2 inhibits BMP/Smad1 pathway and promotes dorsal fates in *Xenopus* embryos

In response to TGFβ, an activated Smad complex can interact with transcriptional co-repressors, such as TGIF, TGIF2, c-Ski or SnoN, which displace co-activators and limit the extent of TGFβ transcriptional activation (Massague et al., 2005). Human TGIF2

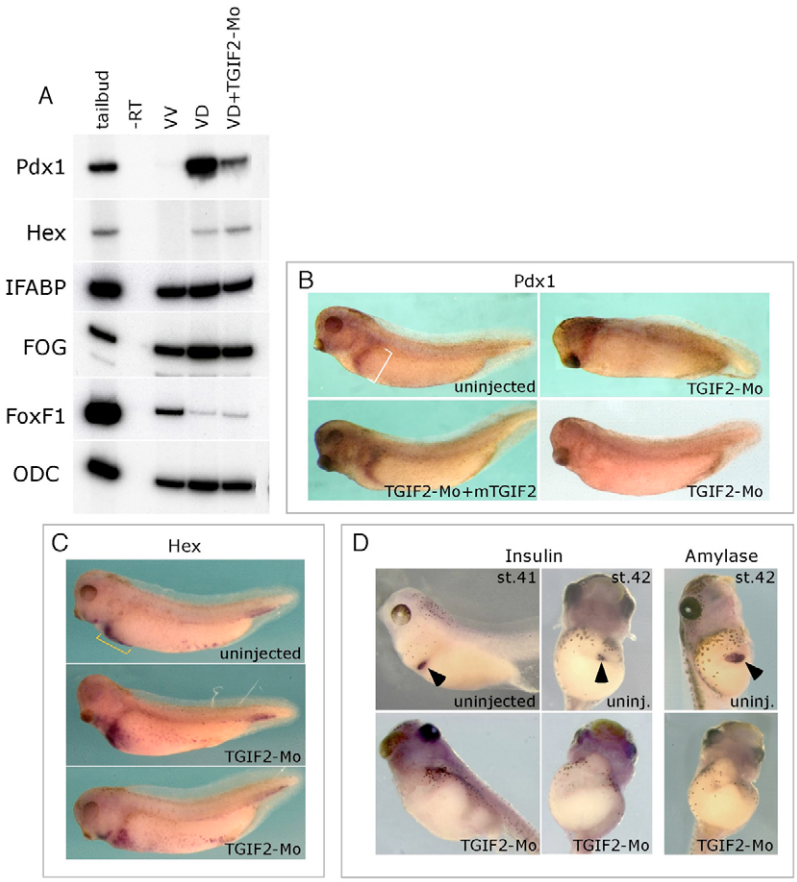

Fig. 4. xTGIF2 is required in vivo for establishing the pancreatic region within the endoderm. (**A**) Both dorsal vegetal (VD) blastomeres of eight-cell stage embryos were injected with a combination (5 ng each) of two antisense morpholino oligonucleotides (TGIF2-Mo) targeting both *Xenopus laevis TGIF2* pseudoalleles (see Fig. S3 in the supplementary material). TGIF2-Mo-injected VD, uninjected VD and ventral vegetal (VV) explants were dissected at stage 10 and assayed for expression of the indicated markers by RT-PCR analysis. (**B**) Whole-mount in situ hybridization using *Pdx1* probe. Embryos injected with TGIF2-Mo showed reduction of *Pdx1* expression domain (80%; *n*=40). *Pdx1* staining was rescued in embryos injected with TGIF2-Mo and mouse *Tgif2* (*mTgif2*; 500 pg) mRNA (70%; *n*=25). Embryos left untreated show normal *Pdx1* expression domain in the pancreatic/duodenum endoderm, as indicated by the white bracket. (**C**) Whole-mount in situ hybridization with *Hex*. Embryos injected with TGIF2-Mo or left untreated were stained at stage 34. *Hex* expression domain in the hepatic endoderm is indicated by the yellow bracket. (**D**) Analysis of the expression of the pancreatic differentiation markers, *insulin* and *amylase*, by whole-mount in situ hybridization. Arrowheads indicate normal domains of expression of *insulin* and *amylase* in the pancreatic bud of tadpole gut tubes.

has been characterized as a transcriptional co-repressor for TGFβ-activated Smads, being able to interact physically with activated SMAD3 (Melhuish et al., 2001).

