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ORIGINAL PAPER

Expression of Id1 in adult, regenerating and developing pancreas

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Abstract Several key transcription factors are necessary for alpha cell development in the pancreas. In this study, we describe the expression of Inhibition of DNA-binding protein 1 (Id1) in the developing as well as the normal adult pancreas. We found co-expression of Id1 with bone morphogenetic protein (BMP) receptor in alpha cells. Inhibition of BMP4 signaling with a specific neutralizing antibody slightly decreases the proportion of glucagon cells in the adult pancreas but had a significant effect in a model of pancreas regeneration. In late embryonic pancreas, Id1 co-localized with GATA4, a transcription factor known for its critical function in glucagon cell development. However, in early postnatal period, the expression of Id1 and GATA4 diverged with Id1 identified in glucagon cells and GATA4 restricted to the acinar pancreas.

Keywords Id1 · Glucagon · BMP4 · IFNgNOD · GATA4

Introduction

Pancreatic islet consists of five cell types, alpha, beta, delta, PP, and epsilon, secreting specific peptide hormones, glucagon, insulin, somatostatin, pancreatic polypeptide, and ghrelin, respectively. These hormones are important for glucose homeostasis and energy metabolism. Multiple transcription factors regulate pancreas development. For alpha cells [1–3] these include MafB [4], Nkx2.5 [5], and GATA4 [3].

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Inhibition of DNA-binding proteins comprising of four members (Id1–Id4), are a family of proteins that are implicated in the control of proliferation and differentiation [6, 7]. The major role of Id proteins is to bind and inhibit the function of bHLH transcription factors [8, 9]; however, non-bHLH targets of Id have been described [10]. Different Id proteins act with differential tissue specificity. For example, Id4 is required for neuronal development, whereas Id3 is involved in lymphocyte development. We have shown previously that Id2 is associated with epithelial cell expansion that was regulated by BMP4, in a model of pancreatic islet regeneration [11]. Id1 overexpression has been related to tumor angiogenesis in human pancreatic cancer [12]. However, the participation of Id1 during pancreas development has not been reported, although its expression has been noted in pancreas gene chip array analysis [13]. In this report, we studied the expression pattern of Id1 in the pancreas and the possible signaling pathways that regulate its function(s), specifically BMP and GATA.

One upstream regulator of Id1 expression is bone morphogenetic protein (BMP) [14]. BMPs are pleiotropic proteins that regulate proliferation, differentiation, and migration of numerous cell types [15]. BMPs signal through heteromeric complex of type 1 and 2 serine/threonine kinase receptors and their downstream nuclear effectors, SMADs [15]. RT-PCR analysis of mouse embryonic pancreas demonstrated expression of BMP4, 5, and 7, as well as BMP type 1 and 2 receptors [16]. In addition, BMP signaling molecules, including SMADs 1, 2, and 4, are expressed in neonatal and adult pancreatic islets. Here, we looked at the expression and effects of BMP4 and Id1 in the pancreas.

GATA proteins constitute evolutionary conserved zinc finger transcription factors that are critical in cell fate specification and differentiation. GATA4 has been ascribed to development of glucagon cells in the pancreas [3].

Importantly, a relationship between BMP signaling and GATA transcription factors has been described. Two hybrid assays demonstrate a direct interaction between GATA4 and Smad1/4 [17]. Moreover, Id proteins inhibited differentiation of P19 cells to myocytes as a consequence of binding to GATA4 and Nkx2.5 [18].

In this study, we described the expression of Id1 in the developing as well as the normal adult pancreas. We found co-expression of Id1 with BMP-R2 receptor and glucagon-positive cells in the adult pancreas. Neutralizing BMP4 signaling with a specific neutralizing antibody significantly reduced the number of glucagon cells in a model of pancreas regeneration but only marginally in the adult pancreas. At embryonic day 18.5 (E18.5) pancreas, Id1 has a similar immunoreactive pattern as GATA4. In early neonatal pancreas, Id1 is found in alpha cells but not GATA4. It is possible that glucagon cell fate requires participation of BMP4 and Id1, which may interact with GATA4, a transcription factor known for regulating glucagon cell development.

Materials and methods

IFNgNOD mice were backcrossed onto the NODShi background and were maintained in the specific pathogen-free barrier facility at The Scripps Research Institute. All studies were conducted in strict accordance with the TSRI Animal Care and Use Committee guidelines.

