

# Expansion of mesenchymal stem cells from human pancreatic ductal epithelium

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**Fibroblast-like cells emerging from cultured human pancreatic endocrine and exocrine tissue have been reported. Although a thorough phenotypic characterization of these cells has not yet been carried out, these cells have been hypothesized to be contaminating fibroblasts, mesenchyme and/or possibly beta-cell progenitors. In this study, we expanded fibroblast-like cells from adult human exocrine pancreas following islet isolation and characterized these cells as mesenchymal stem cells (MSCs) based on their cell surface antigen expression and ability to differentiate into mesoderm. Analysis by flow cytometry demonstrated that pancreatic MSCs express cell surface antigens used to define MSCs isolated from bone marrow such as CD13, CD29, CD44, CD49b, CD54, CD90 and CD105. In addition, utilizing protocols used to differentiate MSCs isolated from other somatic tissues, we successfully differentiated pancreatic MSCs into: (1) osteocytes that stained positive for alkaline phosphatase, collagen, mineralization (calcification) and expressed osteocalcin, (2) adipocytes that contained lipid inclusions and expressed fatty acid binding protein 4 and (3) chondrocytes that expressed aggrecan. We also demonstrated that pancreatic MSCs are multipotent and capable of deriving cells of endodermal origin. Pancreatic MSCs were differentiated into hepatocytes that stained positive for human serum albumin and expressed endoderm and liver-specific genes such as *GATA 4* and *tyrosine aminotransferase*. In addition, preliminary protocols used to differentiate these cells into insulin-producing cells resulted in the expression of genes necessary for islet and beta-cell development such as *Pax4* and *neurogenin 3*. Therefore, multipotent MSCs residing within the adult exocrine pancreas could represent a progenitor cell, which when further manipulated could result in the production of functional islet beta-cells.**  
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Currently, there is an inherent shortage of cadaveric donors necessary to meet organ and tissue transplantation requirements in all disciplines, particularly in the area of clinical islet transplantation. Based on the Edmonton protocol, as many as two to three donor pancreases are required to obtain sufficient islets for a single type I diabetic patient to become insulin independent.<sup>1,2</sup>

In order to address the shortage of islets for clinical transplantation, research is currently directed towards identifying the elusive pancreatic beta-cell progenitor. Possible progenitors have been hypothesized to reside within the pancreatic ductal

epithelium,<sup>3–6</sup> acinar tissue and/or pancreatic small cells.<sup>7,8</sup> Although transplantation of islet grafts containing higher numbers of ductal–epithelial cells results in better clinical outcome,<sup>9</sup> several *in vitro* attempts to isolate beta-cell progenitors thought to reside within the human ductal epithelium have been inconsistent.<sup>3,6,10,11</sup> Recently, we have described an *in vitro* method that enriches the ductal–epithelial cell fraction while depleting the acinar component of the pancreatic exocrine tissue following human islet purification.<sup>12</sup> However, in our culture conditions, we had been unable to expand insulin-producing cells from this enriched ductal cell fraction. Interestingly, we have observed an outgrowth of adherent ‘fibroblast-like’, spindle-shaped cells from the ductal–epithelial cell aggregates, which we characterize in the current study as mesenchymal stem cells (MSCs).

MSCs have been derived from a number of human somatic tissues such as bone marrow (BM), adipose,

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dermis, muscle, peripheral blood and compact bone.<sup>13–16</sup> *In vitro* isolation and characterization of MSCs is based on their adherence, rapid expansion in serum-containing medium, expression of specific cell surface antigens as well as their ability to differentiate into various mesodermal tissues such as fat, bone, cartilage and muscle.<sup>15–18</sup> MSCs have also been reported to cross the mesodermal lineage and give rise to endoderm both *in vitro* and *in vivo*,<sup>18–20</sup> and when implanted at a site of injury or systemically delivered in experimental animal models, they demonstrate the ability to migrate to the injury site, initiate tissue repair and restore function.<sup>18,21,22</sup> We hypothesize that since MSCs are capable of deriving endoderm,<sup>19,20,22,23</sup> they could be directed to become beta-cells and potentially represent an abundant source of insulin-producing cells for transplantation.

By adopting methodology that is currently used to characterize MSCs isolated from various human adult tissues, we report the isolation and characterization of MSCs from the adult human pancreas. In this study, we demonstrate that pancreatic-derived MSCs express cell surface antigens reported to be present on MSCs isolated from various somatic tissues and show that pancreatic MSCs can be differentiated into mesoderm (bone, adipose and cartilage) *in vitro*. In addition, we also demonstrate that pancreatic MSCs are multipotent, and capable of *in vitro* differentiation into endoderm (hepatocytes and pancreatic endocrine cells).

## Materials and methods

### Pancreatic Ductal Cell Isolation

Human pancreatic digests from three independent adult donors ranging from 29 to 58 years of age were obtained following islet purification and cultured as described previously.<sup>12</sup> Briefly, the islet-depleted tissue fraction (89% exocrine, 20% ductal epithelium, 2% beta-cells) is cultured for 4 days in RPMI 1640 (Roswell Park Memorial Institute) medium (Invitrogen, Burlington, Canada), supplemented with 10% fetal calf serum (FCS) (Sigma-Aldrich, Oakville, Canada), at 37°C in 5% CO<sub>2</sub>, 95% air in order to produce cellular aggregates composed primarily of ductal cell epithelium staining greater than 80% for the ductal–epithelial cell markers, cytokeratin 19 (CK19) and cytokeratin 7 (CK7). The resulting cellular aggregates were then used to derive and expand MSCs.

### MSC Expansion and Differentiation

For expansion of MSCs, pancreatic ductal cell aggregates (day 0) were transferred to 100 mm tissue culture-treated plates (Fisher Scientific, Nepean, Canada) at an average cell density of 8.0 cellular aggregates/cm<sup>2</sup>, then cultured at 37°C in 5% CO<sub>2</sub>,

95% air in expansion medium consisting of RPMI 1640, supplemented with 20 ng/ml each of basic fibroblast growth factor (bFGF) (Invitrogen) and epidermal growth factor (EGF) (Invitrogen), 10 mM HEPES (Invitrogen), 1 mM sodium pyruvate (Invitrogen), 10% FCS, 100 U penicillin, 1000 U streptomycin (Invitrogen) and 71.5 μM beta-mercaptoethanol (Sigma). Once adherent fibroblast-like cells migrated out from the ductal cell aggregates and confluency greater than 80% (14 days) was obtained, the monolayer was detached with 0.25% trypsin–EDTA (Invitrogen). Cells were then counted and replated at a density of 2500 cells/cm<sup>2</sup> for up to 13 passages (98 days) in the same culture conditions. For mesoderm differentiation, pancreatic MSCs from passage(s) 2–12 (*n* = 3) were differentiated into bone, fat and cartilage using protocols previously reported for the differentiation of MSCs isolated from BM, adipose and cartilage tissues.<sup>16,24</sup> For endoderm differentiation (*n* = 3), pancreatic MSCs from passage(s) 2–6 were differentiated into hepatocytes using a multistep protocol described by Lee *et al.*<sup>19</sup> For pancreatic endocrine differentiation (*n* = 3), we describe a novel multistep protocol.

