Inhibitor of DNA binding (Id) proteins bind to and inhibit the function of basic helix-loop-helix (bHLH) transcription factors including those that regulate pancreatic development. Moreover, bone morphogenetic proteins (BMPs) regulate the expression of Ids. We hypothesized that BMP4 and Id proteins play a role in the expansion and differentiation of epithelial progenitor cells. We demonstrate that BMP4 induces the expression of Id2 along with the expansion of AR42J pancreatic epithelial cells. Furthermore, neutralization of BMP4 significantly reduced duct epithelial cell expansion in a null mouse model of islet regeneration. BMP4 stimulation promotes Id2 binding to the bHLH transcription factor NeuroD, which is required for the differentiation of pancreatic islet cells. Therefore, our results indicate that BMP4 stimulation blocks the differentiation of endocrine progenitor cells and instead promotes their expansion thereby revealing a novel paradigm of signaling explaining the balance between expansion and differentiation of pancreatic duct epithelial progenitors. Understanding the mechanisms of BMP and Id function elucidates a key step during pancreas embryogenesis, which is important knowledge for expanding pancreatic progenitors in vitro.

The progression of pancreatic progenitors into the differentiated endocrine or exocrine lineage is governed by the sequential expression of key transcription factors. Several basic helix-loop-helix (bHLH) transcription factors, which bind to regulatory E box segments (CANNTG) of target genes, are important for pancreas development. For example, Ngn3 is specifically required for the development of the endocrine cells, as was demonstrated in null mice (1). Hes-1, another bHLH factor, is a critical repressor of endocrine differentiation, because in its absence accelerated differentiation of endocrine cells occurs (2, 3). Moreover, NeuroD regulates the survival and terminal differentiation of pancreatic beta cells (4). However, the mechanisms regulating the biological activities of these bHLH transcription factors are not completely understood.

Inhibition of differentiation proteins (Ids), comprised of four members (Id1–Id4), is a family of proteins that are implicated in a number of cellular processes, including control of proliferation and differentiation (5, 6). The major role of Id proteins is to bind to and inhibit the function of bHLH transcription factors such as those described above that are critical for pancreatic development. Id proteins lack basic DNA-binding domains and heterodimers between Id and bHLH proteins cannot bind DNA, allowing them to inhibit the biological activity of these transcription factors (7, 8). In this sense, Ids inhibit transcription factor binding and may serve to regulate the timing of key events during pancreatic ontogeny. Indeed, the expression of Ids has been detected in the fetal pancreas in a microarray study (9). Importantly, Id2 has been shown to prevent NeuroD from binding to its DNA target (10). Because NeuroD is required for pancreatic islet development, we hypothesized that Id proteins may be important in regulating its biological function, thereby affecting the kinetics of cell growth and differentiation.

Bone morphogenetic proteins (BMPs) are pleiotropic proteins that regulate proliferation, differentiation, and migration of various cell types (reviewed in Ref. 11). BMPs signal through heteromeric complex combinations of types I and II serine/threonine kinase receptors and their nuclear effectors, SMADs (11). Binding of BMP to at least one type I and type II receptor is necessary for signaling (12). Interestingly, reverse transcription (RT) PCR analysis of mouse embryonic pancreas demonstrates expression of BMP4, -5, and -7, as well as BMP type I and II receptors (13). In addition, BMP signaling molecules, including SMADs 1, 2, and 4, are expressed in neonatal and adult pancreatic islets (14), and BMP4, -5, and -6 promote the proliferation and development of epithelial islet-like structures in vitro (15). Several studies also implicate BMPs as pluripotency factors. In particular, BMP4 has been reported to enhance mouse embryonic stem cell self-renewal (16, 17) and is necessary for the production of hematopoietic progenitors (18, 19). As SMAD-binding elements are found in the promoter of Id proteins (20), BMPs have been shown to regulate the expression of Id proteins in several cell types, including embryonic stem cells (20–23). This indicates that BMPs can control the expression of Ids, which in turn, could regulate the biological activity of bHLH transcription factors. However, the role of BMP and Id protein signaling in regulating expansion of pancreatic progenitors has not been explored. Clearly, a more complete knowledge of the function of this family of proteins in pancreatic islet development would allow us to understand the biology behind the maintenance of progenitor cells and the regulation of differentiation in the pancreas.

