Published in final edited form as:

Dev Dyn. 2008 May ; 237(5): 1255–1267. doi:10.1002/dvdy.21527.

Nodal and Lefty signaling regulates the growth of pancreatic cells

You-Qing Zhang, Lori Sterling, Aleksandr Stotland, Hong Hua, Marcie Kritzik, and Nora Sarvetnick*

Department of Immunology, The Scripps Research Institute, La Jolla, CA 92037

Abstract

Nodal and its antagonist, Lefty, are important mediators specifying the laterality of the organs during embryogenesis. Nodal signals through activin receptors in the presence of its co-receptor, Cripto. In the present study, we investigated the possible roles of Nodal and Lefty signaling during islet development and regeneration. We found that both Nodal and Lefty are expressed in the pancreas during embryogenesis and islet regeneration. In vitro studies demonstrated that Nodal inhibits, whereas Lefty enhances, the proliferation of a pancreatic cell line. In addition, we showed that Lefty-1 activates MAPK and Akt phosphorylation in these cells. In vivo blockade of endogenous Lefty using neutralizing Lefty-1 monoclonal antibody results in a significantly decreased proliferation of duct epithelial cells during islet regeneration. This is the first study to decipher the expression and function of Nodal and Lefty in pancreatic growth. Importantly, our results highlight a novel function of Nodal-Lefty signaling in the regulation of expansion of pancreatic cells.

Keywords

Nodal; Lefty; Activin; Cripto; pancreas; proliferation; regeneration; MAPK; Akt

Introduction

Nodal, a member of the TGF- β family, induces the formation of mesoderm and endoderm, and determines left-right asymmetry in vertebrates (Zhou et al., 1993; Conlon et al., 1994; Schier, 2003). Nodal signals by binding to activin receptors, and this process is mediated through its co-receptor Cripto (Yeo and Whitman, 2001; Vincent et al., 2003). Lefty proteins, which inhibit Nodal, are atypical members of the TGF- β family because they lack an α -helix and a crucial cysteine residue essential for formation of homo- or heterodimers. Lefty blocks Nodal signaling by binding to Nodal directly or to Cripto, preventing the assembly of an active Nodal/activin receptor complex (Hamada et al., 2002; Chen and Shen, 2004; Cheng et al., 2004). Loss of Lefty activity leads to an increased range and intensity of Nodal signaling during mesoderm induction and left-right axis determination (Meno et al., 1998; Meno et al., 1999). Because of the early death of Nodal and Lefty-deficient embryos,

*Correspondence: Nora Sarvetnick, Ph.D., Department of Immunology, IMM 23, The Scripps Research Institute, 10550 North Torrey Pines Rd, La Jolla, CA 92037, Tel: (858)-784-9066, Fax: (858)-784-9096, noras@scripps.edu.

nothing is known about their function during pancreatic development or in the adult pancreas.

Activins, members of TGF- β family, display similar activities to Nodal in their mesendoderm induction activity (Schier and Shen, 2000). Activins have been shown to be crucial in the terminal differentiation of pancreatic beta cells. Activin A has been shown to promote endocrine differentiation in human fetal islets (Demeterco et al., 2000). Mice deficient for activin receptors (Kim et al., 2000) or transgenic mice that express a dominant-negative mutation of activin type II receptors (Yamaoka et al., 1998; Shiozaki et al., 1999) display reduced levels of differentiated islet cells. Furthermore, we previously demonstrated that inhibition of activins significantly enhances survival and expansion of pancreatic cells but decreases the number of differentiated beta cells (Zhang et al., 2004b). The Activin antagonist, Cripto is expressed in the pancreas during islet development and islet regeneration and is important in the expansion of pancreatic epithelial cells (Zhang et al., 2004b). Given the important role of Activin receptors during pancreatic development, we hypothesized that Nodal and Lefty, which modulate Activin signaling, could be involved in the development of the pancreas. We tested this hypothesis using both the fetal pancreas and in an islet regeneration mouse model in which interferon-gamma (IFN- γ) is expressed under the control of the insulin promoter. In this model, the pancreas displays strikingly high proliferative activity in ductal epithelial cells. These ductal epithelial cells have been shown to differentiate into insulin-producing beta cells (Sarvetnick and Gu, 1992; Gu and Sarvetnick, 1993). Our results demonstrate that Nodal and Lefty are expressed in the pancreas during development and regeneration. Exposure to recombinant mouse Nodal (rmNodal) inhibited the proliferation of pancreatic cells in vitro, and conversely, recombinant mouse Lefty-1 (rmLefty-1) stimulated the proliferation of these cells. We also found that administration of a neutralizing Lefty-1 antibody in vivo resulted in decreased proliferation of duct epithelial cells, suggesting that Lefty-1 is necessary for epithelial cell expansion during islet regeneration. Taken together, our data provide evidence for a novel role of Nodal-Lefty signaling during pancreatic development and regeneration.

Results

Nodal is expressed during pancreatic ontogeny and islet regeneration

Nodal signals through Activin receptors and plays a critical role in regulating differentiation, although its effects on the pancreas have not been documented. To ask whether Nodal participates in the growth of the pancreas, we first investigated the expression of Nodal during pancreatic islet development and islet regeneration. In C57BL/6 mice, Nodal immunoreactivity was found in E13.5, E15.5 and E18 embryos during ontogeny, when expansion of the pancreas is robust. In these embryos, Nodal immunoreactivity was found in cells adjacent to duct-like structures in the pancreas, although the expression in E13.5 embryos was weaker than in E15.5 and E18 embryos (Figure 1, A–C). In E15.5 and E18 embryos, Nodal reactivity was localized within islet clusters. In adult C57BL/6 mice, relatively modest staining of Nodal was detected inside the islets, and there was no significant staining of Nodal in the ductal or acinar cells (Figure 1, D). Interestingly, in the IFN- γ mouse pancreas, the expression of Nodal demonstrated strong immunoreactivity in

individual cells associated with ducts where endocrine cells are often found in this model (Figure 1, E and F). These combined data suggested that Nodal expression might be augmented during islet development and islet regeneration.

To investigate the specificity of the anti-Nodal antibody, we performed antigen-blocking experiments with the same peptide used to raise the polyclonal antibody. As shown in Figure 2A, immunostaining with the Nodal antibody was intense in the IFN- γ pancreas, however this Nodal reactivity was completely blocked in the presence of the Nodal peptide. Next, we tested the antibody specificity on AR42J cells transiently transfected with a mouse Nodal cDNA in a pcDNA3 expression vector containing a His-tag sequence. Our staining results demonstrated that the transfected cells had strong immunostaining to the anti-Nodal antibody in cells that also expressed the His-tag protein (anti-V5 antibody), indicating that the antibody was specific. There was also very weak staining in non-transfected cells representing endogenous expression of Nodal in AR42J cells (Figure 2B). In addition, Western blotting experiments using pancreatic protein lysates and Nodal-transfected AR42J cells revealed a ~15kDa band corresponding to the mature form of Nodal, and an approximately 40kDa band corresponding to the precursor form (Figure 2C, left and middle panels), consistent with the molecular size of Nodal (Uniprot, the universal protein resource) (Bianco et al., 2002). Purified recombinant mouse Nodal elicited the same size band for the mature form (Figure 2C, right panel). To confirm our results we performed RT-PCR with mouse Nodal-specific primers on islets isolated from C57BL/6 mice; these analyses demonstrated the expression of Nodal in islets (Figure 2D). Together, these results confirmed the specificity of the reagent used in our immunohistochemical analyses.