Immunocytochemistry

C57Bl/6J or IFNgNOD whole pancreas was fixed in 10% (neutral buffered formalin) NBF, and paraffin sections were processed for immunocytochemistry using an avidin–biotin immunoperoxidase system to visualize bound antibody (Vectastain ABC Kit, Vector laboratories, Burlingame, CA) and 3,3-diaminobenzedine (Sigma Chemicals, St. Louis, MO) was used as the chromagen. Otherwise, sections were stained for the indicated antibodies and analyzed using confocal microscopy (BioRad 2100). The following antibodies were used: glucagon and insulin (DAKO), Id1, BMPR2, GATA4 (Santa Cruz, CA).

BMP4-neutralizing antibody treatment

To ascertain the function of BMP4 signaling, we injected C57Bl/6J or IFNgNOD mice intravenously with a neutralizing antibody to BMP4 (200 μg) (R&D Systems Inc., Minneapolis, MN) three times a week for 2 weeks and the tissue was harvested and fixed for histology [11]. Sections were stained for glucagon and the number of glucagon-positive duct cells was counted and expressed as percentage of total duct cells in the field of view [19]. Total number of cells counted from control were 2,026 from four animals and total number of cells counted from BMP4 antibody treated were 3,561 from four animals. For normal adult pancreas, glucagon cells were counted and expressed as a percentage of islet area as determined using Image J. Results were compared using Student's *t*-test in Instat (GraphPad Software Inc.) where $P < 0.05$ was considered significant.

Results

Expression of Id1 in the adult pancreas

To ask whether Id1 is expressed in the adult pancreas, we performed immunostaining using a specific Id1 antibody. Figure 1 shows that Id1 is present in glucagon cells of the pancreas. Since BMP4 is known to induce the expression of Id1 and Id2, we sought to determine if BMP receptors were also expressed in Id1-positive cells in the pancreas. Confocal microscopy showed that BMP-R2 immunoreactivity, like Id1, co-localized with glucagon cells (Fig. 1d–f). To demonstrate the specificity of our results, we also performed immunostaining using serial sections (Fig. 1g, h) (i.e., the sections are consecutive sections separated by 2 μm, but each is stained using BMP-R2 or glucagon antibody). This allows us to show staining of two antibodies in the same cell. We found similar observations with this method. To ascertain whether BMP4 signaling affected Id1 and hormone peptide expression in the adult pancreas, we injected a neutralizing BMP4 antibody intravenously over a 2-week treatment period, as we have published previously [11]. We stained sections of the treated pancreata and quantitated the expression of Id1 and glucagon. We found by confocal microscopy that neutralizing BMP4 reduced Id1 staining intensity in the pancreas (Fig. 2a, b). Next, we counted the number of glucagon-positive cells as a percentage of islet area. We found that control animals had $2.1 \pm 0.2\%$ (SEM, $N = 5$ animals, 46 islets counted) glucagon cells, whereas BMP4 antibody-treated islets had $1.9 \pm 0.1\%$ (SEM, $N = 5$ animals, 37 islets counted) glucagon cells ($P = 0.7$, $N = 46$ islets for control and $N = 37$ islets for BMP4 antibody treated) (Fig. 2c–e). Therefore, in the adult pancreas, BMP-R2 co-localized with both Id1. However, our antibody neutralizing studies demonstrated that blockade of BMP4 only marginally decreased proportion of glucagon-positive cells.

Expression of Id1 in the regenerating pancreas

Transgenic mice expressing IFNg (IFNgNOD) under the control of the insulin promoter demonstrate pancreatic

