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Snail2, a mediator of epithelial-mesenchymal transitions, expressed in progenitor cells of the developing endocrine pancreas

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Abstract

The mammalian pancreas develops by the expansion and morphogenesis of the epithelial cells of the foregut endoderm via the sequential activation of transcription factors that direct differentiation into the various pancreatic lineages. Implicit in this growth and differentiation are the temporal and spatial processes of cell migration and three-dimensional organization, which cooperate to form a properly functioning organ. In many organ systems, such as the kidney, heart, and neural crest derivatives, migration and tissue morphogenesis is accomplished by the transient conversion of stationary epithelial cells to migratory mesenchymal-like cells in a process known as epithelial-mesenchymal transition (EMT). We report the identification of the expression of the transcription factor Snail2/Slug, a known inducer of EMT and cell movement, in both the endocrine and exocrine cells of the developing mouse pancreas. Snail2 is expressed in Neurogenin3-positive endocrine progenitor cells, and expression is maintained during endocrine cell differentiation where it becomes increasingly restricted to the insulin-producing beta cells and somatostatin-producing delta cells. In the adult pancreas, the expression of Snail2 is maintained at low but detectable levels in all beta cells, indicating a latent role for Snail2 in the mature islet. These findings of Snail2 expression during endocrine pancreas development are relevant to the recent evidence demonstrating the involvement of EMT in the expansion of human islet tissue *in vitro*. EMT-like events appear to be involved in the development of the mammalian pancreas *in vivo*.
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1. Results and discussion

The mammalian pancreas develops from an evagination of the gut tube epithelium at approximately embryonic day (E) 9.5 of mouse development (Habener et al., 2005; Slack, 1995). The initial period of pancreatic growth from E9.5 to approximately E12.5, termed the first transition, involves the proliferation and branching of the embryonic ductal epithelium (branching morphogenesis), accompanied by limited cytodifferentiation. The following period beginning at E13, known as the second transition, is characterized by the rapid expansion of endocrine and exocrine precursor cells from the duct epithelium and their spatial and temporal differentiation into the exocrine and endocrine lineages (Pictet et al., 1972; Przybyla et al., 1979; Slack, 1995). During the second transition, the pancreas is extensively remodeled by the combination of: (1) proliferation and branching morphogenesis of the primitive ductal epithelium, (2) differentiation of exocrine cells into acini from ductal precursors, and (3) the formation and delamination of endocrine cells, consisting of the alpha, beta, delta, and PP cells (that produce the hormones, glucagon, insulin, somatostatin, and pancreatic polypeptide, respectively) (Kim and MacDonald, 2002).

The Snail family is comprised of a group of four highly related zinc-finger transcription factors: Snail1, Snail2/Slug,

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Snail3/Smuc, and Scratch. They regulate cell migration, left-right symmetry, stem cell survival, and epithelial-to-mesenchymal transitions (EMT) during metazoan development (Barrallo-Gimeno and Nieto, 2005; Boyer et al., 2000; Nieto, 2002), as well as dedifferentiation and regeneration in the mature adult (Savagner et al., 2005; Zhao et al., 2002). Members of this family, particularly Snail1 and Snail2, function primarily as repressors of gene transcription, regulating a variety of epithelial-specific genes involved in cell adhesion and epithelial cell identity (Bolos et al., 2003; Kajita et al., 2004; Tripathi et al., 2005). Expression of members of the Snail family have been identified in various *in vitro* cell systems for the expansion of pancreatic endocrine cells (Choi et al., 2004; Gershengorn et al., 2004 Rukstalis and Habener, unpublished observation), and therefore we sought to examine the expression of one member of the Snail family, Snail2, during normal pancreas development.

1.1. Snail2-expressing cells are sparsely distributed in the early embryonic pancreatic anlage

The mouse pancreas is specified within a region of the gut tube at approximately embryonic day (E) 9.5 of development. This region is defined, in part, by the expression of the transcription factor PDX-1, which is present in most if not all cells destined to develop into the pancreas (Habener et al., 2005). Over the ensuing four days of development (first transition, E9.5 to E13.5) the pancreas undergoes a rapid expansion and branching morphogenesis of the epithelial cells, with only a limited differentiation of exocrine and endocrine tissue. Immunohistochemical analysis of E12.5 pancreas sections using an antiserum to PDX-1 marks this expanding pancreatic epithelium (Fig. 1). Snail2 is detected in a small number of cells scattered within the pancreatic epithelium (Fig. 1, solid arrows). Most PDX-1 positive cells do not express Snail2; an occasional cell co-expresses PDX-1 and Snail2 (solid arrows). Consistent with the findings of Jensen et al. (2000) we also observed the co-expression of the proendocrine transcription factor neurogenin 3 (Ngn3) and PDX-1 in a subpopulation of cells at E12.5 (data not shown).