For osteogenic differentiation, pancreatic MSCs were allowed to become 80% confluent and treated with osteogenic-selective medium (Stem Cell Technologies, Vancouver, Canada) according to the manufacturer's protocol. The medium was replaced every 3–4 days for 20–31 days. To achieve adipogenic differentiation, pancreatic MSCs were allowed to become 90% confluent and treated with adipogenic-selective medium (Stem Cell Technologies). The medium was replaced every 3–4 days for 14–20 days. For chondrogenic differentiation, pancreatic MSCs were transferred to a sterile 15 ml conical tube (Fisher Scientific), centrifuged at 200 *g* for 5 min and washed twice with phosphate-buffered saline (PBS). Pancreatic MSC pellets were resuspended in Chondrogenic Differentiation Medium (R&D Systems Inc., Minneapolis, MN, USA), centrifuged and cultured in the 15 ml conical for 21–28 days with medium changes every 3–4 days. Negative controls consisted of expanded pancreatic MSCs from the same passage number, cultured in expansion medium and not treated with osteogenic, adipogenic or chondrogenic medium.

For hepatogenic differentiation, pancreatic MSCs from passage(s) 2–6 were allowed to become 100% confluent and differentiated using a three-step protocol (recently described by Lee *et al.*<sup>19</sup>) for differentiating human MSCs isolated from umbilical cord blood and BM. In step 1, the cell monolayer was treated with serum-free IMDM (Iscove's modified Dulbecco's medium) (Invitrogen), supplemented with 20 ng/ml of EGF and 10 ng/ml bFGF for 2 days. In step 2, the medium was replaced with IMDM, supplemented with 20 ng/ml of hepatocyte growth factor (Chemicon International Inc., Temecula, CA, USA), 10 ng/ml bFGF and 5 mM nicotinamide (Sigma) for 1 week with medium

changes every 2–3 days. In step 3, IMDM was supplemented with 20 ng/ml of oncostatin-M (Chemicon), 1  $\mu$ M dexamethasone (Stem Cell Technologies) and 50 mg/ml ITS (insulin, transferrin, selenous acid) + Premix (BD Biosciences, Mississauga, Canada) for 2–5 weeks with medium changes every 2–3 days.

For endocrine differentiation, expanded pancreatic MSCs from passage(s) 2–6 were allowed to become 100% confluent and differentiated using a three-step, 9-day protocol followed by cellular aggregation in serum-free medium. In step 1, the cell monolayer was treated for 3 days with high glucose DMEM (Dulbecco's modified Eagle's medium) (Invitrogen) supplemented with 1% FCS. In step 2, the medium was switched to low glucose DMEM (Invitrogen), supplemented with 1% FCS and 10 mM nicotinamide for 3 days. In step 3, the low glucose medium was supplemented with 1% FCS, 10 mM nicotinamide and 10 nM exendin-4 (Sigma) for 3 days. After 9 days of treatment, the cell monolayer was detached with 0.25% trypsin-EDTA. Cells were aggregated by reconstituting cells at 400 000 cells/ml in low glucose medium, supplemented with 10 nM exendin-4, 10 mM nicotinamide and 10 ng/ml transforming growth factor beta-1 (R&D Systems Inc.) and transferred to a nontissue culture-treated plate for 72 h.

### Immunohistochemistry (IHC) and Histology

Intact ductal cell aggregates, cell suspensions from each passage and differentiated tissue were fixed in Z-fix (Anatech Ltd, Battle Creek, MI, USA), and embedded in 2% low melting point agarose (Sigma). Once the agar had solidified, the agar blocks were paraffin embedded, cut into 3  $\mu$ m sections and immunostained as described previously.<sup>12</sup> Primary antibodies were 1/50 CK19 (Dako Cytomation, Mississauga, Canada), 1/1000 insulin (Dako), 1/5000 glucagon (Cedarlane Laboratory Ltd, Hornby, Canada), 1/100 amylase (Sigma), 1/1000 human serum albumin (Cedarlane) and 1/50 GATA 4 (Cedarlane). Positive controls were sections of human pancreas and hepatocytes. Negative controls were sections incubated without primary antibody. IHC of osteocytes, adipocytes and chondrocytes was assessed as per the manufacturer's protocol, with primary antibodies to 1/200 osteocalcin (R&D), 1/200 fatty acid binding protein 4 (FABP4; R&D) and 1/200 aggrecan (R&D), which are included in the Human Mesenchymal Stem Cell Functional Identification Kit (R&D).

For histological assessment, differentiated and control tissues were either snap-frozen in Cryomatrix embedding compound (Thermo Shandon, Pittsburgh, PA, USA) and 5  $\mu$ m cryosections were prepared or stained directly on the tissue culture surface. Prior to staining, tissue sections were fixed with 1% formaldehyde (BDH Laboratory Supplies, UK), except those sections stained for Oil Red O

(ORO). Von Kossa staining<sup>25</sup> was used to detect mineralization (calcification) of osteocytes. Tissue sections were washed three times in PBS, immersed in water, then immersed in 2% silver nitrate (Sigma) and exposed to ultraviolet light for 45 min. The slides were then rinsed in water, followed by 5% sodium thiosulfate (Fisher Scientific) for 3 min and washed in running tap water before counterstained with Zymed Hematoxylin (Zymed Laboratories, San Francisco, CA, USA). Vector Red Alkaline Phosphatase Substrate Kit (Vector Laboratories, Burlingame, CA) was used to detect alkaline phosphatase (AP) activity of osteocytes. Tissue sections were washed three times in PBS and stained in the dark as per the manufacturer's protocol. Gomori's One-Step Trichrome stain<sup>25</sup> was used to detect collagen matrix deposition in bone cultures. Briefly, sections were immersed in water, warmed in Bouin's solution (60°C) for 30 min, cooled to room temperature in the same Bouin's solution for another 30 min and washed in warm running tap water. Finally, slides were Trichrome stained for 20 min, then immersed in 0.5% acetic water. ORO stain was used to detect lipid droplets in adipogenic cultures.<sup>25</sup> Sections were washed three times in PBS, briefly treated with 70% ethanol and immersed in 0.4% ORO (Sigma). Slides were then rinsed with water and counterstained with Zymed Hematoxylin (Zymed Laboratories). Alcian blue stain was used to detect acid mucopolysaccharides<sup>25</sup> in chondrocytes. After fixation in 1% formaldehyde, sections were placed in 3% acetic acid (Fisher Scientific) for 2 min, immersed in 1% alcian blue (Sigma), pH 2.6, and washed in running tap water.

