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Developmental pathways during *in vitro* progression of human islet neogenesis

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Abstract

Islet neogenesis, or the differentiation of islet cells from precursor cells, is seen *in vitro* and *in vivo* both embryonically and after birth. However, little is known about the differentiation pathways during embryonic development for human pancreas. Our previously reported *in vitro* generation of islets from human pancreatic tissue provides a unique system to identify potential markers of neogenesis and to determine the molecular mechanisms underlying this process. To this end, we analyzed the gene expression profiles of three different stages during *in vitro* islet generation: the Initial Adherent-, Expanded-, and Differentiated- stages. Samples from four human pancreases were hybridized to Affymetrix U95A GeneChips, and data analyzed using GeneSpring 7.0/9.0 software. Using Scatter plots we selected genes with a 2-fold or greater differential expression.. Of the 12,000 genes/ESTs present on these arrays, 295 genes including 38 acinar–enriched genes were selectively lost during the progression from the Initially Adherent stage to the Expanded stage; 468 genes were increased in this progression to Expanded tissue; and 529 genes had a two-fold greater expression in the Differentiated-stage than in the Expanded tissue. Besides the expected increases in insulin, glucagon and duct markers (mucin 6, aquaporin 1 and 5), the beta cell auto-antigen IA-2/phogrin was increased 5-fold in Differentiated. In addition developmentally important pathways, including Notch/jagged, Wnt/Frizzled, TGFβ superfamily (follistatin, BMPs and SMADs), retinoic acid (COUP-TFI, CRABP1, 2 and RAIG1) were differentially regulated during the expansion/differentiation. Two putative markers for islet precursor cells, UCHL1/PGP9.5 and DMBT1, were enhanced during the progression to differentiated cells, but only the latter could be a marker of islet precursor cells. We suggest that appropriate manipulation of these differentiation-associated pathways will enhance the efficiency of differentiation of insulin-producing beta cells in this *in vitro* model.

Introduction

During the last five years the interest in finding islet precursor/cells has increased dramatically. The evidence that such precursors exists is based on experimentally induced islet neogenesis (Bonner-Weir et al., 1983;Bonner-Weir et al., 1993;Boquist and Edstrom, 1970;Gu and Sarvetnick, 1993;Hultquist et al., 1979). Even though there is no conclusive evidence of a pancreatic stem cell, many studies favor the hypothesis that there are islet progenitor within the ducts in the adult pancreas (Bonner-Weir et al., 1993;Gu and Sarvetnick, 1993;Rafaeloff

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et al., 1996;Stoffers et al., 2000;Wang et al., 1993;Weaver et al., 1985;Xu et al., 1999;Yamamoto et al., 2000). Using an *in vitro* model, we earlier demonstrated that islet-depleted human pancreatic tissue, remaining after islet isolation, can differentiate into glucose-responsive insulin-producing cells (Bonner-Weir et al., 2000). Digested pancreatic tissue that was normally discarded after human islet isolation was cultured under conditions that allowed expansion of the ductal cells as a monolayer whereupon the cells were overlaid with a thin layer of Matrigel. With this manipulation, the monolayer of epithelial cells formed 3-dimensional structures of ductal cysts from which budded islet-like clusters of pancreatic endocrine cells. Over 3–4 weeks *in vitro*, the insulin content increased 10–15 folds as the DNA content increased up to 7 fold. The cultivated human islet buds were shown to consist of cytokeratin-19 positive duct cells and hormone-positive islet cells. When challenged with glucose going from basal 5 mM glucose to stimulatory 20 mM glucose, these buds had a 2.3 fold increase in secreted insulin. Thus, ductal tissue from human pancreas can be expanded in culture and be directed to differentiate into glucose-responsive islet tissue (Bonner-Weir et al., 2000;Gao et al., 2003). This *in vitro* model provides an excellent opportunity to investigate the molecular events regulating islet neogenesis from adult human pancreatic cells. *In vitro* neogenesis may recapitulate development of the embryonic pancreas, so it would be of interest if known developmental pathways, such as wnt/frizzled, TGF-β and notch/delta, were involved in this process. In addition, knowledge of relevant pathways may allow their manipulation to increase the efficiency of islet differentiation.

Wnt proteins, the mammalian analogs of *wingless* initially discovered in *Drosophila*, constitute a family of cysteine-rich glycoproteins involved in intercellular signaling during vertebrate development. Wnt signaling is believed to activate genes that promote stem cell fate and inhibit the cellular differentiation (De Boer et al., 2004). Secreted Wnt-proteins are involved in epithelial-mesenchymal interactions (Kengaku et al., 1997). The Wnt-pathway has been implicated in growth of the pancreas during development (Papadopoulou and Edlund, 2005) and in the specific development of endocrine cells in the chick (Pedersen and Heller, 2005).

Many members of the TGF-β pathway are expressed in embryonic pancreas. Dichmann et al. using multiplex PCR to screen TGF-β superfamily genes contributing to murine pancreatic endocrine formation found that TGF-β, Activin, BMP 4, 5 and 7 were expressed (Dichmann et al., 2003). BMP 4, 5 and 6 treatment of dissociated fetal mouse pancreatic cells grown on the extracellular matrix protein laminin-1 resulted in epithelial cysts with insulin and glucagon-positive cells (Jiang et al., 2002). Follistatin, an inhibitor of TGF-β signaling, was found in adult pancreatic rat islets (Ogawa et al., 1993) and to regulate embryonic endocrine development (Miralles et al., 1998). Following reduction of β-cell mass (Zhang et al., 2002) follistatin inhibited activin signaling and promoted expansion of pancreatic progenitor cells (Zhang et al., 2004).

Notch signaling has also been proposed to be involved in pancreatic endocrine and exocrine cell fate (Apelqvist et al., 1999;Jensen et al., 2000;Lammert et al., 2000). Jensen and co-workers, using a multiplex RT-PCR approach on dissected embryonic gut, demonstrated expression of Notch1, 2 and 3 in pancreas, stomach and gut and that of ligands Jagged1, Jagged2, Dll1 and Dll3 and downstream target, Hes1, in the pancreas. These results first suggested a role of the Notch pathway in pancreatic development in mammals (Jensen et al., 2000). In developing mouse and zebrafish pancreas Notch has been shown to inhibit acinar cell differentiation and to drive an acinar-to-ductal phenotypic change. Recently, Notch signaling was suggested to act downstream of FGF10 in maintaining the proliferative potential of pancreatic precursor cells during development (Miralles et al., 2006).

Inflammatory-response and mucosal-defense related genes have roles in regeneration and, perhaps development in other systems. Mucosal defense genes encoding Trefoil factor (TFF)

1, 2 and 3, deleted in malignant brain tumor 1 (DMBT1), surfactant proteins (SP) A and D have been implicated during growth and repair of the liver and intestine (Bisgaard et al., 2002;Leung et al., 2002;Tino and Wright, 1999). Chemokines, CSF-1 and eotaxin, members of the inflammatory response, influence branching morphogenesis in the mammary gland (Gouon-Evans et al., 2000).

In this study, we have examined the gene expression profiles at three well-defined time points of adult human pancreatic tissue during the *in vitro* progression to new islet formation. Four distinct clusters based on expression pattern were found. Additionally we found that genes of the developmentally important Wnt/frizzled, Notch/Delta and the TGF-β superfamily pathways were differentially expressed, suggesting that the *in vitro* differentiation of human adult pancreatic ductal tissue may, in part, recapitulate embryonic pancreatic development. Two putative markers for endocrine precursors, Protein Gene Product 9.5 (PGP9.5)/UCHL1, a member of the ubiquitin family of hydrolases, and deleted in malignant brain tumor 1 (DMBT1), were expressed during this *in vitro* islet differentiation. We further examined whether either could be used as markers of in vitro/in vivo differentiation.

Research design and methods

Cell expansion and differentiation

Human islets were isolated according to the Edmonton protocol (Shapiro et al., 2000). After purification on a continuous Ficoll gradient, the islet-depleted pellet fraction was cultured as previously described (Bonner-Weir et al, 2000). In brief, immediately following islet isolation, the remaining tissue (0.25ml tissue/50ml) was cultured in CMRL 1066 (5.5mM glucose) media plus 10% fetal bovine serum and 1% penicillin/streptomycin in Falcon non-treated T-75 flasks (#3012 Becton Dickinson). As previously reported (Bonner-Weir et al, 2000) after 2–3 days, about 10 % of the tissue had adhered and the remaining floating tissue was removed; we had found that this adherent tissue was selectively ductal with most of the acinar tissue having either died or lost its phenotype by the time the tissue was adherent. The islet contamination of the starting material was usually less than 1% of the tissue with the adherent tissue having less than 11% of the insulin content of the starting tissue. The adherent tissue was the first studied time point, the Initially Adherent stage. After an additional 4–7 days of culture, the tissue had expanded by proliferation and covered more than 80 % of the surface; this was the second time point, the Expanded stage. The media was then changed to serum-free DMEM/F12 (glucose 9mM) with 10 uM nicotinamide, insulin/transferrin/selenium (ITS), keratinocyte growth factor (FGF7) and penicillin/streptomycin and the tissue was overlaid with a thin coat of Matrigel (BD Biosciences, NJ, USA) a commercially available murine basement membrane. The third time point, the Differentiated stage, was 2 weeks later.

Gene Profiling

Total RNA from four pancreatic preparations was isolated using Trizol reagent (Life Technologies, Gaithersburg, MD, USA) at the above three time points: Initially Adherent, Expanded and Differentiated stage. RNA was not pooled, so there were four replicas at three different time points giving us a total of 12 samples. Total RNA was converted to cDNA following the manufacturer's instructions (Superscript Choice cDNA); cDNA were transcribed into cRNA following the manufacturer's protocol (IVT, Enzo), and hybridized to Affymetrix Human genome U95Av2 GeneChip® (Affymetrix, Santa Clara, CA, USA) by the Joslin DERC Genomics Core. Primary data were processed using Affymetrix Microarray Suite 4.0 software to determine the average difference value and assess signal intensity for each probe set. Affymetrix GCOS (gene chip operating software) were used to globally scale (all probe sets) the data to a target intensity of 1500 using the MAS 5.0 algorithm. Further analysis used GeneSpring 7.0 (Silicon Genetics, Redwood CA) software and GeneSpring 9.0 (Heat Map).