1.2. Snail2 co-expression with the pro-endocrine transcription factor Neurogenin3

The detection of Snail2 expression in embryonic endocrine cells prompted us to examine Snail2 expression at E15.5, a period of pancreas development when proendocrine cells undergo a rapid expansion and robust differentiation (commonly referred to as the second transition). During this transition period, the pro-endocrine transcription factor Neurogenin 3 (Ngn3) is highly expressed in a population of partially differentiated cells derived from the undifferentiated, multipotent epithelial cells of the pancreatic duct-like epithelium (Maestro et al., 2003), and it promotes their transition into the endocrine cell lineage. The expression of Ngn3 subsequently defervesces as cells further differentiate into distinct endocrine cell lineages expressing the hormones insulin (beta cells), glucagon (alpha cells), somatostatin (delta cells), and pancreatic polypeptide (PP cells).

Ngn3 is expressed in cells dispersed throughout the E15.5 pancreas (Fig. 2) and reside either adjacent to or within the primitive, undifferentiated duct-like epithelium (Gu et al., 2002; Maestro et al., 2003; Schwitzgebel et al., 2000). At this stage of pancreas development (E15.5), Snail2 is broadly expressed throughout the pancreatic epithelium and is found co-expressed in a large proportion (greater than 80%) of Ngn3 cells (yellow, Fig. 2c), as well as in differentiated insulin-positive beta cells (Fig. 2d, solid arrowhead) and other hormone-positive endocrine cells (data not shown). Weak but detectable Snail2 expression is also present in the exocrine cells, identified by co-immunostaining with amylase. (Fig. 3h and data not shown). Notably, there is a tiered expression of Snail2 levels; Snail2 is most strongly expressed in Ngn3-positive progenitor cells, slightly less in beta cells, and low but detectable levels in all exocrine cells (Fig. 2b and d, compare arrows).

Endocrine cell formation occurs via the activation of Ngn3 within cells of the primitive ductal epithelium, and the subsequent delamination of these cells from this epithelial layer as the cells differentiate and form islets (Apelqvist et al., 1999; Grapin-Botton et al., 2001; Jensen et al., 2000; Pedersen and Heller, 2005). Using the transcription factor TCF2/HNF1β as a marker of the

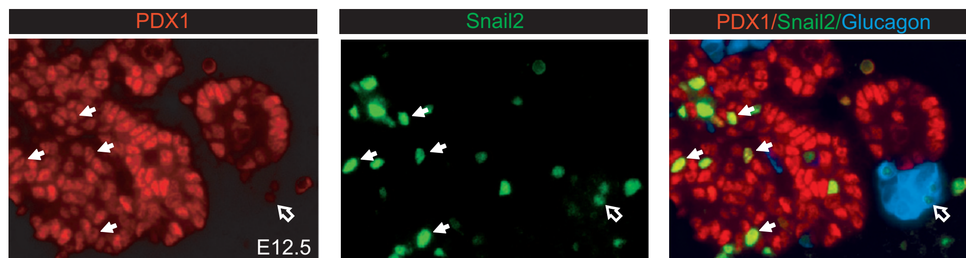

Fig. 1. Snail2 is expressed in scattered cells of the first transition pancreatic epithelium. At E12.5 of pancreas development, PDX-1, a master regulator of pancreatic development, is present in all epithelial cells of the developing pancreas (red). Snail2 is detected in a small subpopulation of pancreatic cells located amongst the PDX1-positive cells (green). Occasional cells appear that co-express PDX-1 and Snail2 (solid arrows). A cell is observed that expresses both glucagon (blue) and Snail2 (green) (open arrow).

