One process for pancreatic β-cell coalescence into islets involves an epithelial–mesenchymal transition

Lori Cole[1], Miranda Anderson[1], Parker B Antin[2] and **Sean W Limesand[1]**

[1]Department of Animal Sciences, Agricultural Research Complex and [2]Department of Cell Biology and Anatomy, University of Arizona, 1650 East Limberlost Drive, Tucson, Arizona 85719, USA

(Correspondence should be addressed to S W Limesand; Email: limesand@ag.arizona.edu)

Abstract

Islet replacement is a promising therapy for treating diabetes mellitus, but the supply of donor tissue for transplantation is limited. To overcome this limitation, endocrine tissue can be expanded, but this requires an understanding of normal developmental processes that regulate islet formation. In this study, we compare pancreas development in sheep and human, and provide evidence that an epithelial–mesenchymal transition (EMT) is involved in β-cell differentiation and islet formation. Transcription factors know to regulate pancreas formation, pancreatic duodenal homeobox factor 1, neurogenin 3, NKX2-2, and NKX6-1, which were expressed in the appropriate spatial and temporal pattern to coordinate pancreatic bud outgrowth and direct endocrine cell specification in sheep. Immunofluorescence staining of the developing pancreas was used to co-localize insulin and epithelial proteins (cytokeratin, E-cadherin, and β-catenin) or insulin and a mesenchymal protein (vimentin). In sheep, individual β-cells become insulin-positive in the progenitor epithelium, then lose epithelial characteristics, and migrate out of the epithelial layer to form islets. As β-cells exit the epithelial progenitor cell layer, they acquire mesenchymal characteristics, shown by their acquisition of vimentin. *In situ* hybridization expression analysis of the *SNAIL* family members of transcriptional repressors (*SNAIL1*, *-2*, and *-3*; listed as *SNAI1*, *-2*, *-3* in the HUGO Database) showed that each of the *SNAIL* genes was expressed in the ductal epithelium during development, and *SNAIL-1* and *-2* were co-expressed with insulin. Our findings provide strong evidence that the movement of β-cells from the pancreatic ductal epithelium involves an EMT.

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Introduction

A central therapeutic goal for treating diabetic patients is to restore adequate β-cell function; however, the quantity of donor tissue for islet transplantation is inadequate (Shapiro *et al.* 2000, Grapin-Botton 2005, Nir & Dor 2005). To combat this limitation a renewable source of β-cells is required, and a possible solution includes *in vitro* β-cell production. A prerequisite for expanding β-cells *in vitro* is to understand the normal developmental processes involved in islet formation. Although some recent reports indicate that endocrine cytodifferentiation in the human pancreas is similar to that of the mouse, detailed analyses have revealed important differences between human and mouse with respect to the timing of β-cell differentiation (Piper *et al.* 2004, Sarkar *et al.* 2008). Therefore, to better understand pancreatic morphogenesis in humans, who have longer pregnancy than rodents, comparative animal systems will be needed. The sheep is a long standing large animal model for studying fetal physiology, and we have observed that the progression of pancreas development in the fetal sheep closely parallels the progression observed in human. This has allowed us to monitor the multi foci differentiation pattern to examine islet formation in a species with a longer gestation period (Limesand *et al.* 2005, Cole *et al.* 2007).

It is generally accepted that islets originate from epithelial progenitor cells because emerging endocrine cells transiently retain epithelial characteristics and are usually located close to or within the pancreatic duct epithelium (Slack 1995, Bonner-Weir *et al.* 2000, Yatoh *et al.* 2007). The mechanism for how pancreatic islets arise from this epithelial cell layer remains unresolved, but appears to involve two distinct processes. In the rat, the most prominent mechanism for islet morphogenesis involves the formation of large ductal cell aggregates, termed islet-forming units, the cells of which begin expressing insulin and other endocrine hormones, while still associated with the epithelium (Bouwens & De 1996). These aggregates gradually lose contact with the epithelium and ultimately form morphologically recognizable islets. In the human, groupings of polyhormonal expressing cells are also observed connected to pancreatic ducts (Bocian-Sobkowska *et al.* 1999), which supports this islet-forming unit theory. Such a mechanism might explain a role for lateral communication in coordinating the differentiation and formation of islet structures.

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A second mechanism of islet cell development was suggested by the work of Pictet & Rutter (1972), which showed that individual endocrine cells first appear within the ductal epithelium, then leave the epithelial layer, and coalesce to form islets of Langerhans. A similar process has been described in the mouse (Jensen 2004), where it was postulated that after the secondary transition individual endocrine cells leave the ductal epithelium and migrate to form aggregates. In both human (Piper *et al*. 2004, Sarkar *et al*. 2008) and sheep (Limesand *et al*. 2005), individual endocrine cells are observed within and adjacent to the duct throughout gestation indicating a common mechanism for the origin of at least some endocrine cells. Although the general changes in cell morphology that occur during this single cell migration (or 'budding') suggest that an epithelial–mesenchymal transition (EMT) is involved, the process has not been carefully defined in any species.

EMT is an important developmental process by which migratory mesenchymal cells arise from an epithelium, ultimately forming new structures in many embryonic tissues (Kang & Massague 2004, Radisky 2005). There is evidence to support an EMT as a plausible mechanism for the origin of pancreatic endocrine cells. In a single-cell transcript analysis during mouse pancreas development, all neurogenin 3 (Ngn3) expressing cells co-expressed the epithelial cell marker E-cadherin, and a majority also expressed the mesenchymal cell marker vimentin (Chiang & Melton 2003). Furthermore, approximately a quarter of the insulin$^+$ cells co-expressed vimentin and the epithelial cell marker cytokeratin. These findings indicate that endocrine progenitor cells typically express both epithelial and mesenchymal cell markers, indicating a transitory period between epithelial and mesenchymal phenotypes. In addition to the expression of Snail-2, a transcriptional repressor that mediates an EMT, is present in the endocrine progenitor cells and differentiated β-cells during mouse pancreas development (Rukstalis & Habener 2007). Finally, an EMT was also shown to promote expansion of human islet cells and nonendocrine epithelial cells *in vitro* by β-cell dedifferentiation to a mesenchymal phenotype, which in some circumstance could be reversed to produce a population of insulin$^+$ cells (Gershengorn *et al*. 2004, Ouziel-Yahalom *et al*. 2006, Seeberger *et al*. 2009). This dedifferentiation phenomenon was not observed in adult islets from rodent and most likely reflects species differences that are further supported by our studies in sheep (Russ *et al*. 2008).

Together, these findings provide support to the idea that an EMT is associated with β-cell differentiation, but as of yet this process has not been defined in any species. Here we compare the origin of pancreatic endocrine cells in the developing human and sheep. We find that the timing and mechanisms of origin of β-cells in the sheep and human are highly similar. In both human and sheep, we identify two processes for islet formation; the so called islet-forming unit in which large aggregates of ductal epithelial cells become insulin-positive and bud off from the duct as one unit, and a single cell process in which individual insulin-positive cells emerge from the ductal epithelium. Immunofluorescence and *in situ* hybridization analyses provide strong evidence that this latter process involves an EMT.