Heatmap were used to visualize the apparent differential expression for genes across treatments for each sample and Scatter Plots were used to identify genes with a 2-fold or greater differential expression between time points. Student's unpaired t-test was used to obtain p-value of selected genes between two time points (e.g. Initially Adherent versus Expanded Stage) not considering all three stages. This statistical test could only be used as guidance to search for interesting genes. When performing Bonferroni Hochberg across all stages only 172 genes pasted testing. With 12000+ genes a p-value from ANOVA or t-test would have to be corrected for false discovery rate. However we still feel confident that the study brings value to the field and we therefore continued with testing specific genes in PCR, genes that were picked from the fold changes and statistical testing.

PCR

Total RNA was diluted to a concentration of 0.2 μg/μl and transcribed to cDNA using Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA).

Reverse Transcriptase PCR (RT-PCR) was performed using *Taq* polymerase (Invitrogen, Carlsbad, CA, USA). The optimal PCR conditions for correct amplification of cDNAs were determined as previously described (Jonas et al., 1999). Radio-labeled PCR products were resolved on 6 % acrylamide-gels, dried gels were exposed on PhosphoImager screen, and band intensities were quantified using ImageQuant Software (Amersham Biosciences Corp. Pennsylvania).

Quantative PCR (q-PCR) was performed using 10 ng of total cDNA per well (TaqMan Applied Biosystems); in addition to some of the samples used for microarray, samples from other pancreases were used; each stage had samples from 4 pancreases, with 2 Initially Adherent from each pancreas. Primers used in RT-PCR and q-PCR are listed in Table 6.

Immunohistochemistry

Immunohistochemistry were performed on sections of frozen human pancreas and paraffin embedded rat pancreas. Tissue for cryostat was embedded in OCT (Tissue-Tek®, Sakura, USA) and immersed in chilled isopentane. Frozen sections were air-dried and fixed in 4% paraformaldehyde (PFA) for 20 min, and then washed in phosphate-buffered saline (PBS). For UCHL1/PGP9.5, microwaving in citrate-buffer was used for antigen retrieval, followed by rabbit anti-human PGP9.5 (Chemicon, 1:600) overnight at 4°C, goat anti-rabbit-biotin-IgG (1:50) 30 min at room temperature, and ABC development (Vector Labs). For DMBT1/gp340 on frozen human pancreas, mouse anti-human-DMBT1/gp340 (1:200, kind gift of Dr. Uffe Holmskov, University of Odense, Denmark) was incubated overnight at 4°C, followed by goat anti-mouse-biotin-IgG (1:50) 30 min at room temperature, and ABC kit development; for paraffin embedded rat pancreas, the same primary antibody, dilution and incubation time was used, followed by Texas Red-conjugated donkey anti-mouse IgG (1:100) as secondary antibody for 30 min at room temperature.

Results

To have a better understanding of the mechanisms involved during this *in vitro* islet generation we analyzed the gene expression profiles at different stages of the expansion/differentiation of these pancreatic cells: the Initial Adherent time point, the Expansion before changing to serum-free media and the final Differentiation stage at 4 weeks of culture (2 weeks after Matrigel) (Figure 1). The two time points that are the most interesting are the Expanded and Differentiated stages. As we reported previously, the Expanded stage is characterized by an almost confluent monolayer of epithelial cells that express CK19 and Pdx-1 with a minor component of small, triangular stromal cells. By the Differentiated stage, the epithelial cells have coalesced into

thickened plaques and 3-dimensional structures of ductal cysts and budding islet-like structures. Between these structures, stromal cells have formed a near-confluent lawn. The tissue of a whole flask was taken for each sample, so the gene expression must be taken as of the whole system rather than of individual cells.

Presentation of Gene Expression data as a *Scatter Plot and Heat Map*

In Figure 2, genes are organized into Scatter Plot (Figure 2A, top and bottom) and Heat Map (Figure 2B) according to expression level. In the Heatmap, the genes (across) are shown as normalized log scale values for each sample (down). In Scatter Plots, each dot represents the normalized average expression for one gene (The average expression of the four replicas, one from each pancreas). The diagonal lines represent the fold changes between the two stages, with the center diagonal line representing one fold change and the two outer diagonal lines either 2-fold increased or decreased. From the Scatter Plot four different scenarios were investigated: A: genes that lost expression from Initially Adherent to Expanded stage, B: genes with an increased expression with progression from the Initially Adherent though Expanded stage; C: genes that lose expression from the Expanded stage through the Differentiated stage; and D: genes that are more than 2-fold increased in Differentiated stage compared to Expanded stage. The following Tables do not list all of these genes but rather some of the more interesting ones with the p-value from t-test and fold change. The complete lists of differentially expressed genes with fold changes are given in the Supplementary data. The complete data sets have been deposited and can be accessed at http://www.cbil.upenn.edu/RAD/php/displayStudy.php?study_id=710&&download=1. As shown in Table 1, 1061 genes were more than 2 fold down regulated from Initially Adherent to Expanded stage. Similarly the table shows the overall genes present in the 3 other scenarios; lists of these genes are in Tables (2–5).

LIST A: Genes turned-off upon initial culturing—Table 2 or List A presents a selection of 151 genes that are down regulated more than 2-fold from the Initially Adherent (after 2–3 days of culture) to the Expanded stage (after 10–14 days of culture). Highly expressed acinar-enriched enzymes, such as *amylase, elastase, lipase* and *chymotrypsin*, have very dramatic decreases in expression often more than 20-fold. Such decrease is consistent with the loss in culture of acinar cells both by apoptosis and loss of their differentiation. A number of islet-associated genes such as *insulin, glucagon, NeuroD1, Ipf-1, IRS1, GATA6, and IGF2* were more moderately decreased. List A also includes other genes with a similar profile to the acinar-enriched enzymes but these are not prototypical acinar genes such as *kallikrein1, notch, mucin1, endothelin, TGFβ1, sFRP1* and *reg1b*.

LIST B: Genes associated with expansion of the tissue—In contrast to LIST A, which is mainly comprised of genes encoding acinar-enriched enzymes, the genes in LIST B (Table 3) all have an increase in expression during expansion of the ductal epithelium (Expanded compared to the Initially Adherent stage). Genes regulating cell cycle progression such as *CyclinD1, cyclin dependent kinases and cyclin dependent kinase inhibitors* as well as numerous extracellular matrix genes (collagens and laminins), are present in this list. Genes of the TGFβ superfamily, such as *TGFβ2*, BMP and its antagonist *gremlin, Smad 3 and 6*, and members of Wnt family are also in this list. One genes of interest, DMBT-1 (deleted in malignant brain tumor 1), had greater than 5-fold increase in Expanded compared to the Initially Adherent stage. This gene encodes the protein for which a role in liver repair and recruitment of oval cells (liver stem cells) has been suggested (Bisgaard et al., 2002). We confirmed its expression in human pancreatic tissue by semi-quantitative RT-PCR (Figure 3) and by immunostaining. On frozen sections of human pancreas, DMBT-1 protein was only immunolocalized to scattered cells of the main duct and smaller peripheral ducts (Fig 4). In rat pancreas DMBT protein was only found in the epithelium of the small proliferating ductules

of the newly forming lobes of regenerating rat pancreas (3 days after partial pancreatectomy) (Figure 5).

LIST C Genes down-regulated as differentiation progresses—List C (Table 4) reports a broad range of genes that showed reduction in expression during the changes from expanded monolayer into the three-dimensional structures in the Differentiated stage. Several of these genes are present in both List B and C; as being up-regulated and activated during expansion but then turned off in response to differentiation these include BMP 1, 2 and Wnt7A. In this list we also find several collagens and laminins as in List B, growth factor related proteins, members of TGF-β superfamily and Wnt/frizzled pathway, retinoic acid related proteins retinoic acid binding genes, retinoic acid induced and retinoic acid receptor, and *notch3, jagged 1 and 2*, members of Notch/delta family

LIST D Genes activated during Differentiation—The final list (Table 5), List D, include those genes that are up-regulated during the Differentiated stage. As expected, the list includes genes encoding markers of terminally differentiated cells of the pancreas such as *glucagon, insulin, IA2/phogrin* and *CFTR, mucin6 and 9* (ducts). Both glucagon and insulin were expressed at the Initially Adherent stage unlike the other members of the cluster, but their expressions decreased by Expanded stage before a more than 2-fold (re)induction by Differentiated stage. Interesting genes of this list include differentiation factors such as TGF-β superfamily member *Gremlin, TGFβ1, BMP4 and 6,* Wnt/frizzled genes *Frizzled 1,2* and *7,* Homeobox genes and the transcription factor *c-maf* known to play a role in differentiation β and α-cells. One gene of note PGP9.5/UHCL1, an ubiquitin carboxyl-terminal hydrolase, is a known neuronal marker and has been suggested to be a marker for islet precursor cells. PGP9.5 is expressed as early as E11.5 during development of the growing murine pancreas and later in adult islets (Yokoyama-Hayashi et al., 2002). Its expression was increased (more than 15-fold) from Expanded stage to the Differentiated stage. Semi-quantitative RT-PCR was performed using human PGP9.5 primers to confirm the array data (Figure 3). Similarly, immunohistochemical analysis was used to confirm the presence of PGP9.5 protein in both normal human and regenerating rat pancreas. Staining of cryostat sections of human pancreas revealed that islets and the epithelia of both small and large ducts were PGP9.5 positive (Figure 6). Additionally, the buds of endocrine tissue in the *in vitro* generated islet buds were positive (Figure 6C), and by double immunostaining, the expression of PGP9.5 was shown to be in the insulin positive cells (Figure 6D) rather than the glucagon positive cells (Figure 6E). In the rat regeneration model of 90 % partial pancreatectomy (Px + 3 days), PGP9.5 positive cells included most, if not all, islet cells, scattered cells in large ducts and in the pre-existing acini (possibly centroacinar cells) (Figure 7). In the newly forming lobes (Figure 7D, E), many of the epithelial cells in the small ductules were PGP9.5 positive.