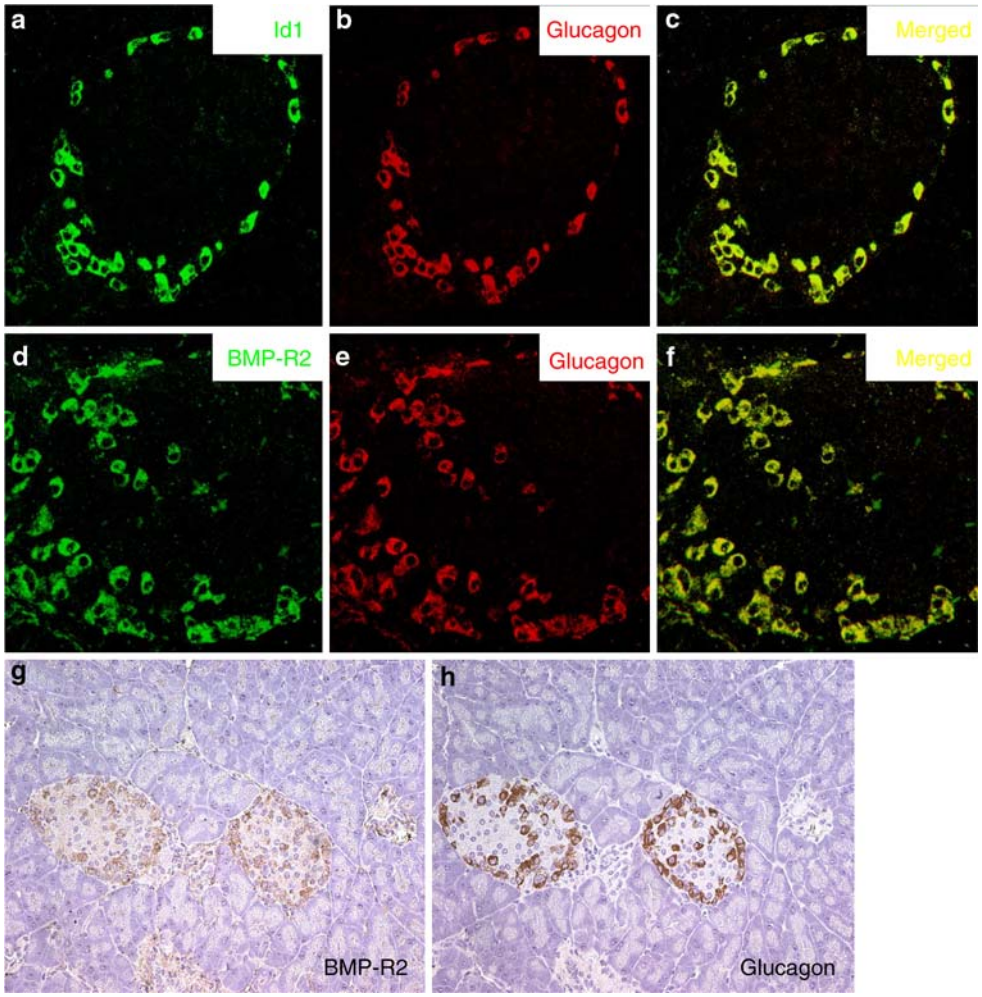

Fig. 1 Id1 and BMP receptor (BMP-R2) expression in adult pancreas. Micrographs are representations of at least three independent experiments. (**a**) Confocal microscopy reveals immunoreactivity of Id1 using FITC-conjugated secondary antibody in islet of adult pancreas. (**b**) Immunoreactivity of glucagon-positive cells in adult pancreas using Texas Red-conjugated secondary antibody. (**c**) Merged confocal image shows Id1 co-localize with glucagon cells in adult pancreas. (**d**) Confocal microscopy reveals immunoreactivity of BMP-R2 using FITC-conjugated secondary antibody in islet of adult pancreas. (**e**) Immunoreactivity of glucagon-positive cells in adult pancreas using Texas Red-conjugated secondary antibody. (**f**) Merged confocal image shows BMP-R2 co-localize with glucagon cells in adult pancreas. (**f**) Immunoreactivity of BMP-R2 and glucagon using serial sections. Adult pancreas tissue was serially sectioned (separated by 2 μm) so that the same cells are depicted by consecutive sections. This technique also shows that BMP-R2 immunoreactive cells (**g**) are glucagon positive (**h**)

ductal hyperplasia and the formation of new islets, and recapitulates several aspects of pancreas development [20–22]. For example, the duct cells express transcription factors found during pancreas development, including PDX-1 and hepatocyte nuclear factor-3beta (HNF3beta) [21]. They can also be used as an in vivo model to look at the impact of inhibition of specific growth factor on the expansion of epithelial duct progenitors, either through FAC analysis of sorted duct cells or through immunohistochemical imaging [11, 19, 22, 23]. Thus, this is an ideal model for studying pancreatic growth during reparation morphogenesis in adults.

We have previously shown that BMP4 immunoreactivity is expressed in the proliferating IFNgNOD and that BMP-R2 is strongly expressed in small ducts and in "islet-like" areas of the IFNgNOD transgenic pancreas [11]. In this study, thin serial section staining demonstrated that some Id1-positive cells express glucagon. Likewise, some BMP-R2-positive cells are glucagon-expressing cells (Fig. 3a–d). To ask whether BMP4 was required for the expression of glucagon cells, we injected neutralizing BMP4 antibody into IFNgNOD mice using the protocol referenced above. However, since IFNgNOD pancreas does not have structured islets, we counted the percentage of glucagon cells in comparison to the total number of duct cells as previously published by our lab [23]. We found that control animals had 15 ± 1.5% (SEM, $N = 5$ animals, 42 ducts counted) glucagon-positive cells within the duct