Materials and Methods

Pancreatic collection and processing

Pancreata were dissected from sheep fetuses and fixed in 4% paraformaldehyde (PFA) in PBS (137 mM NaCl, 2·7 mM KCl, 4·3 mM $Na_2HPO_4{\cdot}H_2O$, 1·4 mM KH_2PO_4, pH 7·3) at 4 °C for 14–24 h. The tissues were immersed in 30% sucrose overnight at 4 °C and then equilibrated in a 1:1 30% sucrose: optimal cutting temperature (OCT) compound (volume:volume) mixture for an additional 24 h at 4 °C. Tissues were embedded with OCT (Tissue Tek manufactured for Sakura Finetek USA, Inc., Torrance, CA, USA), frozen, and stored at −80 °C. Six micrometres thick tissue sections were cut with a cryostat (Microm HM 520) for immunostaining and *in situ* hybridization procedures. Human pancreas tissue sections were collected by Prof. John C Hutton and Dr Suparna Sarkar, Barbara Davis Center for Childhood Diabetes (UCDHSC, Aurora, CO, USA), and fetal mouse sections were collected by Dr Lori Sussel, Department of Genetics and Development (University of Columbia, New York, NY, USA).

Immunofluorescent staining

Pancreas sections were dried to Superfrost Plus slides for 30 min at 37 °C and then washed twice with water for 5 min. Antigen retrieval was performed by microwaving tissues in 10 mM citric acid buffer, pH 6·0 for 10 min. The tissues were cooled for 20 min, washed three times in PBS for 5 min, and non-specific binding sites were blocked with 0·5% NEN blocking buffer reagent (0·1 M Tris–HCl, 0·15 M NaCl; Perkin–Elmer) for 45 min. Primary antiserum was diluted in 1% BSA supplemented PBS, applied to the section, and incubated at 4 °C overnight in a humidified chamber; exclusion of primary antiserum served as the negative control.

Pancreatic β-cells were identified in the fetal sheep pancreas with guinea pig anti-porcine insulin (1:500; Dako, Carpinteria, CA, USA), mouse anti-sheep insulin C-peptide (1:500; S W Limesand), rabbit anti-mouse pancreatic duodenal homeobox factor 1 (Pdx1; 1:1000; Millipore, Billerica, MA, USA), and rabbit anti-mouse Nkx6.1 (1:500; J Jensen, Cleveland, OH, USA). Epithelial cells were identified in the fetal sheep pancreas with mouse anti-human pan-cytokeratins 4, 5, 6, 8, 10, 13, 18 (1:250; Research Diagnostic, Inc., Concord, MA, USA), rabbit anti-β-catenin (1:100; Lab Vision Neomarkers, Fremont, CA, USA), and guinea pig anti-E-cadherin (1:100; J C Hutton). Mesenchymal cells were identified with mouse anti-vimentin IgM (1:11 000; Sigma–Aldrich). Antibody specificity for epithelial and mesenchymal proteins was verified by immunoblot analysis, and all antiserum-detected proteins

of appropriate molecular weight in fetal sheep pancreas. After incubation with the primary antiserum, the tissue sections were washed three times for 10 min with PBS. Immunocomplexes were detected with affinity-purified secondary antiserum conjugated to Cy2, Cy3, Texas Red, or 7-amino-4-methylcoumarin-3-acetic acid (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) diluted 1:500 in 1% BSA–PBS for 60 min at 25 °C. The tissues were then washed with PBS (3× for 10 min) and mounted in 50% glycerol with 10 mM Tris–HCl, pH 8.

Chromogen immunohistochemistry

Pancreas sections were washed twice with distilled water for 5 min. Endogenous peroxidases were quenched in 0·3% H_2O_2 in water during two 10-min incubations. The Vectastain Elite ABC Kits (Vector Laboratories, Burlingame, CA, USA) were used for immunostaining, as per the manufacturer's instructions, and detected with 3,3′-diaminobenzidine (DAB) peroxidase substrate (Vector Laboratories). Tissues were rinsed in water and dehydrated through a series of increasing ethanol washes, cleared with Histo-clear, and mounted with Securemount (Thermo Fisher Scientific, Waltham, MA, USA).

Morphometric analysis

Fluorescent images were visualized on a Leica DM5500 Microscope System and digitally captured with a Spot Pursuit 4 Megapixel CCD camera (Diagnostic Instruments, Inc., Sterling Heights, MI, USA). Confocal fluorescent images (0·7 μm thick) were captured on a Zeiss LSM 510NLO-Meta Multiphoton/Confocal Microscope System (Carl Zeiss, Inc., Thornwood, NY, USA). Morphometric analysis was performed with Image Pro Plus 5.1 software (Media Cybernetics, Silver Spring, MD, USA). To determine the percentage of β-cells expressing vimentin, 300–1400 β-cells were counted per pancreas ($n \geq 3$/gestational age group). The human data set consisted of ten individuals, four adults, and six fetal subjects (ranging from 11 to 23 weeks gestational age (wGA) previously reported (Sarkar *et al*. 2008). Five adult rat pancreata from two strains, Sprague–Dawley (8 months old, $n=3$), or Fisher (5 months old, $n=2$) were examined alongside seven pancreata of FVB/N mice at 4 weeks of age. All values are presented as mean ± S.E.M.

In situ *hybridization*

Ovine *SNAIL1* (*SNAI1*), *SNAIL2* (*SNAI2*), *SNAIL3* (*SNAI3*), *NGN3* (*NEUROG3*), and *NKX2-2* DNA products were amplified from total RNA extracted from fetal sheep pancreas by reverse transcription-PCR. Synthetic oligonucleotide primers were designed from the bovine sequences: SNAIL1, 5′-CGA CAC TCA TCT GGG ACT CTC-3′ and 5′-ACC CAG GCT GAT GTA CTC CTT-3′; SNAIL2, 5′-CCT GGT CAA GAA GCA TTT CAA-3′ and 5′-CAG GCT CAC GTA TTC CTT GTC-3′; SNAIL3, 5′-TCA GAG ACA GCG TGA ACC AC-3′ and 5′-TGC CGT ACT CCT TGT CAC AG-3′; NGN3, 5′-AAG GAT GRC GCC TCA WCC CT-3′ and 5′-ATG TAG TTG TGG GCG AAG C-3′ (nest PCR with 5′-AAG GAT GRC GCC TCA WCC CT-3′ and 5′-TTT CAC AGR AAR TCT GAG A-3′ in first reaction); and Nkx2.2, 5′-ATG TCG CTG ACC AAC ACA AAG A-3′ and 5′-TGT ACT GGG CGT TGT ACT GC-3′. The DNA products were cloned and sequenced as previously described (Limesand *et al*. 2007). All nucleotide sequences were submitted to GenBank: *SNAIL1*, EU081875; *SNAIL2*, EU081876; *SNAIL3*, EU081877; *NGN3*, DQ072379; and *NKX2-2*, DQ054804.

Plasmid DNA, linearized using the appropriate restriction endonuclease enzyme, was extracted with phenol:chloroform:isoAmyl (25:24:1), chloroform:isoAmyl (24:1), precipitated in ethanol with ammonium acetate, washed with 70% ethanol, and dried. The linear DNA was re-suspended in nuclease-free water, and the integrity and concentration were measured by agarose gel electrophoresis and spectrophotometry with a Nanodrop Spectrophotometer ND-100 (Thermo Fisher Scientific). The digoxigenin (DIG)-labeled RNA probe was generated with SP6 or T7 RNA polymerase (Promega) with the reaction containing 1 μg linearized DNA in 1×txn buffer, 2 ng/μl DIG labeling mix (Roche), 0.5 U/μl RNAsin, 1 mmol/l dithiothreitol, and 1 U/μl T7 or 0·7 U/μl SP6 RNA polymerase (Promega) at a final volume of 20 μl. The reaction was incubated for >3 h at 37 °C. The DIG-labeled RNA probe was ethanol precipitated with ammonium acetate and 0·2 μg glycogen. The RNA pellet was washed with 70% ethanol, dried, and re-suspended in 100 μl sterile water. RNA concentrations were determined by measuring the absorbance at A260, and DIG-labeled RNA integrity was assessed on an agarose gel.