Developmental important pathways

During development many important pathways have been found to regulate proliferation and subsequent differentiation. During progression of the *in vitro* neogenesis as seen in this study, multiple members of two of these pathways, TGF-β Superfamily and the Wnt/Frizzled, were differentially expressed (Figure 8 and 9 respectively) suggesting that the pathways are important during *in vitro* islet formation from human pancreatic tissue. Selected genes in these pathways were confirmed with q-PCR: BMP1, BMP2A, FZD2, Gremlin and TGFB1. In addition FZD3, also a member of Wnt/frizzled pathway, which were not present on the genearray, showed a similar and interesting profile when using q-PCR (Figure 10).

Discussion

We have used microarray technology to profile gene expression during *in vitro* human islet neogenesis. Three time points that represented major stages in the progression were compared. Initially Adherent stage, after a 1–4 day culture, was the tissue that differentially adhered to the flasks and just started to spread. This tissue was preferentially exocrine (ductal and acinar) with almost no endocrine contamination. Without lineage tracing for human tissue, we cannot estimate what percent of the acinar cells dedifferentiated and regained a ductal phenotype. Even so, the initial proportion of ductal tissue is much higher in the human pancreas, being 40–50 % of the human pancreas than the 10% found in rodent pancreas (Bouwens L and DG Pipeleers, Diabetolgia 1998, 41:629–33). The Expanded stage, after 7–11 days of culture, represented the expansion of cells with ductal phenotype, with only a minor contamination of stromal cells at this time. Finally, the Differentiated stage, after 4 weeks of culture with the last two weeks with an overlay of Matrigel, consisted of ductal cysts with buds of endocrine cells and a “feeder-like” layer of stromal cells.

List A consisted of 30+ genes that decreased dramatically from the Initially Adherent stage towards the Expanded stage. Most of these genes encode acinar-enriched enzymes suggesting that acinar phenotype was lost within the first 7 days of culture. We have previously found that amylase gene expression was lost after only 2–3 days culture (data not shown). Such a loss of acinar-enriched genes could occur by the death of acinar cells and/or by a dedifferentiation of acinar cells to a more ductal phenotype as has been suggested by the group of Bouwens (Rooman et al., 2000). Genes of List A are consistent with the 50 most abundant transcripts identified in the exocrine HEXO1 library by Cras-Meneur et al (Cras-Meneur et al., 2004). We also found endocrine-related genes such as those encoding insulin and glucagon suggesting that any initial contaminating endocrine cells do not survive through expansion. It is unlikely that they had expanded sufficiently in the first seven days to lose their insulin gene expression as suggested by Gershengorn et al (Wei et al., 2006).

In lists B and C we find genes that have their highest expression in the Expanded stage compared to the Initially Adherent or Differentiated stages. At this stage, the predominant cell has a ductal epithelial phenotype and has rapidly expanded; the differential genes include those involved in proliferation, migration and cell-cell interactions. Many of the genes in this list represent genes that interact with another gene in the list (e.g., gene and its target). For example, the activity of the cell cycle regulator cyclin D1 is enhanced by galectins (Lin et al., 2002), and both genes are in List B. Since precursors are likely to expand before differentiating, this cluster is most likely one to include markers of such an expanding precursor population. One such putative marker, DMBT-1, has been implicated in recruitment of oval liver stem cells during liver regeneration (Bisgaard et al., 2002). Furthermore, induction of terminal differentiation of a renal intercalated cell line requires the polymerization of hensin, a DMBT1 homologue, by galectin 3 (Hikita et al., 2000). The last list, List D, represents the differentiation process during which precursor cells differentiated into mature duct and hormone-expressing endocrine phenotypes; genes here include insulin and glucagon for islets and CFTR and aquaporin 1 for ducts. This list also includes IA2/Phogrin, an auto-antigen in type 1 diabetes, present during development and later in adult islets (Roberts et al., 2001) and the endocrine-associated transcription factor c-Maf (Nishimura et al., 2006).

Of considerable interest is the differential expression of members of several developmentally important pathways: Wnt/Frizzled- and the TGF-β pathways. Induction of these pathways in our *in vitro* neogenesis model could suggest that these pathways play an important role in inducing expansion of ductal tissue and/or the differentiation into ductal cysts and islet buds. The regulation of these pathways during *in vitro* neogenesis is important for our understanding of the process of islet differentiation. The Wnt pathway, one means of activating the beta-

catenin/TCF4 transcription factor complex involved in cell proliferation and differentiation, has a critical role in pancreas development (Heller et al., 2002). Two of the receptors, Frizzled-7 and Frizzled 2, are induced and differentially expressed. A secreted frizzled-related gene, also in List D, has an inhibitory function since it competes with membrane-bound frizzled for Wnt binding. The TGF-β superfamily has three major groups of genes (TGFβs, activins and BMPs) that all signal through a family of transmembrane serine/threonine kinase receptors classified as type I or type II receptors (Massague, 1998). The Smad family is involved in intracellular signaling of this superfamily (Wrana and Attisano, 2000). Many members of the TGF-β superfamily are found in embryonic pancreatic epithelium and mesenchyme. Transgenic mice with a targeted mutation within the activin receptor have hypoplasia of the pancreatic islets (Yamaoka et al., 1998). Dissociated fetal mouse pancreatic cells cultured in the presence of Laminin-1 and BMP 4, 5 and 6 formed cystic epithelial colonies with insulin and glucagon positive cells; both TGF-β and activin antagonized this effect and inhibited colony formation (Jiang et al., 2002). In our human pancreatic cultures, many of the superfamily members are differentially expressed: BMP2A with a high expression at the Expanded stage, Gremlin (a BMP antagonist) and BMP2B with a high expression at the Differentiated stage. TGF β1, as confirmed by q-PCR, and follistatin, a regulator of endocrine development (Miralles et al., 1998), are highly expressed at the Differentiated stage.

In summary our gene expression analysis demonstrates that culturing exocrine cells is accompanied by a loss of acinar phenotype, proliferation and expansion of ductal tissue, and differentiation toward mature endocrine cells. Gene profiling of the different stages of *in vitro* generation of islets provides a framework of pathways involved in the development of human islets. While further studies will be necessary to fully understand the process, the identification of these developmentally important pathways in *in vitro* neogenesis suggests that manipulating these pathways may enhance the differentiation of islet tissue.

Potential markers of islet precursor cells

Gene expression profiling during *in vitro* islet neogenesis also permitted identification of potential markers for islet precursors. Two genes, PGP9.5 and DMBT1, previously suggested as putative markers of precursor cells (Bisgaard et al., 2002;Yokoyama-Hayashi et al., 2002), were differentially expressed in our gene-profiling study and so were studied further with RT-PCR and immunolocalization.

PGP9.5/UCHL1, a member of the ubiquitin family of hydrolases, has been implicated as a marker of differentiation by its induction during *in vitro* differentiation of human acute lymphoblastic leukemia cell line (al-Katib et al., 1995) and human neuronal cell lines (Satoh and Kuroda, 2001). During rat embryonic pancreas development, PGP9.5 is expressed in E11.5 epithelial buds with declining expression in the epithelial cells after birth; it becomes restricted to islets in adults (Yokoyama-Hayashi et al., 2002). However, in the rat duct ligation model, PGP9.5 is expressed in ducts during islet neogenesis, suggesting it might be a marker for islet precursor cells. In our analysis, PGP9.5 had one of the highest fold changes from Expanded to Differentiated Stages, differential expression confirmed by RT-PCR. In adult rat pancreas we could confirm by immunostaining PGP9.5 protein in islets (α- and β-cells), centroacinar cells and in proliferating ductules of the regenerating pancreas with no presence in normal adult ducts. In normal adult human pancreas PGP9.5 protein was localized in the islets and most epithelial cells of both large and small ducts and in the *in vitro* generated human islet buds PGP9.5 seemed to only co-localize with insulin positive cells, whereas epithelial cells and a-cells were negative. Thus, in human pancreas PGP9.5 appears to be a marker only for differentiated cells rather than of precursor cells as suggested for rats.

The second candidate precursor marking gene, human DMBT1, has been implicated in numerous tissues. DMBT1 has several homologues (hensin, gp-340, CRP-ductin) due to

alternative splicing of a single gene (Holmskov et al., 1999). Its role in epithelial differentiation was first suggested in the rabbit kidney in which hensin regulated terminal differentiation of epithelial cells through interactions with galectin 3 (Al Awqati et al., 1999). This interaction was also found to initiate differentiation both of skin epithelial cells (Mollenhauer et al., 2003) and renal intercalated epithelial cell line (Hikita et al., 2000). The homologue CRP-ductin was found in the stem cell niche of the mouse intestinal crypts as well as in pancreatic and hepatic ducts (Cheng et al., 1996) and as a receptor for trefoil factors (TFF) (Thim and Mortz, 2000). In a liver regeneration model, DMBT1 mRNA and protein expression increased as oval cells were recruited (Bisgaard et al., 2002), suggesting it as a marker for oval (liver bi-potential stem) cells. In our human data, DMBT1 expression peaked at the Expanded stage. The Expanded stage is likely to have increased precursor cells that have yet to differentiate as well as cells in early stages of pancreatic differentiation. Consistent with its expression in a precursor population, immunohistochemical localization demonstrates its presence in only a few ductal cells in the adult human pancreas but in many ductal cells in the regions of regeneration after partial pancreatectomy in the rat. Thus, our results suggest that DMBT1 is a likely candidate marker of precursor cells or cells in early stages of endocrine differentiation in the pancreas.

