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Endocrine Precursor Cells from Mouse Islets Are Not Generated by Epithelial-to-Mesenchymal Transition of Mature Beta Cells

Russell A. Morton, Elizabeth Geras-Raaka, Leah M. Wilson, Bruce M. Raaka, and Marvin C. Gershengorn

Clinical Endocrinology Branch, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, USA

Abstract

We previously presented evidence that proliferative human islet precursor cells may be derived *in vitro* from adult islets by epithelial-to-mesenchymal transition (EMT) and show here that similar fibroblast-like cells can be derived from mouse islets. These mouse cell populations exhibited changes in gene expression consistent with EMT. Both C-peptide and insulin mRNAs were undetectable in expanded cultures of mouse islet-derived precursor cells (mIPCs). After expansion, mIPCs could be induced to migrate into clusters and differentiate into hormone-expressing islet-like aggregates. Although early morphological changes suggesting EMT were observed by time-lapse microscopy when green fluorescent protein-labeled beta cells were placed in culture, the expanded precursor cell population was not fluorescent. Using two mouse models in which beta cells were permanently made either to express alkaline phosphatase or to have a deleted M₃ muscarinic receptor, we provide evidence that mIPCs in long term culture are not derived from beta cells.

Investigators worldwide are conducting pre-clinical research aimed at employing *in vitro* systems to generate large numbers of islets of Langerhans (or beta cells) (1,2). Although beta cells proliferate *in vivo* (3) and *in vitro* (4), well-differentiated beta cells do not proliferate rapidly *in vitro*. Therefore, islet (or beta cell) precursor cells are being studied for this purpose based on the concept that undifferentiated precursor cells can be expanded exponentially and then induced to differentiate into islet-like cells. We (5-7) and others (8-12) have derived islet precursor cells from adult human islet preparations. However, insights into the processes involved in derivation, expansion and differentiation of these human precursor cells are limited. Assuming conservation across species, similar precursor cells derived from nonhuman islets could be used to study these processes further. For example, transgenic or knockout mice could be used to delineate signal transduction pathways involved in proliferation and differentiation of precursor cells and thereby identify targets for regulation by drugs.

We provided evidence previously that precursor cells from human islets may be derived by *in vitro* epithelial-to-mesenchymal transition (EMT) of insulin-expressing cells (5). The origin of these cells was uncertain because individual human insulin-expressing cells could not be identified and followed in culture. In this report, transgenic mice are used for this purpose. We demonstrate that cells similar to human islet-derived precursor cells (IPCs) (5) can be derived from preparations of mouse islets. Like human IPCs (hIPCs), these cells can proliferate for over 20 doublings and after expansion can be differentiated into clusters of hormone-expressing

Corresponding Author: Marvin C. Gershengorn, Scientific Director, NIDDK, National Institutes of Health, 50 South Drive, Bethesda, MD 20892-8029, USA, Tel: 301-451-6305, Fax: 301-480-4214, E-mail: marving@intr.niddk.nih.gov

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cells. Further, we show directly that green fluorescent protein (GFP)-labeled beta cells from transgenic mice (MIP-GFP mice) (13) undergo an initial morphological change to fluorescent fibroblast-like cells in culture but, using two mouse models in which beta cells are permanently marked, show that proliferating mouse IPCs (mIPCs) are not derived from beta cells.

Research Design and Methods

Cell culture

Mouse pancreatic islet isolations were performed according to a modification of the procedure of Shewade et al. (14). Briefly, after washing and mincing, the excised pancreas tissue was digested for 6-10 min at 37°C with shaking in Dulbecco's modified Eagle's medium (DMEM) containing 3 mg/ml collagenase (Gibco, Type IV). Digestion was quenched by addition of fetal bovine serum (FBS) and the digested tissue was washed twice with cold RPMI-1640 medium (Gibco). Tissue from 5 mouse pancreases was placed in a 100 mm tissue culture-treated dish in 10 ml RPMI-1640 with 10% FBS for 3 days. During this period, much of the exocrine and ductal tissue attaches to the culture dish or dies, while islets remain mostly unattached or attached loosely. Following release of any attached islets with gentle agitation, the islets were collected by centrifugation and placed in tissue culture treated dishes in CMRL-1066 medium (Gibco) containing 5.5 mM glucose supplemented with 2mM L-glutamine and 10% FBS (growth medium). Preparations at this stage were composed generally of 60% to 90% mature islets with residual duct and exocrine tissue. The majority of islets attached to the dish within 3 to 7 days. Cultures were re-fed when the majority of islets had attached and thereafter as needed to replenish nutrients and remove debris. "Passage 0" is defined as 10 to 14 days after the islets are placed in culture at a time when the cultures were nearly confluent with fibroblast-like cells. Beginning at passage 0, cells were harvested with trypsin and sub-cultured (1:2) every 3-4 days. To measure the cell doubling rate, one million cells were seeded in a 10-cm dish in growth medium and counted every three days.