To define the cellular specificity of Nodal expression, double and triple immunostaining experiments were performed. As shown in Figure 3A, the expression of Nodal was often observed to be colocalized with either insulin or glucagon immunoreactivity in the embryonic pancreas, in adult islets, and in the regenerating pancreas. There was a small subpopulation of non-insulin and non-glucagon Nodal-positive cells, most of which co-expressed somatostatin (data not shown). We also found that Nodal was co-expressed with PDX-1, a pancreatic transcription factor that is critical for islet development and beta cell function. Co-expression was observed both in E15.5 and E18 embryos as well in adult islets. We found that many of the PDX-1 positive cells are also Nodal positive (Figure 3B). These results suggest that Nodal might be important both in islet development and adult islet function. However, we did not observe co-localization of Nodal with another pancreatic progenitor cell marker, neurogenin 3 (Ngn3) (Figure 4A). Of note, although we found that Nodal positive cells are adjacent in the duct-like structures in E18 embryonic pancreas and in the ducts of the IFN- γ transgenic pancreas, only in very rare cells were Nodal and cytokeratin co-localized. However, in that instance, the cytokeratin immunoreactivity was weak (Figure 4B), suggesting that Nodal expression is restricted to cells exhibiting endocrine differentiation.

Lefty is expressed in the developing pancreas and during islet regeneration

Lefty is secreted and can bind to Nodal, limiting its positive influence on the Activin receptor to discrete regions. We investigated the expression pattern of Lefty in the pancreas

during ontogeny. We found strong Lefty immunoreactivity in individual cells around the islet clusters in E15.5 embryos (Figure 5A, a). In the regenerating pancreas there was strong immunoreactivity in a subset of duct cells, especially in small ducts (Figure 5A, b). We did not observe significant Lefty staining in the normal adult pancreas. However, by RT-PCR, we found the mRNA expression of Lefty using islets derived from C57BL/6 mice (Figure 2D). It should be noted that the anti-Lefty antibody we used recognizes both Lefty-1 and Lefty-2; therefore, either protein could contribute to the staining we observed.

To validate the specificity of the commercially obtained Lefty antibody for use in these experiments, we performed several sets of tests. By Western blotting, we observed a ~42 kDa band in the pancreatic protein extracts from the IFN- γ transgenic pancreas (Figure 5B, left panel) and in cell lysates in Lefty-transfected AR42J cells (Figure 5B, middle panel). The molecular weight of Lefty we observed is consistent with that reported in Uniprot and in a previous report (Ulloa et al., 2001). In addition, Western blotting studies using recombinant Lefty-1 demonstrated a ~42 kDa and a ~34 kDa band, respectively, which represent the precursor and the mature forms of Lefty-1 (Ulloa et al., 2001) (Figure 5B, right panel). Lastly, antigen-blocking experiments using sequential tissue sections demonstrated completely blockade of immunostaining by incubation with Lefty-1 blocking peptides (Figure 5C). Additional experiments were performed to investigate the cellular specificity of Lefty. In the small ducts of the IFN- γ transgenic mice, Lefty staining was found co-localized with ductal marker, cytokeratin (Figure 5D). We did not observe co-localization of Lefty-1 with Ngn3 in developing pancreas, although Lefty was found adjacent to Ngn3 positive cells (data not shown).

Nodal inhibits the proliferation and induces the apoptosis of AR42J cells

To gain insight into the functional roles of Nodal and Lefty, we used the AR42J cell line, which was derived from a chemically induced rat pancreatic acinar carcinoma (Christophe, 1994). AR42J cells provide an excellent cell system to test the effects of the Activin receptor signaling modulators Lefty and Nodal on the growth of pancreatic cells. AR42J cells are responsive to Activins and can be differentiated into insulin-producing cells by treatment with Activin A and Betacellulin (BTC) / hepatocyte growth factor (HGF) (Mashima et al., 1996a; Mashima et al., 1996b). Of note is that Activin treatment of AR42J cells leads to upregulation of Ngn3 and Pax4, which are also critical for pancreatic development in vivo (Zhang et al., 2001; Mamin and Philippe, 2007). To determine whether AR42J cells can respond to Lefty or Nodal, we first investigated the expression of Nodal, Lefty, and Cripto in AR42J cells. By Western blotting, we found that both Nodal and Lefty were weakly expressed in AR42J cells, as well as in a beta cell line, Min6 cells (Figure 6A). We also found that Nodal's co-receptor Cripto was weakly expressed in AR42J cells by immunostaining (Figure 6B, left panel). The specificity of the Cripto antibody was confirmed following transfection of the Cripto cDNA (Figure 6B, middle panel). Additional controls verified that no staining was observed in the sections incubated without Cripto antibody (Figure 6B, right panel). Thus despite the obvious limitations inherent in the use of an immortalized cell line, the AR42J cells provide us with a pancreatic cell line that demonstrates responsiveness to Activin, and induction of Ngn3, which are both relevant for endocrine cell development.

To investigate whether Nodal can regulate the growth of AR42J cells, we quantitated the proliferation of AR42J cells by measuring [^3H] thymidine incorporation. We found that Nodal (at concentrations 5 $\mu\text{g/ml}$ and above) significantly inhibited the proliferation of AR42J cells (Figure 7A). Interestingly, we found that some of the Nodal-treated cells demonstrated apoptotic condensed nuclei (Figure 7B). Increased TUNEL staining was observed in Nodal-treated AR42J cells (Figure 7C), suggesting that the growth inhibition in AR42J cells induced by Nodal may be at least partially enacted through the induction of apoptosis. Interestingly, we did not observe significant morphological changes in AR42J cells after Nodal treatment. Additional experiments were performed by incubating AR42J cells with Nodal and HGF, a protocol which has been previously reported to induce endocrine differentiation of AR42J cells when Activin A and HGF were combined (Mashima et al., 1996b). By RT-PCR, we found that Nodal did not induce the expression of endocrine cell markers such as PP, Pax 4, glucose transporter 2 (GLUT2), and insulin even in the presence of HGF (Figure 7D). Furthermore, we determined that Nodal treatment induced significant Smad2 and Smad3 phosphorylation, and that, importantly, although its coreceptor Cripto alone did not induce p-Smad2 or p-Smad3, the level of p-Smad2 and p-Smad3 was greatly increased when Cripto was supplemented in the cultures (Figure 7E). However, even with the addition of exogenous Cripto, we did not detect significant morphological changes or differentiation of Nodal-treated AR42J cells. Therefore, the previously demonstrated differentiation inducing activity of Activin A is not shared by Nodal. In addition, our results also showed that, exogenous Cripto was able to inhibit Activin A-induced p-Smad2 and p-Smad3 in AR42J cells suggesting that *in vitro*, Cripto is able to inhibit Activin signaling as well as augment Nodal signaling. The mechanism of growth inhibition induced by Nodal may involve the activation of Smad2 and Smad3.