Intriguingly, the injection of *xTGIF2* mRNA into the ventral vegetal region of the *Xenopus* embryos elicited the induction of a partial posterior secondary dorsal axis (Fig. 5A). This phenotype in amphibian embryos can occur by induction of Smad2/3 by TGFβ-like signaling or by inhibition of ongoing BMP signals. In order to address how *xTGIF2* caused a secondary axis, we performed a series of functional and biochemical experiments in the amphibian embryo. First, we co-injected *xTGIF2* mRNA with the TGFβ members, *activin* (Smith et al., 1990) or *Xnr1* (*nodal-related factor-1*) (Hyde and Old, 2000), and monitored the expression of immediate response genes activated by these signaling molecules in ectodermal explants (Fig. 5B; data not shown). In this assay, *xTGIF2* blocked the induction of a subset of *activin/nodal* target genes, such as the lateroventral mesodermal marker, *Wnt8* (Smith and Harland, 1991), but did not block the induction of the dorsal marker, *chordin* (Sasai et al., 1996), or pan-mesodermal marker, *brachyury* (Smith et al., 1990) (Fig. 5B; data not shown). Thus, a promoter-specific repression of TGFβ-induced activity by *TGIF2* seems to be conserved in vertebrates (Melhuish et al., 2001).

Second, we considered the possibility that *xTGIF2* might regulate the BMP/Smad1 branch of the pathway. As BMP signaling normally promotes ventral fates, *xTGIF2* modulation of this pathway could explain the double dorsal axis phenotype (Fig. 5A) and the dorsalized character of the endoderm observed upon overexpression of x*TGIF2* into the ventral vegetal pole (Fig. 3A). In intact uninjected ectodermal explants (animal caps), BMP signaling is active and induces ventral epidermal fate, as judged by the induction of its immediate early response genes, *msx1* and *msx2*, and *epidermal keratin* (EK) (Wilson and Hemmati-Brivanlou, 1995). Fig. 5C shows that injection of *xTGIF2* into animal caps strongly downregulated *msx1*, *msx2* and *EK* at gastrula stage, whereas no induction of mesoderm (*xbra*) was detected (Fig. 5C). In line with this, *xTGIF2* behaves as a weak neural inducer, being able to induce *sox2* at early gastrula stage, but only a subset of anterior neural markers, such as *xAG* (Bell et al., 2003), at later stages (Fig. 5C; data not shown). Taken together, these results suggest that *xTGIF2* has the ability to inhibit BMP signaling. Next, we investigated the effect of *xTGIF2* on BMP-activated transcriptional responses using the BMP-responsive xVent-2 promoter-luciferase (Vent2-Luc) reporter (Hata et al., 2000) in *Xenopus* embryos. As shown in Fig. 5D, overexpression of *xTGIF2* into the animal pole of the embryos robustly abrogated the activation of Vent2-Luc transcription in response to BMP4. Expression of *xTGIF2* had no effect on this reporter in the absence of BMP4 (data not shown). Taken together, these data strongly suggest that *xTGIF2* inhibits the BMP/Smad1 branch of the pathway and modifies the TGFβ/Smad2 branch of the pathway by selective inhibition of a subset of regional markers.

