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Pancreatic Tissue Resident Mesenchymal Stromal Cell (MSC)-Like Cells as a Source of In Vitro Islet Neogenesis

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ABSTRACT

Insufficient β-cell mass is a common denominator for both type1 and type 2 diabetes. In vitro generation of β-cells from islet precursor cells, exocrine cells or ductal epithelia provide an alternative source of insulin-producing cells. However the presence of multipotent precursor cells within the pancreas is also deliberated. In this study we isolated mesenchymal stromal cell (MSC)-like cells from adult mouse pancreas by collagenase digestion. We used Knockout DMEM for our isolation procedure and the floating islets and acini were removed after 48 h. This strategy permitted the adhesion of stromal cells with typical mesenchymal morphology. These cells not only expressed MSC-specific markers like Sca-1, CD90.2, CD73, and CD44 but also generated osteocytes, adipocytes, and neurons when induced with specific growth media. Upon exposure to islet differentiation serum-free cocktail a significant upregulation of pancreatic markers like Nkx2.2, Nkx6.1, Pdx1, insulin, and somatostatin was seen. The differentiated islet-like cell aggregates (ICAs) secreted insulin which increased over the days in culture in presence of basal glucose levels. Taken together, our data strongly indicate that there is a tissue-resident precursor population within the pancreas that can be exploited for islet neogenesis in vitro. J. Cell. Biochem. 114: 2240–2247, 2013.

KEY WORDS: β-CELL ENRICHMENT; MESENCHYMAL STROMAL CELL DIFFERENTIATION; ISLET NEOGENESIS; INSULIN SECRETION

The functional mass of β-cells is reduced in most forms of diabetes, hence replacing the lost β-cells or triggering their regeneration can be an alternate research focus bearing both basic and applied value in diabetes treatment. Therefore expansion of the β-cell mass from endogenous sources; either in vivo or in vitro is of soaring interest and have raised tremendous optimism among both scientists and clinicians. The most promising approach includes the replacement of the β-cell mass, which is currently practiced through ectopancreatic transplantation. However there is a chronic shortage of donors which calls for other possible alternatives [Shapiro et al., 2000]. The presence of pluripotent/multipotent adult stem cells in most, if not all organs presents a potential source for new islet cells. Nevertheless "pancreatic stem cells" is an elusive area till date. Majority believe that a facultative role is assumed by the differentiated epithelial cells in the pancreas, which then dedifferentiates and proliferates repeatedly to serve a stem cell role [Bonner-Weir and Sharma, 2002].

Ex vivo derivation of pancreatic β-cells, acinar cells, and ductal epithelial cells in order to produce insulin-producing functional islets have met with considerable success [Venkatesan et al., 2011]. The use of autologous tissue-specific stem cells is now challenging the conventional wisdom that once established; damage is permanent to organs, and that, once lost, tissues cannot be restored. Although a true pancreatic stem cell has not yet been identified, several candidate cell types are in the forefront [Lechner and Habener, 2003] which have been classified by various studies on islet neogenesis in rodent injury models [Bonner-Weir et al., 1993; Fernandes et al., 1997; Guz et al., 2001]. In this report we describe the generation of insulin producing islet-like cell aggregates (ICAs) from mesenchymal stromal cell (MSC)-like cells of pancreatic origin.

MATERIALS AND METHODS

ISOLATION AND EXPANSION OF MESENCHYMAL STROMAL CELL (MSC)-LIKE CELLS FROM ADULT MOUSE PANCREAS

All animal experiments were performed in accordance with the guidelines of Manipal University Animal Ethics Committee. The protocol for the isolation of islets from the pancreas was carried out as

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described earlier [Shewade et al., 1999]. Groups of two to four mice were killed by cervical dislocation, and pancreata were removed aseptically without ductal injection and distension. Here we used 12–14 weeks old CF-1 mice weighing 25–30 g. The pancreata were cut into small pieces and washed thrice with HBSS. The resulting pieces were subjected to collagenase digestion using a magnetic stirrer. The dissociation medium consisted of Dulbecco's modified minimum essential medium (DMEM) supplemented with 1 mg/ml collagenase type V (Sigma) and 2% BSA fraction V (Sigma). The tissue digest was then transferred to a 50 ml conical flask, and digestion was stopped by addition of chilled DMEM with 10% fetal calf serum. The mixture was then centrifuged at 1,000 rpm for 10 min, vortexed and the pellet was seeded in T25 culture flasks. A notable modification was the usage of DMEM Knockout media (Invitrogen) with 10% FBS (Hyclone) for the isolation procedure instead of RPMI-1640. The same media was then used for the propagation and expansion of these cells. The cells seeded in T25 flasks were incubated at 37°C in an atmosphere comprising of 5% CO_2 for 48 h. The flasks were rinsed by removing the supernatant and flushing out the islets with media. Fibroblastoid-like cells seen attached to the surface were passaged every 3rd to 4th day once they acquired 80–90% confluency. The cells were thereafter maintained under similar conditions. Cell growth was analyzed by direct cell count to determine the log, lag, and stationary phases. For this purpose, the cells were seeded onto a 12-well tissue culture dish at a density of 3×10^3 cells/cm^2. Viability of cells was checked with trypan blue dye exclusion test using 0.4% (w/v) trypan blue in 1× PBS (ICN Pharmaceuticals, Inc., USA). Cells stained blue were scored as non-viable while, unstained (due to exclusion of the dye) were scored as viable cells using hemocytometer. Viable cells were counted in triplicates every 48 h using the trypan blue (Sigma) dye exclusion test.