Pancreas sections were fixed in fresh 4% PFA in PBS for 10 min, washed three times in PBS for 3 min, digested with 10 μg/ml proteinase K (50 mmol/l Tris, pH 7·5, 5 mmol/l EDTA) for 8 min, and fixed again in fresh 4% PFA in PBS for 5 min. After three 3 min 1×PBS washes, pancreatic tissue sections were acetylated (102 mmol/l triethanolamine, 0·01 mmol/l HCl, 27 mmol/l acetic anhydride) for 10 min at room temperature and washed thrice in PBS for 5 min. Nonspecific binding was blocked by incubating the tissues in hybridization buffer (50% formamide, 5×SSC pH 4·5, 50 μg/ml yeast tRNA, 1% SDS, 50 μg/ml heparin) for 2 h at 55 °C. The DIG-labeled RNA was added to the hybridization buffer (snail-1: anti-sense 110 ng/μl, sense 78 ng/μl; snail-2: anti-sense 98 ng/μl, sense 115 ng/μl; snail-3: anti-sense 83·6 ng/μl, sense 38·6 ng/μl), heat-denatured at 80 °C for 5 min, cooled for 5 min, and applied to the tissue, which was then covered with a glass cover slip and incubated overnight at 70 °C in a humidified chamber. Cover slips were removed and pancreas sections were washed in pre-warmed, 70 °C 5×SSC (1×SSC:150 mmol/l NaCl, 15 mmol/l sodium citrate, pH to 7·0) for 30 min at room temperature, then incubated in pre-warmed, 70 °C

0·2×SSC for 3 h at 70 °C. The tissues were washed at room temperature in 0·2×SSC for 5 min, then equilibrated in Malic acid buffer (1×MAB; 100 mmol/l maleic acid, 150 mmol/l NaCl, pH 8·0) for 5 min. Pancreas sections were incubated in blocking buffer (2% blocking reagent (Roche Applied Science), 10% heat inactivated sheep serum, 0·1% Tween-20 and brought to a final volume with 1×MAB) for 1 h at room temperature, and then incubated overnight at 4 °C in blocking buffer containing anti-DIG-AP Fab fragments antibody (Roche Applied Science; 1:1000). Following the incubation, the pancreatic sections were washed for 15 min three times with MAB containing 0·1% Tween-20, and then washed in distilled water with 0·1% Tween-20 for 20 min. Pancreas sections were developed in BM Purple AP Substrate (Roche Applied Science) supplemented with 0·1% Tween-20 for 3–36 h.

Results

Conserved processes for endocrine lineage specification in sheep

From the mouse, a hierarchy of transcription factors involved in endocrine cell determination from pluripotent pancreatic progenitors has been established (Jensen 2004). Using this knowledge, we determined *PDX1*, *NGN3*, and Nkx homeobox proteins, *NKX2.2* and *NKX6.1*, expression in sheep.

At 24 days gestational age (dGA; 0·16 of gestation), the dorsal pancreatic bud was organized as a dense outgrowth (Fig. 1A). Robust PDX1 staining was detected in a majority of the epithelial cell nuclei of the pancreatic bud and antral stomach. After elongation into the surrounding mesenchymal tissue, occurring between 24 and 29 dGA in the sheep, the strong PDX1 nuclear staining became restricted to β-cells (insulin$^+$), but less intense nuclear staining and diffuse cytoplasmic staining was still present in the pancreatic endoderm at 33 dGA (0·22 of gestation; Fig. 1B). By 131 dGA (0·9 of gestation), the PDX1 staining was predominantly localized to cells expressing insulin in either β-cell clusters or individual β-cells (Fig. 1C).

In the mouse, *Ngn3* is required for endocrine cell determination committing pancreatic epithelial progenitor cells to an endocrine fate (Gradwohl *et al.* 2000). We cloned the ovine *NGN3* gene, which shares 83% identity with human, to perform *in situ* hybridizations on sheep pancreas. Anti-sense *NGN3* was expressed in either solitary cells or grouped into small clusters in the pancreatic epithelium at 24 dGA, 63 dGA, and lambs at postnatal day 10 (Fig. 1D). No specific staining was found with the DIG-labeled sense strand of *NGN3* (Fig. 1D, insert). Similar to the mouse and human, the Ngn3$^+$ cells do not co-express mature endocrine hormones, insulin or glucagon, further substantiating its role as an islet cell precursor in sheep (Fig. 1E and F; Jensen *et al.* 2000, Sarkar *et al.* 2008). To validate *NGN3*'s temporal expression in pre-endocrine cells, we also evaluated *NKX2.2* expression in sheep. In the mouse, *Nkx2.2* is expressed downstream of *Ngn3* in the sequence of transcription factors that determine endocrine cell differentiation from pancreatic progenitors (Sussel *et al.* 1998). The nucleotide sequence for ovine *NKX2.2* shared 94% identity with human *NKX2.2*. In sheep, the expression pattern of *NKX2.2* appears to overlap *NGN3*, but was more extensive because *NKX2.2* expression persists in both insulin$^+$ cells and most glucagon$^+$ cells (Fig. 1G and H). Another transcription factor downstream of *NKX2.2* in the lineage of β-cell specification is *NKX6.1* (Sander *et al.* 2000) and in the sheep pancreas, we found that NKX6.1 was predominantly localized to insulin$^+$ cells, but occasionally NKX6.1$^+$ insulin$^-$ cells were observed (Fig. 2Q–T). Strikingly, even though the secondary transition (e.g. endocrine cell differentiation) was extended in the sheep, similar to the human (Piper *et al.* 2004, Sarkar *et al.* 2008), the progression through the previously defined cascade of critical transcription factors was conserved.

Two processes for islet formation in the sheep and human pancreas

Fetal sheep and human pancreata were immunostained for insulin, vimentin, and β-catenin to identify β-cells, mesenchymal cells, and epithelial cells respectively. In fetal sheep, β-cells were observed in three distinct morphological structures: the ductal epithelium, the mesenchymal stroma, and endocrine cell clusters (Fig. 3). Solitary β-cells were found in and immediately adjacent to the ductal epithelium and in the mesenchymal stroma at all gestational ages examined (29–142 dGA). β-Cell clusters were also present in the pancreatic parenchyma at all gestational ages examined. Some clusters were closely associated with the ductal epithelium and likely represent the islet-forming units previously described (Bocian-Sobkowska *et al.* 1999). Other β-cell clusters appeared as isolated structures, likely representing coalescing islets. These β-cell morphologies were also observed in the human fetal pancreas at 11 and 23 wGA (0·28 and 0·55 of gestation; Fig. 3D–G). Individual β-cells and β-cell clusters were identified in both the mesenchymal and epithelial compartments of the developing pancreas.

These findings suggest that in both sheep and humans, β-cell clusters arise through two independent processes; as individual cells that move from the ductal epithelium to the stroma, and as large aggregates of cells that emerge within the ductal epithelium and then bud off to form isolated clusters.