To determine whether TGIF2 is able to interact with the mediator of the BMP signals, Smad1 (Massague et al., 2005), we performed immunoprecipitation assays. Fig. 5E shows that Flag-tagged *xTGIF2* injected into the ventral vegetal zone (the region where BMP signal is active during gastrulation) (Faure et al., 2000) of the embryos interacted with endogenous Smad1 (Fig. 5E). In parallel, we performed the same assay on the dorsal vegetal pole, and found

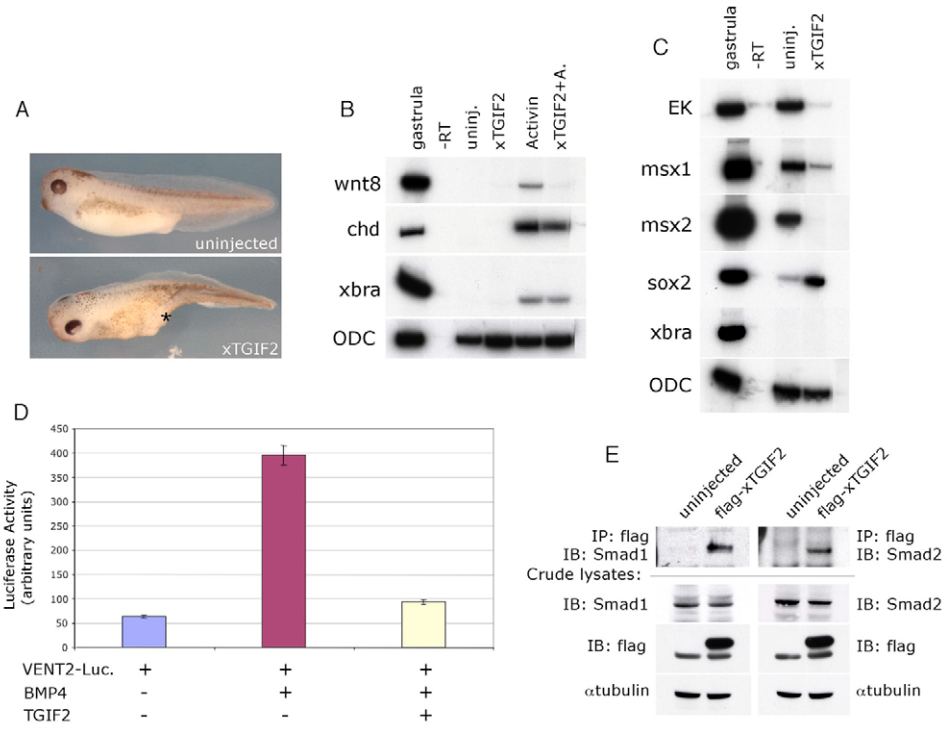

Fig. 5. Inhibitory effects of *xTGIF2* on TGFβ and BMP signalings in *Xenopus*. (**A**) Partial secondary axis (indicated by *) was observed in tadpole stage embryos injected with *xTGIF2* (1 ng) mRNA into one ventral vegetal cell. (**B**) *xTGIF2* (1 ng) mRNA and *activin* (100 pg) mRNA were injected separetely or together into the animal pole of two-cell stage embryos. Animal caps were analyzed at gastrula stage (stage 11) for the expression of indicated markers by RT-PCR. (**C**) Animal caps injected with *xTGIF2* (1 ng) mRNA were analyzed at gastrula stage (stage 11) for the expression of indicated markers by RT-PCR. (**D**) Luciferase assay with BMP inducible VENT2-luciferase (VENT2-Luc.) reporter construct. Two-cell stage embryos were injected with VENT2-Luc. alone or in combinations with *BMP4* (200 pg) and/or *xTGIF2* (1 ng) mRNAs, as indicated. Embryos were harvested at the onset of gastrulation and assayed for luciferase activity. (**E**) Immunoprecipitation (IP) of flag-xTGIF2 and endogenous Smad1 or Smad2. Four-cell stage embryos were injected into the the vegetal pole (ventrally for Smad1 IP; dorsally for Smad2 IP) with flag-*xTGIF2* (1 ng), lysates were prepared at gastrula stage (stage 11) and immunoprecipitated with anti-flag antibody and analyzed by immunoblot (IB) with anti-Smad1 and anti-Smad2 antibodies. The expression of flag-xTGIF2, Smad1 and Smad2 was checked by immunoblotting on the crude extracts used for the IP reaction. As loading control, the membranes were stripped and reprobed with anti-α-tubulin. Chd, *chordin*; EK, *epidermal keratin*; xbra, *brachyury*.

an interaction between Flag-xTGIF2 and endogenous Smad2 (Faure et al., 2000) (Fig. 5E). Similar results were obtained upon immunoprecipitating Smad1 or Smad2 and immunoblotting to detect the tag on xTGIF2 (see Fig. S4 in the supplementary material). These results showed that xTGIF2 is able to interact with both intracellular mediators of the TGFβ signaling pathway.