To induce differentiation, fibroblast-like cells at various passages were monodispersed with trypsin and 3×10^5 cells were added to each well of 6-well tissue culture-treated plates in serum-free CMRL-1066 medium supplemented with 1% bovine serum albumin, insulin (10 µg/ml), transferrin (5.5 µg/ml) and sodium selenite (6.7 ng/ml) (differentiation medium). During differentiation, cells form islet-like cell aggregates (ICAs). The medium was replaced every other day using unit gravity to retain ICAs.

Time-lapse microscopy

Islets were prepared from MIP-GFP transgenic mice (13). Following the initial 3 day culture in RPMI-1640 medium with 10% FBS, islets were handpicked and placed in 8-well Permanox dishes (LAB-TEK #177445) at 8-10 islets per dish in 0.25 ml RPMI-1640 containing 10% FBS and 10 mM HEPES, pH 7.2. Cultures were overlaid with mineral oil to retard evaporation and maintained at 37°C. Images were acquired with a Zeiss Axiovert 200M inverted microscope (25x objective) equipped with xenon and halogen lamps and an Orca-ER (Hamamatsu) CCD camera. GFP fluorescence was detected using a 535/40 nm bandpass filter. Multiple x, y, z coordinates were captured every 15 min for up to 7 days using MetaMorph™ software (Ver. 6.1, Molecular Devices, Downingtown, PA).

Quantitative RT-PCR

Total RNA was purified using RNeasy Mini kits (Qiagen). First strand cDNA was prepared using a High Capacity cDNA Archive Kit (Applied Biosystems). RT-PCR was performed in 25 µl reactions using cDNA prepared from 25-100 ng of total RNA and Universal PCR Master Mix (Applied Biosystems). Primers and probes were Assay-on-Demand (Applied Biosystems).

Quantitative RT-PCR results were normalized to mouse Gapdh to correct for differences in RNA input.

Antibodies and immunostaining

Mouse anti-glucagon, rabbit anti-mouse C-peptide 1 and rabbit anti-mouse C-peptide 2 (all from Beta Cell Biology Consortium, www.betacell.org) were used at 1:100 dilution in blocking buffer (4% donkey serum in DPBS). Alexa-Fluor 488, 546 and 633 F(ab')₂ secondary antibodies (Molecular Probes) were used at 1:200 dilution. For antigen retrieval, samples were incubated in citrate buffer (10 mM sodium citrate, pH 6.0) for 20 min at 95°C. After incubation in blocking buffer for 30 min at room temperature, sections were incubated with primary antibodies for 2 h at 37°C, washed 3 times with DPBS and incubated with secondary antibodies at 37°C for 1 h. Slides were then washed extensively with DPBS and mounted in Prolong Gold antifade reagent (Invitrogen) supplemented with DAPI. Confocal images were captured with a Zeiss LSM 510 Meta NLO laser scanning inverted microscope.

Measurement of alkaline phosphatase activity

Paraffin-embedded sections were de-paraffinized following standard procedures. Cells growing in monolayers were fixed with 4% paraformaldehyde. Sections or fixed cells were incubated at 70°C for 30 min in PBS to inactivate endogenous alkaline phosphatases followed by staining with 0.17mg/ml BCIP and 0.34mg/ml NBT in NTM buffer (100mM NaCl, 100mM Tris pH9.5, 5mM MgCl) for 30-45 min at 37°C in the dark.

Analysis of genomic DNA

Genomic DNA from islets or cells was prepared using DirectPCR lysis reagent (Viagen Biotech Cat #301-C) containing 0.5 mg/ml proteinase K according to the manufacturer's protocol. PCR conditions using 200 ng of genomic DNA and Hotstart 2x Master Mix (Qiagen) were 95°C for 15 sec; 40 cycles at 94°C for 45 sec, 54°C for 30 sec, 68°C for 45 sec; and a final extension at 68°C for 5 min. Primers for PCR were as described (15). Primer pair 1/2 yields a 290 bp product that identifies the M₃ receptor locus after excision by Cre recombinase. Primer pairs 3/4 (720 bp) and 5/6 (704 bp) are within and outside the region of the M₃ locus targeted for Cre excision, respectively.