Our previously reported *in vitro* generation of islets from islet depleted human pancreatic tissue provides a unique system to identify the differentiation pathways important for islet neogenesis. With gene profiling analysis, we report differential gene expression of pathways, normally known to be important in embryonic pancreatic development, present in this *in vitro* system. Further characterization of these pathways should lead to identification of steps that may be manipulated to improve the efficiency of islet differentiation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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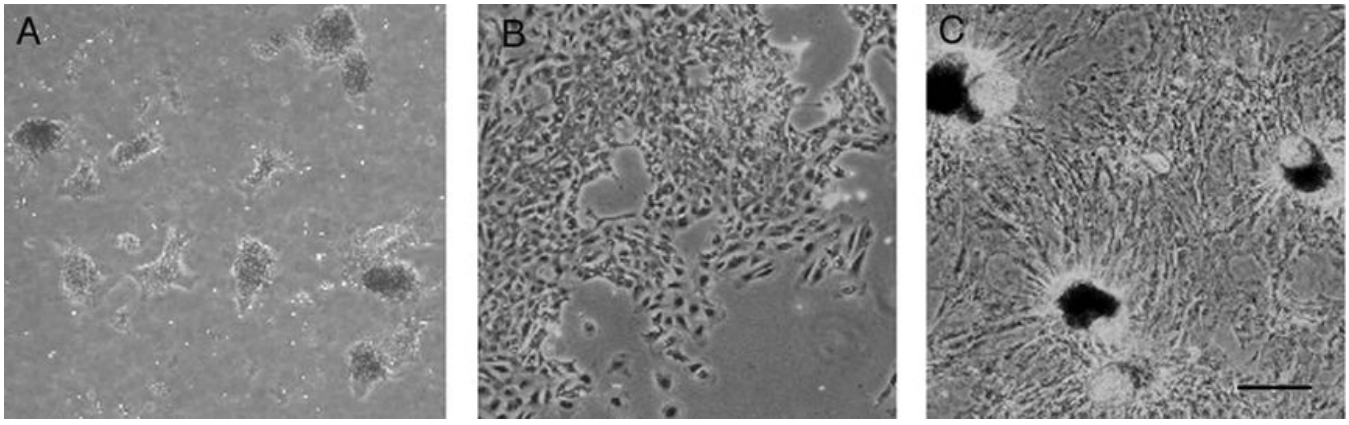
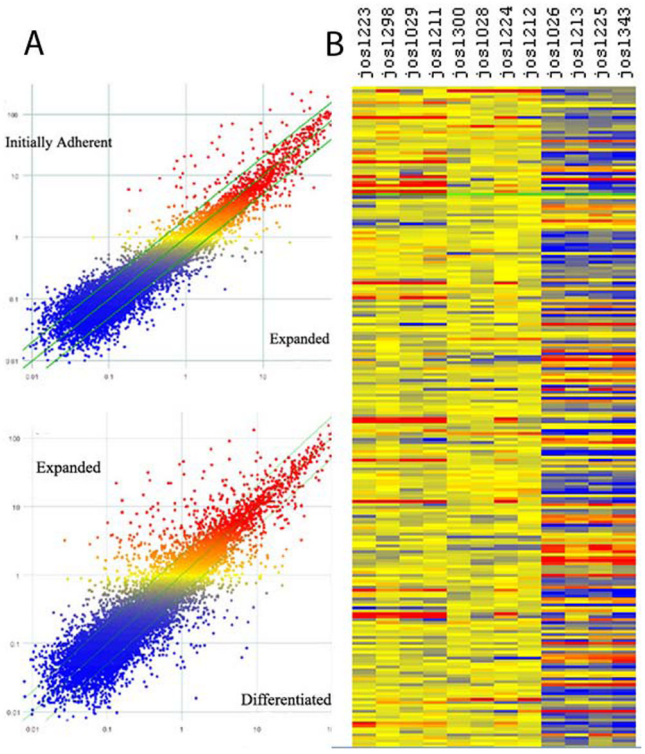


Figure 1.



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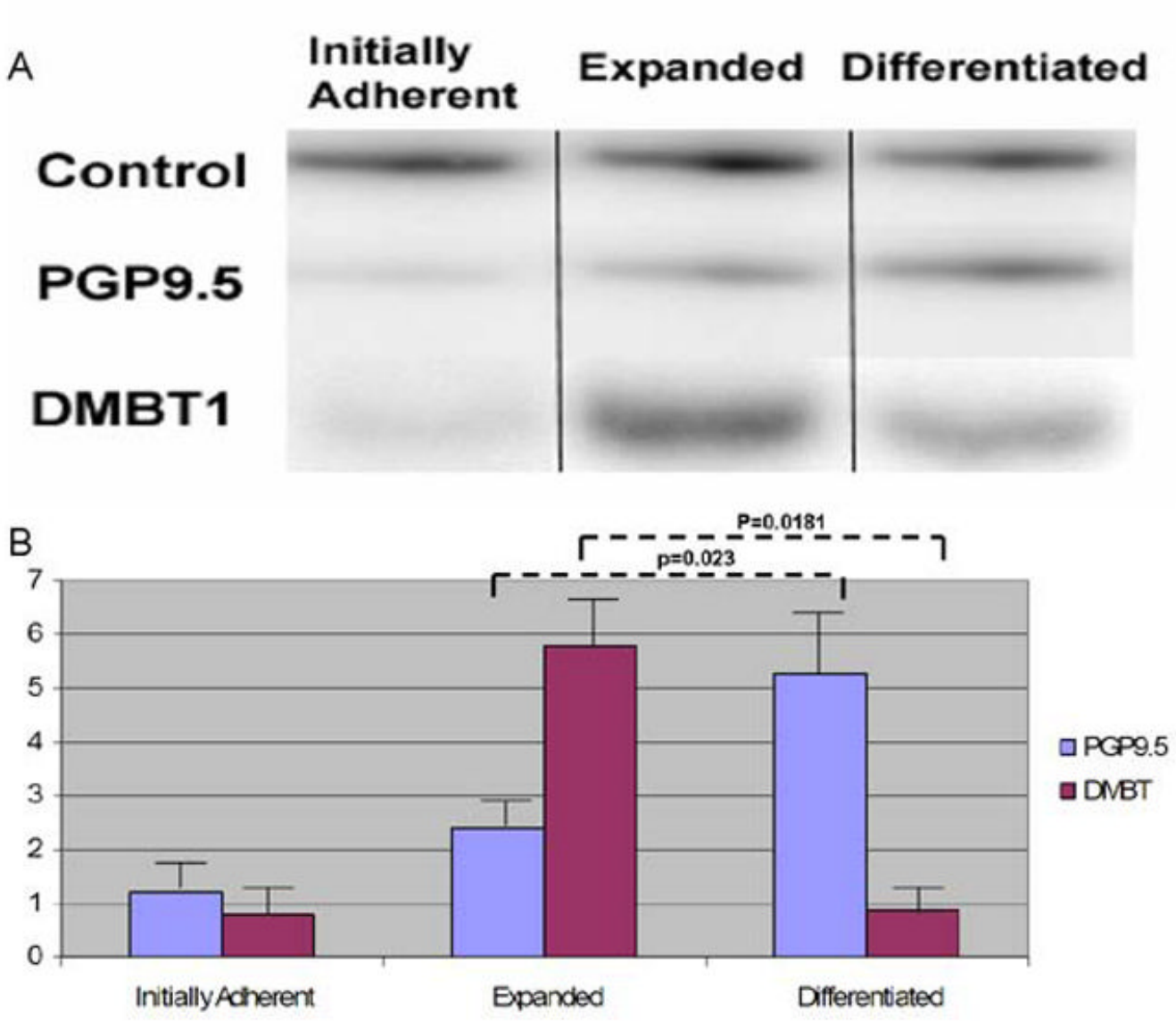