### RNA Isolation and Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Ductal cell aggregates from the start of expansion, cell isolates from each passage and differentiated tissue were dissolved in 1 ml of Trizol reagent (Invitrogen) and RNA extracted according to the manufacturer's protocol. cDNA was constructed from 1  $\mu$ g of total RNA using TaqMan Reverse Transcription Reagents (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocol and 1  $\mu$ l of cDNA was amplified for 35 cycles using *Taq* DNA polymerase (Invitrogen). The PCR conditions were as follows: 95°C for 30 s, 59°C for 30 s and 72°C for 30 s with a final extension of 72°C for 8 min utilizing primer pairs that span at least one intron (Table 1). PCR products were separated on an ethidium bromide-stained (Sigma) agarose gel (1.5%) and images captured with Alpha Digidoc software (Perkin-Elmer, Boston, MA, USA). Bands of the expected size were ligated in the pCR5-TOPO vector (TOPO TA Cloning Kit for Sequencing, Invitrogen), sequenced (University of Alberta DNA Core Lab) and compared with known GenBank sequences. Positive control consisted of human islet

**Table 1** Primers used for PCR amplification

<i>Gene</i>	<i>Size (bp)</i>	<i>Strand</i>	<i>Sequence 5'–3'</i>	<i>GenBank</i>
Amylase	300	F	GAC TTT CCA GCA GTC CCA TA	NM_020978
		R	GTT TAC TTC CTG CAG GGA AC	
Beta-2 microglobulin	268	F	CCA GCA GAG AAT GGA AAG TC	NM_004048
		R	GAT GCT GCT TAC ATG TCT CG	
GATA 4	366	F	CTC CTT CAG GCA GTG AGA GCC	NM_002052
		R	GGT CCG TGC AGG AAT TTG ACC	
Glucagon	221	F	CCG AAG ATT TTG TGC AGT GGT T	NM_002054
		R	GCG GCC AAG TTC TTC AAC AAT	
Insulin	263	F	GCA GCC TTT GTG AAC CAA CA	AY138590
		R	GTT GCA GTA GTT CTC CAG GTG	
Ngn3	313	F	GGT AGA AAG GAT GAC GCC TC	NM_020999
		R	CCG AGT TGA GGT CGT GCA T	
NeuroD	439	F	ATC CCA ACC CAC CAC CAA CC	NM_002500
		R	CAG CGG TGC CTG AGA AGA TT	
Pax 4	496	F	AGG AGG ACC AGG GAC TAC CGT	NM_006193
		R	TTT AGG TGG GGT GTC ACT CAG	
PDX1	262	F	CCC ATG GAT GAA GTC TAC C	NM_000209
		R	GTC CTC CTC CTT TTT CCA C	
Tyrosine Aminotransferase	358	F	TGA GCA GTC TGT CCA CTG CCT	NM_000353
		R	ATG TGA ATG AGG AGG ATC TGA G	

**Table 2** Antibodies for the characterization of cell surface antigens

<i>Anti-human</i>	<i>Dilution</i>	<i>Fluorochrome</i>	<i>Source</i>
CD13	3/50	PE-Cy5	Caltag Laboratories, Burlingame, CA, USA
CD29	3/50	PE-Cy5	Caltag Laboratories, Burlingame, CA, USA
CD34	2/5	PerCP-Cy5.5	BD Biosciences, Mississauga, CA, USA
CD44	1/10	Fluorescein isothiocyanate (FITC)	Santa Cruz Biotechnology, CA, USA
CD45	3/50	Phycoerythrin (PE)	Caltag Laboratories, Burlingame, CA, USA
CD49b	1/10	FITC	Sanquin, Amsterdam, The Netherlands
CD54	1/50	FITC	Caltag Laboratories, Burlingame, CA, USA
CD90	1/50	PE	BD Biosciences, Mississauga, Canada
CD105	3/50	PE	Caltag Laboratories, Burlingame, CA, USA
CD117	1/5	FITC	Chemicon International, Temecula, CA, USA

or hepatocyte cDNA, while the negative control consisted of water in place of experimental cDNA and beta-2 microglobulin (housekeeping gene) ensured cDNA integrity.

### Immunophenotyping by Flow Cytometry

To determine the phenotype of the cell monolayer, cell surface antigen expression of single cells obtained from each passage was analyzed by flow cytometry. Briefly, cells were fixed with cold 1% formaldehyde (BDH Laboratory Supplies), washed once with PBS and stained with the primary antibodies summarized in Table 2. Cell populations were analyzed on the FACS Calibur (BD Biosciences, Mississauga, Canada) using Cell Quest Pro software and compared to the isotype control.

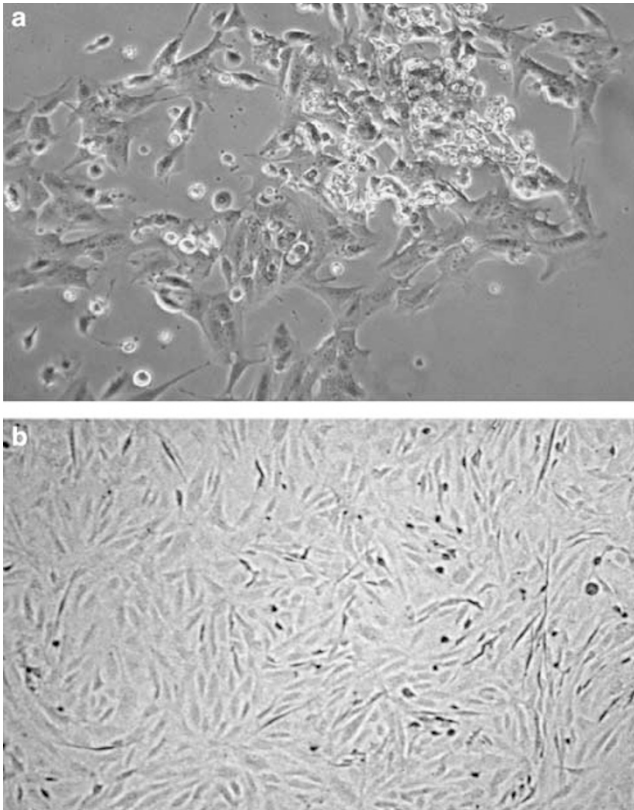
## Results

### Isolation and Characterization of Cell Preparations

Human adult pancreatic tissue ( $n = 3$ ) prepared from islet-depleted digests was cultured for 4 days in

RPMI supplemented with 10% FCS. As reported previously, this procedure results in cellular aggregates<sup>12</sup> composed primarily of ductal cell epithelium. To isolate MSCs, the ductal cell aggregates (day 0) were plated onto tissue culture-treated dishes and cultured with expansion medium. Within 5 days, adherent fibroblast-like cells were observed growing out of the ductal cell aggregates (Figure 1a). Confluency was achieved by day 14 when the cell monolayer was detached by trypsin-EDTA treatment and transferred to new 100 mm tissue culture-treated dishes. After this first passage, the adherent cells divided rapidly and became confluent within 7 days, for a total of 21 days in expansion medium (Figure 1b). The cells were passaged again (passage 2) and cultures were maintained for 13 passages (98 days). During this culture period, at least a 12-fold expansion in cell number was observed between successive passages until passage 9 (70 days) when the fold increase in cell number decreased by 20%. Passage(s) 10–12 had only a five-fold increase, and by passage 13, proliferation had ceased.