Results and Discussion

Similar to our findings with human islet preparations (5), cells migrated out in monolayers from mouse islets placed in serum-containing medium (Fig. 1). After 3 days, most islets had adhered to the tissue culture dish and cells had begun to migrate away from the adherent islets (Fig. 1a). After 9 days, the islets had flattened further with many cells retaining immunopositivity for C-peptides or glucagon (Fig. 1b). However, it could not be determined whether all the fibroblast-like cells in the cultures were derived from islets. After harvesting with trypsin and re-plating, cells re-adhered to the tissue culture dish and proliferated, appearing morphologically homogeneous and fibroblast-like as illustrated by a typical culture after 50 days (Fig. 1c). These cultures expand exponentially with a doubling time of approximately 90 hr (Fig. 1d).

As the cultures containing mature cells within islets changed to cultures of fibroblast-like cells, the levels of transcripts expressed typically in endocrine/epithelial cells including insulin 1, insulin 2, glucagon, Ipfl (Pdx1), somatostatin, E-cadherin, claudin 3 and claudin 4, decreased whereas the levels of transcripts characteristic of mesenchymal cells, such as, vimentin, Thy1, smooth muscle alpha-actin and smooth muscle gamma-actin, increased (Table 1). These observations in cultures of mouse cells were similar to those previously made in cultures of

human cells (5). It is noteworthy that the levels of insulin 1, insulin 2 and glucagon mRNAs decreased more than one million-fold from the levels in mature endocrine cells to those in the proliferative fibroblast-like cultures. These changes in gene expression patterns are consistent with transition from stationary endocrine/epithelial cells that exhibit cell-cell contacts within islets to proliferative, migratory mesenchymal cells in tissue culture.

To show that these fibroblast-like cells are islet precursors, passage 5 and passage 7 cells (32- and 128-fold expanded, respectively) were cultured in differentiation medium to induce hormone-expression. Table 1 illustrates that upon culture in differentiation medium, the cells exhibited decreased levels of two of four mesenchymal transcripts and increased levels for most of endocrine/epithelial transcripts. It is important to note that while endocrine transcripts did increase during *in vitro* differentiation, all are still substantially below those of the normal mouse islet, similar to our previous findings with human islet-derived precursor cells (5). We conclude that these fibroblast-like cells are mIPCs based on their ability to differentiate into endocrine/epithelial cells that expressed insulin 1, insulin 2 and glucagon mRNAs and exhibited expression of C-peptide and glucagon peptide by immunostaining (see Fig. 2 below). Thus, islet precursor cells can be derived *in vitro* from adult islets of mice as well as humans.

We next attempted to obtain direct evidence that mIPCs were derived from beta cells using islets from MIP-GFP mice in which beta cells are labeled with green fluorescent protein (13) that could be followed as living beta cells continuously as they adapted to culture. Cultures of islet cells from MIP-GFP mice undergo the same change from endocrine/epithelial to fibroblast-like cells in culture as from nontransgenic mice (not shown). Thereafter, MIP-GFP cells proliferate as fibroblast-appearing cells also. Figure 2 illustrates that MIP-GFP mesenchymal cells, which had expanded 4000-fold and exhibited no detectable hormone expression by transcript or protein (not shown), differentiated into C-peptide- and glucagon peptide-expressing clusters after 7 days in differentiation medium. Thus, mIPCs can be derived from MIP-GFP mice. However, we did not observe expression of GFP from the MIP-GFP transgene, perhaps indicating positional effects or differential activity of the promoter regions included in the transgene.