The mouse *Tgif2* displays a conserved role

Although we found that *Xenopus* TGIF2 can modulate both BMP and TGFβ signalings, human TGIF2 has been found to regulate only the TGFβ branch of the pathway (Melhuish et al., 2001). To address whether the BMP antagonistic activity of TGIF2 was conserved in mammals, we performed reporter assays for the mouse TGIF2 using BMP-responsive promoters both in *Xenopus* embryos and C2C12 mouse myoblast cells. Following transfection of *mTgif2* DNA in C2C12 cells, we observed a significant repression of the activation of the BMP-responsive promoter [BMP response element (BRE)] (Hata et al., 2000) by BMP4 addition (Fig. 6A). Similar observations were made in *Xenopus* ectodermal explants injected with *mTgif2* mRNA (data not shown). These results indicate that TGIF2 is an inhibitor of the BMP pathway in both systems.

In order to investigate whether the mouse homolog of *TGIF2* was also able to induce ectopic expression of *Pdx1*, we tested *mTgif2* mRNA in the context of the *Xenopus* system using vegetal explant assay, as described above (Fig. 6B). Importantly, we observed that *mTgif2* was able to induce *Pdx1* expression in the ventral vegetal explants to the same extent as *xTGIF2* (Fig. 6B). Next, we moved to a mammalian system, such as the mouse pancreatic cell line BTC6 (Poitout et al., 1995), that expresses differentiated pancreatic markers, such as *Pdx1* and insulin, as well as *Tgif2* (Poitout et al., 1995) (data not shown). By a loss-of-function approach using silencing short hairpin RNAs targeting *mTgif2* (shTGIF2), we showed that *mTgif2* is required for the maintenance of the expression of *Pdx1* and insulin (Fig. 6C). Transfection of increasing dose of shTGIF2 into BTC6 cells resulted in a reduction of the respective mRNA levels of *Tgif2* itself, *Pdx1* and insulin, as judged by real-time RT-PCR analysis, whereas the shRNA control vector had no effect (Fig. 6C). These results suggest that the ability of *TGIF2* to control the early pancreatic regional marker *Pdx1* is conserved across species.

BMP signaling controls dorsoventral regionalization in the endoderm

Based on our findings, we reasoned that the specification of the pancreatic territory within the dorsal endoderm might be dependent on the inhibition of BMP signaling in vivo. To test this hypothesis, we challenged the BMP signaling activity in the context of endoderm regionalization by two independent approaches. First, we knocked down the level of chordin, a robust BMP antagonist released by the organizer (Harland and Gerhart, 1997; Oelgeschlager et al., 2003; Sasai et al., 1996), using the same assay as for TGIF2-Mo in *Xenopus* embryos (Fig. 4; Fig. 7A). As shown in Fig. 7A, by real-time RT-PCR analysis, we detected a reduction of the level of expression of *Pdx1* in dorsal/vegetal cells injected with chordin antisense morpholinos (Chd-Mo) (Oelgeschlager et al., 2003), whereas the expression of the pan-endodermal marker *Endodermin* remained unchanged. Second, we inhibited endogenous BMP signaling in the ventral vegetal region of the embryo by using a dominant-negative BMP4 receptor I (DN-*Alk3*), which robustly blocks BMP signaling (Mishina et al., 1995; Suzuki et al., 1995). Fig. 7B shows that *Pdx1* expression was induced in