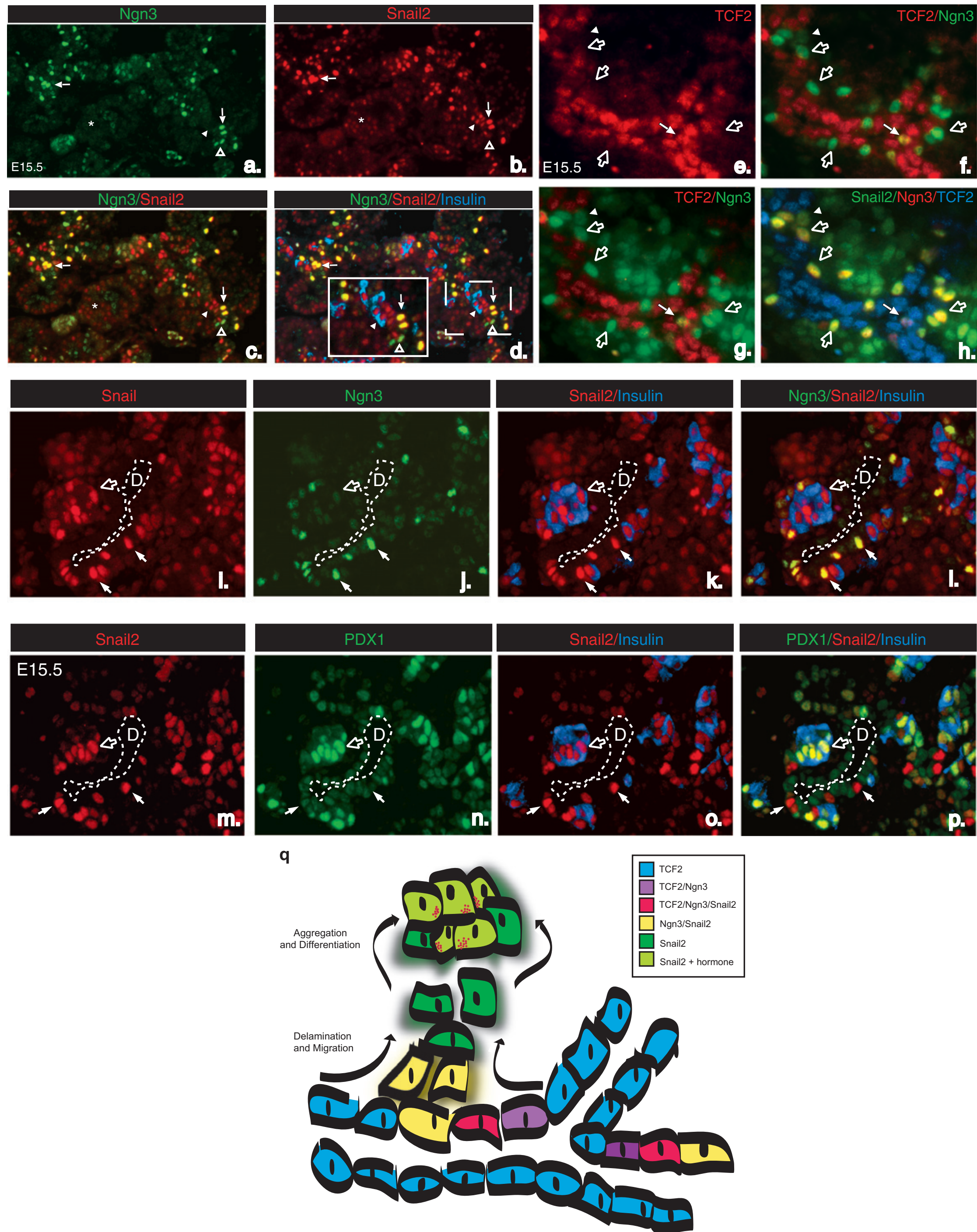

Fig. 2. Robust Snail2 expression in developing endocrine cells of second transition. Snail2 is highly expressed in Ngn3+ endocrine progenitor cells. At E15.5 of mouse pancreas development a wave of endocrine cell commitment occurs, as visualized by expression of the pro-endocrine transcription factor Ngn3 (green) (a). Snail2 expression (red) is detected at this stage throughout the pancreas (b), with highest levels of Snail2 present within cells co-expressing Ngn3 (c) (yellow, solid arrow). Less Snail2 expression is present in differentiated beta cells (blue) (d). Lowest levels of Snail2 expression are found in the acinar cells of the exocrine pancreas (asterisk). Snail2 is expressed in endocrine cells delaminating from the primitive ductal tubules, defined in part by the expression of TCF2 (e). Ngn3-positive endocrine progenitor cells are interdigitated with TCF2 cells (f) and co-localizes with Snail2 (open arrows) (g). Occasional co-expression of all three transcription factors can also be detected (solid arrow) (h). Snail2 is maintained as endocrine cells differentiate. When the E15.5 duct-like tubules (D) are viewed in cross-section, Snail2-positive cells are seen co-localizing with Ngn3 (solid arrow) and delaminating from the primitive tubules (i–l, dotted line outlines primitive duct lumen). Snail2 expression is maintained in differentiated beta cells (open arrow). Snail2 is co-expressed with PDX-1 (m–p) (solid arrows), which is expressed at low levels in the undifferentiated tubules of the pancreatic rudiment, but is strongly induced in differentiated beta cells (n and p, open arrows). Potential model for Snail2 expression during endocrine development. (q) Ngn3 cells arise from the TCF2 primitive tubular epithelium, and soon after initiate the expression of Snail2. TCF2 expression is then extinguished in these cells as they delaminate from the tubules and migrate into the parenchyma. Ngn3 expression declines while Snail2 expression is maintained as endocrine differentiation occurs.