We hypothesize that BMP4 and Id2 proteins play prominent roles in pancreatic ontogeny and regeneration by affecting islet progenitor cell expansion during the differentiation of pancreatic islet cells. In this report, we show that BMP4 and Id proteins regulate the expansion of AR42J cells, a pancreatic epithelial cell line with the potential to differentiate into endocrine cells in vitro (24, 25). Furthermore, we found that BMP4 is necessary for the hyperplasia of duct cells during pancreatic islet regeneration. To define the functional mechanism of BMP4 regulation of progenitor cell expansion, we demonstrate that BMP4 exposure enhances binding of Id proteins to the bHLH transcription factor NeuroD. Our work dissects a critical signaling pathway for the regulation of cell growth and differentiation of endocrine progenitors.

EXPERIMENTAL PROCEDURES

Animal Housing and Cell Lines—IFNγ NOD mice were backcrossed onto the NODShi background and were maintained in the specific pathogen-free barrier facility at The Scripps Research Institute. AR42J...
cells were a generous gift from Dr. Itaru Kojima (Institute for Molecular and Cellular Regulation, Gunma University, Maebashi, Japan) and were maintained in Dulbecco’s modified Eagle’s medium at 37 °C and 5% CO₂. All studies were conducted in strict accordance with the TSRI Animal Care and Use Committee guidelines.

**Immunocytochemistry**—Tissue sections (4 μm) were subjected to immunohistochemistry using commercial polyclonal antibodies including Id2 (1:600, 1:1000), BMPR2 (1:50), BMP4 (1:50) (Santa Cruz Biotechnology, Santa Cruz, CA), insulin (1:1000) (Dako, Denmark), BrdUrd (1:50) (Accurate Chemical, San Diego, CA), PAX6 (1:100) (Convance, CA), and NeuroD (1 μg/ml) (Cemines, La Jolla, CA and Chemicon, Temecula, CA). An avidin-biotin immunoperoxidase system was used to visualize bound antibody (Vectastain ABC Kit, Vector laboratories, Burlingame, CA), and 3,3-diaminobenzedine (Sigma) was used as the chromagen. Samples were counterstained with hematoxylin. In some cases, images were acquired using a Zeiss 2100 confocal microscope with a 60× objective, and RGB micrographs were compiled with Image J software.

**Western Immunoblotting**—AR42J cells were lysed in 2× sample buffer (0.13 mol/liter Tris-base, 192 mmol/l glycine, pH 8.3, and 20% methanol). The membranes were blocked in 5% skim milk powder in Tris buffer containing 0.05% Tween 20, then probed with the indicated antibody and visualized with enhanced chemiluminescence. Equal loading of protein was assessed by reprobing the membrane for actin. The densitometry analysis was carried out using Image J software. Briefly, the intensity of images of scanned Western blots was determined, and a ratio of each band to its actin control was calculated.

**Co-immunoprecipitation**—AR42J cells were growth-arrested in medium lacking fetal calf serum, stimulated with BMP4 (100 ng/ml) for 18 h, and the cells were lysed in radioimmune precipitation assay buffer. Equal amounts of protein (400–600 μg) were precleared with 50 μl of TrueBlot anti-rabbit Ig IP beads (eBioscience, San Diego, CA), immunoprecipitated with 10 μg of Id2 antibody (2h, 4 °C), and then pulled down with TrueBlot anti-rabbit Ig IP beads. The samples were lysed in 2× sample buffer, and equal volumes of protein were separated by SDS-PAGE and probed for the indicated antibody. The protein levels were determined by reprobing the blot with the Id2 antibody.