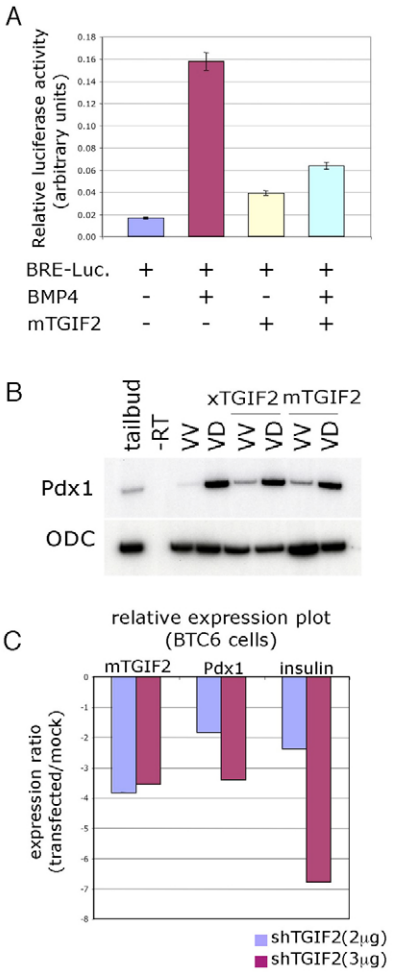

Fig. 6. Conserved role for the mouse *Tgif2*. (**A**) BRE-luciferase activity in mammalian cells. Mouse C2C12 cells were transfected with *Renilla* luciferase and BRE-Luc. reporter constructs alone or in combination with *mTgif2*, as indicated. Transfected cells were stimulated 48 hours after transfection with BMP4 recombinant protein, as indicated. *Renilla* luciferase was used for normalization. (**B**) Eight-cell stage embryos were injected into both ventral (VV) or dorsal vegetal (VD) blastomeres with *xTGIF2* or *mTgif2* mRNAs. Explants were collected at tailbud stage and assayed for expression of the indicated markers by RT-PCR analysis. (**C**) BTC6 cells were transfected with increasing amount of shRNA targeted against *mTgif2* or with control shRNA, and analyzed 48 hours later by real-time RT-PCR for the expression of endogenous *mTgif2* level and indicated markers. The plot shows the regulation (expression ratio) of target genes in shTGIF2-transfected cells versus shControl-transfected cells. All the values were normalized to the reference gene SDHA (succinate dehydrogenase), and calculated using the software REST (Pfaffl et al., 2002). Data were determined in triplicate.

ventral vegetal pole explants injected with DN-*Alk3* mRNA as well as with *xTGIF2*. A similar induction was observed also for additional anterior endodermal and pancreatic markers, such as *Foxa2* and *Ptf1a* (Kawaguchi et al., 2002) (Fig. 7B). Consistently, the injection of constitutive active BMP4 receptor I (CA-*Alk3*) into the dorsal vegetal half of the embryo led to a significant downregulation of *Pdx1* expression level, while the expression of gut-surrounding mesoderm markers was unaffected (Fig. 7C; data not shown).

Altogether, our findings suggest that ongoing inhibition of the ventralizer signal, BMP, is required and sufficient to define the pancreatic rudiment within the endoderm. This inhibition is mediated endogenously by extracellular factors, such as chordin, and by intracellular endodermal effectors, such as TGIF2.

DISCUSSION

Using a microarray approach, we have uncovered a crucial step in the definition of the pancreatic rudiment within the endoderm, mediated by *TGIF2*, which operates between *Gata5* and *Pdx1* expression (Fig. 7D). This finding connects *Gata5* to *Pdx1*, covering the gap of knowledge in the window of time between endoderm induction, patterning and organogenesis.

By performing both gain- and loss-of-function experiments, we have shown that *TGIF2* behaves as a modifier, imparting a dorsal character to the endoderm, and is required for the induction of the pancreatic regional marker, *Pdx1*, in *Xenopus* endoderm as well as in mammalian cell culture. Mouse *Tgif2* mimics its *Xenopus* counterpart in inducing *Pdx1*, pointing to an evolutionarily conserved role of *TGIF2* within the context of endodermal patterning. This observation reflects the finding that *TGIF2* transcripts show a similar expression profile in mouse and frog embryos (Jin et al., 2005) (our unpublished results).