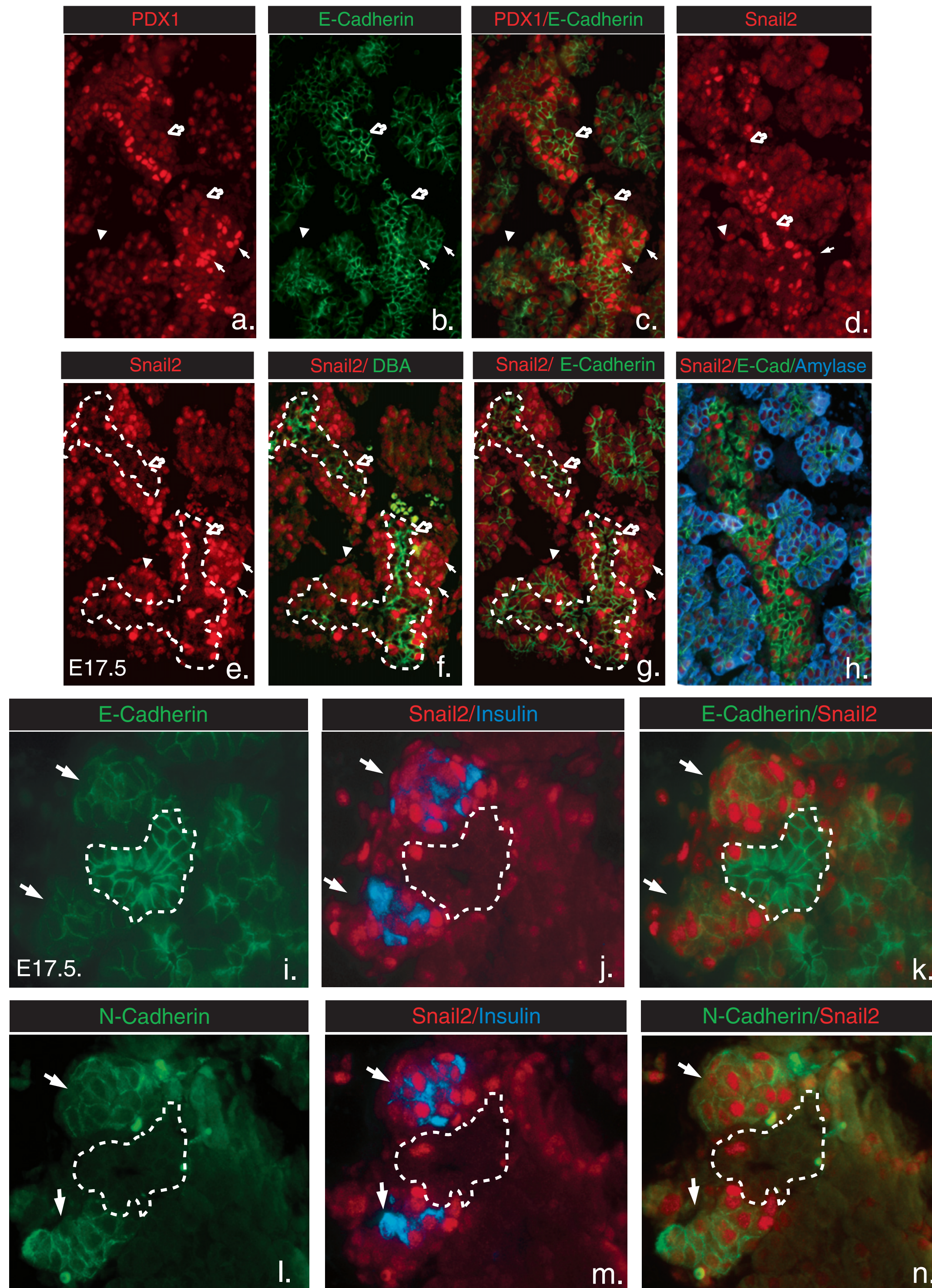

Fig. 3. Snail2 expression is maintained during endocrine cell maturation and islet aggregation. Using PDX-1 as a marker of the pancreatic tubules (open arrow), acinar cells (arrowhead), and coalescing beta cells (solid arrow) (a), strong expression of E-cadherin can be seen in the primitive ductal tubules (b and c, open arrows) as compared to the other cell types of the pancreas at E17.5 of mouse development. Immunostaining of serial sections for Snail2 (e–g and d, h), DBA (f), E-cadherin (g), and amylase (h) reveals that Snail2 is specifically excluded from the strongly E-cadherin positive cells of the ductal tubules (dotted line, open arrows). Cadherin expression is altered in Snail2 cells. E-Cadherin expression (i and k) is reduced in Snail2 endocrine cells (j and k) that are clustering into islets (arrows) adjacent to the duct-like tubular epithelium (dotted line). Reduction in E-cadherin expression in Snail2 cells is accompanied by a corresponding increase in N-cadherin expression (l–n).

proliferating duct-like epithelium that gives rise to Ngn3 cells (Maestro et al., 2003), we detect both Ngn3-positive and Snail2-positive cells adjacent-to and interdigitated-with the cells of the primitive ductal epithelium (Fig. 2e–h, open arrows). Cells are detected that co-express all three transcription factors (Fig. 2e–h, solid arrows) indicating

that the Snail2/Ngn3 cells arise from TCF2-expressing duct like precursors.