**IFNγ Duct Cell Isolation**—Pancreatic duct cell isolation was performed as described previously (26). Briefly, each IFNγNOD pancreas was treated with 1 mg/ml collagenase P in Dulbecco’s modified Eagle’s medium F-12 with slight agitation at 37 °C for 30 min. The duct preparation was then filtered through a 200-μm mesh, washed in cold phosphate-buffered saline, and treated with 0.05% trypsin, EDTA at 37 °C for 10–15 min. Fetal calf serum was added to stop the trypsin treatment.
and the duct preparation was washed in cold phosphate-buffered saline. Finally, duct cells were filtered over a 70-/H9262 m cell strainer and cultured in 10% fetal calf serum Dulbecco’s modified Eagle’s F-12 medium overnight. For BMP4 treatment, duct cells were growth-arrested in 0.5% fetal calf serum Dulbecco’s modified Eagle’s F-12 medium for 24 h before the addition of BMP4 (1–100 ng/ml) for 18 h. For some of the experiments, the duct epithelial cells isolated from IFN/H9253 NOD pancreas were pretreated with the neutralizing antibody to BMP4 (1–10 g/ml) for 2 h before the addition of recombinant BMP4.

BMP4 Neutralizing Antibody Treatment and BrdUrd Count—To ascertain whether BMP4 signaling affects pancreatic duct cell expansion, we injected mice intravenously with a neutralizing antibody to BMP4 (200 g) (R&D Systems, Inc., Minneapolis, MN) three times a week for 2 weeks. These mice were injected with BrdUrd (100 g/g) intraperitoneally on day 15 and sacrificed on day 16, and the tissue was harvested and fixed for histology. After sections were cut, the tissues were analyzed by immunohistochemistry. Every fifth section was stained with antibody to BrdUrd, and the cells were counted for BrdUrd-positive duct cells in at least 10 fields per section. The labeled cells were evaluated as the percentage of BrdUrd-labeled cells out of the total number of epithelial cells per duct (27, 28).

Statistics—Data were expressed as mean ± S.D. and analyzed using Instat (GraphPad Software Inc.). Comparisons were made by Student’s t test. A value of p < 0.05 was considered statistically significant.

RESULTS

BMP4 Regulates Id Expression in AR42J Cells and Induces Cell Proliferation—The AR42J cell line is a pluripotent cell line derived from a rat pancreatic acinar carcinoma, which are acinar-like cells when exposed to dexamethasone (29), and in contrast, convert into insulin-producing cells with betacellulin and activin A treatment (24). Therefore, AR42J cells have the potential to become exocrine or endocrine cells, making them a valuable in vitro correlate to study the mechanisms of beta cell differentiation as well as for expansion of pancreatic progenitors. We tested the hypothesis that BMP4 regulates the expression of Id protein and is required for cell expansion in AR42J pancreatic cells. To this end, AR42J cells were growth-arrested and then treated with recombinant BMP4 for 18 h, and the expression of Id was quantitated by Western immunoblotting (Fig. 1A). Id1 and Id3 were not detected (data not shown), whereas Id2 expression was inducible by recombinant BMP4. Densitometric analysis showed that BMP4 increased Id2 expression by 2±0.6-fold over untreated control cells (Fig. 1B). BMP antagonists, such as noggin, regulate the activities of BMPs by forming a complex with them and preventing their binding to BMP receptors (30, 31). We found that pretreatment of AR42J cells with noggin (5 g/ml) significantly reduced BMP4-dependent stimulation of Id2 protein expression (Fig. 1, A and B). Therefore, our data are consistent with the hypothesis that Id protein expression is induced by BMP4 signaling in pancreatic epithelial cells.
BMP4 and Id2 Regulate Progenitor Cell Expansion

To determine whether BMP4 regulates AR42J cell proliferation, we growth-arrested the cells and stimulated them with BMP4 for 18 h with the addition of [3H]thymidine during the last 2 h. The growth index of the cultured AR42J cells was determined by quantitatively measuring [3H]thymidine uptake. We found that BMP4 increased [3H]thymidine counts after 18 h of BMP4 stimulation compared with control cells grown without BMP4 ($p < 0.0001$) (Fig. 1C). We confirmed these results utilizing BrdUrd incorporation experiments. After 2 h of pulsing with BrdUrd, we observed an increased proportion of AR42J cells stimulated with BMP4 that were BrdUrd positive as determined by immunofluorescence staining (Fig. 1, $D$ and $E$). These results demonstrate that BMP4 stimulates the expansion of pancreatic progenitor cells.