Lefty-1 enhances the proliferation of AR42J cells

To investigate whether Lefty can regulate the growth of AR42J cells, we quantitated the proliferation of AR42J cells using two methods. First, [^3H] thymidine incorporation was measured in the presence or absence of rmLefty-1. As shown in Figure 8A, we observed that rmLefty-1 stimulated the proliferation of AR42J cells in a dose-dependent manner, and the effect was significant from 5ng/ml ($p=0.01$) and was saturated at 50ng/ml ($p=0.001$). Importantly, the rmLefty-1 mediated proliferation of AR42J cells was completely blocked by incubation with a neutralizing anti-mouse Lefty-1 monoclonal antibody (500ng/ml), demonstrating that the effect of Lefty-1 on proliferation was specific (Figure 8B). We further confirmed the Lefty-1 mediated expansion of AR42J cells using a BrdU incorporation assay. The results of this experiment showed that addition of 20ng/ml of rmLefty-1 caused an increase in BrdU-positive cells compared to control cells incubated in the absence of rmLefty-1 (Figure 8C). Although the magnitude of increased proliferation is not exactly the same in these two assays, both analyses revealed a similar trend towards increased proliferation in AR42J cells. The differences we observed might be due to the differing sensitivities of these two different assays, with BrdU incorporation require antibody staining for quantitation, which may be less sensitive than direct quantitation of the thymidine.

Lefty-1 stimulates MAPK and Akt phosphorylation in AR42J cells

To elucidate the possible signaling pathways through which Lefty affects the proliferation of AR42J cells, we investigated whether Lefty-1 could stimulate the phosphorylation of ERK/ MAPK, which can occur downstream of Lefty in other cell types (Ulloa et al., 2001). As shown in Figure 9A, Lefty-1 (20ng/ml) significantly stimulated ERK1/2 phosphorylation following 30min of treatment (3.1 fold over control, as determined by the ratio of phosphorylated ERK1/2 to actin by densitometry, $P=0.003$, $n=3$). Sixty minutes after rmLefty-1 treatment, the increase of phospho-ERK1/ERK2 was an average of 1.9 fold over controls ($P=0.02$, $n=3$). The quantity of unphosphorylated ERK1/2 was unchanged during the stimulation period.

Akt activation suppresses TGF- β responses (Conery et al., 2004; Remy et al., 2004; Song et al., 2006). We found that Akt phosphorylation was significantly increased after 60 minutes of rmLefty1 treatment (an average of 3.5 fold increase over control, $P=0.04$, $n=3$). Peak Akt phosphorylation was found at 24 hours following rmLefty stimulation (an average of 7.8 fold over control, $P=0.0006$, $n=3$). There were no significant changes in the amount of unphosphorylated Akt during the stimulation period (Figure 9B). Furthermore, we investigated the expression of the anti-apoptotic protein, Bcl-2. Our results demonstrated that the expression of Bcl-2 was significantly increased 60 minutes after rmLefty-1 treatment (an average of 1.6 fold over control, $P=0.018$, $n=3$), and peak at 6 hours after Lefty-1 treatment (Figure 9C, 2 fold over control, $P=0.04$, $n=3$). The phosphorylation of ERK1/2 and Akt in response to Lefty-1, as well as the up-regulation of Bcl-2 expression, suggest that activation of these intracellular signaling factors is involved in mediating the proliferation and survival of pancreatic AR42J cells that occurs following exposure to Lefty-1.

Lefty-1 stimulates the proliferation of a pancreatic beta cell line

Recent studies have shown that beta cell turnover results from duplication of pre-existing beta cells, suggesting that differentiated beta cells may have the ability to divide (Dor et al., 2004). Based upon the expression of Nodal and Activin receptors in pancreatic islets, we hypothesized that Lefty might affect the expansion of a pancreatic beta cell line. To test this, we used a mouse beta cell line, Min 6 cells. As shown in Figure 10A, rmLefty-1 stimulated the [^3H] thymidine incorporation of Min 6 cells in a dose-dependent manner, and the effect was significant at a concentration of 5ng/ml ($p=0.02$) and greater. The proliferation was completely blocked by the addition of 500ng/ml of the monoclonal antibody to mouse Lefty-1 (Figure 10B). Interestingly, we found that addition of the Lefty-1 antibody alone inhibited the proliferation of Min 6 cells, suggesting that these cells produce some endogenous Lefty, which was confirmed by Western blotting (Figure 6A).

Neutralization of Lefty-1 inhibits the mitosis of epithelial cells in the IFN- γ transgenic pancreas

To test the role of endogenous Lefty-1 on pancreatic epithelial cell expansion, we used the islet regeneration model, IFN- γ transgenic mice, where new islets are continuously developing. We administered neutralizing Lefty-1 mAb or isotype control antibody through the tail vein for two weeks. We chose a two-week treatment interval since previous studies

demonstrated that this treatment time frame is sufficient for us to observe changes in the mitosis and cellular composition of the growth response in this model (Kayali et al., 2003; Zhang et al., 2004b; Hua et al., 2006). Sixteen hours prior to sacrifice, BrdU was injected intraperitoneally to label the proliferating cells, and BrdU-positive cells were visualized by immunostaining of histological sections. The results showed a significantly decreased number of BrdU positive cells in the ducts in Lefty-1 mAb injected mice compared to that of the IgG-injected control mice (Figure 11A). In control IgG-treated mice, an average of 19.4 % cells in the duct epithelium were BrdU positive, compared with 12.4 % BrdU-positive cells in the ducts of mAb-treated mice (n=27 fields from three different mice, p=0.0012) (Figure 11B). The epithelial nature of the BrdU positive cells in the ducts was determined by double staining of cytokeratin and BrdU in both large and small ducts of the IFN- γ pancreas (Figure 11C). This significant decrease in the proportion of BrdU-positive duct cells indicated that Lefty-1 has a critical role in the net expansion of ductal epithelium in this model. In NOD and C57BL/6 mice that received the same amount of neutralizing Lefty monoclonal antibody as the transgenic mice did, we did not observe significant changes in BrdU incorporation in the pancreas compared with IgG-injected control mice (data not shown). Two factors might contribute to our observations. Lefty might regulate growth processes primarily during active cell proliferation, as is observed during islet regeneration, or alternatively, the low proliferation rate observed in the adult non-transgenic pancreas might limit our ability to detect such an effect.