1.3. Snail2 expression is maintained in differentiated endocrine cells

As endocrine precursors exit from within the primitive duct, Ngn3 expression becomes downregulated and endocrine cytodifferentiation occurs (Grapin-Botton et al., 2001). This pattern of expression is seen when the primitive pancreatic duct-like tubules are visualized in cross section and immunostained for Ngn3, Snail2, and insulin (Fig. 2i–l). Snail2 and Ngn3 are co-expressed in insulin-negative cells within the duct-like epithelium, but Ngn3 is absent from the differentiated, Snail2-positive beta cells (Fig. 2i–l, open arrows). These differentiated beta cells strongly express the transcription factor PDX1 (Fig. 2m–p, open arrows). Snail2 is also expressed in some weak-PDX-1/insulin-negative cells of the undifferentiated tubular epithelium (Fig. 2m–p, solid arrows). This pattern of expression indicates a model in which Snail2 is expressed soon after Ngn3 cells become specified from TCF2-positive duct cells, and then its expression is maintained as cells delaminate from the duct, migrate into the parenchyma to differentiate into insulin-expressing endocrine cells and form islets (Fig. 2q).

1.4. Snail2 expression is maintained in the E17.5 pancreas

The pancreatic epithelium is visualized in the E17.5 mouse embryo by the widespread expression of the transcription factor PDX-1 (Fig. 3a). PDX1 is weakly expressed in both duct (open arrow) and exocrine cells (arrowhead), whereas it is strongly expressed in the nascent beta cells that are arrayed along the primitive ductal tubules, as identified by staining with the marker Dolichos biflorus agglutinin (DBA) (compare staining of serial sections in panels a and f).

The canonical epithelial cell adhesion molecule E-cadherin is also broadly expressed throughout the pancreatic epithelium at this stage (Fig. 3b). Strongest expression of E-cadherin is seen amongst cells of the tubular duct-like precursor epithelium (open arrows). The signal intensity of E-cadherin is consistently less in the developing endocrine and exocrine cells of the pancreas that are adjacent to the primative ductal tubules.

Immunostaining of serial sections for Snail2, a known repressor of E-cadherin expression, reveals that Snail2 is present in most cells of the pancreas including the acinar cells of the exocrine pancreas (Fig. 3d–h) but is specifically excluded from the undifferentiated primative ductal tubules, as marked by DBA immunoreactivity (panels e and f, dotted line). This expression pattern of Snail2 is inversely correlated with that of E-cadherin, as highest levels of E-cadherin expression are present within the Snail2-negative stationary epithelial cells of the ducts (Fig. 3e and g).

1.5. Snail2 expression is associated with a change from E-cadherin to N-cadherin expression

The gradation of intensity of E-cadherin expression is seen at higher magnification when the ductal tubules (E17.5) are viewed in cross section and immunostained for E-cadherin, Snail2, and insulin (Fig. 3i–k). E-Cadherin is strongly expressed in the cells that comprise the duct, but expression is reduced in those cells outside the ductal domain, particularly in the nascent islets (Fig. 3i, arrows). These cells contain reduced E-cadherin expression and are positive for Snail2 (Fig. 3j and k, arrows).

It has been reported previously that endocrine cells express N-cadherin in addition to E-cadherin during islet formation (Dahl et al., 1996). N-Cadherin is expressed in migratory cells which are undergoing EMT, and has been shown to increase cell motility even in the presence of E-cadherin (Hazan et al., 2000; Nieman et al., 1999). During EMT the expression of E-cadherin defervesces and the expression of N-cadherin increases (Nakajima et al., 2004). We therefore reasoned that because Snail2 is a known inducer of EMT, expression of Snail2 may correlate with a change in expression of E-cadherin to N-cadherin expression during islet development. Immunostaining of E17.5 pancreas sections reveals that N-cadherin and Snail2 are co-expressed in the developing endocrine cells as they exit from the duct and coalesce into islet-like clusters (Fig. 3l–n, arrows). These cell clusters that express N-cadherin correspond to those cells that contain reduced levels of E-cadherin in serial sections (compare panels a and d) indicating that Snail2 is associated with the change in cadherin expression that occurs during islet formation.

1.6. Snail2 becomes restricted to beta and delta cells

We next characterized the expression of Snail2 in endocrine cells as islet development progresses throughout the second transition and into postnatal life. During the process of islet morphogenesis and endocrine cell cytodifferentiation, the transcription factor ISL1 becomes expressed early on in all cells within the endocrine lineage where it enhances transcription of the genes for the endocrine hormones (insulin, glucagon, sand omatostatin) gene promoters (Habener et al., 2005). Because of the broad expression of ISL1 in all early endocrine cell types, we compared the expression of ISL1 with Snail2 to test whether Snail2 expression correlates with a specific endocrine lineage.