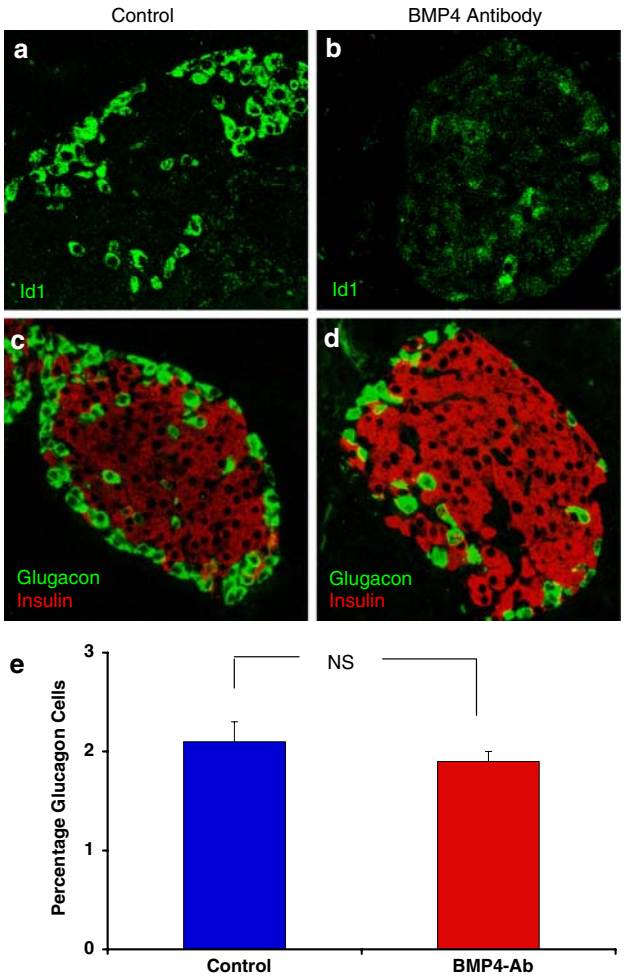

Fig. 2 Effect of neutralizing BMP4 on glucagon expression in adult pancreas. Adult mice were intravenously treated with neutralizing antibody for 2 weeks and pancreas sections were analyzed for Id1 expression. (**a**) Id1 expression in a representative control pancreas and (**b**) reduced Id1 expression in a BMP4 neutralizing antibody-treated mouse. Confocal microscopy shows that insulin staining was not altered in these mice (**c**–**d**). Neutralizing BMP4 slightly reduced the number of glucagon cells in these mice (**c**–**d**). (**e**) Graphical representation of the number of glucagon cells counted with respect to islet area. Five mice were used for each group with 46 islets counted for controls and 37 islets counted from the BMP4 antibody-treated group. $P = 0.7$ control versus BMP4 antibody treated

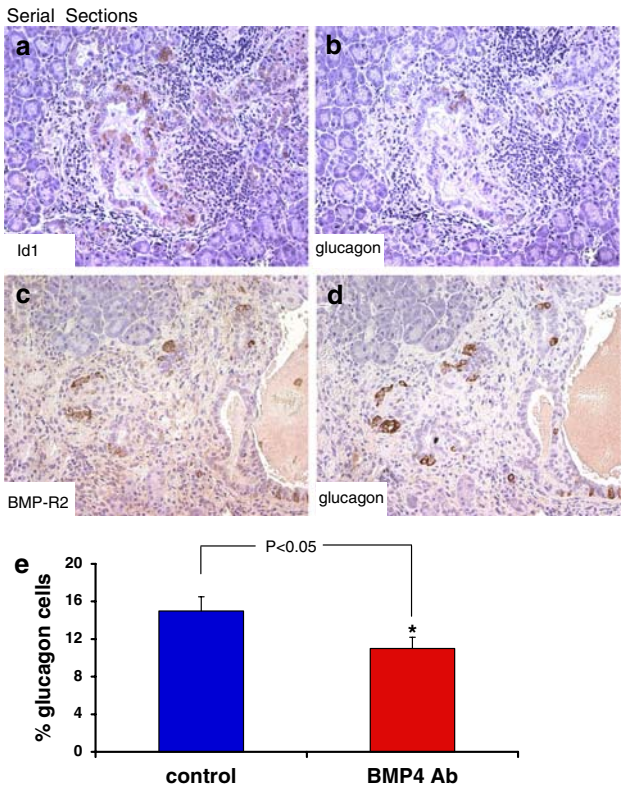

Fig. 3 Id1 and BMP-R2 expression in the IFNgNOD pancreas. (**a**–**b**) Micrographs show that Id1 is expressed in duct cells of IFNgNOD pancreas and serial sections demonstrates that not all Id1 (**a**) cells are glucagon (**b**) immunoreactive. (**c**–**d**) Some duct cells are also BMP-R2 (**c**) immunoreactive and that these are also glucagon- (**d**) expressing cells. (**e**) Graphical representation showing neutralizing BMP4 reduced the number of glucagon cells in the IFNgNOD pancreas. The data were derived by counting the percentage of glucagon cells from the total number of cells of a given duct. From the control group, 42 ducts were counted from a total of five animals, and for the antibody-treated group, 53 ducts were counted from five animals. $P < 0.05$ control versus BMP4 antibody treated

epithelium, whereas in BMP4 antibody-treated animals, this was significantly decreased to $11 \pm 1.2\%$ (SEM, $N = 5$ animals, 53 ducts counted) ($P < 0.05$) (Fig. 3e). Therefore, in the regenerating pancreas, transient inhibition of BMP4 was sufficient to diminish glucagon cell population.