Discussion

We showed that Nodal, as well as its antagonist, Lefty, are expressed in pancreatic cell lines and in the pancreas during development and islet regeneration. Nodal is secreted and signals through activin receptors with its co-receptor Cripto (Vincent et al., 2003). We have shown that Activin receptors are expressed in the embryonic pancreas (Zhang et al., 2004b) and adult islets (Zhang et al., 2002). Our previous studies also demonstrated that Cripto is expressed during islet development and its expression is up-regulated in a model of islet regeneration (Zhang et al., 2004b). Interestingly, in the present study, by RT-PCR, Cripto transcript was also detected in the adult islet (Figure 2D). Therefore, it is likely that Nodal exerts its function through modulating activin receptor signals in the pancreas. Nodal was found to be expressed in duct associated islet cell clusters whereas Lefty was found mainly in duct cells with an epithelial phenotype. Within the developing pancreas, the juxtaposition of these two factors would limit their respective range of influence within the pancreatic tissue. Whereas Lefty would act to maintain cell survival and growth in ductal areas, its range of effects would be diminished as the new islet clusters migrated away from the pancreatic ducts. Once these islet clusters leave the ducts the Nodal signal would slow cell division. A previous study by Dichmann et al demonstrated that Nodal and Lefty are not expressed in the developing pancreas (Dichmann et al., 2003), the discrepancy between their data and our results is likely due to the different conditions and primers used for RT-PCR. In addition, data from the beta cell consortium website also demonstrated that Nodal transcripts are expressed during embryonic development.

Activin A inhibited the proliferation and induced morphological changes in AR42J cells that are associated with neuronal or endocrine differentiation (Ohnishi et al., 1995; Zhang et al.,

1999). Like Activin A, Nodal inhibited the proliferation and induced the apoptosis of these cells. In addition, Nodal's induction of Smad2/3 was partially dependent on supplementation with additional exogenous Cripto, supporting its specificity in our assay. However, Nodal did not induce overt morphological differentiation in AR42J cells, indicating a divergence of functions that need to be clarified in the future. Interestingly, we observed the activated p-Smad2 and p-Smad3 following Nodal or Activin A stimulations. A role for Smad2/3 during the differentiation of pancreatic islets was implicated by elegant transgenic studies where their inhibitor Smad7 was expressed under the control of the PDX-1 promoter (Smart et al., 2006). These transgenic mice demonstrate a dramatic decrease in islet beta cells when Smad7 is expressed. Therefore the induction of Smad2/3 by Nodal may also be linked to the differentiation of pancreatic beta cells.

The modulation of cell survival mechanisms is a critical component of differentiation process. The serine/threonine kinase Akt (or protein kinase B) has been implicated in mitogen-regulated control of cell growth and survival (Kandel and Hay, 1999). A previous report demonstrated that beta cell specific overexpression of Akt significantly increased islet mass, improved glucose tolerance and conferred resistance to experimental diabetes (Tuttle et al., 2001). Interestingly, recent studies show that Akt suppresses TGF- β -induced apoptosis by directing binding to TGF- β downstream mediators, such as Smad3 (Conery et al., 2004; Remy et al., 2004; Song et al., 2006). Therefore, it is possible that Lefty, by activating Akt, inhibits TGF- β -like signaling of Nodal in the pancreas, maintaining the homeostasis between cell growth and death. However, further investigations are needed to fully elucidate the signaling pathways involved. Bcl-2 is a well known anti-apoptotic protein which is involved in promoting cell survival (Merry and Korsmeyer, 1997). Studies have shown that Akt signaling promotes cell survival through up-regulation of Bcl-2 expression (Pugazhenthil et al., 2000), which represents an important mechanism by which growth factors promote cell survival (Singleton et al., 1996; Minshall et al., 1997). Here, we present evidence that Akt phosphorylation and Bcl-2 up-regulation occur following Lefty-1 treatment. We hypothesize that Lefty-1 induced proliferation of the AR42J pancreatic progenitor cell line involves phosphorylation of MAPK and Akt, as well as enhanced expression of Bcl-2.

Members of the TGF- β family modulate diverse cellular responses in numerous cell types. TGF- β signals through two distinct mechanisms, MAPK activation (Yue and Mulder, 2000) and Smad phosphorylation (Massague and Chen, 2000). Lefty-1, which we observed to activate MAPK in AR42J cells, may directly induce MAPK activation as previously reported (Ulloa et al., 2001). Alternatively, it is possible that when Lefty-1 binds to Cripto, it activates Cripto signal transduction in an Activin receptor independent manner. Indeed, Cripto itself has been reported to activate MAPK and Akt (Kannan et al., 1997; Ebert et al., 1999), suggesting a distinct mechanism for Cripto's participation in the regulation of cell proliferation. The exact binding mechanisms used by these molecules to exert their functions needs to be clarified in future studies.

Lefty antagonizes EGF-CFC co-receptor dependent TGF- β signaling, affecting the biological actions of Nodal and Vg1/GDF (growth and differentiation factor). This antagonism is achieved via interaction of Lefty with Nodal or Nodal's co-receptor, Cripto (Branford and Yost, 2004; Chen and Shen, 2004). In fact, we have performed Western blot

to detect changes in endogenous Nodal expression, and found that with rmLefty-1 treatment, endogenous Nodal expression was partially inhibited in the AR42J cells (data not shown), suggesting that in these cells, it is possible that, at least partially, the effect of Lefty on proliferation may be induced by inhibition of endogenous Nodal. Interestingly, Lefty has been shown to stimulate the expression of matrix metalloproteinases (MMPs) in the endometrium, which promote matrix remodeling (Cornet et al., 2005). The ability to modulate the composition of the extracellular matrix could promote cell migration. Cell migration is an essential feature of pancreatic islet development and its regulation underlies pancreas formation. Therefore, it will be of interest to investigate the impact of Lefty on the migration of pancreatic progenitor cells in future studies.

Our work shows that the Nodal/Lefty pathway participates in the process of pancreatic growth in both cultured cells and during islet regeneration. Further study of this area is warranted since it represents a novel pathway for the generation of pancreatic beta cells.

Experimental Procedures

Mice

The IFN- γ transgenic mice were on the Non Obese Diabetic (NODShi) background. All mice were maintained in a specific pathogen-free facility at The Scripps Research Institute (TSRI). All studies were carried out in strict accordance with guidelines from the Animal Care and Use Committee at TSRI.

Antibodies and reagents

Antibodies used in this study are included in the supplemental data of Table 1. RmLefty-1, rmNodal and rhCripto were purchased from R&D systems (Minneapolis, MN). The amount of rmNodal used in these experiments was based on the manufacturer's data sheet and a previous report (Kumar, JBC, 2001). RhActivin A was a generous gift from Dr. Yuzuru Eto (Pharmaceutical Research Laboratories, Ajinomoto Co., Kawasaki, Japan).