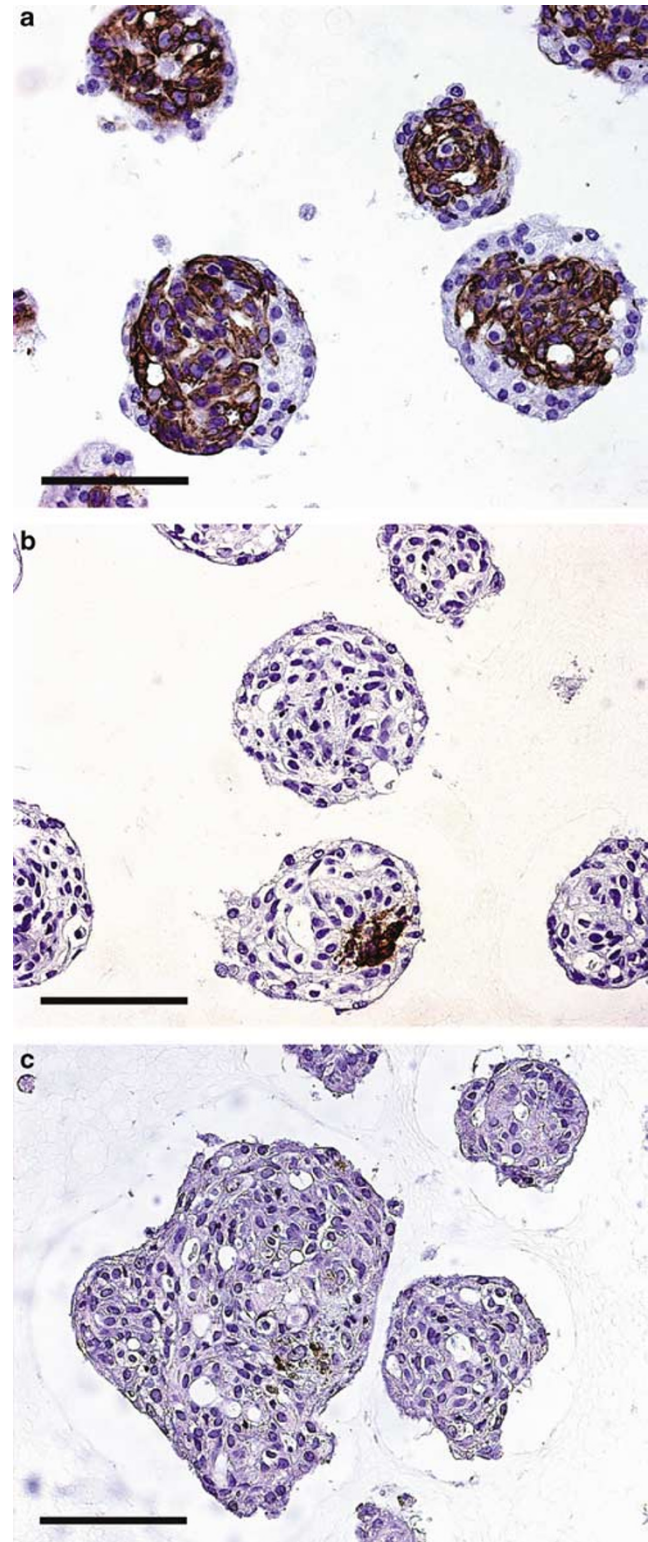
The cellular composition of the ductal cell aggregates (day 0) and passaged cells was deter-



**Figure 1** Outgrowth of fibroblast-like cells from pancreatic ductal cell aggregates. After 5 days in expansion medium, adherent, fibroblast-like cells were observed emerging from cultured ductal cell aggregates (a). Magnification  $\times 100$ . Confluent monolayer of adherent, fibroblast-like cells prior to passage 2 (b). Magnification  $\times 40$ .

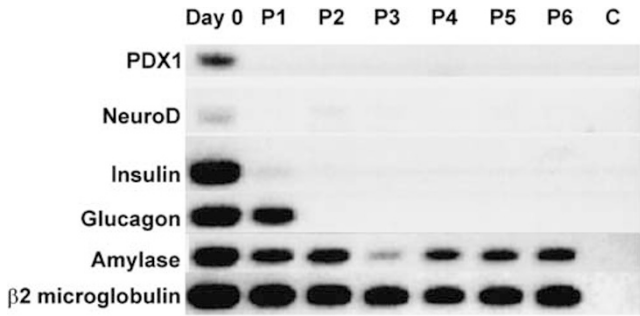
mined by IHC. At day 0, the ductal cell aggregates consisted of  $81.3 \pm 9.6\%$  ductal-epithelial cells (CK19),  $2.0 \pm 0.9\%$  beta-cells (insulin),  $6.8 \pm 1.1\%$  acinar cells (amylase) and no alpha-cells (glucagon) were observed (Figure 2a-c). During cellular expansion, immunoreactivity decreased for both exocrine and endocrine markers. Single cells obtained from passage 3 (28 days) consisted of  $1.33 \pm 2.3\%$  CK19,  $0\%$  beta-cells and  $1.23 \pm 1.1\%$  acinar cells (not shown). Single cells from passage 5 (42 days) were negative for CK19, insulin and glucagon staining, but were still comprised of  $7.0 \pm 8.0\%$  acinar tissue (not shown).

Amplification of mRNA confirmed that the fibroblast-like cells expanded from the ductal cell aggregates were not comprised of endocrine cells. Only the initial aggregates (day 0) expressed mRNA for insulin and the insulin transcription factors *PDX1* and *NeuroD* (Figure 3). Although message for glucagon was still present at the time of the first passage (14 days), the mRNA was not translated, since glucagon protein was not detected by IHC. mRNA for amylase was detected in all passages measured correlating with the IHC staining results for amylase. Neither the initial ductal cell aggregates



**Figure 2** Immunohistochemical staining results of pancreatic ductal-epithelial cell aggregates (day 0) prior to mesenchymal cell expansion. Pancreatic ductal cell aggregates stained positive (a) for the ductal-epithelial cell marker CK19; one aggregate in the field is positive for insulin (b) and amylase (c) staining. Positive immunoperoxidase stain is brown and hematoxylin counterstain is blue. Scale bar represents  $50 \mu\text{m}$ .