From a mechanistic point of view, it has been previously reported that *TGIF2* exerts a promoter-specific repression on TGFβ/Smad2-induced activity (Melhuish et al., 2001). Our present study complements this finding, by showing that *TGIF2* strongly antagonizes BMP signaling in both *Xenopus* and mammalian cells. The balance between Smad co-activators and co-repressors has been proposed to refine the TGFβ-mediated response (Massague et al., 2005). Our study suggests that the main role of TGIF2 in vivo is to bias this balance more toward BMP/Smad1 inhibition.

From an embryological point of view, we show that overexpression of *TGIF2* into the ventral vegetal blastomeres, where the Smad1 signal is normally active from gastrulation onward (Faure et al., 2000), leads to dorsalization of the endoderm, inducing ectopic expression of *Pdx1*. Interestingly, concomitant to *Pdx1* induction, we observed a strong downregulation of another transcription factor, *Hex*. While *Pdx1* demarcates the future pancreatic territory, *Hex* is an endodermal marker that demarcates the future hepatic territory. Interestingly, in line with our observations *Hex* has been described as a BMP-responsive gene in vertebrates (Zhang et al., 2002) and in amphioxus (Yu et al., 2007).

BMP signaling has also been shown to play a crucial role in specifying gut regions in mouse embryos (Bachiller et al., 2003; Rossi et al., 2001). For example, BMPs released from the septum transversum mesenchyme are needed to induce ventral endoderm to adopt hepatic fate and exclude pancreatic fate (Rossi et al., 2001). In explants of mouse foregut endoderm cultured in the presence of the BMP antagonist noggin, *Pdx1* is activated, whereas albumin (a liver marker) is not (Rossi et al., 2001). Similarly, *Tgif2* seems to counteract the expression of *Hex* in favor of *Pdx1*, suggesting a potential fine regulatory role of *Tgif2* over the choice between pancreatic fate versus hepatic fate. This is consistent with the fact that both the ventral pancreatic bud and the liver originate from the same anterior/ventral endodermal cells (Chalmers and Slack, 2000; Deutsch et al., 2001). In line with these observations in the mouse embryo, a recent study has shown that exposure of mouse embryonic stem cells to BMP4 also induces differentiation along the hepatic lineage (Gouon-Evans et al., 2006).