BMP Signaling Molecules and Id2 Are Expressed in the Pancreatic Epithelium of Embryonic,Adults, and Regenerating IFNγ/NOD Pancreas—BMP4 and its receptors are expressed in the embryonic pancreas (13, 15). Because we observed that BMP4 exposure promotes Id2 expression in AR42J progenitor cells, we next asked whether these factors are expressed in the developing and adult pancreas. We performed immunolocalization experiments and found that Id2-expressing cells were located in the embryonic pancreas but not strikingly colocalized with insulin (Fig. 2).

Next, we studied the expression of BMP signaling proteins in mice that express IFNγ/NOD (IFNγ/NOD) driven by the insulin promoter. These mice are ideal for studying pancreatic regeneration as they demonstrate ductal hyperplasia and recapitulate several aspects of pancreas development (32, 33). Immunoreactive BMP4 and BMP2R expression were found to be localized to “islet-like” regions and in the small ducts (Fig. 2, $D$ and $E$) of this transgenic strain. Likewise, in the regenerating IFNγ/NOD pancreas, Id2 expression was strong in duct epithelial cells (Fig. 2F). Moreover double-labeling experiments demonstrated that the majority of Id2-positive cells do not express insulin (Fig. 2, $G–I$). To ascertain the potential relevance of Id2 in epithelial cell expansion in the IFNγ/NOD pancreas, we stained the pancreas for BrdUrd and Id2 and performed confocal analysis. We found that a subset of Id2 positive cells also incorporated BrdUrd (Fig. 2, $J–L$). Therefore, cells harboring BMP, its receptor, and Id2 are found within the epithelial regions of the developing and regenerating mouse pancreas, suggesting that these molecules may regulate expansion of duct epithelial progenitor cells.

BMP4 Regulates Id Protein Expression and Pancreatic Duct Cell Expansion in IFNγ/NOD—We asked whether Id proteins are induced by BMPs in pancreatic duct cells. We isolated IFNγ/NOD duct epithelial cells (because this strain expressed the BMPR2 in the epithelium), growth arrested the cultures, and then stimulated them with recombinant BMP4 protein. We focused on the effect of BMP4 on Id2, because it is found exclusively in duct epithelial cells of the IFNγ/NOD pancreas. We found that Id2 expression was enhanced in the presence of BMP4 by 2-fold (Fig. 3A). We further found that when these duct epithelial cells were treated with a neutralizing BMP4 antibody, Id2 expression was reduced, supporting the notion that Id2 propagates BMP4 signals in the pancreatic epithelium (Fig. 3). We next asked whether BMP4 regulates epithelial cell expansion in vivo. Because the IFNγ/NOD strain displays ductal hyperplasia, it represents a model to test whether BMP4 regulates cellular proliferation in the pancreas. To ascertain whether BMP4 signaling affects pancreatic duct epithelial cell expansion, we injected mice intravenously with the neutralizing antibody to BMP4 (34). Fig. 3, $B$ and $C$, depicts a representative histological section of the IFNγ/NOD pancreas tissue stained for BrdUrd in the control and BMP4 neutralizing...
BMP4 and Id2 Regulate Progenitor Cell Expansion

**FIGURE 5. Model of BMP4 and Id protein signaling in the expansion of pancreatic epithelial progenitors.** In the presence of BMP4 ligand, BMP receptors transmit the signal that leads to phosphorylation (P) of SMAD1 and -5 as well as co-SMAD4. Activated SMADs bind to SMAD-binding elements (SBE) on the Id promoter resulting in transcription of Id proteins. These bind to bHLH transcription factors such as NeuroD, preventing it from binding to the E-box elements of endocrine-specific genes. Therefore, pancreatic epithelial progenitors do not differentiate along the NeuroD-specific endocrine lineage but may instead expand, as Id proteins are involved in cell proliferation. In the absence of BMP4 or the presence of noggin, SMAD-dependent expression of Id does not occur, allowing NeuroD to activate transcription of endocrine-specific genes.

antibody–treated groups, demonstrating a reduction in BrdUrd-positive epithelial cells following anti-BMP treatment. We determined the percentage of BrdUrd-positive cells and found that in control animals, the average percentage of BrdUrd-positive cells is 17 ± 7, whereas treatment with the BMP4 neutralizing antibody reduced expansion to 8 ± 3% (n = 5 animals/condition, p = 0.003) (Fig. 3D). Our cumulative data demonstrate that BMP4 regulates the expansion of pancreatic duct epithelial cell progenitors in vivo.