Immunohistochemical staining, Western blotting and TUNEL assays

Tissues from the pancreas were fixed in Bouin's solution, embedded in paraffin and stained as previously described (Zhang et al., 2002). DAB images were obtained using a Zeiss Axioskop (West Germany). Fluorescence images were observed using a BioRad MRC 1024 scanning confocal microscope (Richmond, CA). Negative controls were performed without the primary antibodies. Western blotting was performed as described previously (Kayali et al., 2003). Densitometry analysis was carried out using Image J software. For detection of apoptosis, AR42J cells were incubated with Nodal or Activin A for 24 hours. TUNEL staining was performed using the Roche In Situ Cell Death Detection POD kit (Roche, Indianapolis, IN). To determine antibody specificity, the antibody was incubated with excess peptide (50 fold over the antibody concentration according to the manufacturer's instructions) for 24 hours at 4°C. After centrifugation, the supernatant was used as the primary antibody.

Reverse transcriptase polymerase chain reaction (RT-PCR)

Pancreatic islets were obtained by hand picking after collagenase digestion (Zhang et al., 2004a). RT-PCR was performed as previously described (Zhang et al., 2002). The oligonucleotide primers used are summarized in the supplemental data of Table 2.

CDNA transfection

Mouse Nodal and Lefty-1 cDNAs were kindly provided by Dr. Hiroshi Hamada (Graduate School of Frontier Biosciences, Osaka University, Japan) and Dr. Michael M. Shen (Center for Advanced Biotechnology and Medicine, UMDNJ-Robert Wood Johnson Medical School, Piscataway, NJ), respectively. These cDNAs were subcloned into a pcDNA3.1/V5-His vector (Invitrogen). Mouse Cripto cDNA (mCR-1/pEF) was kindly provided by Dr. David Salomon (Mammary Biology and Tumorigenesis Laboratory, National Institutes of Health, Bethesda, MD). Transient transfections were performed using FuGENE 6 (Roche Diagnostics, Indianapolis, IN).

Cell culture and measurement of cell proliferation

AR42J and Min 6 cells were generously provided by Dr. Itaru Kojima (Institute for Molecular and Cellular Regulation, Gunma University, Maebashi, Japan). AR42J cells were maintained in DMEM low glucose with 10% FBS. Min 6 cells were cultured in DMEM high glucose with 15% FBS. All cultures were kept at 37°C under humidified conditions of 95% air and 5% CO₂. To measure [³H] thymidine incorporation, cells were plated in 96-well dishes and the growth of cells was arrested in serum free medium for 24 hours, and at which point the indicated treatments were started. 10μCi/ml [³H] thymidine was added at 42 hours after treatment and the culture was incubated for an additional 4 hours. [³H] Thymidine incorporation was quantitated on a 1205 BetaplateTM liquid scintillation counter (Perkin Elmer, Shelton, CT). For determination of the labeling index, BrdU (10mM) was included from 42–46 hours during the incubation. Cells were then fixed in 4% paraformaldehyde and stained with anti-BrdU antibody. The labeled cells were tabulated as the percentage of BrdU positive cells out of the total number of nuclei stained with Topro-3.

Lefty-1 neutralizing monoclonal antibody (Lefty-1mAb) treatment and identification of BrdU-labeled cells

To test the role of endogenous Lefty in epithelial cell expansion, neutralizing monoclonal antibody for mouse Lefty-1 (Lefty-1 mAb) was used. The Lefty-1 mAb was dissolved in PBS, and 20 μg per mouse was injected through the tail vein every other day for two weeks. The dose of monoclonal antibody used was deduced from the concentration yielding the neutralizing capacity on AR42J cells *in vitro*, and the manufacturer's data. We found that 500ng/ml is enough to block the exogenous effect of Lefty-1 (10ng/ml) *in vitro*, and the data sheet indicated that 18–26ng/ml of this antibody results in 50% reduction in Lefty-1-induced cardiac reversals in *Xenopus* embryos. We doubled the concentration from our own data to achieve effective neutralization. Control mice were injected with rat IgG (Sigma, 20μg/mouse). To examine the regeneration of duct epithelial cells, BrdU (100μg/g of body weight) was injected 16h before sacrifice. The labeled cells were evaluated as the percentage of BrdU-positive cells out of the total number of duct epithelial cells. Ten fields were

counted per section and four sections from different levels were counted per mouse. In total, 3 mice were examined for each test group.

Statistical analysis

All results from this study were expressed as mean $\pm$ SD and statistical analysis was carried out using the Student's *t* test for two-tailed unpaired data. Differences with $P < 0.05$ were considered to be significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We are grateful to Dr. Itaru Kojima for AR42J and Min 6 cell lines; Dr. Yuzuru Eto for the recombinant Activin A; Dr. Michael German for the rabbit anti-neurogenin 3 antibody; Dr. David Salomon for Cripto cDNA; and Dr. Hiroshi Hamada and Dr. Michael M. Shen for Nodal and Lefty-1 cDNAs. This work was supported by the National Institute of Health grant DK 60746 to Nora Sarvetnick. Hong Hua was supported by a fellowship from the Larry L. Hillblom Foundation. You-Qing Zhang was supported by a fellowship from NIH training grant T32 HL00795 and a career development award from the CROHN'S & COLITIS FOUNDATION OF AMERICA. This is manuscript number 18195-IMM from the Scripps Research Institute.

References

- Bianco C, Adkins HB, Wechselberger C, Seno M, Normanno N, De Luca A, Sun Y, Khan N, Kenney N, Ebert A, Williams KP, Sanicola M, Salomon DS. Cripto-1 activates nodal- and ALK4-dependent and -independent signaling pathways in mammary epithelial Cells. *Mol Cell Biol.* 2002; 22:2586–2597. [PubMed: 11909953]
- Branford WW, Yost HJ. Nodal signaling: CrypticLefty mechanism of antagonism decoded. *Curr Biol.* 2004; 14:R341–343. [PubMed: 15120085]
- Chen C, Shen MM. Two modes by which Lefty proteins inhibit nodal signaling. *Curr Biol.* 2004; 14:618–624. [PubMed: 15062104]
- Cheng SK, Olale F, Brivanlou AH, Schier AF. Lefty blocks a subset of TGFbeta signals by antagonizing EGF-CFC coreceptors. *PLoS Biol.* 2004; 2:E30. [PubMed: 14966532]
- Christophe J. Pancreatic tumoral cell line AR42J: an amphicrine model. *Am J Physiol.* 1994; 266:G963–971. [PubMed: 7517639]
- Conery AR, Cao Y, Thompson EA, Townsend CM Jr, Ko TC, Luo K. Akt interacts directly with Smad3 to regulate the sensitivity to TGF-beta induced apoptosis. *Nat Cell Biol.* 2004; 6:366–372. [PubMed: 15104092]
- Conlon FL, Lyons KM, Takaesu N, Barth KS, Kispert A, Herrmann B, Robertson EJ. A primary requirement for nodal in the formation and maintenance of the primitive streak in the mouse. *Development.* 1994; 120:1919–1928. [PubMed: 7924997]
- Cornet PB, Galant C, Eeckhout Y, Courtoy PJ, Marbaix E, Henriët P. Regulation of matrix metalloproteinase-9/gelatinase B expression and activation by ovarian steroids and LEFTY-A/endometrial bleeding-associated factor in the human endometrium. *J Clin Endocrinol Metab.* 2005; 90:1001–1011. [PubMed: 15536155]
- Demeterco C, Beattie GM, Dib SA, Lopez AD, Hayek A. A role for activin A and betacellulin in human fetal pancreatic cell differentiation and growth. *J Clin Endocrinol Metab.* 2000; 85:3892–3897. [PubMed: 11061554]
- Dichmann DS, Miller CP, Jensen J, Scott Heller R, Serup P. Expression and misexpression of members of the FGF and TGFbeta families of growth factors in the developing mouse pancreas. *Dev Dyn.* 2003; 226:663–674. [PubMed: 12666204]