Id1 expression in the developing and early neonatal pancreas

We next asked if Id1 is expressed in the embryonic pancreas. By immunostaining of the E18.5 pancreas tissue, we found the expression of Id1-positive cells (Fig. 4a). In contrast to the adult pancreas, our confocal microscopy studies revealed that Id1 did not co-localize with insulin or glucagon in E18.5 pancreas (Fig. 4b, c). However, we observed a striking similarity of Id1's expression to that of GATA4 (Fig. 4d, e). GATA4 is a zinc finger transcription factor critical for development of glucagon cells in the pancreas [3]. Like Id1's expression, GATA4 also did not co-localize with insulin-positive cells in the embryo. More interestingly, we did not find GATA4 expression in adult pancreas islets but it is present in the acinar tissue. Moreover, some duct cells in the regenerating pancreas express GATA4 (Fig. 4f, g). In the early neonatal pancreas, the expression of Id1 and GATA4 has already diverged. Id1 is found in glucagon-expressing areas whereas GATA4 expression is found in the acinar regions (Fig. 5). Therefore, there may be a reciprocal pattern of expression

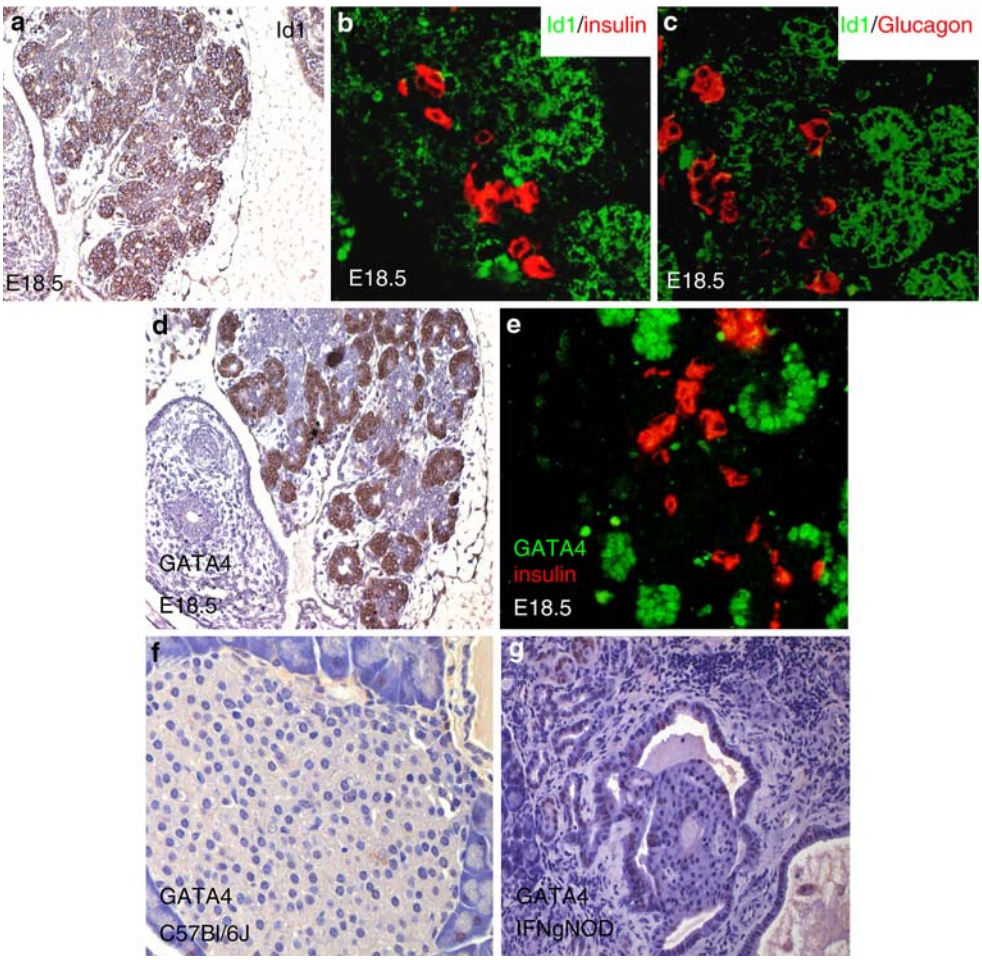

Fig. 4 Id1 expression in E18.5 pancreas. (**a**–**c**) Micrographs depicting Id1 (**a**) immunoreactivity in clusters in E18.5 pancreas. However, confocal microscopy shows that Id1-positive cells at this stage did not express insulin (**b**: Id1–FITC, insulin-Texas Red) or glucagon (**c**: Id1–FITC, glucagon-Texas Red). We noted that Id1 expression was strikingly similar to that of GATA4 (**d**). Like Id1, GATA4 positive was also not insulin positive (**e**: GATA–FITC, insulin-Texas Red). GATA4 immunoreactivity was not detected in islets of adult pancreas (**f**) but was found in some duct cells of the IFNgNOD pancreas (**g**). Micrographs are representations of at least three separate experiments

between Id1 and GATA4. This may account for the decrease of glucagon cells observed in the regenerating but not in the adult pancreas.

Discussion

In this report, we found that Id1 co-localized with glucagon cells in the adult pancreas along with BMP-R2. However, neutralizing BMP4 signaling only marginally inhibited the number of glucagon cells in the normal adult pancreas. In contrast, in the regenerating pancreas, a subset of Id1 and BMP-R2 cells co-express glucagon and inhibition of BMP4 significantly reduced the proportion of glucagon-positive cells within the duct epithelium. Histochemical studies in the embryonic pancreas reveal that there is a possible link between Id1 and GATA4 expression and the subsequent glucagon cell fate. This is reiterated by the absence of GATA4 immunoreactivity in the adult islet compared to the regenerating pancreas.

Very little is known regarding the expression and function of Id1 in the pancreas. Gene array and transcription factor analysis have demonstrated Id proteins in the developing pancreas [13, 24]. Id1 enhanced vascular neogenesis in pancreatic cancer [12], but its role in normal pancreas has not been described. Id proteins inhibit the functions of bHLH transcription factors as well as atypical targets. Some bHLH transcription factors involved in pancreas development include NeuroD [25], NGN3 [26], Hes1 [27], and Mist1 [28]. We have found by co-immunoprecipitation that Id2 binds to NeuroD and that this interaction was affected by BMP4 signaling [11]. Therefore, in this study, we wanted to know the expression pattern and function of Id1. We found that Id1 is expressed in differentiated glucagon cells of the normal adult pancreas, in late embryonic pancreas as well as in a model of