We monitored islets from MIP-GFP mice continuously as they adapted to culture by fluorescence and phase contrast time-lapse microscopy. As reported previously by Hara *et al.* (13), there was co-expression of GFP and C-peptide in freshly isolated islets (not shown). Thus, fluorescent cells were beta cells that expressed insulin *in situ* and could be followed as living cells in culture by their fluorescence. Figure 3 illustrates individual snapshots of a MIP-GFP islet over a 4-day period. Fluorescent cells initially had an epithelial morphology within islets. Over time, the fluorescent epithelial cells elongated as they became adherent to the tissue culture surface. At later times, these cells migrated out from the disaggregating islet and became separated from each other. Thus, fluorescently-labeled beta cells were observed to undergo morphological changes from an epithelial/endocrine to a fibroblast-like phenotype. However, in most cells, fluorescence was lost within 7 to 14 days and it could not be determined whether all of the fibroblast-like cells were derived from fluorescently-labeled cells. Thus, these data show an initial change of beta cells from an epithelial to a fibroblast-like morphology but do not show that mIPCs are derived from fluorescently labeled beta cells.

We attempted to demonstrate a direct transition of beta cells to precursor cells by lineage analysis using two different mouse models – mice in which beta cells were permanently made to express human placental alkaline phosphatase (RIP/CreER:Z/AP mice) (3) and mice in which the M₃ muscarinic receptor was permanently deleted specifically in beta cells (15). In the first set of experiments, islets were isolated from RIP/CreER:Z/AP mice treated with tamoxifen for 2 weeks to induce alkaline phosphatase expression or without tamoxifen treatment (controls), and precursor cells were generated. More than 50% of the cells within the

islets stained positively for human alkaline phosphatase (not shown). mIPC cultures were stained for alkaline phosphatase after multiple passages beginning at passage 4. We found that less than 1% of cells stained positively for alkaline phosphatase in cultures from both tamoxifen-treated and control mice (not shown). In the second set of experiments, mIPCs were generated from beta cell-specific M₃ muscarinic receptor knock-out mice and control littermates. Three primer sets (15) were used to probe genomic DNA obtained from precursor cells and from the islets by PCR. We found the 290 bp PCR product expected after beta cell-specific, Cre recombinase-mediated deletion of part of the M₃ muscarinic receptor gene in islets from the knock-out mice and a time-dependent loss of this product in cells as they transitioned to a culture of mIPCs (Fig. 4). Because the 290 and 720 bp products reflect beta and non-beta cell origins respectively, we would have expected the 290 bp product to increase relative to the 720 bp product if the proliferative population had derived from beta cells. Thus, we found no evidence that proliferative precursor cells retained either marker of beta cell origin, alkaline phosphatase in the case of RIP/CreER:Z/AP mice or deletion of a segment of the M₃ muscarinic receptor gene from beta cell-specific M₃ receptor knockout mice. In both cases, these markers were readily detected in beta cells within the murine islets from which the proliferative cells were derived.

In conclusion, we have demonstrated that IPCs can be derived from preparations of adult islets of nontransgenic and transgenic mice using the same protocol as we previously used with human cadaveric islets. It is important to note that islet preparations from mice and humans are not homogeneous but are contaminated by non-islet tissue. Transgenic and knockout mouse models allow approaches not available in humans that will enhance our ability to understand the processes by which these cells are derived, expand and undergo differentiation. In this report we used transgenic animals in which beta cells are fluorescently labeled or permanently marked *in situ* to follow these cells during their generation *in vitro*. Using fluorescently labeled beta cells, we showed that beta cells can adapt to culture under our incubation conditions by becoming fibroblast-like but we were not able to follow these cells after several days in culture because they lost expression of green fluorescent protein. Moreover, we could not determine whether fibroblast-like cells in these cultures were derived from islets or whether some of these cells were from contaminating non-islet tissues. Using two different mouse models to permanently mark beta cells and determine whether mIPCs were derived from beta cells, we found no evidence that mouse precursor cells were derived from beta cells. A recent study described fibroblast-like cells derived from mouse islets which could differentiate into adipocytes and had properties similar to bone-marrow derived mesenchymal stem cells (16). Although their potential for islet endocrine differentiation was not explored, it was shown that these islet-derived cells, like those reported here, were not derived from beta cells (16). There are differences between mIPCs and hIPCs in their rates of generation from islets, in their morphologies and in their rates of proliferation. Thus, although we did not find that mIPCs are derived from mature mouse beta cells, the possibility can not be excluded that hIPCs are derived by EMT from human endocrine/epithelial cells; however this hypothesis remains unproved.

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Figure 1.