- Dor Y, Brown J, Martinez OI, Melton DA. Adult pancreatic beta-cells are formed by self-duplication rather than stem-cell differentiation. *Nature*. 2004; 429:41–46. [PubMed: 15129273]
- Ebert AD, Wechselberger C, Frank S, Wallace-Jones B, Seno M, Martinez-Lacaci I, Bianco C, De Santis M, Weitzel HK, Salomon DS. Cripto-1 induces phosphatidylinositol 3'-kinase-dependent phosphorylation of AKT and glycogen synthase kinase 3 β in human cervical carcinoma cells. *Cancer Res*. 1999; 59:4502–4505. [PubMed: 10493495]
- Gu D, Sarvetnick N. Epithelial cell proliferation and islet neogenesis in IFN- γ transgenic mice. *Development*. 1993; 118:33–46. [PubMed: 8104143]
- Hamada H, Meno C, Watanabe D, Saijoh Y. Establishment of vertebrate left-right asymmetry. *Nat Rev Genet*. 2002; 3:103–113. [PubMed: 11836504]
- Hua H, Zhang YQ, Arnaud-Dabernat S, Kritzik M, Dietz D, Sterling L, Sarvetnick NE. BMP4 regulates pancreatic progenitor cell expansion through ID2. *J Biol Chem*. 2006
- Kandel ES, Hay N. The regulation and activities of the multifunctional serine/threonine kinase Akt/PKB. *Exp Cell Res*. 1999; 253:210–229. [PubMed: 10579924]
- Kannan S, De Santis M, Lohmeyer M, Riese DJ 2nd, Smith GH, Hynes N, Seno M, Brandt R, Bianco C, Persico G, Kenney N, Normanno N, Martinez-Lacaci I, Ciardiello F, Stern DF, Gullick WJ, Salomon DS. Cripto enhances the tyrosine phosphorylation of Shc and activates mitogen-activated protein kinase (MAPK) in mammary epithelial cells. *J Biol Chem*. 1997; 272:3330–3335. [PubMed: 9013573]
- Kayali AG, Van Gunst K, Campbell IL, Stotland A, Kritzik M, Liu G, Flodstrom-Tullberg M, Zhang YQ, Sarvetnick N. The stromal cell-derived factor-1 α /CXCR4 ligand-receptor axis is critical for progenitor survival and migration in the pancreas. *J Cell Biol*. 2003; 163:859–869. [PubMed: 14638861]
- Kim SK, Hebrok M, Li E, Oh SP, Schrewe H, Harmon EB, Lee JS, Melton DA. Activin receptor patterning of foregut organogenesis. *Genes Dev*. 2000; 14:1866–1871. [PubMed: 10921901]
- Mamin A, Philippe J. Activin A decreases glucagon and arx gene expression in alpha-cell lines. *Mol Endocrinol*. 2007; 21:259–273. [PubMed: 16988001]
- Mashima H, Ohnishi H, Wakabayashi K, Mine T, Miyagawa J, Hanafusa T, Seno M, Yamada H, Kojima I. Betacellulin and activin A coordinately convert amylase-secreting pancreatic AR42J cells into insulin-secreting cells. *J Clin Invest*. 1996a; 97:1647–1654. [PubMed: 8601630]
- Mashima H, Shibata H, Mine T, Kojima I. Formation of insulin-producing cells from pancreatic acinar AR42J cells by hepatocyte growth factor. *Endocrinology*. 1996b; 137:3969–3976. [PubMed: 8756573]
- Massague J, Chen YG. Controlling TGF- β signaling. *Genes Dev*. 2000; 14:627–644. [PubMed: 10733523]
- Meno C, Gritsman K, Ohishi S, Ohfuji Y, Heckscher E, Mochida K, Shimono A, Kondoh H, Talbot WS, Robertson EJ, Schier AF, Hamada H. Mouse Lefty2 and zebrafish antivin are feedback inhibitors of nodal signaling during vertebrate gastrulation. *Mol Cell*. 1999; 4:287–298. [PubMed: 10518210]
- Meno C, Shimono A, Saijoh Y, Yashiro K, Mochida K, Ohishi S, Noji S, Kondoh H, Hamada H. lefty-1 is required for left-right determination as a regulator of lefty-2 and nodal. *Cell*. 1998; 94:287–297. [PubMed: 9708731]
- Merry DE, Korsmeyer SJ. Bcl-2 gene family in the nervous system. *Annu Rev Neurosci*. 1997; 20:245–267. [PubMed: 9056714]
- Minshall C, Arkins S, Straza J, Conners J, Dantzer R, Freund GG, Kelley KW. IL-4 and insulin-like growth factor-I inhibit the decline in Bcl-2 and promote the survival of IL-3-deprived myeloid progenitors. *J Immunol*. 1997; 159:1225–1232. [PubMed: 9233617]
- Ohnishi H, Ohgushi N, Tanaka S, Mogami H, Nobusawa R, Mashima H, Furukawa M, Mine T, Shimada O, Ishikawa H, et al. Conversion of amylase-secreting rat pancreatic AR42J cells to neuronlike cells by activin A. *J Clin Invest*. 1995; 95:2304–2314. [PubMed: 7537763]
- Pugazhenthai S, Nesterova A, Sable C, Heidenreich KA, Boxer LM, Heasley LE, Reusch JE. Akt/protein kinase B up-regulates Bcl-2 expression through cAMP-response element-binding protein. *J Biol Chem*. 2000; 275:10761–10766. [PubMed: 10753867]