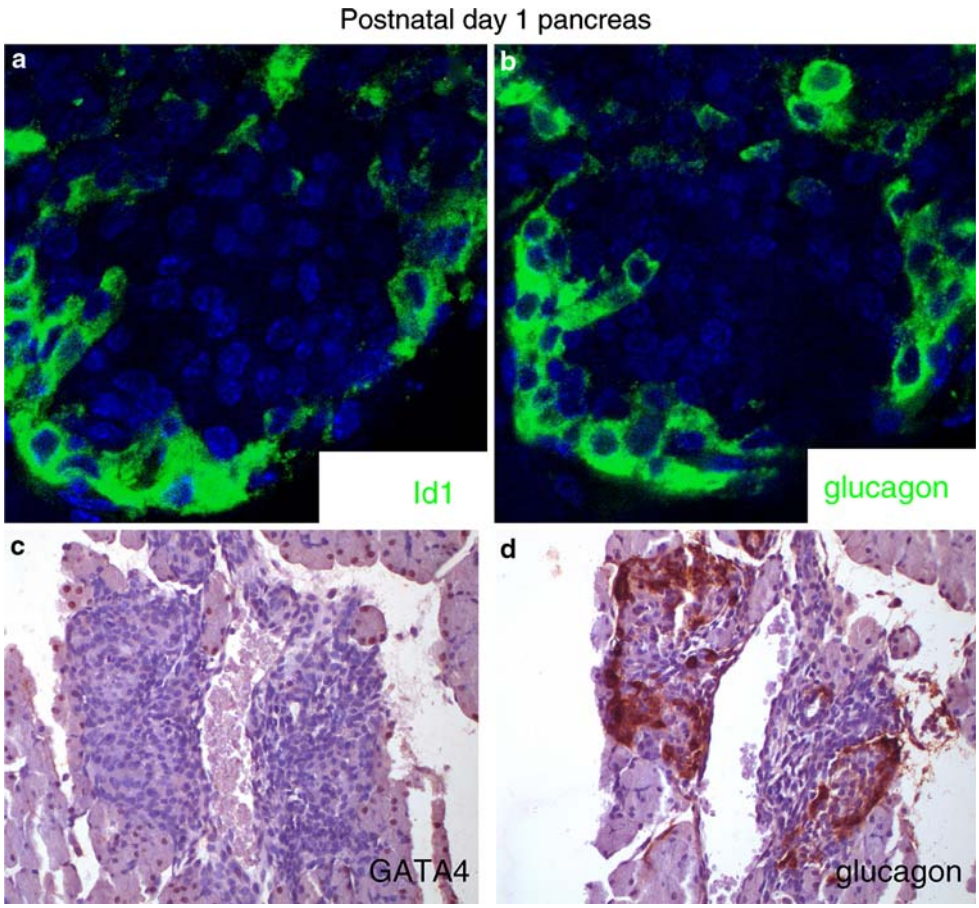

Fig. 5 Id1 and GATA4 expression in early neonatal pancreas. Postnatal day 1 pancreas was harvested, stained for Id1 (**a**) or glucagon (**b**), and analyzed by confocal microscopy. Consecutive sections show that Id1 (FITC) has the same pattern of expression as glucagon (FITC). Nuclei are stained with DAPI. Postnatal pancreas was also stained for GATA4 using DAB as the chromagen. In contrast to Id1, GATA4 is found in acinar tissue (**c**), not in glucagon-positive areas (**d**)

regenerating pancreas (although in the IFNgNOD we found less expression of Id1 compared to Id2). We found that neutralizing BMP4 signaling significantly reduced the percentage of glucagon cells in the regenerating pancreas but had only a modest effect in the adult. It is possible that neutralizing BMP4 signaling has no effect on committed, differentiated alpha cells; thus, we can see an effect in the regenerating IFNgNOD model but not in the adult pancreas. Perhaps 2 weeks is insufficient for the neutralizing antibody to have an effect in wild-type mice. This could be due to the difference in the structure of the islets between wild-type and IFNgNOD mice. The IFNgNOD pancreas contains a lot of ducts and therefore is more permeable to the antibody. We cannot exclude the possibility that redundant signaling averted the effects of neutralizing BMP4 or that BMP4 does not play a significant role in the differentiation of alpha cells.

GATA4 is able to transactivate glucagon gene in glucagon-producing InR1G9 cells [3]. GATA4 is found in glucagon-positive cells at E12.5 but in the adult is limited to the exocrine pancreas [3]. We also observed GATA4 expression in the adult exocrine pancreas but not in islets. Thus, most glucagon-positive cells express GATA4, but as development proceeds, the number of glucagon+/GATA4+ cells decreases. It is possible that during embryonic pancreas development, GATA4 co-localizes with Id1, but in the adult GATA4 becomes restricted to the exocrine