Immunohistochemical staining of E17.5 pancreatic sections with antisera to Snail2, ISL1, and either insulin or glucagon, reveals extensive but incomplete overlap between Snail2 and ISL1 expression in the coalescing islets, with considerable variability in the relative expression of Snail2 and ISL1 from one cell to the next (Fig. 4a–h). This variability does not appear to be directly related to cell differentiation, however, as cells can be found that express equivalent levels of ISL1 and glucagon but either express (solid arrow) or lack (open arrow) Snail2. This variation in Snail2 expression in

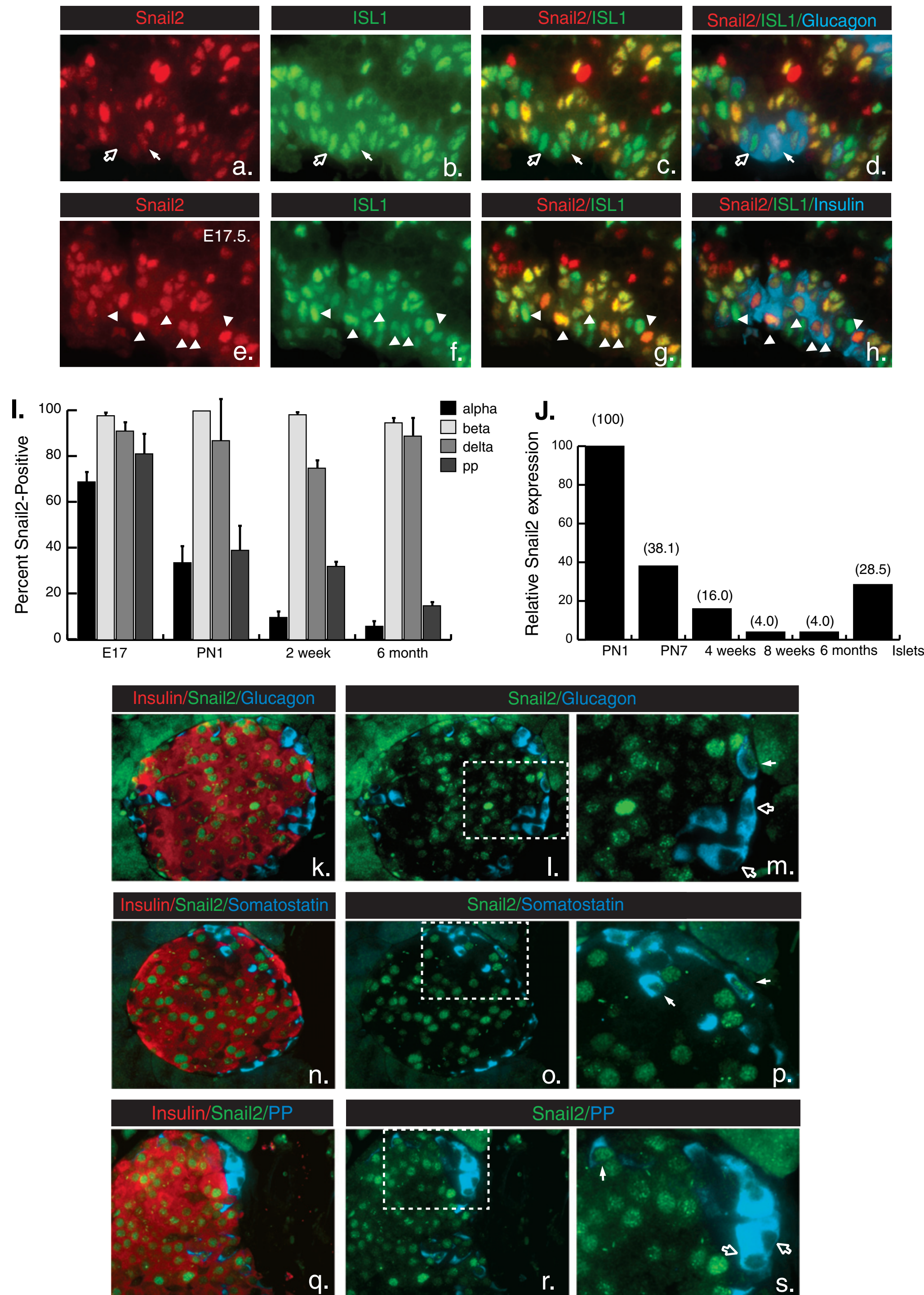

Fig. 4. Snail2 expression becomes restricted to a subset of endocrine cells. E15.5 pancreas sections were immunostained for ISL1 (green), which is expressed in all endocrine cells, Snail2 (red), and glucagon (blue, a–d) or insulin (blue, e–h). Snail2 expression is not completely coincident with ISL1, with more Snail2/ISL co-expression in insulin-producing beta cells (e–h, arrow heads) than in glucagon-producing alpha cells (a–d, compare open and closed arrows). Morphometric analysis of Snail2 expression in each of the endocrine cell types reveals that Snail2 becomes progressively restricted to insulin-producing beta cells and somatostain-producing delta cells as the pancreas matures (i). Real time-PCR analysis of Snail2 expression reveals that Snail2 expression declines during postnatal pancreas development and becomes restricted to the islets of Langerhans (j). Relative expression values are noted above each bar and displayed relative to the Snail2 expression level at post-natal day 1 (PN1). In the adult pancreas, Snail2 is expressed primarily in beta cells (k, n, and q) and delta cells (o, and p), and in only a small percentage of alpha cells (n) and PP cells (r and s, arrow).

glucagon-expressing alpha cells is not seen in beta cells, which express essentially equivalent amounts of Snail2, ISL1, and insulin (Fig. 4e–h, arrowheads).

One interpretation of the variability of Snail2 expression in glucagon-positive alpha cells is that Snail2 is expressed in all Ngn3-positive progenitor cells and early endocrine cells, but

as the cells mature, Snail2 becomes restricted to beta cells. During this process of cell-type restriction, Snail2 expression is seen as variegated among the non-beta cell endocrine fraction of the pancreas but remains consistently expressed in beta cells. To address this notion, pancreas tissue sections from various times in embryonic and postnatal pancreas development were immunostained with Snail2 and each of the endocrine hormones. The percentage of each endocrine cell type that expresses Snail2 was scored and displayed graphically to compare how Snail2 