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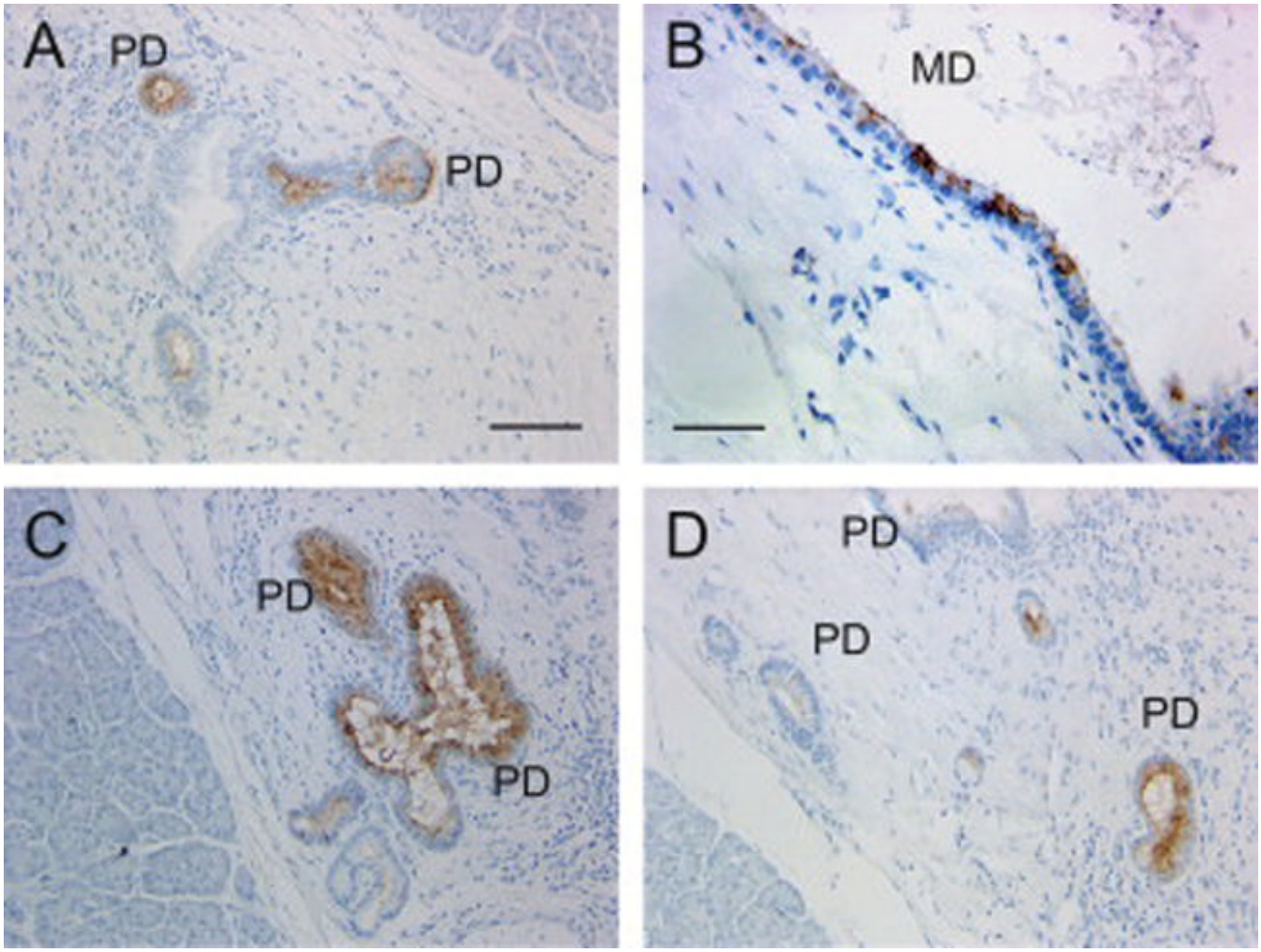

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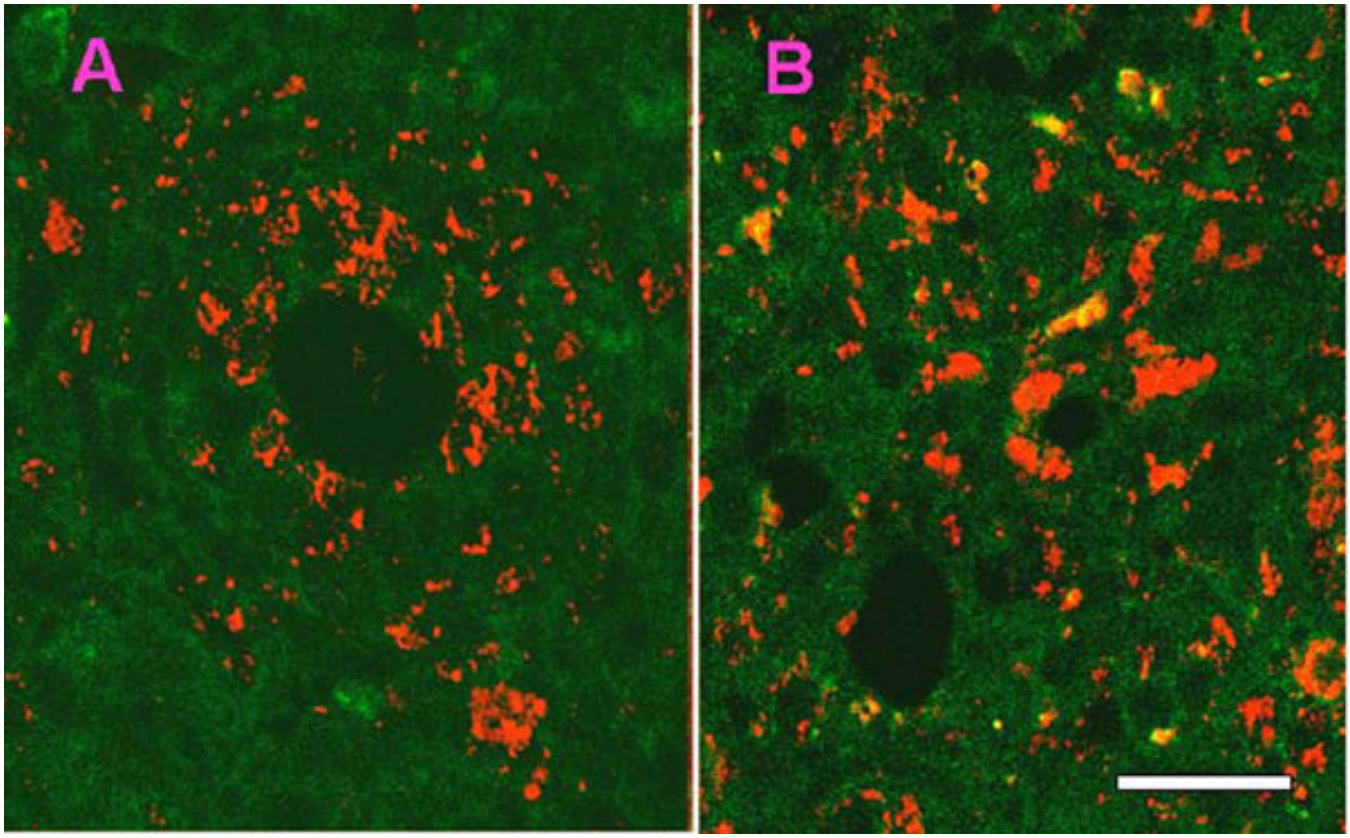

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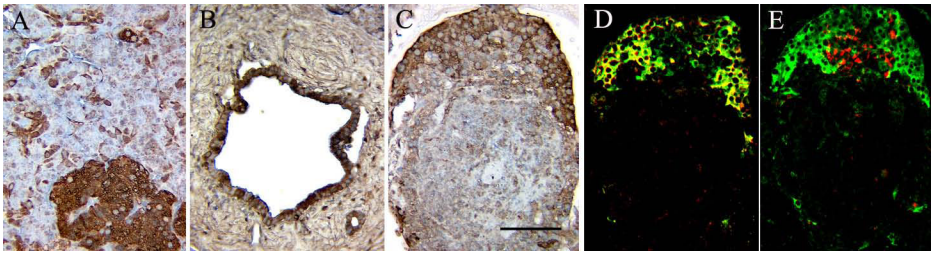

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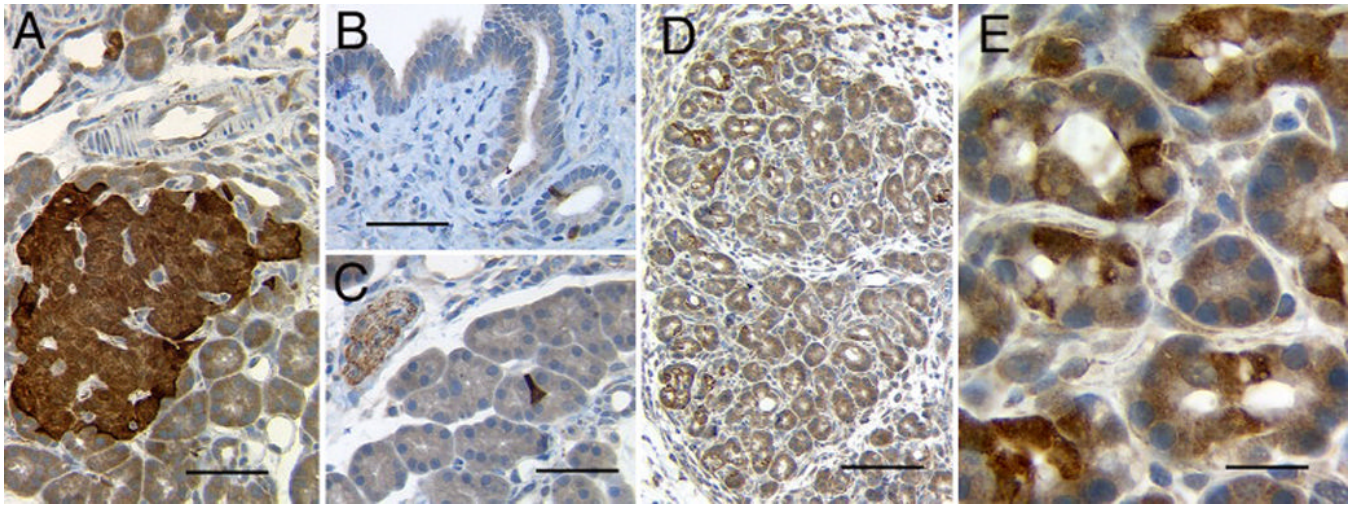