β-Cells differentiate from pancreatic ducts and lose epithelial characteristics

Fetal sheep pancreata were immunostained for insulin, PDX1, and cytokeratin proteins to demarcate β-cells (insulin$^+$ and PDX1$^+$) and epithelial cells (cytokeratin$^+$). Individual β-cells within the ductal epithelium and immediately adjacent to the lumen typically showed distinct cytokeratin staining along the plasma membrane (Fig. 2A–D). Single β-cells that were not adjacent to the lumen of the pancreatic ducts but located within the ductal

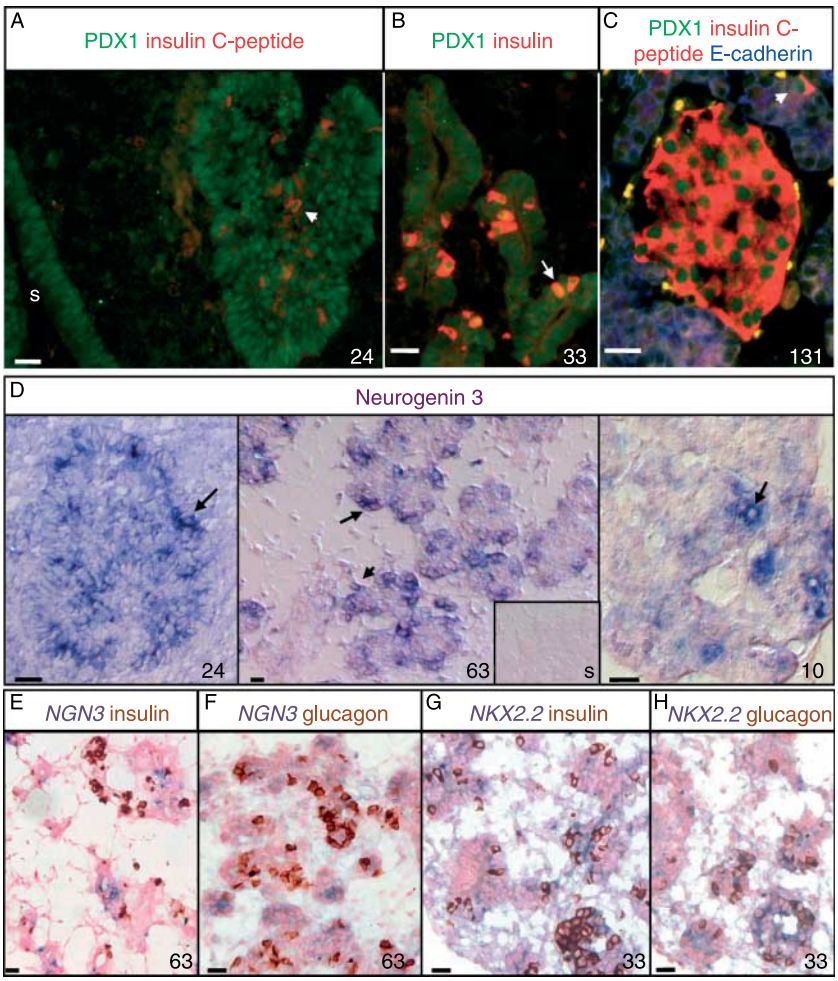

Figure 1 Developmental progression of transcriptional regulatory factors in sheep. Immunofluorescent staining for PDX1 (Cy2, green) and insulin (Texas Red) was determined in the sheep pancreas at several stages of gestation (A–C). A longitudinal cross section at 24 dGA of the dorsal pancreatic bud and stomach (s) are shown (A), and the arrow identifies a PDX1$^+$ insulin$^+$ cell. At 33 dGA elongated ducts composed of PDX1$^+$ epithelium were surrounded by a mesenchymal bed (B), solitary insulin$^+$ cells with nuclear PDX1 staining are apparent, whereas insulin$^-$ cells exhibit a variety of PDX1 staining localized in the nucleus and/or cytoplasm. Both mature islet-like structures and solitary insulin$^+$ cells (arrow) are shown at 131 dGA (C), and E-cadherin staining in blue is observed in the epithelium. Representative photographs of sheep pancreas with differential interference contrast microscopy are shown for *in situ* hybridization with antisense or sense (insert) DIG-labeled neurogenin 3 RNA for fetuses at 24 and 63 dGA, and a lamb at postnatal day 10 (D). The black arrows identify examples of NGN3$^+$ cells stained with BM purple. The inserted picture illustrates the negative control, *NGN3* sense strand (s), for the 63 dGA fetus and encompasses an area equivalent to the area of the antisense picture. A 63 dGA, sheep pancreatic section was stained for *NGN3* by *in situ* hybridization techniques and subsequently immunostained for insulin (E) or glucagon (F). Pancreas section from a sheep fetus at 33 dGA was stained for *NKX2.2* using *in situ* hybridization methodologies and then immunostained for insulin (G) or glucagon (H). In all photographs, the factors are labeled above in their respective color, the gestational or postnatal age is indicated in the right corner, and a 20 μm scale bar is present in the lower left corner.

epithelium acquired a more diffuse cytosolic distribution of cytokeratin (Fig. 2E–H). Individual β-cells that were located outside the ductal epithelium also showed low level diffuse cytosolic staining for cytokeratin proteins, and β-cells clusters independent of ductal epithelium showed low or undetectable cytokeratin levels (Fig. 2I–L).

Similar immunostaining results were obtained for β-catenin (Fig. 3) and E-cadherin (Fig. 2Q–T). Insulin$^+$