**BMP4 Promotes the Binding of Id2 with NeuroD and Increases PAX6 Expression**—Id proteins regulate the biological activity of bHLH transcription factors. NeuroD is a bHLH transcription factor that is important for the terminal differentiation of the endocrine lineage in the pancreas (4). Because NeuroD is required for the terminal differentiation of pancreatic endocrine cells, we asked whether it was found in the pancreatic epithelium during regeneration, and whether Id proteins might affect the expansion of pancreatic epithelial cells by inhibiting the differentiation program promoted by NeuroD. We found that NeuroD immunoreactivity was present in ductal cells, and in islet-like areas of the regenerating pancreas, but the overall level of immunoreactivity was not altered by inhibiting BMP4 signaling (Fig. 4, A and B). We next asked whether BMP treatment could enhance the binding of Id2 and NeuroD. Using immunoprecipitation experiments, we determined that recombinant BMP4 treatment increased the amount of Id2 bound to NeuroD. The ratio of bound NeuroD to the immunoprecipitated Id2 was 0.8 compared with 0.5 in untreated samples (Fig. 4, C and D). Interestingly, we did not observe any increase in NeuroD levels in the total cell lysate (data not shown), suggesting that regulation is enacted through protein–protein interactions or through induction of Id2 expression, as demonstrated above. These data therefore reveal that BMP4 regulates the accumulation and expansion of ductal progenitor epithelial cells through the activity of Id proteins and their bHLH targets.

PAX6 is an important transcription factor in the regulation of pancreatic morphogenesis (35). Importantly, it has been demonstrated that NeuroD binds specifically to E boxes in the promoter of PAX6, regulating its expression (36). We asked whether neutralization of BMP4 affected PAX6 expression. We stained the BMP4-neutralized IFNγNOD mouse pancreas with an antibody to PAX6. We found increased numbers of PAX6-positive cells and enhanced intensity of PAX6 expression in the BMP4-neutralized pancreata compared with controls (Fig. 4, E and F). These results demonstrate the BMP pathway affects the expression of this critical factor for islet differentiation through NeuroD regulation.

**DISCUSSION**

Id proteins are important in the regulation of cell proliferation and inhibition of differentiation. Our studies demonstrate the importance of BMP4 induction of Id2 in ductal progenitor cell expansion in the pancreas. We showed that BMP4 stimulated Id2 expression and induced cellular proliferation in AR42J cells. Furthermore, BMP signaling proteins and Id2 are expressed in the developing and regenerating mouse pancreas. The importance of BMP4 in epithelial duct cell expansion was ascertained by neutralization of BMP4, which significantly reduced BrdUrd incorporation during pancreatic regeneration. Therefore, our results are consistent with the paradigm that BMP4 signaling is important for ductal expansion. Moreover, we unraveled a link between BMP4 and Id2, which contributes to endocrine-specific differentiation in the pancreas. We have observed that BMP4 induces Id2 expression and its binding to NeuroD, a bHLH transcription factor critical for endocrine differentiation during pancreatic development. Furthermore, in the IFNγNOD pancreas, neutralizing BMP4 enhanced PAX6 immunoreactivity, a transcription factor that has been previously demonstrated to be a target of NeuroD, thereby indicating enhanced NeuroD activity in the absence of BMP4. Our results support the hypothesis that BMP4 is critical for the expansion of pancreatic duct epithelial progenitors and perhaps in the maintenance of their predifferentiated state (Fig. 5).

**BMPs and Ids in Cell Growth**—The importance of BMP signaling in growth has been amply demonstrated. For example, SMAD1 regulates lung morphogenesis in an explant culture model by reducing lung epithelial branching, cell proliferation, and differentiation (37). In addition,
exposure to low concentrations of BMP4 promoted proliferation of subventricular zone progenitors (38). Furthermore, BMP4 promotes proliferation of committed neuronal progenitors, as well as facilitating their commitment into astrocytes (38). Indeed, we found that BMP4 is essential for the growth of pancreatic epithelial cells in AR42J cells in vitro as well as in a regenerating pancreas model.