Derivation of islet precursor cells from preparations of adult mouse islets. a) A phase contrast image of a mouse islet after 3 days of in vitro culture. b) A mouse islet after 9 days in vitro. Left: Differential interference contrast (DIC) image. Right: Confocal image (1 μ m optical slice) after immunostaining with anti-C-peptides 1 and 2 and anti-glucagon antibodies. c) Phase contrast image of mesenchymal mouse islet-derived precursor cells (mIPCs) after 50 days in culture. d) Growth curves for 3 preparations of mouse mesenchymal cells. Ms01 and Ms02 were derived from normal mice and MIP-GFP01 was derived from a MIP-GFP transgenic mouse.

Figure 2.

Differentiation of islet precursor cells from MIP-GFP mice. Micrographs depict clusters of passage 12 mesenchymal cells that had been expanded at least 4000-fold. Cells were cultured in differentiation medium for 7 days prior to fixation, embedding in paraffin and sectioning. Left: DIC image. Right: Confocal image (0.6 μ m optical slice) after immunostaining with anti-C-peptides 1 and 2 and anti-glucagon antibodies.

Figure 3.

Selected images from time-lapse microscopy during *in vitro* culture of MIP-GFP islets. Images of a single MIP-GFP islet were acquired from days 3 through 7 at 15 minute intervals. Single images from each day from day 3 (d3) through day 7 (d7) are presented. Left: Phase contrast; Right: GFP epifluorescence.

Figure 4.

PCR analysis of genomic DNA from M_3 muscarinic receptor knockout mouse. Islets were prepared from floxed M_3 muscarinic receptor mutant mice (15) expressing Cre recombinase from the rat insulin promoter. Genomic DNA was isolated from a freshly isolated pancreas digest (Pancreas), from islet clusters after 2 days in RPMI media (Day 2), from adherent cells after 12 days (Day 12) and two days later (Day 14). PCR results using primer pairs 1/2 (290 bp), 3/4 (720 bp) and 5/6 (704 bp) are shown. The 290 bp product from primer pair 1/2 identifies the M_3 receptor locus after excision by Cre recombinase and is present in genomic DNA from pancreas and after 2 days in culture but is lost by days 12 and 14 in the resulting mesenchymal cell cultures.

Expression of representative epithelial/endocrine and mesenchymal mRNAs in mouse islets (Islets), proliferative mesenchymal mouse islet precursor cells (mouse islet precursor cells) (mIPCs) at passages 2, 5, 10 and 20 (p2, p5, p10, p20), and at initiation (Day 0) and after 4 days (Day 4) of differentiation of passage 5 (p5) and 7 (p7) mIPCs (Differentiation). All values are cycle thresholds from quantitative real time PCR (qRT-PCR) reactions using cDNA prepared from 50-100 ng total RNA and were normalized to mouse Gapdh to correct for differences in input RNA. Ins1, insulin 1; Ins2, insulin 2; Gcg, glucagon; Ipf1, insulin promoter factor 1; Sst, somatostatin; Glp1r, glucagon-like peptide 1 receptor; Gck, glucokinase; Glut2, solute carrier family 2 (facilitated glucose transporter), member 2; Cdh1, E-cadherin; Cldn3, claudin 3; Cldn4, claudin 4; Vim, vimentin; Thy1, thymus cell antigen 1; Acta2, actin, alpha 2; Actg2, actin, gamma 2.

Table 1

Islets	qRT-PCR cycle threshold									
	Mouse islet precursor cells					Differentiation				
	p2	p5	p10	p20	Day 0	Day 4	Day 0	Day 4	p7	
Endocrine/Epithelial										
Ins1	15	30	>38	>38	32	25	>38	>38	29	Day 4
Ins2	13	30	>38	>38	>38	27	>38	>38	29	
Gcg	19	27	>38	>38	31	25	36	36	27	
Ipf1	24	34	36	>38	36	32	35	35	34	
Sst	22	30	>38	>38	37	30	>38	>38	>38	
Glp1r	24	30	34	>38	31	34	34	34	33	
Gck	28	35	37	>38	>38	34	>38	>38	35	
Glut2	26	33	31	>38	31	32	>38	>38	>38	
Cdh1	20	30	31	32	32	31	34	34	33	
Cldn3	20	31	31	29	29	26	29	29	25	
Cldn4	18	29	30	28	28	24	28	28	23	
Mesenchymal										
Vim	22	17	17	16	15	16	16	16	15	
Thy1	27	20	20	19	18	18	19	19	18	
Acta2	25	21	21	21	20	25	20	20	23	
Actg2	35	20	21	21	19	25	20	20	24	