- Remy I, Montmarquette A, Michnick SW. PKB/Akt modulates TGF-beta signalling through a direct interaction with Smad3. *Nat Cell Biol.* 2004; 6:358–365. [PubMed: 15048128]
- Sarvetnick NE, Gu D. Regeneration of pancreatic endocrine cells in interferon-gamma transgenic mice. *Adv Exp Med Biol.* 1992; 321:85–89. discussion 91–83. [PubMed: 1449085]
- Schier AF. Nodal signaling in vertebrate development. *Annu Rev Cell Dev Biol.* 2003; 19:589–621. [PubMed: 14570583]
- Schier AF, Shen MM. Nodal signalling in vertebrate development. *Nature.* 2000; 403:385–389. [PubMed: 10667782]
- Shiozaki S, Tajima T, Zhang YQ, Furukawa M, Nakazato Y, Kojima I. Impaired differentiation of endocrine and exocrine cells of the pancreas in transgenic mouse expressing the truncated type II activin receptor. *Biochim Biophys Acta.* 1999; 1450:1–11. [PubMed: 10231550]
- Singleton JR, Dixit VM, Feldman EL. Type I insulin-like growth factor receptor activation regulates apoptotic proteins. *J Biol Chem.* 1996; 271:31791–31794. [PubMed: 8943217]
- Smart NG, Apelqvist AA, Gu X, Harmon EB, Topper JN, MacDonald RJ, Kim SK. Conditional expression of Smad7 in pancreatic beta cells disrupts TGF-beta signaling and induces reversible diabetes mellitus. *PLoS Biol.* 2006; 4:e39. [PubMed: 16435884]
- Song K, Wang H, Krebs TL, Danielpour D. Novel roles of Akt and mTOR in suppressing TGF-beta/ALK5-mediated Smad3 activation. *Embo J.* 2006; 25:58–69. [PubMed: 16362038]
- Tuttle RL, Gill NS, Pugh W, Lee JP, Koeberlein B, Furth EE, Polonsky KS, Naji A, Birnbaum MJ. Regulation of pancreatic beta-cell growth and survival by the serine/threonine protein kinase Akt1/PKBalpha. *Nat Med.* 2001; 7:1133–1137. [PubMed: 11590437]
- Ulloa L, Creemers JW, Roy S, Liu S, Mason J, Tabibzadeh S. Lefty proteins exhibit unique processing and activate the MAPK pathway. *J Biol Chem.* 2001; 276:21387–21396. [PubMed: 11278322]
- Vincent SD, Dunn NR, Hayashi S, Norris DP, Robertson EJ. Cell fate decisions within the mouse organizer are governed by graded Nodal signals. *Genes Dev.* 2003; 17:1646–1662. [PubMed: 12842913]
- Yamaoka T, Idehara C, Yano M, Matsushita T, Yamada T, Ii S, Moritani M, Hata J, Sugino H, Noji S, Itakura M. Hypoplasia of pancreatic islets in transgenic mice expressing activin receptor mutants. *J Clin Invest.* 1998; 102:294–301. [PubMed: 9664070]
- Yeo C, Whitman M. Nodal signals to Smads through Cripto-dependent and Cripto-independent mechanisms. *Mol Cell.* 2001; 7:949–957. [PubMed: 11389842]
- Yue J, Mulder KM. Requirement of Ras/MAPK pathway activation by transforming growth factor beta for transforming growth factor beta 1 production in a smad-dependent pathway. *J Biol Chem.* 2000; 275:35656. [PubMed: 11063751]
- Zhang H, Nagasawa M, Yamada S, Mogami H, Suzuki Y, Kojima I. Bimodal role of conventional protein kinase C in insulin secretion from rat pancreatic beta cells. *J Physiol.* 2004a; 561:133–147. [PubMed: 15388777]
- Zhang YQ, Cleary MM, Si Y, Liu G, Eto Y, Kritzik M, Dabernat S, Kayali AG, Sarvetnick N. Inhibition of activin signaling induces pancreatic epithelial cell expansion and diminishes terminal differentiation of pancreatic beta-cells. *Diabetes.* 2004b; 53:2024–2033. [PubMed: 15277382]
- Zhang YQ, Kanzaki M, Furukawa M, Shibata H, Ozeki M, Kojima I. Involvement of Smad proteins in the differentiation of pancreatic AR42J cells induced by activin A. *Diabetologia.* 1999; 42:719–727. [PubMed: 10382592]
- Zhang YQ, Mashima H, Kojima I. Changes in the expression of transcription factors in pancreatic AR42J cells during differentiation into insulin-producing cells. *Diabetes.* 2001; 50(Suppl 1):S10–14. [PubMed: 11272164]
- Zhang YQ, Zhang H, Maeshima A, Kurihara H, Miyagawa J, Takeuchi T, Kojima I. Up-regulation of the expression of activins in the pancreatic duct by reduction of the beta-cell mass. *Endocrinology.* 2002; 143:3540–3547. [PubMed: 12193568]
- Zhou X, Sasaki H, Lowe L, Hogan BL, Kuehn MR. Nodal is a novel TGF-beta-like gene expressed in the mouse node during gastrulation. *Nature.* 1993; 361:543–547. [PubMed: 8429908]

Figure 1.

Identification of Nodal expression in the pancreas of C57BL/6 and IFN- γ transgenic mice. Sections were stained with anti-Nodal antibody and counterstained with hematoxylin. Magnification: 40 $\times$ objective. Nodal immunoreactivity was found in islet clusters during ontogeny, E13.5 (A), E15.5 (B), E18 (C), in islets of adult pancreas of C57BL/6 mice (D) and in ductal areas of IFN- γ transgenic pancreas (E and F). i: islet; d: duct; E: embryonic day. Arrows represent the Nodal immunoreactivity area.

Figure 2.

Validation of the anti-Nodal antibody. A: Sections from IFN- γ transgenic pancreas were stained with anti-Nodal antibody treated without or with blocking peptide. *: Same area from the sequential sections. Magnification: 40 $\times$ objective. B: AR42J cells were transfected with mouse Nodal cDNA in a His-tagged pcDNA3 vector and stained with anti-Nodal (green) and anti-V5-His (red) antibodies. The transfected Nodal was detected using anti-Nodal antibodies and confirmed by colocalization with anti-V5-His antibodies (yellow in the merged panel). Magnification: 40 $\times$ objective. C: Western blotting was performed using pancreatic tissue lysate (left panel); vector control (C) and Nodal cDNA-transfected (Tr) AR42J cell lysates (middle panel) and purified rmNodal (right panel). D: Expression of Nodal, Lefty, and Cripto mRNA in islets from C57BL/6 pancreata was demonstrated by RT-PCR. RT (+): reverse transcriptase; RT (-): without reverse transcriptase; M: 100bp DNA size marker.

Figure 3.

The cellular specificity of Nodal expression in the pancreas. A: Tissue sections from C57BL/6 embryonic pancreas (E18), adult C57BL/6 pancreas (2 months), and adult IFN- γ pancreas (2 months) were triple stained with anti-Nodal (green), anti-insulin (red), and anti-glucagon (blue) antibodies. Nodal-positive cells were found colocalized with both insulin and glucagons. B: Sections from the embryonic pancreas (E18) and adult C57BL/6 pancreas were double stained with anti-Nodal (green) and anti-PDX-1 (red) antibodies. White arrows indicate the colocalization of Nodal and PDX-1. Scale bars = 50 μ m.

Figure 4.