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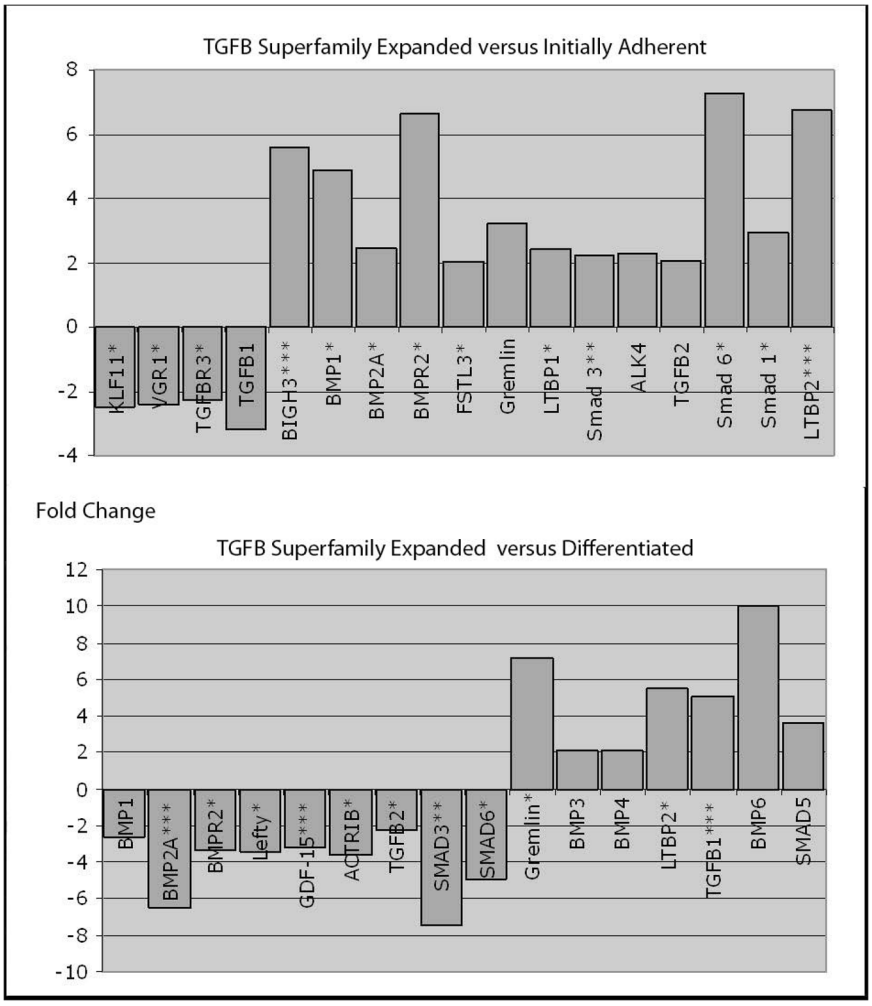

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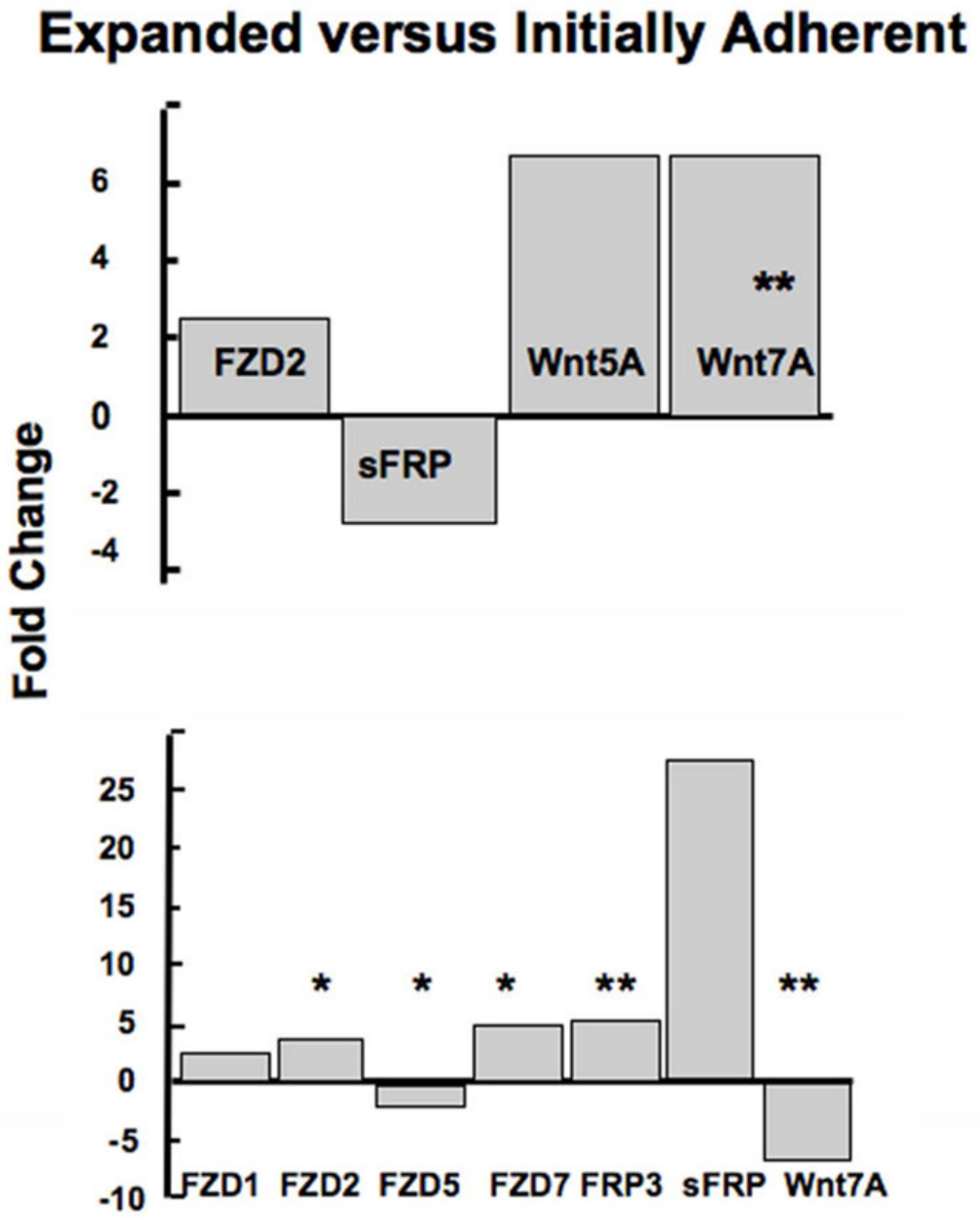

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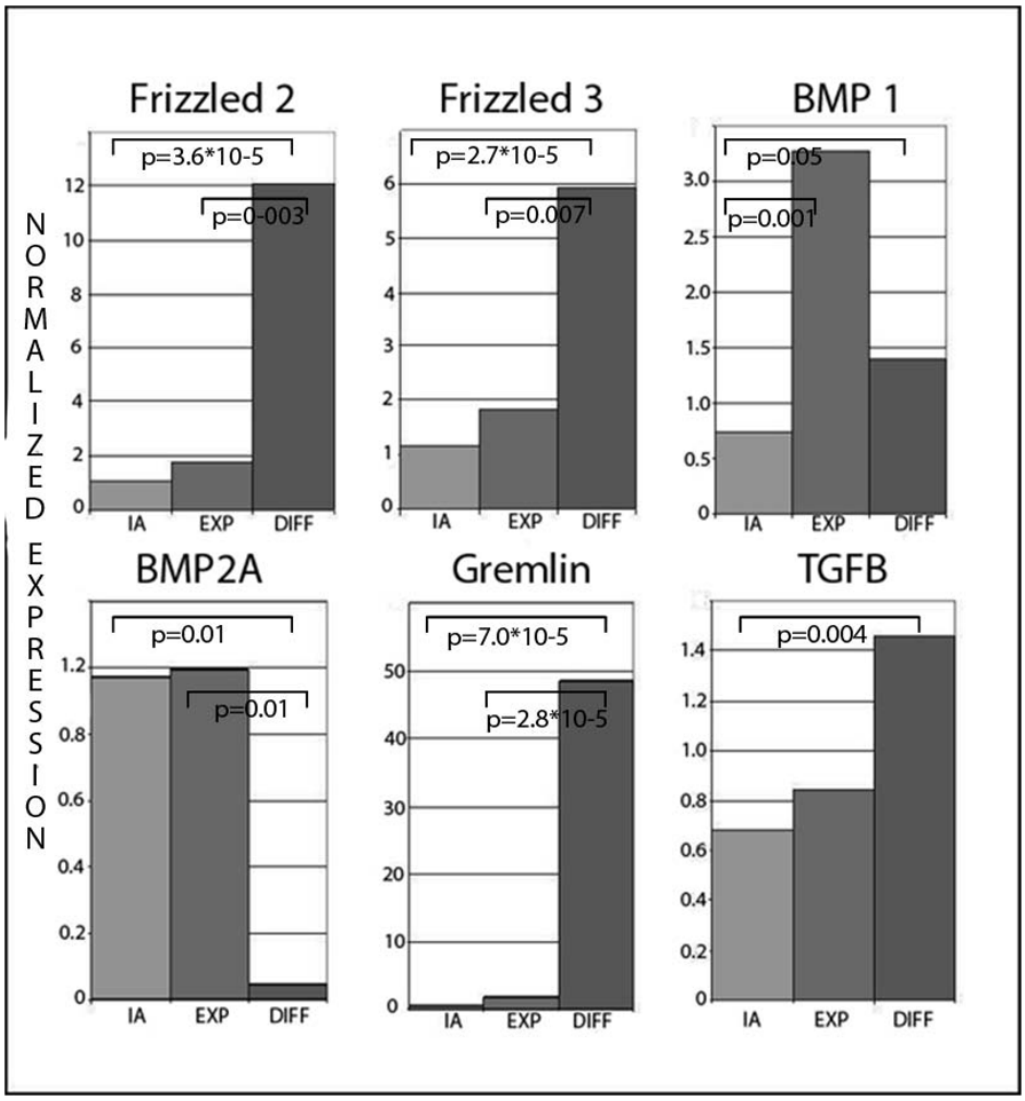

Figure 10.