expression changes during mouse pancreas development (Fig. 4i). At E17.5 of pancreas development, Snail2 is expressed in virtually all beta and delta cells, as well as in a high percentage of alpha and PP cells. As the mouse ages and the pancreas matures, Snail2 expression becomes increasingly restricted to beta and delta cells, with a small percentage of alpha and PP cells continuing to express Snail2, even in the mature adult. It is worth noting that the intensity of Snail2 expression per cells is significantly higher in beta cells as compared to the other endocrine cell types, however the meaning of this difference is not clear at this time. Based on this temporal pattern of Snail2 expression as the pancreas matures, it appears likely that Snail2 is present in most, if not all, endocrine cells as they differentiate from Ngn3-positive precursors, but is maintained only in differentiated beta and delta cells. Formal linage tracing experiments will be required to more fully address this question.

1.7. Snail2 expression decreases as the pancreas matures

During the first 4 weeks of postnatal mouse development, the pancreas undergoes a period of growth and maturation as both the endocrine and exocrine portions of the pancreas become competent to respond to the increasing nutrient demands required as the animal transitions from a liquid (milk) to a solid food diet. Real-time RT-PCR analysis of Snail2 expression during this period of transition reveals that Snail2 expression declines by approximately 80% over the first 4 weeks of postnatal life, and then declines further as the mouse reaches adulthood (Fig. 4j). Snail2 expression in the adult also becomes exclusively expressed in the islets of Langerhans with no detectable expression in the exocrine pancreas (Supplemental Fig. 4).

Immunostaining of 6 month adult mouse islets for Snail2 and each of the endocrine hormones reveals that Snail2 is expressed at low but detectable levels in all beta cells of the mature islet (Fig. 4k–s), as well as in most delta cells and in only a small number of alpha- and PP-cells (Fig. 4k–s, solid arrows). Expression of Snail2 is maintained in the islets, predominantly in beta cells, throughout the lifetime of the adult, up to the oldest time point tested (17 months, data not shown).

1.8. Conclusion

Epithelial- mesenchymal transition (EMT) is a conserved developmental mechanism utilized during the gastrulation of the embryo, organogenesis, and derivation of the neural crest that allows for cell migration, tissue morphogenesis, and organ remodeling. We have demonstrated here for the first time that the transcription factor Snail2, a core component of the EMT machinery, is expressed in both the embryonic and adult pancreas, with