A: Sections from an embryonic pancreas (E15.5) were double stained with antiNodal (green) and anti-Ngn3 (red) antibodies. Nodal positive cells were not found to colocalize with Ngn3. B: Sections from pancreas were double stained with anti-Nodal (green) and anti-pan CK (red) antibodies. Nodal positive cells were not found to colocalize with cytokeratin. E: embryonic day; CK: cytokeratin; Ngn3: neurogenin 3. Scale bars = 50 μ m.

Figure 5.

Expression of Lefty in the pancreas. A: Sections from the embryonic pancreas (E15.5) (left panel) and IFN- γ pancreas (right panel) were stained with anti-Lefty antibody and counterstained with hematoxylin. Arrows indicate the Lefty immunoreactivity area. Magnification: 40 $\times$ objective. B: Western blotting was performed using pancreatic tissues from adult pancreas (left panel), AR42J cell lysates (middle panel), vector control (C); Lefty cDNA-transfected (Tr) and purified rmLefty (right panel). C: Sequential sections from IFN- γ pancreas were stained with anti-Lefty antibody in the absence (a, c) or presence (b, d) of Lefty blocking peptide. The staining for Lefty was effectively reduced by pre-incubation of the antibody with blocking peptide. Black arrows indicate the same area from sequential sections. Magnification: 40 $\times$ objective. D: Sections from IFN- γ pancreas were double stained with anti-Lefty (green) and anti-pan CK (red) antibodies. Some Lefty positive cells colocalized with cytokeratin (yellow in the merge). CK: cytokeratin. Scale bar = 50 μ m.

Figure 6.

A: Identification of Nodal and Lefty expression in AR42J and Min6 cells. Cell lysates from AR42J or Min6 cells were extracted and Western blotting was performed. B: Expression of Cripto in AR42J cells. Vector control (right panel) and Cripto-transfected (middle panel) AR42J cells were fixed and stained with an anti-mouse Cripto antibody (green) and Topro-3 to detect the nuclei (blue). Negative control (right panel): incubated without Cripto antibody. Scale bar = 50 μ m.

Figure 7.

Effect of Nodal and Activin A in AR42J cells. A: Effect of Nodal and Activin A on the proliferation of AR42J cells. AR42J cells were incubated with different concentrations of Nodal or Activin A, and 10μCi/ml [³H] thymidine was added at 42 hours and the incubation was continued for a further 4 hours. [³H] Thymidine incorporation was quantitated. The data is shown as mean ± SD of three independent experiments. *P< 0.05; **P< 0.01 vs. control. B: Effect of Nodal and Activin A on the morphology in AR42J cells. Serum starved AR42J cells were incubated with Nodal or Activin A for 48 hours in medium containing 0.5% FBS. Activin A treatment induced the morphology of AR42J cells to change into neuron-like cells, whereas Nodal treatment induced apoptosis in some cells (black arrows) but no

obvious morphological changes in AR42J cells. C: Effect of Activin A and Nodal on the survival of AR42J cells. AR42J cells were incubated with Activin A or Nodal for 24 hours, and tunnel staining was performed. Apoptotic cells appear as dark brown. a: Untreated control cells; b: No enzyme, AR42J cells treated with Activin A, and the staining was performed without terminal transferase; c-d: Cells treated with Activin A (50ng/ml); e-f: Cells treated with Nodal (5 μ g/ml). Scale bars = 50 μ m. D: Expression of differentiated endocrine markers in treated AR42J cells. AR42J cells were incubated with Activin A or Nodal for 48–72 hours, total RNA was extracted and RT-PCR was performed. M: 100bp DNA size marker. 1: Untreated AR42J cells; 2: Nodal (5 μ g/ml); 3: Nodal (5 μ g/ml) + HGF (10ng/ml); 4: Activin A (50ng/ml); 5: Activin A (50ng/ml)+HGF (10ng/ml); 6: RT (-); 7: rat islet. E: Phosphorylation of Smad2 and Smad3 induced by Nodal or Activin A. AR42J cells were incubated with Nodal (5 μ g/ml), Activin A (50ng/ml) and Cripto (250ng/ml) for 30 min. Whole cell lysates were immunoblotted with phopho-Smad2, phosho-Smad3, total Smad2/3 and anti- β -actin antibodies. Control: no treatment.

Figure 8.

Effect of rmLefty on the proliferation of AR42J cells. AR42J cells were incubated with the different concentrations of rmLefty-1 for 42 hours. 10 μ ci/ml [³H] thymidine (A) or 10mM BrdU (C) was added after 42 hours incubation and incubated for an additional 4 hours. [³H] Thymidine incorporation was quantitated and BrdU positive cells were counted. A: Representative experiment of dose-dependent rmLefty-1 stimulated AR42J cell proliferation; data are shown as mean $\pm$ SD (n=4). Similar results were obtained from four independent experiments. B: Effect of rmLefty-1 (20ng/ml) on the proliferation of AR42J cells in the presence or absence of Lefty mAb (500ng/ml). Data are shown as mean $\pm$ SD of three independent experiments. C: Percentage of BrdU positive cells in the presence or absence of rmLefty-1 (20ng/ml). Data are shown as mean $\pm$ SD of three different experiments. *P<0.05; **P<0.01 vs. control. Scale bar = 50 μ m.

Figure 9.

Kinetics of rmLefty-1 stimulated phosphorylation of MAPK and Akt and induction of Bcl-2 expression. AR42J cells were incubated with rmLefty-1 (20ng/ml) for the indicated times. Whole cell lysates were immunoblotted with antibodies to phospho-ERK1/2 (p-44/p-42), total ERK1/2 (t-44/t-42) (A), phospho-Akt, total Akt (B), and Bcl-2 (C). The blots were stripped and reblotted with anti-β-actin antibody to normalize for differences in protein loading. Data shown is a representative of three independent experiments with similar results.

Figure 10.

A: Representative data from dose-dependent rmLefty-1 stimulation of Min 6 cell proliferation; data are mean $\pm$ SD (n=4). Similar results were obtained from three independent experiments. B: Effect of rmLefty-1 (20ng/ml) on the proliferation of Min 6 cells in the presence or absence of Lefty neutralizing monoclonal antibody (Lefty-1 mAb). Data are shown as mean $\pm$ SD (n=4) of a representative experiment. Similar results were obtained from 3 independent experiments. *P< 0.05; **P< 0.01 vs. control.

Figure 11.

A: Representative data of Lefty-1 neutralizing monoclonal antibody (Lefty-1 mAb) on the proliferation of pancreatic progenitor cells from BrdU histological analyses. Sections from Lefty-1 mAb-treated and IgG-treated control mice were stained with an anti-BrdU antibody (brown color). Magnification: 40× objective. B: The ratio of BrdU-positive ductal cells to the total number of ductal cells. Data are shown as mean $\pm$ SD (n=3 mice). ** $P < 0.01$ vs. control. C: Sections from IFN- γ pancreas were double stained with anti-BrdU (green) and anti-pan CK (red) antibodies. White arrows indicate the BrdU and Pan-CK positive cells. CK: cytokeratin. Scale bars = 50μm