		Total probe sets in lists ≤ 2 fold	Probe sets in list with p-value ≤ 0.05 obtained from student t-test (not FDR corrected)
FOLD CHANGE BY SCATTERPLOT	**List A** < 2 fold ↓ from Initially Adherent to Expanded	**1061**	**295**
	LIST B < 2 fold ↑ from Initial Adherent to Expanded	**1336**	**468**
	LIST C < 2 fold ↓ from Expanded to Differentiated	**1445**	**671**
	LIST D < 2 fold ↑ from Expanded to Differentiated	**1435**	**529**

NCBI Official name	Symbol	NCBI ID	p-value	Fold
Amylase, alpha 2B	AMY2B	M24895	0.0073	24.8
Aquaporin 8	AQP8	AB013456	0.0012	4.18
Bile salt-activated lipase	BSSL BAL	X54457	0.0200	40.9
Carboxyl ester lipase pseudogene	CELP	L14813	0.0429	22.7
Carboxypeptidase A1	CPA1	X67318	0.0028	24.1
Carboxypeptidase A2	CPA2	U19977	0.0264	24.4
Carboxypeptidase B1	CPB1	M81057	0.0205	19.0
Chymotrypsinogen B1	CTRB1	M24400	0.0343	12.9
Colipase precursor	CLPS	AA921399	0.0159	59.9
Elastase 2A	ELA2A	M16652	0.0044	27.3
Elastase 3A	ELA3A	M18700	0.0174	23.0
Endothelin 3	EDN3	J05081	0.0165	2.72
Frizzled related protein 1, secreted	sFRP1	AF056087	NS	2.75
GATA binding protein 6	GATA6	X95701	0.0254	2.53
Glucagon	GCG	J04040	0.0291	5.30
Glucagon-like peptide 1 receptor	GLP1R	U01156	0.0819[NS]	3.25
Glutathione S-transferase A2	GSTA2	M16594	0.043	3.33
Growth hormone receptor	GHR	X06562	NS	3.51
Indian hedgehog homolog	IHH	L38517	NS	2.98
Insulin	INS	L15440	0.0398	2.96
Insulin promoter factor 1	IPF1	X99894	0.0925[NS]	3.34
Insulin receptor substrate 1	IRS1	S62539	0.0496	5.43
Insulin-like 4	INSL4	L34838	0.0392	3.51
Insulin-like growth factor 2	IGF2	J03242	0.0144	4.41
Kallikrein 1	KLK1	M25629	0.031	20.2
Keratin 76 (HUMCYT2a)	KRT76	M99063	0.0369	3.21
Kruppel-like factor 11	KLF11	AF028008	NS	2.49
Mucin 1	MUC1	M21868	0.0098	14.3
Neurogenic Differentiation 1	NEUROD1	D82347	NS	2.06
Notch homolog 4 (Notch 3)	NOTCH4	U95299	0.0331	2.11
Pancreatic lipase-related protein 1	PNLIPRP1	M93283	0.0032	24.8
Phospholipase A2, group 1B	PLA2G1B	M21056	0.0289	36.9
Regenerating islet-derived 1 beta	REG1B	D17291	0.0450	11.9
Solute carrier 9 (Na+/H+ exchanger)	SLC9A3	U28043	0.0237	2.11
Solute carrier family 2 (GLUT2)	SLC2A2	J03810	NS	2.22
Transforming growth factor, beta 1	TGFB1	X05839	0.0391	3.19
Transforming growth factor, beta receptor III	TGFBR3	L07594	NS	2.30

NCBI Official name	Symbol	NCBI ID	p-value	Fold
Bone morphogenetic protein 1	BMP1	M22488	0.0479	4.85
Bone morphogenetic protein 2	BMP2	M22489	0.0391	2.46
Bone morphogenetic receptor, type II	BMPR2	Z48923	0.0079	6.66
Cadherin 2, type 1, N-cadherin	CDH2	M34064	0.0006	12.9
Catenin, alpha 2	CTNNA2	M94151	0.0463	3.30
Cellular retinoic acid binding protein 2	CRABP2	M97815	0.0295	2.93
Collagen, type I, alpha 1	COL1A1	Y15915	0.0030	11.0
Collagen, type I, alpha 2	COL1A2	J03464	0.0333	11.1
Collagen, type III, alpha 1	COL3A1	X14420	0.0099	5.62
Collagen, type IV, alpha 1	COL4A1	M26576	0.0044	3.79
Collagen, type VI, alpha 3	COL6A3	X52022	0.0292	2.07
Collagen, type XI, alpha 1	COL11A1	J04177	0.0393	6.63
Colony stimulating factor 2	CSF2	M13207	0.0002	17.3
Cyclin D1	CCND1	M73554	0.0469	3.51
Cyclin-dependent kinase 3	CDK3	X66357	N/A	2.48
Cyclin-dependent kinase inhibitor 2A	CDKN2A	U26727	0.0016	3.42
Cyclin-dependent kinase inhibitor 2B	CDKN2B	L36844	0.0042	8.33
Deleted in malignant brain tumors 1	DMBT1	AJ000342	0.0413	5.62
Desert hedgehog homolog	DHH	U59748	N/A	2.68
Disc, large homolog 2	DLG2	U32376	0.0497	3.83
Disc, large homolog 5	DLG5	AB011155	0.0145	2.60
Dishevelled, dsh homolog 3	DVL3	D86963	0.0495	2.97
EGF-like repeats and discoidin I-like domaiN/AEDIL3		U70312	N/A	2.82
3				
Fibroblast growth factor 7	FGF7	M60828	0.0411	2.72
Fibroblast growth factor receptor 2	FGFR2	Z71929	N/A	2.75
Follistatin like 3	FSTL3	U76702	N/A	2.02
Frizzled homolog 2	FZD2	L37882	N/A	2.59
Gap junction protein, alpha 1 (CX43)	GJA1	X52947	0.0119	4.13
G-protein coupled receptor, family C, group5, member A	GPRC5	AF095448	0.0148	2.97
Gremlin 1	GREM1	AF045800	0.0950[N/A]	3.24
Growth factor receptor-bound protein 2	GRB2	M96995	0.0284	2.01
Laminin, alpha 2	LAMA2	Z26653	N/A	4.33
Laminin, beta 2	LAMB2	X79683	0.0135	2.34
Laminin, beta 3	LAMB3	U17760	0.0083	2.51
Laminin, gamma 2	LAMC2	Z15008	0.0002	2.68
Latent TraN/Aforming Growth factor beta binding protein 1	LTBP1	M34057	0.0171	2.24
Latent TraN/Aforming Growth factor beta binding protein 2	LTBP2	Z37976	0.0002	6.76
Midkine (neurite outgrowth promoting factor 2)	MDK	M94250	0.0472	4.05
Mucin 6	MUC6	U97698	N/A	2.54
Notch homolog 3	NOTCH3	U97669	0.0103	3.38
Nuclear receptor subfamily 2, group F, member 1	NR2F1	X16155	0.0650 [N/A]	2.23
Retinoic acid receptor responder	RARRES1	U27185	0.0361	4.74
SMAD3, mothers agaiN/At DPP homolog 3	SMAD3	U68019	0.0094	2.24
SMAD6, mothers agaiN/At DPP homolog 6	SMAD6	AF035528	0.0222	7.30
TraN/Aforming growth factor, beta receptor 2	TGFBR2	D50683	0.0020	2.82
TraN/Aforming growth factor, beta 2	TGFB2	Y00083	N/A	2.08
TraN/Aforming growth factor, beta-induced	TGFBI	M77349	0.0188	5.59
TraN/Aforming growth factor, beta receptor associated protein 1	TGFBRAP1	AF022795	N/A	2.16
Tripartite motif-containing 22	TRIM22	X82200	0.0322	7.09
Ubiquitin carboxyl-terminal esterase L1 (PGP9.5)	UCHL1	X04741	N/A	2.05
Wingless-type MMTV integration site family, member 5A	WNT5A	L20861	0.0794 [N/A]	5.68
Wingless-type MMTV integration site family, member 7A	WNT7A	U53476	0.0014	5.78