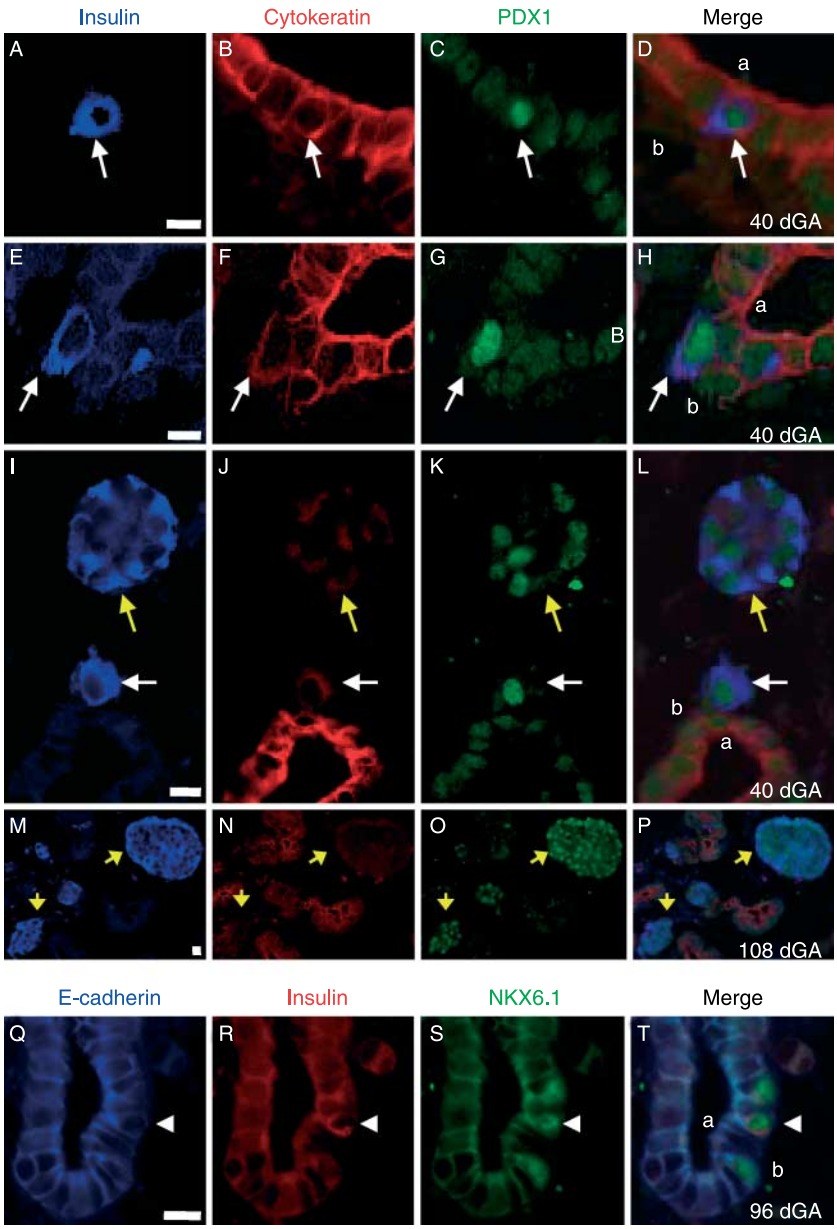

Figure 2 β-Cells lose cytokeratin expression during islet formation. Representative photographs from sheep pancreatic sections immunostained for insulin (AMCA, blue), cytokeratin (Texas Red), and PDX1 (Cy2, green) illustrate cytokeratin expression in β-cells with respect to their location (A–P). The merge image is shown on the right, and the fetal age (dGA) is indicated in the bottom corner. The scale bars are presented on the insulin photograph and represent 10 μm. Solitary β-cells (white arrows) were observed adjacent to the epithelial lumen (A–D), within the epithelium but not adjacent to the lumen (E–H), and distinct from the epithelial layer (I–L). Small and large β-cell clusters were also independent of the epithelial layer and primarily cytokeratin- (I–P; yellow arrows). E-cadherin (AMCA), insulin (Texas Red), and NKX6.1 (Cy2) immunofluorescent staining is shown in Q–T and the arrow head points to a bottle-shaped β-cell that has lower E-cadherin expression in its plasma membrane. In figures D, H, L and T, the 'a' identifies the apical surface and 'b' the basolateral surface of the pancreatic epithelium.

cells within the ductal epithelium show distinct β-catenin framing of the plasmalemma, whereas cells adjacent to the ducts show a diffuse cytosolic β-catenin distribution, which disappears in β-cells that are located within β-cell clusters (Fig. 3A–C). Immunostaining with E-cadherin also declines in insulin$^+$ cells as they differentiate within the pancreatic epithelial layer (Fig. 2Q–T). These β-cells appear to be losing E-cadherin expression as they migrate to the basal surface of the epithelium and acquire a 'bottle-shaped' appearance that is reminiscent of a cell undergoing an EMT. Together,

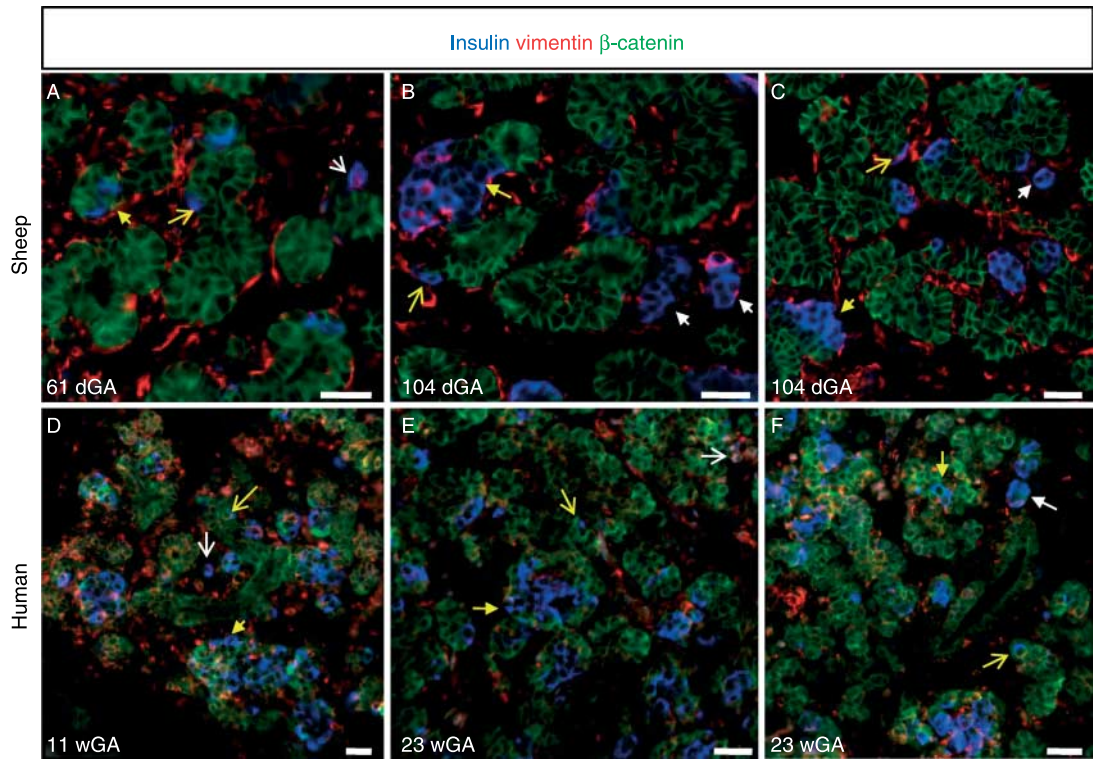

Figure 3 Two independent processes in sheep and human for β-cell coalescence into islets. Fetal pancreas tissues collected from sheep (top row) and human (bottom row) fetuses were immunostained for insulin (AMCA, blue), vimentin (Texas Red), and β-catenin (Cy2, green). The days (dGA) or weeks (wGA) of gestational age are indicated at the bottom of each image along with a scale bar that equals 25 μm. The white arrows identify solitary β-cells (open arrowhead) or β-cells clusters (closed arrowhead) located in the mesenchymal stroma independent from the ductal epithelium. The yellow arrows identify solitary β-cells (open arrowhead) or β-cells clusters (closed arrowhead) within the ductal epithelium.

these data show a transition in localization and expression levels of several proteins that are highly similar to those occurring during an EMT in other cell types (Thiery & Sleeman 2006). Following cytodifferentiation (expression of insulin), β-cells within the ductal epithelium lose their epithelial phenotype and appear to exit the epithelium.

β-Cells emerging from the ductal epithelium gain mesenchymal characteristics

Vimentin, an intermediate filament protein that demarcates mesenchymal cells, was occasionally co-expressed with insulin in both the sheep and human pancreas (Fig. 3). While vimentin staining was rarely observed within insulin$^-$ductal cells, some insulin$^+$ cells still associated with the ductal lumen were vimentin$^-$, indicating differentiation takes place prior to developing a mesenchymal phenotype. Insulin$^+$ cells within the epithelium but not in contact with the lumen, or located outside of the ductal epithelium, almost always showed vimentin staining (Fig. 4; Table 1). These findings suggest that vimentin expression becomes detectable as insulin$^+$ cells acquire a mesenchymal phenotype.