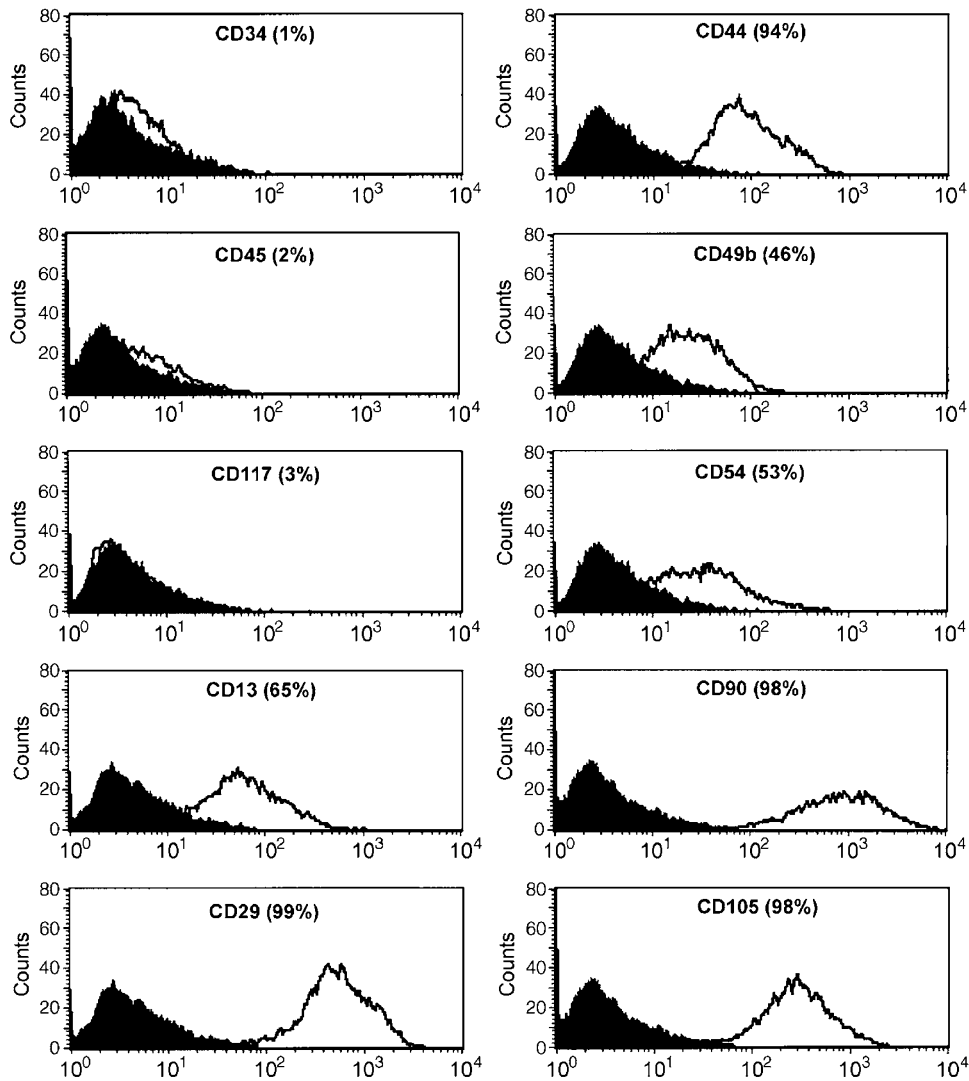




**Figure 3** RT-PCR of pancreatic ductal cell aggregates (day 0) and cells from passage(s) 1–6. Message for insulin, and insulin transcription factors PDX1 and NeuroD was only detected in the initial ductal (day 0) tissue and not in subsequent passages, while message for glucagon persists until passage 1 (14 days). Message for amylase is present in all passages shown. Beta-2 microglobulin-positive control denotes RNA and cDNA integrity. Water blank (C) is negative control.

(day 0) nor expanded cells expressed mRNA for transcription factors *neurogenin 3* (*ngn3*) or *Pax4* (not shown).

Cell surface antigen expression on adherent fibroblast-like cells from all 13 passages ( $n=3$ ) was analyzed by flow cytometry. Primary antibodies used to describe the phenotype of MSCs isolated from BM, adipose and umbilical cord blood<sup>13,16,23,24,26</sup> were used in the analysis. Adherent fibroblast-like cells expanded from ductal cell aggregates did not express CD34, CD45 and CD117 cell surface antigens, indicating that they were not of hematopoietic origin (Figure 4). These cells did, however, express CD13, CD29, CD44, CD49b, CD54, CD90 and CD105 cell surface antigens, thereby demonstrating an MSC phenotype. This MSC phenotype persisted until passage 13 (98 days). The cells at this time lost their spindle shape,



**Figure 4** Representative cell surface antigen expression profile of pancreatic mesenchymal stem cells analyzed by flow cytometry. Fibroblast-like cells expanded from the ductal cell aggregates are less than 3% positive for CD34, CD45 and CD117 and express cell surface antigens CD13, CD29, CD44, CD49b, CD54, CD90 and CD105. Plots show isotype control staining profile (solid) vs specific antibody staining profile (line).

broadened, lost expression of CD49b and CD13 (not shown) and were no longer capable of proliferation or differentiation.

### ***In Vitro* Differentiation of Pancreatic MSCs into Osteocytes, Adipocytes and Chondrocytes**

Osteogenic differentiation was successful when attempted after passage 2 when the cells expressed a predominantly MSC phenotype as demonstrated by flow cytometry (Figure 4). Correspondingly, at passage 13 when these cells no longer expressed an MSC phenotype, differentiation was not possible. During osteogenic differentiation, changes in monolayer cell density were observed after 12 days and in addition to a lattice appearance dense foci were evident within the outer edge of the cell monolayer at 16 days (not shown). After 16 days in osteogenic medium, the monolayer appeared to fold in upon itself and form a single large adherent aggregate (not shown). Tissue harvested and stained at 20 days of differentiation had abundant AP (Figure 5a) and some evidence of mineralization (calcification) demonstrated by Von Kossa staining (Figure 5c). Moderate collagen deposition was demonstrated by Gomori's Trichrome stain (Figure 5e) and IHC for osteocalcin was also positive (Figure 5g). To assess if the developing bone could mature, adherent tissue was treated with osteogenic medium for an additional 11 days. Tissue collected after 31 days of differentiation had scant AP activity (Figure 5b), a substantial amount of mineralization (Figure 5d) and was composed entirely of collagen (Figure 5f). IHC for osteocalcin was also positive (Figure 5h). Undifferentiated controls had slight AP activity, no evidence of mineralization, collagen or osteocalcin expression (not shown).

Differentiation of pancreatic MSCs into adipocytes was also attempted after passage 2 using adipocyte-selective medium; however, after passage 4, pancreatic MSCs could not be differentiated into adipocytes. During adipogenic differentiation, lipid droplets were observed after 7 days by light microscopy (not shown). At 14 days, lipid inclusions were evident by ORO staining (Figure 6a) and differentiated cells expressed FABP4 as measured by IHC (Figure 6b). Pancreatic MSCs not treated with adipogenic medium did not develop lipid droplets and did not express FABP4 (not shown).

Differentiation of pancreatic MSCs into chondrocytes was successful when attempted at the same passages as osteogenesis (passages 2–12) using chondrocyte-selective medium. Differentiated cell pellets harvested at 21 and 28 days of treatment stained with alcian blue (Figure 6c) and expressed aggrecan as measured by IHC (Figure 6d). Undifferentiated tissue did not express aggrecan and cytoplasm did not stain for alcian blue.

