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,
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 chorion 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 sequences of the clones identified in the array were 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+. The 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 AslI-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 SalI and transcribed using T3 polymerase. Two different xTGIF2 in situ probes were used: one full-length (SalI/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, Ncol/SpeI); Hex-KSII (BanHI/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 an 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, Endoderm (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 identification of the temporal hierarchy of Gata5 target activation, by using a hormone-inducible version of Gata5, referred to as Gata5-GR (Afloua 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 ([F7]; 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 TGF2 [(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 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
Interestingly, 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 \( \text{xTGIF2} \) was presented together with VegT did we observe induction of the pancreatic marker \( \text{Pdx1} \), which was accompanied by downregulation of \( \text{Hex} \) (Fig. 3B). These results suggest that \( \text{xTGIF2} \) 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 \( \text{IFABP} \), or of mesodermal and neural fates, as judged by the absence of expression of the mesodermal-specific marker \( \text{MyoD} \) and neural-specific marker \( \text{NCAM} \) (Fig. 3B). More specifically, we also investigated a potential effect of \( \text{xTGIF2} \) 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

tissue) explants, as embryological assay. Fig. 3A shows that expression of \( \text{xTGIF2} \) strongly induced the expression of \( \text{Pdx1} \) 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 \( \text{xTGIF2} \) (Fig. 3A). Interestingly, the transcription factor Foxa2, a marker of anterior endoderm and hepatic bud (Zorn and Mason, 2001), was slightly induced by \( \text{xTGIF2} \) 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 \( \text{xTGIF2} \) has a modifier activity within the endoderm, changing the character of ventral to dorsal.

To determine whether \( \text{xTGIF2} \) has an inducer activity in addition to its modifier activity within the endoderm, we injected \( \text{xTGIF2} \) 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 stained for the expression of the endodermal markers, such as \( \text{IFABP} \) and \( \text{Pdx1} \) by RT-PCR (Fig. 3B). Only when \( \text{xTGIF2} \) was presented together with VegT did we observe induction of the pancreatic marker \( \text{Pdx1} \), which was accompanied by downregulation of \( \text{IFABP} \) (Fig. 3B). These results suggest that \( \text{xTGIF2} \) 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 \( \text{IFABP} \), 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 \( \text{xTGIF2} \) 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
induction of Pdx1 in ventral vegetal cells injected with xTGIF2 was not accompanied by induction or modulation of gut-surrounding mesoderm markers, such as Foxa1, 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 (*Foxa1* 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 *amylose* 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 TGIFβ, 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 TGFB transcriptional activation (Massague et al., 2005). Human TGIF2
has been characterized as a transcriptional co-repressor for TGFβ-activated Smads, being able to interact physically with activated SMAD3 (Melhuih 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/H9252-inhibiting molecules in ectodermal explants (Fig. 5B; data not shown). Thus, a promoter-specific repression of TGFβ signaling normally promotes ventral fates, 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 (Vent-2-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 Vent-2-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 separately 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, Smad1 and Smad2. Four-cell stage embryos were injected with 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 TGFβ can modulate both BMP and TGFβ signalings, human TGFβ has been found to regulate only the TGFβ branch of the pathway (Melhuish et al., 2001). To address whether the BMP antagonistic activity of TGFβ2 was conserved in mammals, we performed reporter assays for the mouse TGFβ2 using BMP-responsive promoters both in Xenopus embryos and C2C12 mouse myoblast cells. Following transfection of mTgf2 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 mTgf2 mRNA (data not shown). These results indicate that Tgf2 is an inhibitor of the BMP pathway in both systems.

In order to investigate whether the mouse homolog of TGFβ2 was also able to induce ectopic expression of Pdx1, we tested mTgf2 mRNA in the context of the Xenopus system using vegetal explant assay, as described above (Fig. 6B). Importantly, we observed that mTgf2 was able to induce Pdx1 expression in the ventral vegetal explants to the same extent as xTGF2 (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 Tgf2 (Poitout et al., 1995) (data not shown). By a loss-of-function approach using silencing short hairpin RNAs targeting mTgf2 (shTGF2), we showed that mTgf2 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 Tgf2 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 TGF2 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
ventral vegetal pole explants injected with DN-Alk3 mRNA as well as with \(x\)Tgf2. 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 Tgf2 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, Tgf2 seems to counteract the expression of Hex in favor of Pdx1, suggesting a potential fine regulatory role of Tgf2 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).
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 dorsal-ventral 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.
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/i451/DC1

References


TGF2 induces Pdx1 and inhibits BMP