Because the temporal expression pattern of vimentin persisted in larger β-cell clusters (Table 1), we evaluated its expression in adult islets (Fig. 5). Strikingly, vimentin$^+$ β-cells were found in adult sheep islets prompting further examination across different species. In human, vimentin$^+$ β-cells were observed in the islets of Langerhans (Fig. 5). However, β-cells in rodents did not co-express insulin and vimentin, even though dual staining was observed during pancreas development in the mouse at embryonic day 15·5 (data not shown). These findings indicate that sheep and human islets have a small proportion of β-cells with a mesenchymal phenotype, which might provide a mechanism for continued islet expansion or remodeling in adulthood.

SNAIL family members are expressed in the ductal epithelium

To examine expression of SNAIL family members during sheep pancreas development, cDNAs coding for *SNAIL1*, *SNAIL2*, and *SNAIL3* were cloned from fetal sheep pancreatic RNA. The sheep *SNAIL1*, *SNAIL2*, and *SNAIL3* nucleotide sequences were 87, 85, and 71% identical to the human orthologs respectively (data not shown).

In situ hybridization analysis showed that mRNAs coding for *SNAIL1*, *SNAIL2*, or *SNAIL3* were localized predominantly to ductal epithelial invaginations or budding

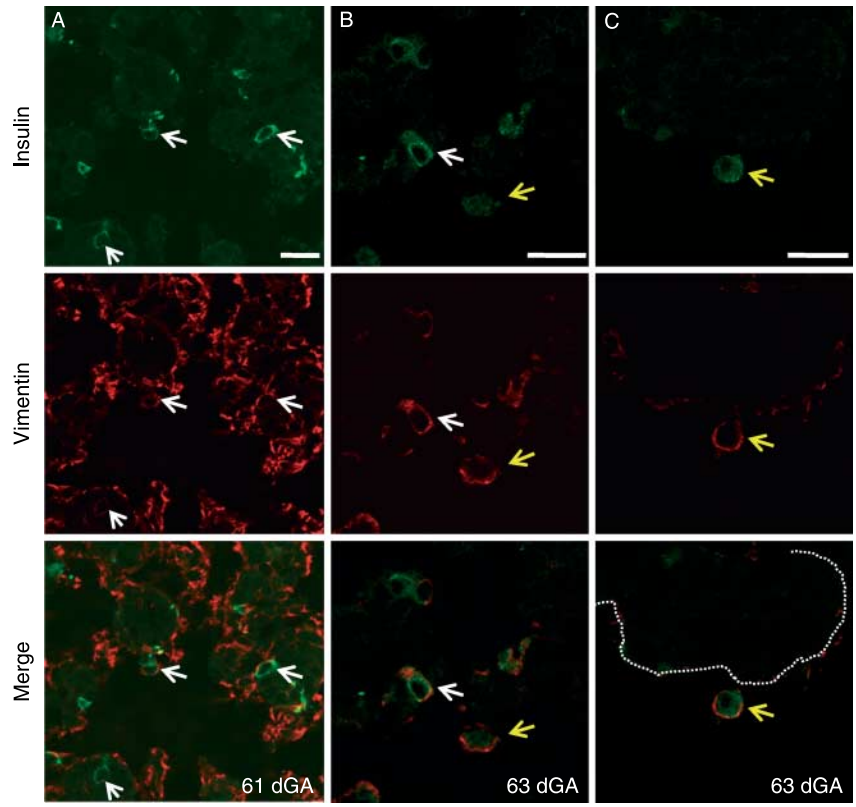

Figure 4 Vimentin expression in solitary β-cells. Fetal sheep pancreata were immunostained for vimentin (Cy3, red) and ovine insulin C-peptide (Cy2, green), and digital images were captured using confocal microscopy. Three representative images are shown (A–C) for insulin, vimentin, and the merged image at 61 and 63 dGA. The arrows identify individual β-cells located within (white) or outside (yellow) of the epithelial layer, which is marked with a dashed line in C. The scale bars on the insulin stained images represent 20 μm.

structures (Fig. 6A–C), indicating these transcriptional repressors might play a role in branching morphogenesis within the pancreas as well as facilitating β-cell migration through an EMT. Sections stained for *SNAIL1* mRNA or *SNAIL2* mRNA were subsequently immunostained for insulin (Fig. 6A/D, B/E and F/G). A majority of the β-cells within clusters express *SNAIL1* and *-2* transcripts; however, the *SNAIL1* and *-2* expression was not exclusive because insulin$^+$ SNAIL1$^-$or insulin$^+$ SNAIL2$^-$cells were also found in the pancreas (Fig. 6). Solitary β-cells not associated with the pancreatic ducts had low-level SNAIL1 and -2 staining (Fig. 6). β-Cell clusters that were associated with the ducts expressed *SNAIL1* and *-2* mRNA. Together, these findings place the SNAIL family members correctly in the pancreatic progenitor epithelium and newly formed β-cells.

Table 1 Proportion of vimentin$^+$ β-cells relative to the cluster size

	Days gestational age				
	29 (%)	33–40 (%)	61–63 (%)	95–109 (%)	129–142 (%)
Cell number in cluster					
1	70±5	62±7	43±7	27±5	24±7
2–3	56±5	63±8	42±5	23±5	21±5
4–10	48±6	51±8	42±4	24±8	24±7
11–50		46±5	46±5	34±8	21±3
50–100		29[a]		64±17	43±10
>100				55±13	63±15

[a]Only one cluster found in the age group.