### ***In Vitro* Differentiation of Pancreatic MSCs into Hepatocytes and Beta-Cells**

Differentiation of pancreatic MSCs into hepatocytes was successful at the passages attempted (passages 2–6). During differentiation, changes in cell morphology were observed after 16 days, or 7 days after the addition of oncostatin-M and dexamethasone. When compared to undifferentiated pancreatic MSCs (Figure 6e), cells acquired a cuboidal morphology (Figure 6f) that persisted throughout differentiation. Differentiated cells harvested at 16 days were human serum albumin immunoreactive (not shown) and cells harvested after 27 days expressed both human serum albumin and GATA 4 demonstrated by IHC (Figure 6g and h). Undifferentiated cells did not express human serum albumin or GATA 4 (not shown). After 27 days of differentiation, cuboidal cells expressed mRNA for both the endoderm-specific gene *GATA 4* and liver-specific gene tyrosine aminotransferase (*TAT*) (Figure 7). At 21 and 24 days only *TAT* was expressed and prior to 21 days neither gene was expressed (Figure 7). Undifferentiated pancreatic MSCs did not express *GATA 4* or *TAT*, while hepatocytes expressed both *GATA 4* and *TAT* (Figure 7).

The preliminary protocol used in this study to differentiate pancreatic MSCs into beta-cells was somewhat successful when attempted at passage(s) 2–6 ( $n = 3$ ). Changes in cell morphology were not observed during differentiation, but after 9 days of differentiation, the following genes were expressed, as measured by RT-PCR: *NeuroD*, insulin and glucagon (Figure 8). Following cellular aggregation, in addition to *NeuroD*, insulin and glucagon, mRNA for *ngn3*, *PDX1* and *Pax4* were also expressed (Figure 8) with three patterns of expression observed: (1) *ngn3*, (2) *PDX1* with *Pax4* or (3) *PDX1* with *ngn3*. Undifferentiated pancreatic MSCs and cell preparations analyzed after 3 and 6 days of differentiation did not express the above genes (not shown). Although RT-PCR revealed the expression of genes necessary for beta-cell development and function, IHC staining for the gene products was negative (not shown), indicating the mRNA was not translated.

## **Discussion**

In the present study, we report the *in vitro* isolation of MSCs from adult human exocrine tissue. We expanded pancreatic MSCs based on their ability to adhere and rapidly divide in the presence of serum, while endocrine and exocrine cell populations declined. These pancreatic MSCs expressed the same cell surface antigens reported to be on MSCs isolated from human BM, adipose and umbilical cord blood.<sup>13,16,18,23,24</sup> Pancreatic MSCs expressed CD13, CD29, CD44, CD49b, CD54, CD90 and CD105, but did not express CD34, CD45 and CD117. However, since cell surface antigen expression alone

is not sufficient to characterize MSCs,<sup>15</sup> we also demonstrated that pancreatic MSCs could be differentiated into mesoderm (osteocytes, adipocytes and chondrocytes). In addition, pancreatic MSCs were shown to be multipotent since they were also capable of deriving endoderm (hepatocytes and beta-cells).

We successfully derived osteocytes from passages 2 to 12 when cells expressed a MSC phenotype. During osteogenesis, tissue was collected at an early time point of 20 days postdifferentiation and later time point of 31 days postdifferentiation. Although osteocalcin expression was detected at both time points during differentiation, a change in the amount of AP activity, mineralization (calcification) and collagen deposition was observed. Only in the earlier time point (20 day) was a large amount of AP activity observed, while only a modest amount calcification and collagen deposition was detected. However, in contrast to the early time point, we repeatedly observed little or no AP activity in the later time points (31 day) that always coincided with a substantial increase in mineralization and collagen deposition. This pattern of AP activity declining as mineralization and collagen deposition increases has been observed when differentiating MSCs isolated from BM<sup>27</sup> and is reflective of active osteogenesis and mature compact bone formation despite tissue lifting off during differentiation.

We also successfully differentiated adult pancreatic MSCs into adipocytes and chondrocytes. Although pancreatic MSCs were not capable of adipocyte differentiation after passage 4, osteocyte and chondrocyte differentiation was not affected until changes in cell morphology and phenotype were observed at passage 13. Changes in pancreatic MSC morphology were observed after passage 12, when cells became broad and flat in appearance and ceased to divide. In addition, analysis by flow cytometry demonstrated that cells from passage 13 no longer expressed CD49b or CD13 cell surface antigens. In other studies, MSCs isolated from adult BM cultured in similar conditions were reportedly able to maintain both their phenotype and ability to differentiate throughout multiple passages.<sup>15,22,24</sup> However, similar studies have reported changes in MSC morphology and phenotype with repeated passages *in vitro*<sup>28–30</sup> and have also emphasized the loss of adipogenic potential with higher passage numbers. One study of MSCs isolated from human BM summarized that most MSCs undergoing *in vitro* expansion lose telomeres with each cell division

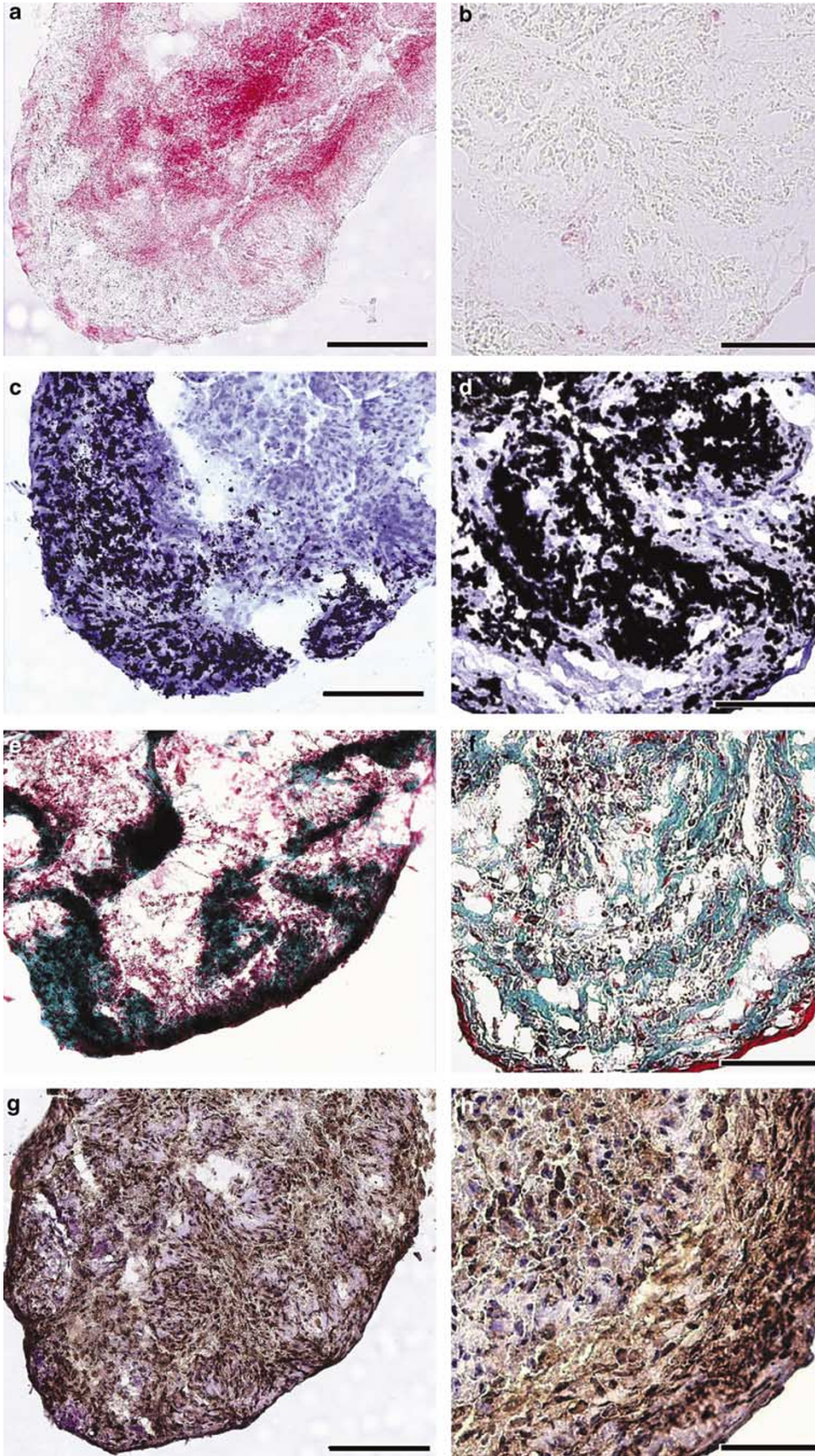
until reaching a threshold where division ceases and cells assume a senescent phenotype.<sup>30</sup> Another *in vitro* study correlated changes in morphology, progressive loss of adipogenic potential and senescent phenotype with an increase in caveolin-1 expression.<sup>29</sup> In addition, the authors of the latter study concluded that the capacity of adult stem cells could be reflective of donor age, tissue source and biological aging.<sup>29</sup> Therefore, the phenotypic changes we observed at higher passages and the loss of adipogenic potential are not unique features that can be attributed to pancreatic MSCs or the culture conditions used in our study. Rather, this common characteristic of loss of potential serves to emphasize the limitations faced when expanding adult stem cells.