pancreas whereas Id1 is maintained in glucagon cells. We observed that GATA4 and Id1 co-localized but were not endocrine cells at E18.5. By postnatal day 1, GATA4 is already found in the exocrine pancreas and Id1 is expressed in glucagon cells. Likewise, in the adult, Id1 is restricted to glucagon cells, whereas GATA4 immunoreactivity was found in the exocrine pancreas. Both Id1 and GATA4 are found in the regenerating pancreas. It should be noted that in IFNgNOD duct cells, there are more GATA4-positive cells than there are Id1 cells; therefore, more than likely they probably have dissimilar functions as well.

In accordance to our hypothesis, other studies have shown a relationship between BMP signaling and GATA transcription factors. Two hybrid assays demonstrate a direct interaction between GATA4 and Smad1/4 [17]. BMPs are shown to induce ectopic expression of cardiac transcription factors. In an in vitro cardiomyocyte differentiation system that overexpresses noggin, an inhibitor of BMP signaling, P19CL6 cells did not differentiate into beating cardiomyocytes, but the effect is rescued by overexpressing cardiac transcription factors Nkx2.5 and

GATA4, suggesting that these are downstream factors of BMP signaling [29]. Moreover, Id proteins inhibited differentiation of P19 cells to myocytes as a consequence of binding to GATA4 and Nkx2.5 [18]. Both Nnkx2.5 [5] and GATA4 [3] are important transcription factors in glucagon cell development.

In summary, our observations suggest an interesting paradigm wherein glucagon cell development requires GATA4 expression, which is influenced by BMP4 signaling. As development progresses, GATA4 expression is diminished in alpha cells, perhaps through the interplay with Id1 binding. Thus, in the IFNgNOD model, which resembles embryonic stage, where morphogenesis is an ongoing process, more glucagon cells express BMP-R2 than Id1 and are still pervious to BMP signals. In the adult, Id1 expression dominates and perhaps excludes the expression of GATA4.