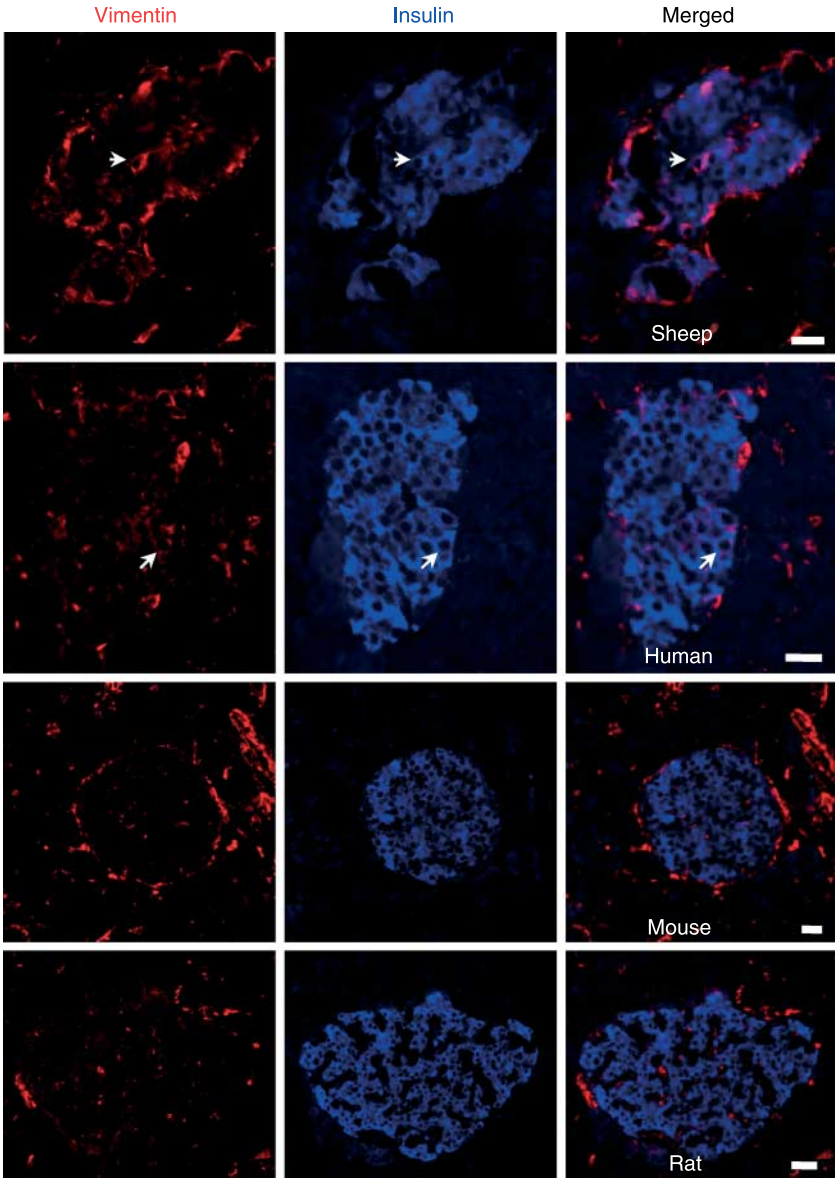

Figure 5 Vimentin$^+$ β-cells in adult islets. Pancreas tissue from adult sheep, human, rat, and mouse were immunostained for insulin (AMCA, blue) and vimentin (Texas Red). A representative islet is shown for each species, which is labeled on the bottom of the merged image. White arrows identify a cell that is immunopositive for insulin and vimentin in the sheep and human, while dual staining was not observed in rodents ($n \geq 5$ islets from at least five different mature individuals). In the merged images, the scale bars represent 20 μm.

Discussion

In this study, we show that the cascade of transcription factors mediating sheep endocrine cell specification parallels the genetic network defined in the mouse. Additionally, we distinguish two independent processes by which β-cells arise in the ductal epithelium and form islets of Langerhans. The first involves aggregates of ductal cells (the islet-forming unit) that begin to express insulin and then detach in mass from the duct, while the second involves individual insulin$^+$ cells that appear within the ductal epithelium and then exit the epithelium before migrating to form islets (Fig. 7). Through the use of a variety of cytoskeletal and transcription factor markers, we provide strong evidence that these latter cells exit the ductal epithelium via a classical EMT. Evidence to support this includes the localization of insulin$^+$ cells within

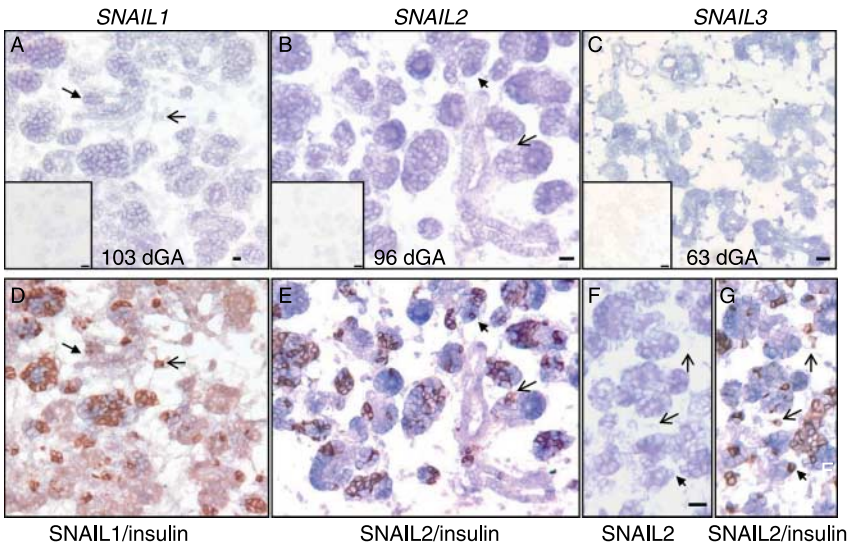

Figure 6 Expression of the SNAIL family members in the sheep pancreas. On sheep pancreatic sections, *in situ* hybridizations were performed with antisense and sense (inserts) *SNAIL1* (A), *SNAIL2* (B and F), and *SNAIL3* (C) cRNA. After capturing images for *SNAIL1* and *SNAIL2*, the sections were subsequently immunostained for insulin to co-localize SNAIL1 (D) and SNAIL2 (E and G) to β-cells. The black filled arrows identify β-cells co-expressing SNAIL1 or SNAIL2. The open arrowheads identify SNAIL- insulin$^+$ cells. Scale bars in the antisense *in situ* hybridization pictures represent a 20 μm, and bars in the inserts (sense strand, negative controls) are 50 μm long.

the ductal epithelium, at the periphery of the epithelium, and as individual cells within the pancreatic stroma, and corresponding transitions in expression and subcellular localization of proteins characteristic of epithelial and mesenchymal cells. β-Cells within the epithelium in contact with the lumen show E-cadherin, β-catenin, and cytokeratin expression closely associated with the plasma membrane. β-Cells still within the epithelium that have lost contact with the lumen show more diffuse staining of these proteins, and some also exhibit the bottle shape that is characteristic of cells undergoing an EMT (Thiery & Sleeman 2006). Individual β-cells within the stroma show diffuse or undetectable levels of E-cadherin, β-catenin and cytokeratin. Conversely, insulin$^+$ cells often express vimentin, with

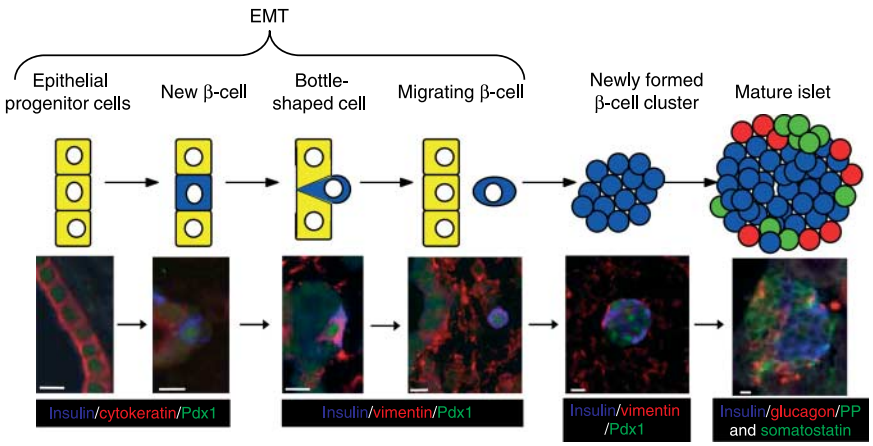

Figure 7 Single cell migration model of islet formation. A schematic with representative photographs is shown for the EMT process in sheep. In the model, β-cells (in blue) differentiate within the epithelial progenitor layer (yellow cells) and exit the epithelial layer. In the mature, islet cells in green and red represent other pancreatic endocrine cells. The triple immunofluorescent staining is labeled below each picture in the color representing its fluorescent staining and a scale bar representing 10 μm is presented.

proportions increasing in cells no longer in contact with the duct lumen or individual cells in the stroma. Finally, *SNAIL1*, *SNAIL2*, and *SNAIL3* mRNA transcripts are expressed in the pancreatic progenitor epithelium, confirming that facilitators of an EMT are in the correct temporal and spatial location.