Although limitations exist when working with adult stem cells, MSCs derived from various somatic tissues are capable of differentiating into mesoderm both *in vitro* and *in vivo*, and are considered excellent candidates for cell-based therapy.<sup>15,18,21,28,31</sup> In experimental animal models, after myocardial infarction, MSCs when injected at the site of ischemia or systemically delivered migrate to the site of injury, initiate tissue repair and, in some instances, restore function.<sup>18,32</sup> Therefore, promising therapeutic applications in cardiology are emerging and clinical trials have commenced.<sup>33</sup> Other clinically relevant cell therapy applications reside with the MSCs ability to cross the mesodermal lineage and derive cells of an endodermal origin. For example, MSCs isolated from BM, umbilical cord blood and adipose are able to differentiate into functional hepatocytes capable of expressing liver-specific genes.<sup>19,20,23</sup> The same is true for pancreatic MSCs. In this study, we derived cuboidal hepatocytes that expressed liver- and endoderm-specific genes. Similar to other studies, we observed a time-dependent manner in which changes to cell morphology and gene expression were observed.<sup>19,23</sup> Changes in cell morphology and gene expression were not evident until several days after the addition of oncostatin-M and dexamethasone to the medium. When these same growth factors were omitted, hepatocyte differentiation was not apparent. In addition, IHC demonstrated that these differentiated cells expressed human serum albumin as early as day 16; however, similar to RT-PCR results, GATA 4 was not apparent until after 27 days of differentiation.

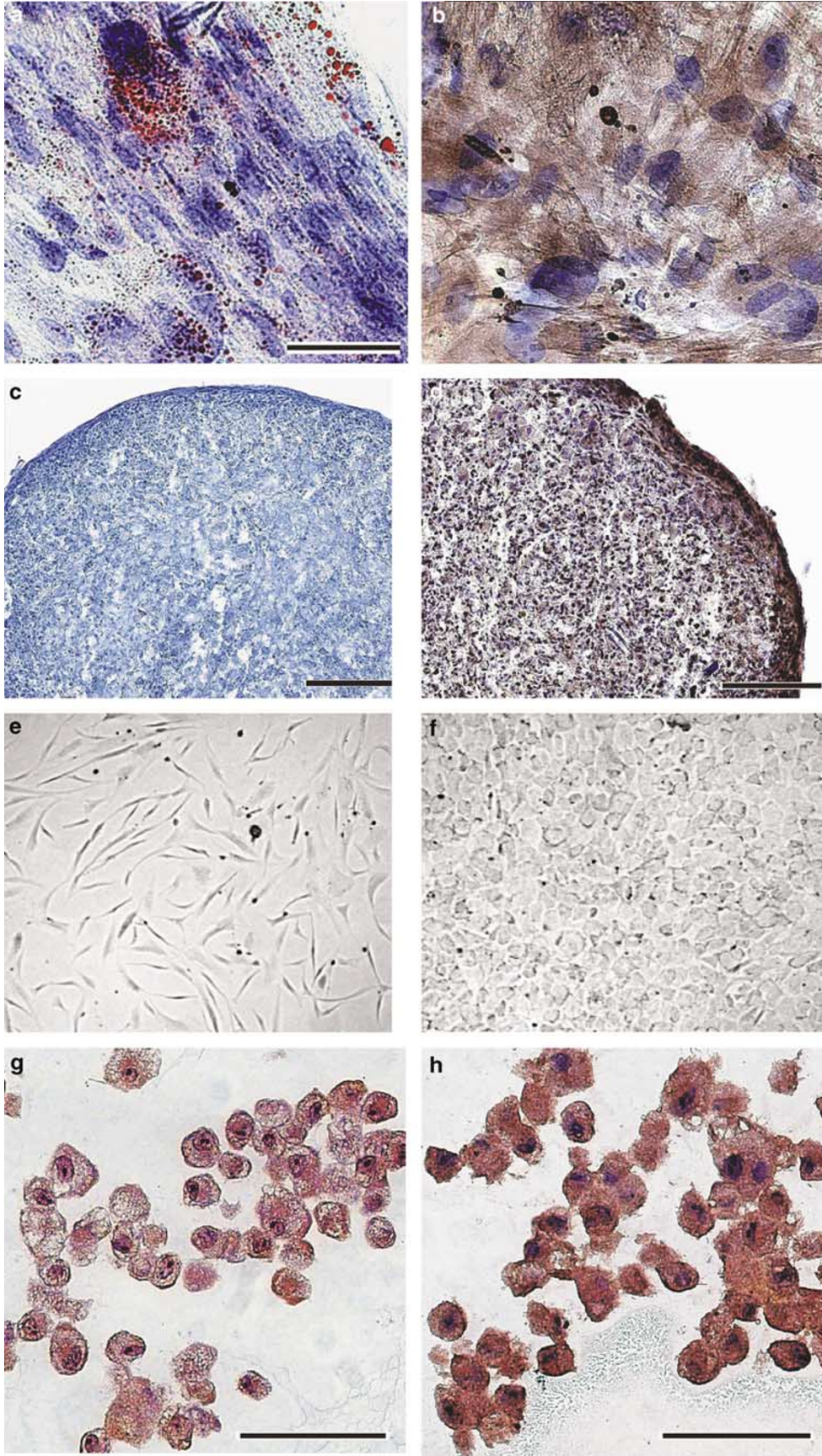
Preliminary data also suggest that pancreatic MSCs have the potential to derive beta-cells.