Id protein expression correlates with cell proliferation and inhibition of differentiation in many cell types. In the pancreas, Id2 expression was demonstrated to be elevated in both cancerous pancreatic tissues and in Panc-1 pancreatic cancer cell line (39). In mammary epithelial cells and breast cancer cells, ectopic expression of Id1 allowed for continued proliferation (40). In addition, Id protein signaling is known to intersect with cell cycle regulatory functions, and deregulation of Ids may lead to tumorigenesis (7, 41, 42). However, the function of Id proteins in the growth of progenitor cells in the pancreas has not previously been studied. Here, using AR42J cells and in vivo studies, we demonstrated that BMP4 acts upstream of Id2 and that these signaling molecules are required for pancreatic epithelial cell expansion.

Id Proteins Are Regulated by BMPs—We found that exposure to BMPs caused the induction of Id protein expression in AR42J cells as well as in isolated IFNγ-NOD epithelial duct cells. Several other interesting studies have also linked BMPs with the expression of Ids (reviewed in Ref. 11). Importantly, SMAD-binding elements are found on Id gene promoters (21, 22, 42). In addition, BMP4 contributes to astrocyte lineage commitment through Id-mediated inhibition of OLIG and E2A transcription factors (43). Id1, Id2, and Id3 are up-regulated following BMP stimulation in osteogenic cells. Furthermore, microarray gene analysis of epithelial cells stimulated with BMP7 showed induction of Ids, which prevented TGF-β-induced growth and epithelial-mesenchymal transition (44). Similarly to our studies in the pancreas, the up-regulated Id genes promoted cell proliferation and inhibited differentiation. Clearly, BMP-dependent Id activity is a positive regulator of proliferation and a modulator of cell differentiation. Our data using AR42J cells and isolated IFNγ-NOD ductal epithelial cells showed that Id2 is induced by BMP4 signaling. Thus BMPs may promote expansion via their ability to suppress terminal differentiation, a process that is mediated by bHLH transcription factors.

BMPs Are Important Factors in Cell Fate Determination—BMPs are important for directing the cell fate of several cell types, including mesenchymal cells (45). NeuroD is a bHLH transcription factor expressed in pancreatic, intestinal, and neural tissue, and plays a prominent role in endotheline lineage commitment and regulation of insulin gene transcription (4, 46). Clinically, mutations in NeuroD cause maturity-onset diabetes of the young (MODY). NeuroD null mice have a striking reduction in the number of beta cells, develop severe diabetes, and die perinatally (4, 47), although the genetic background of the mice determines the severity of the phenotype, as neogenesis of beta cells can occur in NeuroD null mice (48). It is possible that the neogenesis observed in this knock-out strain may arise from the propensity for expansion, which results from the absence of strong signals promoting terminal differentiation. Here, we show the net outcome of BMP4 on the regulation of endothelins differentiation, in which Id2 binding to NeuroD is regulated by BMP4. This relationship may underlie the distinct homeostasis of pancreatic progenitor cells compared with differentiated endocrine cells. NeuroD was previously shown to form heterodimers with E47, resulting in binding to the E-box of the insulin gene promoter and its transactivation (47). Moreover, overexpression of Id2 in COS M6 cells inhibited NeuroD from binding to its E-box target (10). It has been demonstrated that one of the targets of NeuroD in determining pancreas morphogenesis and maintaining endocrine cell type numbers is Pax6 (35, 36). In agreement with this, our data show an enhanced Pax6 immunoreactivity when BMP4 signaling is neutralized and NeuroD binding to Id2 is reduced. Therefore, Id proteins may regulate beta cell-specific gene expression through NeuroD. Overall, this is the first study to demonstrate that BMP4 and Id2 regulate expansion and differentiation of the endocrine lineage in pancreatic duct epithelial progenitors by inducing Id2 binding to NeuroD. These results will enhance our knowledge of the signaling pathways that are necessary for expansion of pancreatic progenitors and, subsequently, for their differentiation into beta cells for future therapeutic treatment of type I diabetes.

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