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References

1. F.G. Andersen, R.S. Heller, H.V. Petersen, J. Jensen, O.D. Madsen, P. Serup, FEBS Lett. **445**, 306–310 (1999)
2. Y. Liu, W. Shen, P.L. Brubaker, K.H. Kaestner, D.J. Drucker, Biochem. J. **366**, 633–641 (2002)
3. B. Ritz-Laser, A. Mamin, T. Brun, I. Avril, V.M. Schwitzgebel, J. Philippe, Mol. Endocrinol. **19**, 759–770 (2005)
4. I. Artner, J. Le Lay, Y. Hang, L. Elghazi, J.C. Schisler, E. Henderson, B. Sosa-Pineda, R. Stein, Diabetes **55**, 297–304 (2006)
5. M.J. Doyle, Z.L. Loomis, L. Sussel, Development **134**, 515–523 (2007)
6. R. Benezra, Trends Cardiovasc. Med. **11**, 237–241 (2001)
7. J.D. Norton, R.W. Deed, G. Craggs, F. Sablitzky, Trends Cell. Biol. **8**, 58–65 (1998)
8. A. Lasorella, T. Uo, A. Iavarone, Oncogene **20**, 8326–8333 (2001)
9. J.D. Norton, J. Cell Sci. **113**(Pt 22), 3897–3905 (2000)
10. A. Iavarone, P. Garg, A. Lasorella, J. Hsu, M.A. Israel, Genes Dev. **8**, 1270–1284 (1994)
11. H. Hua, Y.Q. Zhang, S. Dabernat, M. Kritzik, D. Dietz, L. Sterling, N. Sarvetnick, J. Biol. Chem. **281**, 13574–13580 (2006)
12. K.T. Lee, Y.W. Lee, J.K. Lee, S.H. Choi, J.C. Rhee, S.S. Paik, G. Kong, Br. J. Cancer **90**, 1198–1203 (2004)
13. Q. Zhou, A.C. Law, J. Rajagopal, W.J. Anderson, P.A. Gray, D.A. Melton, Dev. Cell **13**, 103–114 (2007)
14. M. Brorson, D.M. Hougaard, J.H. Nielsen, D. Tornehave, L.I. Larsson, Histochem. Cell Biol. **116**, 263–267 (2001)
15. P. ten Dijke, O. Korchynskyi, G. Valdimarsdottir, M.J. Goumans, Mol. Cell Endocrinol. **211**, 105–113 (2003)
16. D.S. Dichmann, C.P. Miller, J. Jensen, R. Scott, R. Heller, P. Serup, Dev. Dyn. **226**, 663–674 (2003)
17. C.O. Brown 3rd, X. Chi, E. Garcia-Gras, M. Shirai, X.H. Feng, R.J. Schwartz, J. Biol. Chem. **279**, 10659–10669 (2004)
18. B. Ding, C.J. Liu, Y. Huang, J. Yu, W. Kong, P. Lengyel, J. Biol. Chem. **281**, 14893–14906 (2006)
19. Y.Q. Zhang et al., Diabetes **53**, 2024–2033 (2004)
20. N.E. Sarvetnick, D. Gu, Adv. Exp. Med. Biol. **321**, 85–89 (1992), discussion 91–93
21. M.R. Kritzik, T. Krahl, A. Good, M. Krakowski, L. St-Onge, P. Gruss, C. Wright, N. Sarvetnick, Mol. Cell Endocrinol. **164**, 99–107 (2000)
22. S. Arnaud-Dabernat, M. Kritzik, A.G. Kayali, Y.Q. Zhang, G. Liu, C. Ungles, N. Sarvetnick, Diabetes **56**, 96–106 (2007)
23. A.G. Kayali et al., J. Cell Biol. **163**, 859–869 (2003)
24. G. Gu, J.M. Wells, D. Dombkowski, F. Preffer, B. Aronow, D.A. Melton, Development **131**, 165–179 (2004)
25. F.J. Naya, H.P. Huang, Y. Qiu, H. Mutoh, F.J. DeMayo, A.B. Leiter, M.J. Tsai, Genes Dev. **11**, 2323–2334 (1997)
26. G. Gradwohl, A. Dierich, M. LeMeur, F. Guillemot, Proc. Natl. Acad. Sci. USA **97**, 1607–1611 (2000)
27. J. Jensen et al., Nat. Genet. **24**, 36–44 (2000)
28. C.L. Pin, J.M. Rukstalis, C. Johnson, S.F. Konieczny, J. Cell Biol. **155**, 519–530 (2001)
29. K. Monzen et al., Mol. Cell Biol. **19**, 7096–8105 (1999)