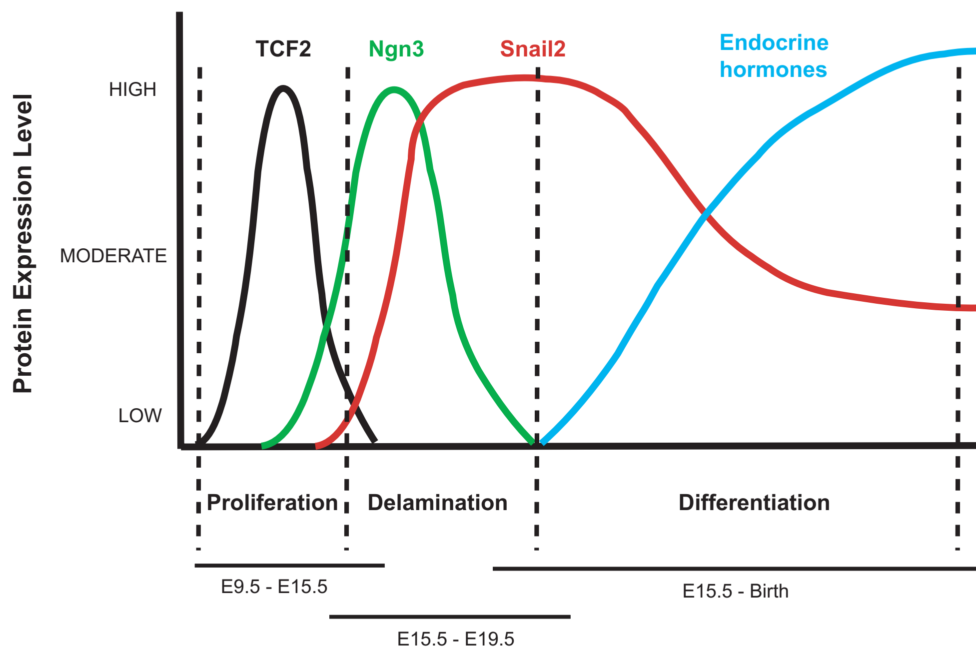

Fig. 5. Proposed model of Snail2 expression during pancreas development. In the embryonic pancreas, TCF2-positive cells of the primative ductal tubules proliferate and expand the pool of cells from which Ngn3 endocrine progenitor cells are derived (Maestro et al., 2003). Soon after cells become committed to the endocrine linage, Snail2 expression becomes induced in the delaminating pro-endocrine cells. Ngn3 levels decline while Snail2 expression is maintained as endocrine differentiation occurs. Snail2 levels then slowly decline as the endocrine cells mature, and Snail2 becomes progressively restricted to adult beta and delta cells.

particularly strong expression in endocrine cells. Based on the comparative expression profiles of the various transcription factors examined in this study, a putative model can be made for the generation of pancreatic endocrine cells from the undifferentiated tubular epithelium (Fig. 5), whereby Ngn3 precedes Snail2 expression in developing endocrine cells. As endocrine differentiation occurs, Ngn3 levels decline, while Snail2 is maintained in differentiated beta and delta cells. Snail2 expression then abates as endocrine cells mature, but is maintained at low but detectable levels in the adult.

2. Experimental procedures

2.1. Animal husbandry and immunohistiochemistry

All studies were performed using C57BL/6 mice purchased from Jackson laboratories. Embryo ages were timed by designating noon of the day the vaginal plug was detected as Embryonic day (E)0.5.

Paraffin embedded tissue was sectioned at 5 μm, rehydrated through graded series of ethanols, and processed for immunostaining in PickCell 2100 (PickCell Labs, Amsterdam, North Holland) retriever using Retrievagen A solution (BS Biosciences, San Jose, CA). Immunohistochemistry was performed using mouse on mouse (MOM) detection system (Vector Labs, Burlingame, CA) according to manufacturer's protocol. Primary antibodies used were: rabbit anti-Snail2 (Covance Research Products), rabbit anti-Snail2 H-140 (Santa Cruz Biotechnology), mouse anti-PDX1 (Beta cell Biology Consortium), rabbit anti-PDX1 (Covance Research Products), guinea pig anti-insulin (Linco), guinea pig anti-glucagon (Linco), guinea pig anti-PP (Linco), sheep anti-somatostatin (Cortex), Fluorescein Dolichos biflorus agglutinin (DBA) (Vector Labs), mouse anti-Ngn3 (Developmental Studies Hybridoma Bank), goat anti-TCF2 (Santa Cruz), mouse anti-E-cadherin (BD Transduction Labs), goat anti-amylase (Santa Cruz), and mouse anti-N-cadherin (US Biological).

2.2. Snail2 antibody production

Because antisera suitable for the detection of Snail2 in paraffin tissue sections are not commercially available, we generated a rabbit polyclonal antibody to a peptide corresponding to the amino-terminus of the mouse Snail2 protein. This region of the protein is highly conserved amongst the mouse, rat, and human Snail2 proteins. Antibodies were generated by Covance Research Products by injection of the immunization peptide (CRMSLLHKHQESGSSGGPR) into two rabbits (HM5291 and HM5292). The amino-terminal cysteine was added to the peptide to allow the conjugation of the peptide to keyhole limpet hemocyanin for immunization. Similar results were seen using sera from both animals, and all experiments in this manuscript were performed using HN5291. These antibodies were tested for specificity by multiple methods (Supplemental Figs. 1–3).

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.modgep.2006.11.001.

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