NCBI Official name	Symbol	NCBI ID	p-value	Fold
Activin A receptor, type IIB	ACVR2B	X77533	0.0193	3.55
Alpha-2-glycoprotein 1, zinc	AZGP1	X59766	0.0001	177
Aquaporin 9	AQP9	AB008775	0.0142	5.30
Betacellulin	BTC	S55606	N/A	2.29
Bone morphogenetic protein 1	BMP1	M22488	N/A	2.61
Bone morphogenetic protein 2	BMP2	M22489	0.0050	6.53
Bone morphogenetic protein receptor, type II	BMPR2	Z48923	0.0292	3.38
Colony stimulating factor 2	CSF2	M13207	0.0001	42.4
Colony stimulating factor 2 receptor, alpha	CSF2RA	M73832	N/A	2.15
Colony stimulating factor 3	CSF3	X03656	0.017	5.63
Cyclin D1	CCND1	M73554	0.0085	2.51
Deleted in malignant brain tumors 1	DMBT1	AJ000342	N/A	3.53
Desert hedgehog homolog	DHH	U59748	0.0183	2.45
Epidermal growth factor receptor	EGFR	X00588	0.0524 N/A	4.25
Fibroblast growth factor 2 (basic)	FGF2	J04513	0.0344	2.20
Fibroblast growth factor 5	FGF5	M37825	N/A	2.47
Fibroblast growth factor 8	FGF8	U47011	0.0315	2.22
Fibroblast growth factor 13	FGF13	U66198	N/A	3.68
Fibroblast growth factor 18	FGF18	AF075292	0.0878 N/A	2.95
Fibroblast growth factor binding protein 1	FGFBP1	M60047	0.0257	3.99
Follistatin-like 3 (secreted glycoprotein)	FSTL3	U76702	0.0025	6.61
Frizzled homolog 5	FZD5	U43318	0.0052	2.18
G protein-coupled receptor, family C, group 5, member B	GPRC5B	AF095448	0.0005	2.80
GATA binding protein 2	GATA2	M77810	0.0510 N/A	2.44
Glucokinase	GCK	M90299	N/A	2.25
Glucokinase (hexokinase 4) regulator	GCKR	Z48475	0.0324	4.97
Glutathione S-traN/Aferase A2	GSTA2	M16594	0.0231	32.0
G-protein coupled receptor, family C, group 5, member A	GPRC5A	AF095448	0.0012	20.7
Growth differentiation factor 15	GDF15	AB000584	0.0021	3.19
Heat shock 70kDa protein 6	HSPA6	X51757	0.0349	7.18
Homeobox D3	HOXD3	Y09980	0.0011	2.14
Inhibitor of DNA binding 1	ID1	X77956	0.0230	3.06
IN/Aulin receptor	IN/AR	X02160	0.0419	3.72
Jagged 1	JAG1	AF003837	0.0310	2.17
Jagged 2	JAG2	AF029778	N/A	3.19
Kallikrein 1	KLK1	M25629	0.0203	21.3
Keratin 15	KRT15	X07696	0.0601 N/A	2.64
Keratin 17	KRT17	Z19574	0.0153	3.56
Keratin 18	KRT18	M26326	0.0286	3.50
Keratin 19	KRT19	Y00503	0.0028	9.47
Keratin 20	KRT20	X73502	N/A	2.60
Laminin, alpha 3	LAMA3	L34155	0.0001	4.45
Laminin, beta 3	LAMB3	U17760	0.0011	34.2
Lectin, galactoside-binding lectin, soluble, 2 (Galectin 2)	LGALS2	AL022315	0.0051	22.4
Lectin, galactoside-binding lectin, soluble, 9 (Galectin 9)	LGALS9	AB006782	0.0003	4.35
Left-right determination factor 1	LEFTY1	AF081507	0.0471	3.45
Lipocalin	LCN2	M90424	0.0033	90.4
SMAD3, mothers agaiN/At DPP homolog 3	SMAD3	U68019	0.0051	7.58
Midkine (neurite growth-promoting factor2)	MDK	M94250	0.0960 N/A	3.38
Mucin 1, cell surface associated	MUC1	J05581	0.0039	13.8
Notch homolog 3	NOTCH3	U97669	N/A	4.01
POU domain, class 4, traN/Acription factor 2 (BRN3B)	POU4F2	U06233	0.0260	3.56
POU domain, class 5, traN/Acription factor 1 (OCT3/OCT4)	POU5F1	Z11898	0.0058	3.64
Regenerating islet-derived 1 beta	REG1B	D17291	0.0186	27.6
Retinoic acid binding protein 2, cellular	CRABP2	M97815	0.0099	3.72
Retinoic acid receptor responder 1	RARRES1	U27185	0.0012	4.69
Retinoic acid receptor, beta	RARB	Y00291	N/A	3.04
Retinol binding protein 1, cellular	RBP1	M11433	0.0003	7.98
Serpin peptidase inhibitor, clade B, member 7 (Megsin)	SERPINB7	AF027866	0.0001	231
Suppressor of cytokine signaling 1	SOCS-1	AB000734	0.0068	6.34
TNF receptor-associated factor 1	TRAF1	U19261	0.0059	9.05
Wingless-type MMTV integration site family, member 7A	WNT7A	U53476	0.0017	12.0

NCBI Official name	Symbol	NCBI ID	p-value	Fold
Activating traN/Acription factor 5	ATF5	AB021663	N/A	4.46
Activating traN/Acription factor 7	ATF7	X52943	0.0890 N/A	4.00
Activin A receptor type II-like 1	ACVRL1	U77713	N/A	2.26
Activin A receptor, type IIA	ACVR2A	D31770	0.0070	2.45
ATP-binding cassette subfamily (CFTR/MRP) member 2	ABCC2	U49248	N/A	2.43
Bone morphogenetic protein 4	BMP4	M22490	0.0040	2.12
Bone morphogenetic protein 6	BMP6	M60315	0.0003	10.2
Cadherin 11, type 2, OB-cadherin	CDH11	D21255	0.0048	9.52
Cathepsin G	CTSG	J04990	0.0244	3.68
Cathepsin O	CTSO	X82153	0.0002	31.9
Chemokine (C-C motif) ligand 1 (eotaxin)	CCL11	U46573	0.0006	57.8
Collagen, type I, alpha 2	COL1A2	J03464	0.0283	6.58
Collagen, type III, alpha 1	COL3A1	X14420	0.0088	4.69
Collagen, type X, alpha 1	COL10A1	X60382	0.0002	3.41
Collagen, type XV, alpha 1	COL15A1	L25286	0.0071	10.3
Collagen, type XVI, alpha 1	COL16A1	M92642	0.0488	3.29
Disabled homolog 2	DAB2	U53446	0.0074	3.40
Distal-less homeo box 2	DLX2	L07919	0.0880 N/A	3.60
EGF-like repeats and discoidin I-like domaiN/A 3	EDIL3	U70312	0.0261	7.36
Endoglin	ENG	X72012	N/A	2.71
Fibroblast growth factor 7	FGF7	M60828	0.0184	33.0
Follistatin	FST	M19481	0.0027	3.38
Forkhead box D1	FOXD1	U59831	0.0043	21.8
Forkhead box F1	FOXF1	U13219	0.0007	3.18
Frizzled homolog 1	FZD1	AB017363	0.0959 N/A	2.48
Frizzled homolog 2	FZD2	L37882	0.0266	3.75
Frizzled homolog 7	FZD7	AB017365	0.0274	5.15
Frizzled-related protein	FRZB	U91903	0.0022	5.23
Frizzled-related protein 1, secreted	SFRP1	AF056087	0.0044	27.8
Glucagon	GCG	J04040	0.0291	7.07
Gremlin 1	GREM1	AF045800	0.0112	7.04
Growth arrest-specific 6	GAS6	L13720	0.0213	2.49
Homeobox A4	HOXA4	M74297	0.0097	4.31
Homeobox B2	HOXB2	X16665	0.0087	3.47
Homeobox B5	HOXB5	M92299	0.0066	2.31
Homeobox B6	HOXB6	X58431	0.0043	2.60
Homeobox C6	HOXC6	S82986	0.0437	3.89
IN/Aulin	IN/A	L15440	0.0398	2.06
iN/Aulin receptor substrate 1	IRS1	S62539	0.0311	3.70
IN/Aulin-like growth factor 1	IGF1	X57025	0.0003	5.85
IN/Aulin-like growth factor binding protein 4	IGFBP4	M62403	0.0002	17.9
iN/Aulin-like growth factor binding protein 5	IGFBP5	AF055033	0.0286	4.78
Kruppel-like factor 10	KLF10	AF050110	0.0479	3.89
Latent traN/Aforming growth factor beta binding protein 1	LTBP1	M34057	0.0486	2.30
Latent traN/Aforming growth factor beta binding protein 2	LTBP2	Z37976	0.0364	5.49
Leptin receptor	LEPR	U50748	N/A	2.82
Lunatic fringe homolog	LFNG	U94354	N/A	2.77
Microfibrillar-associated protein 4	MFAP4	L38486	0.0001	200
Mucin 6	MUC6	U97698	N/A	3.61
Nidogen 1	NID1	M30269	0.0196	4.03
Nuclear receptor subfamily 2, group F, member 1	NR2F1	X16155	0.0247	2.48
Oviductal glycoprotein 1, OVGP1 (MUC9)	MUC9	U09550	0.0955 N/A	4.50
Protein tyrosine phosphatase (IA-2/Phogrin)	PTPRN	L18983	0.0032	5.43
Solute carrier family 2, member 3 (GLUT3)	SLC2A3	M20681	0.0013	7.81
TraN/Aforming growth factor, beta 1	TGFB1	X05839	0.0373	5.00
TraN/Aforming growth factor, beta receptor III	TGFBR3	L07594	N/A	2.53
Trefoil factor 3 (intestinal)	TFF3	L08044	0.0244	4.63
Ubiquitin carboxyl-terminal esterase L1 (PGP9.5)	UCHL1	X04741	0.0208	16.1
v-Maf (c-Maf)	MAF	AF055376	0.0003	3.68

	Gene/Genebank ID	Primers and probes	Sequence
	PGP9.5	Forward	TTGAAGAGCTGAAGGGACAAG
	NM004181	Reverse	TACCCGACATTGGCCTTC
Semi Quantative RT-PCR	DMBT1	Forward	AGTTGAGGCTGACTTGAGAGC
	NM017579	Reverse	TGCAGGGTGGAGAAAGGAAG
	Cyclophilin	Forward	CCCACCGTTTCTTCGAC
	Y00052	Reverse	ATCTTCTTGCTGGTCTTGCC
	FZD2	Forward	TTTTTCTGTCGGGCTGCTACA
	L37882	Reverse	CGCTCCTGGAGCACGAA
		Probe	TGTCGGTGGCCTACATCGCGG
	FZD3	Forward	CATAGGCACGTCCTTCTTGCT
	AF056087	Reverse	GTGCCGTCGTGTTTCATGAT
		Probe	TTCGTGTCCCTCTTCCGTATCCGCA
	BMP1	Forward	CGCTCCTGGAGCACGAA
	M22488	Reverse	GGGCAATGTCCCCAAGAAA
Real Time q-PCR		Probe	CCCCTGCAAGGCGGCTGC
	BMP2A	Forward	TCCCAGCGTGAAAAGAGAGACT
	M22489	Reverse	AATGTCCGTTCCTTTTCTTTGC
		Probe	CCGGCACCCGGGAGAAGGA
	GREMLIN	Forward	TGCTGGAGTCCAGCCAAGA
	AF045800	Reverse	GCACCAGTCTCGCTTCAGGTA
		Probe	CCCTGCATGTGACGGAGCGC
	TGFB1	Forward	CGAGCCTGAGGCCGACTAC
	M384449	Reverse	AGATTTCGTTGTGGGTTTCCA
		Probe	CCAAGGAGGTCACCCGCGTGCTA