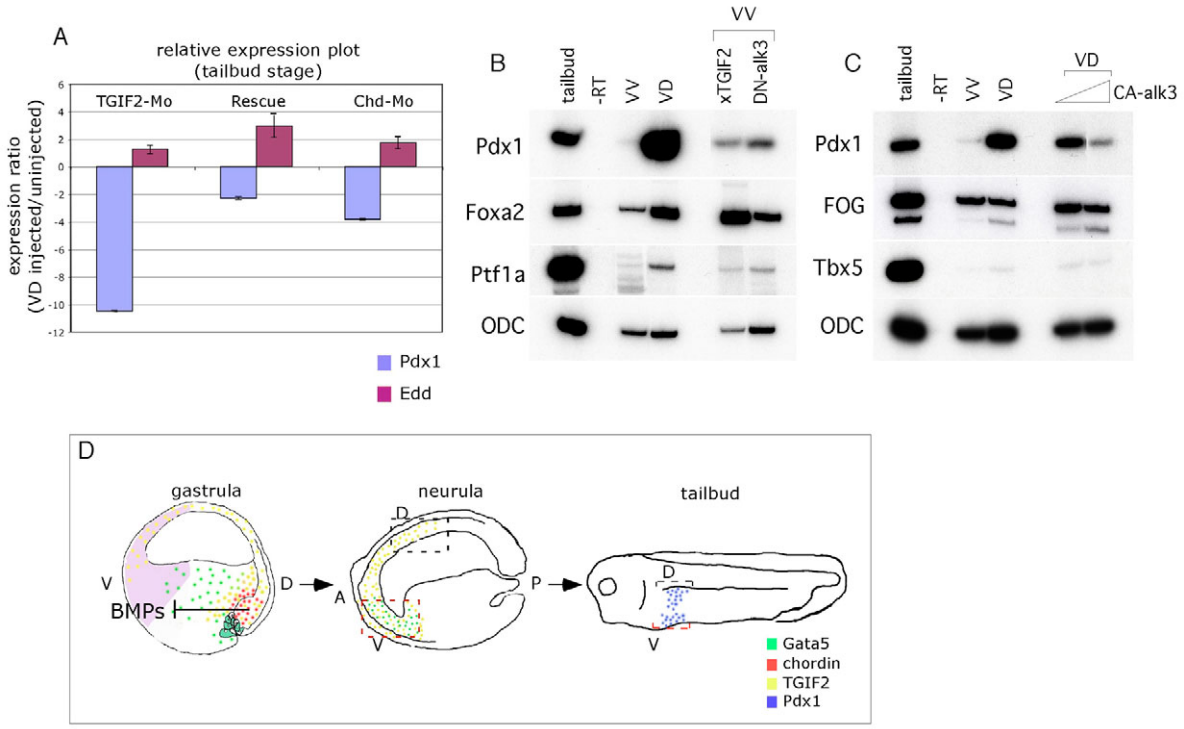

Fig. 7. BMP inhibits pancreatic fate within the endoderm. (**A**) Real-time RT-PCR analysis of dorsal vegetal (VD) explants injected with TGIF2-Mo, TGIF2-Mo in combination with *mTgif2* (500 pg) mRNA for the rescue, and a combination of two antisense morpholino oligonucleotides targeting both *Xenopus laevis chordin* (Chd-Mo) pseudoalleles (Oelgeschlager et al., 2003). The plot shows the regulation (expression ratio) of target genes in VD-injected cells versus VD-uninjected cells. The analysis was done as described in Fig. 6. (**B**) Eight-cell stage embryos were injected into both ventral (VV) blastomeres with *xTGIF2* (1 ng) mRNA and DN-*Alk3* (1 ng). All explants were collected at tailbud stage and assayed for expression of the indicated markers by RT-PCR analysis. (**C**) Eight-cell stage embryos were injected into both dorsal vegetal (VD) blastomeres with increasing amount of CA-*Alk3* (0.5 and 1 ng). All explants were collected at tailbud stage and assayed for expression of the indicated markers by RT-PCR analysis. (**D**) Schematic diagrams of gastrula, neurula and tadpole stages embryos. At gastrula stage, signals from the organizer and dorsal endoderm counteract the ventralizing factor BMP, establishing the region where pancreatic fate is specified. At neurula stage, ongoing intracellular inhibition of BMP signals by *TGIF2* defines the region where prospective pancreatic buds are formed (dorsal bud is boxed in black; ventral bud boxed in red). At tailbud stage, *Pdx1* marks both pancreatic buds. V, ventral; D, dorsal; A, anterior; P, posterior.

Finally, we show that modulation of the BMP pathway itself through TGIF2-independent means modifies the dorsoventral character of the endoderm. As such, pancreatic fate is inhibited by enhanced BMP signaling upon depletion of the BMP antagonist *chordin* and is induced by cell-autonomous BMP inhibition through expression of the dominant-negative receptor, DN-*Alk3*. This latter observation is similar to the endogenous effects of *TGIF2* that we observed. Taken together, these findings propose a more general mechanism by which patterning of the dorsal endoderm towards a pancreatic fate relies on the inhibition of the endogenous BMP signaling (Fig. 7D). This evidence supports the proposal (Harland and Gerhart, 1997; Henry et al., 1996; Sasai et al., 1996; Zorn et al., 1999) that the endoderm might be patterned by the same signals already implicated in mesoderm and ectoderm patterning.