This study provides evidence for an EMT-mediated migration (or 'budding') process of islet formation (Fig. 7). However, β-cells appear to maintain the mesenchymal phenotype as they aggregate into clusters (Table 1). Moreover, in the islet-forming unit process we have also found that fetal β-cells co-express insulin and vimentin (Fig. 3), indicating that an EMT might be involved in islet remodeling. Therefore, an EMT appears to be an important developmental process for β-cell differentiation, irrespective of their mechanism for isletogenesis.

The single cell migration process was originally proposed to result from a parallel versus perpendicular cell division in which the axis of division is parallel to the apical–basal cell axis. Thus, one daughter cell is freed from the apical matrix, while the other daughter cell retains the epithelial junctional complexes and apical connections with its neighboring ductal cells (Pictet & Rutter 1972). This mechanism of β-cell emergence was not disproved in this study, but β-cells were observed at the apical surface or found to have a bottle-shaped appearance (Fig. 2), similar to what is observed during an EMT in gastrulation (Shook & Keller 2003). These bottle-shaped β-cells undergo apical constriction, which reduces the surface area, the apical boundary length with adjacent cells, and tends to push the cytoplasm to the basal region of the cell, thereby causing a change in shape (Shook & Keller 2003). These bottle-shaped β-cells have down-regulated cytokeratin expression and usually express vimentin, indicating that as β-cells leave the progenitor epithelium they acquire mesenchymal characteristics. The exact process by which β-cells bud off of the ductal epithelium remains to be completely defined, but both proposed mechanisms would require the loss of epithelial connections and migration out of the epithelial layer, a process resembling an EMT.

Vimentin expression in β-cells decreases during gestation in size-matched clusters (Table 1), yet as β-cells aggregate into larger clusters, some retain vimentin expression. Surprisingly, very large β-cell clusters (>50 cells), which are usually present in later stages of gestation (95–142 dGA), have a very high proportion of vimentin$^+$ β-cells (40–63%), which may indicate a mesenchymal phenotype, is required for spatial remodeling within a forming islet. Islet remodeling and maturation occurs for 2–3 weeks after birth in the mouse pancreas (Habener *et al.* 2005) and for 6 months after birth in the human pancreas (Kassem *et al.* 2000). Similarly, islet remodeling was observed during the first 10 days in the lamb (Titlbach *et al.* 1985). In this study, we found vimentin$^+$ β-cells in adult sheep and human islets, indicating that even mature islets might continue to remodel, which may require a mesenchymal phenotype. In contrast, adult rodent β-cells did not co-express vimentin. These findings demonstrate a striking variation between mammals that is not fully understood. Together, our data support that an EMT takes place during pancreas development in all species examined demonstrating that it is a conserved developmental process across vertebrate phylogeny, but this phenotype is not necessarily required in adult rodents.

SNAIL mRNA expression is localized to the pancreatic progenitor epithelium and also to β-cells within the epithelial layer (Fig. 6), illustrating they are expressed at the correct temporal and spatial location. β-Cells expressing *SNAIL* mRNA were located within the ductal epithelium and may be characterized as pre-migratory β-cells (e.g. β-cells preparing to leave the ductal epithelium). β-Cells located outside of the ductal epithelium that were SNAIL1$^-$ or SNAIL2$^-$ appear to be migratory β-cells. These migratory β-cells expressed vimentin; thus indicating the EMT has already taken place, which might reflect the downregulation of *SNAIL1* (Peiro *et al.* 2006). Together, these data suggest a transient process for SNAIL1 and -2 proteins that allow β-cells to acquire a mesenchymal phenotype to migrate and then expression is lost until they coalesce into an islet (Komatsu *et al.* 1995).

SNAIL proteins have been found in all EMT processes studied (Nieto 2002), and their expression is regulated by growth factors (DeCraene *et al.* 2005), which also can influence subcellular localization and protein degradataion (Schlessinger & Hall 2004, Zhou *et al.* 2004, Bachelder *et al.* 2005, Yang *et al.* 2005, Yook *et al.* 2006). The molecular mechanism initiating an EMT in islet formation remains unsolved. A potential regulator in the developing pancreas is transforming growth factor β (TGFβ or TGFB1), a member of the TGFβ superfamily, which can induce *SNAIL1* and *SNAIL2 in vivo* and *in vitro* (DeCraene *et al.* 2005). In the pancreatic islet, TGFβ is of particular interest because induces β-cell migration in rat pancreatic islets (Battelino *et al.* 2000). TGFβ enhances matrix metalloproteinase-2, which is necessary for proper morphogenesis of the islets and inhibition of TGFβ activity represses islet morphogenesis (Battelino *et al.* 2000). TGFβ signaling can activate expression of *SNAIL* transcription factors and the EMT process and is a likely candidate to be the initiator of an EMT in the formation of islets. However, this direct link has not been made until now and remains to be fully tested.

Recent reports indicate that an EMT regulates primary human β-cell expansion. Two studies with cultured human pancreatic islet cells found that pancreatic β-cells can undergo EMT and trans- or dedifferentiate into fibroblast-like cells. Following expansion, these cells were induced to differentiate back into hormone expressing cells (Gershengorn *et al.* 2004, Ouziel-Yahalom *et al.* 2006). During the transition from islet cells to proliferating fibroblast-like cells, epithelial cell markers such as E-cadherin, claudins, and occludins as well as endocrine markers declined, and mesenchymal cell markers, including vimentin and SNAIL, increased (Gershengorn *et al.* 2004).

Following the mesenchymal–epithelial transition, the cells were allowed to aggregate; however, the newly-formed islet-like clusters were unable to produce functional quantities of insulin (Gershengorn *et al.* 2004). A similar study reported that proliferating human islet-derived (PHID) cells were induced to dedifferentiate, expand, and redifferentiate with β-cellulin, an epidermal growth factor family member that is mitogenic for β-cells (Ouziel-Yahalom *et al.* 2006). During expansion, the cells continued to express β-cell markers, indicating that PHID cells originated from β-cells rather than from a rare population of stem/progenitor cells. Interestingly, our findings show that a cohort of vimentin$^+$ β-cells exist in the adult human and sheep islets, identifying them as an expandable population.

Conversely, in adult mouse islets a genetic-based cell lineage-tracing experiment showed that β-cells do not undergo an EMT in culture and do not significantly contribute to the proliferating fibroblast-like cell population (Chase *et al.* 2007, Weinberg *et al.* 2007). These studies did not examine this process during development, and so an EMT process may be involved in mouse pancreas development (Rukstalis & Habener 2007). The difference between human and mouse appears to reflect some species divergence (Bliss & Sharp 1992, Gershengorn *et al.* 2004, Ouziel-Yahalom *et al.* 2006, Russ *et al.* 2008). We show that adult human and sheep islets contain a few vimentin$^+$ β-cells and that these cells retain junctional complexes. The maintenance of the mesenchymal phenotype might suggest that adult human and sheep β-cells are capable of remodeling, whereas adult rodent β-cells do not appear to use this mechanism, confirming the species differences found in the *in vitro* data.

In conclusion, we show that β-cells exit the epithelial layer after differentiating by undergoing an EMT, following the conserved pattern of regulatory transcription factors for pancreatic endocrine cell determination. As demonstrated in other developmental systems, this induction appears to involve SNAIL proteins that facilitate the EMT. The expression of multiple epithelial and mesenchymal markers in pre-endocrine and new endocrine cells support this process in vertebrate β-cell differentiation. The information provided connects a large body of literature on pancreatic β-cell development to an extensive literature on EMT and provides promising new insight to refine strategies for β-cell expansion *in vitro* for islet replacement therapy.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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