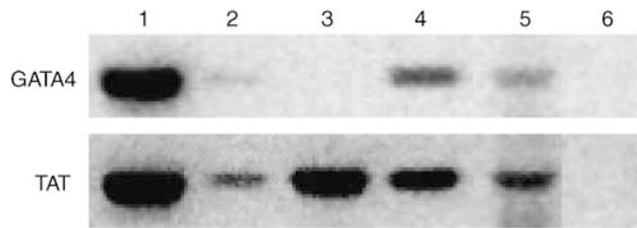
**Figure 5** Osteogenic differentiation of pancreatic mesenchymal stem cells. Tissue collected after 20 days of treatment with osteo-selective medium had abundant alkaline phosphatase (AP) activity stained red (a), some evidence of mineralization stained black by Von Kossa (VK) staining at the left edge of the section (c) and collagen deposition stained green by Gomori's Trichrome stain (e). Immunohistochemical staining for osteocalcin is positive (brown) using the immunoperoxidase method (g). VK stain and osteocalcin are counterstained with hematoxylin (blue). Tissue collected at 31 days of treatment had scant AP activity (b), a significant increase in mineralization stained black (d) compared to images (a and c), overlying the tissue that is composed entirely of collagen, stained green by Trichrome stain (f). Immunohistochemical staining for osteocalcin is still positive (h). Immunoperoxidase stain is brown and hematoxylin counterstain is blue. Scale bar represents 100  $\mu$ m.







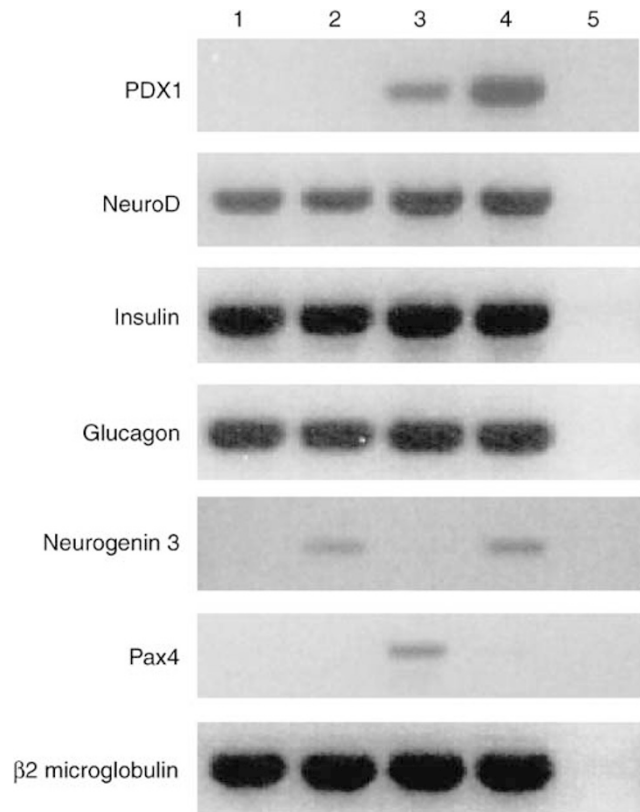




**Figure 7** RT-PCR of pancreatic MSCs differentiated into hepatocytes. Message for TAT was detected at 21, 24, 27 and 40 days of hepatocyte differentiation (lanes 2–5). Message for GATA 4 was detected at 27 and 40 days of differentiation (lanes 4 and 5). Undifferentiated pancreatic MSCs did not express message for either gene (lane 6) and human hepatocytes expressed both TAT and GATA 4 (lane 1).

Analyses of preaggregated tissue during differentiation demonstrated that cells could express islet cell mRNA previously seen in the initial ductal cell aggregates used to expand MSCs. In addition, further differentiation by aggregating in the presence of growth factors resulted in the expression of the transcription factors *PDX1*, *Pax4* and *ngn3*, where *Pax4* and *ngn3* were not expressed in the initial ductal cell aggregates. Although multiple patterns of gene expression were observed in these initial experiments, which could be due to donor variability, such as age, and IHC staining was negative, this preliminary data suggest the potential for deriving functional beta-cells and warrants further investigation. For example, infection of mouse and human duct cell lines and/or isolated duct epithelial cells<sup>34,35</sup> with an *ngn3*-expressing adenovirus or plasmid encoding *PDX1*<sup>36</sup> resulted in the expression of genes critical to islet differentiation and insulin expression that include *Pax4* and *NeuroD*.<sup>34,35</sup> Therefore, the fact that we were able to induce the expression of these genes without adenoviral infection suggests that with further *in vitro* and possible *in vivo* manipulation, pancreatic MSCs could be induced to express functional islet proteins.

Since there is an inherent shortage of donor islets for the transplantation of patients with type I diabetes, our long-term goal is to identify and expand the ‘islet/beta-cell progenitor’ hypothesized to reside within the pancreatic ductal epithelium. Therefore, we are continuing to explore the multipotency of the pancreatic MSC and assess its complete potential. However, if, as postulated recently by Gershengorn *et al*<sup>37</sup> and Linning *et al*,<sup>38</sup> that fibroblast-like cells residing within the pan-



**Figure 8** RT-PCR of pancreatic MSCs differentiated into beta-cells. Differentiation for 9 days resulted in the expression of *NeuroD*, insulin and glucagon (lane 1). Cellular aggregation for 72 h had three outcomes. In addition to *NeuroD*, insulin and glucagons, either *ngn3* (lane 2), *PDX1* and *Pax4* (lane 3) or *PDX1* and *ngn3* (lane 4) were also expressed. Beta-2 microglobulin-positive control denotes RNA and cDNA integrity. Water blank (lane 5) is negative.

creas are multipotent and capable of reversible endoderm to mesoderm transitions, perhaps pancreatic MSCs could represent an abundant source of islet progenitors and potentially derive sufficient numbers of insulin-producing cells required for transplantation.

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**Figure 6** Representative image of pancreatic MSCs differentiated into adipocytes (a, b), chondrocytes (c, d) and hepatocytes (e–h). ORO staining detects lipid droplets (a) and immunohistochemical staining for FABP4 (brown) is positive (b). Lipid droplets are red, positive immunoperoxidase stain is brown and hematoxylin counterstain is blue. Chondrocyte pellet is positive for alcian blue stain (c) and immunohistochemical staining for aggrecan is positive (d). Positive immunoperoxidase stain is brown and hematoxylin counterstain is blue. Scale bar represents 50 μm. Hepatogenic differentiation of pancreatic MSCs (e–h). Phase contrast demonstrates that after 16 days, pancreatic MSCs lose their fibroblast-like morphology (e) and assume a cuboidal morphology (f). Magnification × 40. Immunohistochemical staining for human serum albumin (g) and GATA 4 (h) is positive in cuboidal cells harvested after 27 days of differentiation. Positive immunoperoxidase stain is brown and hematoxylin counterstain is blue. Scale bar represents 50 μm.

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