The developing embryo can modulate BMP activity through the expression of a variety of BMP antagonists. BMP inhibitors have been characterized mostly in the context of the organizer, which acts to impart dorsoanterior fates in the surrounding tissues (Harland and Gerhart, 1997). However, work in neural induction in *Xenopus* has shown that the prospective neural tissue itself produces inhibitors of BMP signaling to sustain a prolonged BMP inhibition (Bell et al., 2003; Munoz-Sanjuan et al., 2002). With regard to the endoderm, very few examples of BMP inhibitors have been detected in this germ layer. For instance, the expression domain of *chordin* in *Xenopus* has been shown to expand from the organizer into deep dorsal endodermal cell and the so-called bottle cells at gastrulation, but its expression in the endoderm dramatically decreases at neurula stage (Sasai et al., 1996; Zorn et al., 1999). Similarly, the secreted BMP inhibitor Cerberus shows a transient expression in the anterior endoderm that does not persist during later development (Piccolo et al., 1999). To this scheme we can add a novel intracellular BMP/Smad1 inhibitor, *TGIF2*, within the endoderm. It is likely that a BMP-BMP antagonist gradient within the endoderm is initiated by an organizer signal, such as *chordin*, with which *TGIF2* might synergize. Subsequently, from neurula stage onward *TGIF2* would maintain and reinforce BMP inhibition intracellularly in order to define the pancreatic region (Fig. 7D).

In the chick and zebrafish, BMP signaling seems instead to promote pancreatic identity (Kumar et al., 2003; Tiso et al., 2002). For instance, in zebrafish *swirl* mutant, deficient in Bmp2b, the expression of the pancreatic marker NeuroD is reduced, whereas *chordino* (a BMP inhibitor) mutant embryos show an enlargement of the pancreas, as detected only by Islet1 expression (Tiso et al., 2002). This study does not conclusively address the role of BMPs in patterning the pancreatic endoderm at early stages, being based on a very limited number of late-stage pancreatic markers. However,

these opposite effects of BMP reported in zebrafish and chick might be ascribed to differences in the origin and positioning of the pancreatic precursors within the endoderm among species. Alternatively, BMPs might have different effects at different stages of pancreatic development, depending on the competence of the endoderm to respond to such signals. An answer to this might come from a temporally and spatially controlled inactivation of BMP signals during development.

Intriguingly, other members of the TALE family of homeodomain proteins, including Meis and Pbx, have been characterized as co-factors of Pdx1 (Moens and Selleri, 2006). These interactions can increase the binding specificity and transcriptional effectiveness of homeodomain proteins. For instance, a Pdx1 complex containing Pbx1 and Meis2 has been described in pancreatic exocrine cells and seems to contribute to the switch between endocrine and exocrine fate (Swift et al., 1998). Similar mechanisms might also account for the effect of *TGIF2* in the context of pancreatic tissue and, for instance, in the BTC6 pancreatic line, analyzed here. In support of this, we have found evolutionarily conserved TGIF-binding sites in the mouse *Pdx1* promoter (our unpublished results). Thus, similarly to other pancreatic factors (Jensen, 2004), *TGIF2* may be involved in the earliest stages of pancreatic induction as well as later in the maintenance of *Pdx1* expression in pancreatic cells.

Although all three GATA factors, *Gata4*, *Gata5* and *Gata6*, clearly play a role in the development of the endoderm, recent findings have indicated a predominant role for *Gata6* in the genetic network orchestrating endodermal programming (Afouda et al., 2005). In our analysis, we have found that all the putative *Gata5* targets that we analyzed here, including *TGIF2*, are also induced by *Gata6* at the same extent and same developmental time, suggesting a functional redundancy among the different members of the subfamily (our unpublished data).

In conclusion, our global molecular analysis has provided new insight into the early mechanisms of endodermal regionalization. Notably, we propose that a graded distribution of BMP activity controls the segregation of endodermal territories, where low BMP levels would define the early pancreatic region within the dorsal endoderm. *TGIF2*, one of the effectors of *Gata5*, is the endodermal factor that can lower BMP signaling at the appropriate time and location during pancreatic formation, establishing a molecular link between dorsal/ventral patterning of the endoderm and pancreatic induction.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/135/3/451/DC1

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