FLOW CYTOMETRY AND CELL CYCLE ANALYSIS

Cell surface markers present on MSC-like cells were analyzed by flow cytometry using an antibody panel comprising of Sca-1, CD90.2, CD44, CD73, and CD45 (all PE labeled, BD). Non-specific fluorescence was determined with appropriate isotype controls. Harvested cells were washed twice with PBS and fixed with 1% PFA for 10 min. Non-specific binding sites were blocked using 5% BSA for 20 min at 4°C after two successive washes with PBS. Cells were then incubated in antibody containing buffer (PBS containing 1% FBS and 0.01% sodium azide) on ice for 45 min. After three washes with 1× PBS containing 0.5% BSA cells were re-suspended in 250 μl of PBS, transferred into FACS tubes and acquired on a BD FACS Calibur flow cytometer. The data was analyzed using the BD Cell Quest Pro software. For cell cycle analysis, cells were fixed in 70% ethanol, rehydrated in PBS, treated with RNase A (1 mg/ml) for 30 min and stained with 1 μg/ml of propidium iodide (PI) for 45 min at dark. The cells were acquired on BD LSR II flow cytometer and analyzed using the WinMDI software.

IMMUNOCYTOCHEMISTRY

The cells were fixed with 4% paraformaldehyde (PFA) diluted in PBS for 20 min at room temperature (RT) on two-well chambered slides. Cells were then permeabilized with 0.2% Triton X-100 (Sigma) and blocked with 0.1% (w/v) bovine serum albumin (BSA; Sigma) to minimize non-specific binding of antibodies. The samples were incubated with primary antibodies for vimentin, α-SMA, nestin, Pdx1 (all from Millipore), GLUT2 (Abcam), and insulin (Abexome) overnight at 4°C. Fluorescein isothiocyanate (FITC) or Alexa Fluor 594 (Millipore) conjugated secondary antibody incubation was performed at RT for 1 h in the dark. Cell nuclei were counterstained with 4′-6-diamidino-2-phenylindole (DAPI, 10 μg/ml; Sigma). Fluorescent images were captured using a Nikon Eclipse 80i (Nikon) microscope and analyzed with Q imaging software.

DIFFERENTIATION CULTURE CONDITIONS

To promote adipogenic differentiation, MSC-like cells were treated to three cycles of induction/maintenance to stimulate optimal adipogenic differentiation as per manufacturer's instructions (Millipore, Cat No: SCR020). Each cycle consisted of feeding the cells with supplemented adipogenesis induction medium and culture for 5 days (37°C, 5% CO_2) followed by 1–3 days of culture in supplemented adipogenic maintenance medium (a total of 21 days). The lipid rich vacuoles in the cells were stained positive by Oil Red O. For osteogenic differentiation, MSC-like cells were plated on vitronectin coated 24-well tissue culture plates. When they were 100% confluent, Osteogenesis Induction Medium was added. The cells were fed every 3–4 days for 2–3 weeks by completely replacing the medium with fresh Osteogenesis Induction Medium as per manufacturer's instructions (Millipore, Cat No: SCR028). Alizarin Red S stain indicated direct evidence of calcium deposits as amorphous accumulation between cells after 3 weeks. For neuronal induction, cells were grown in DMEM containing 1% FBS and supplementary 100 ng/ml bFGF for 14 days. Presence of neural cells in the monolayer cultures was confirmed by staining with β-tubulin III (Sigma) as described in the Immunocytochemistry Section.

ISOLATION OF TOTAL RNA, PREPARATION OF cDNA AND RT-PCR

Cell pellets were collected and total RNA was isolated by the Trizol method (Invitrogen) as per the manufacturer's protocol and was further quantified using spectrophotometry (Agilent, NanoDrop, Technologies, Inc.). The first strand of cDNA was synthesized using 1 μg of RNA treated with RNase-OUT ribonuclease inhibitor and Superscript III First Strand Synthesis system (Invitrogen) as per the manufacturer's instructions. The details of the primers and the amplification program are provided (Table I). Amplified PCR products were analyzed on 1.5% agarose gel stained with ethidium bromide (EtBr, Sigma), viewed under a UV trans-illuminator and photographed on a gel documentation system (Alpha Innotech, CA, USA). Arbitrary band intensities were measured using the densitometry parameter of Alpha Imager software and the values were normalized to GAPDH housekeeping genes for relative quantitative measurements.

DIFFERENTIATION INTO ISLET-LIKE CELL CLUSTERS

The protocol used for differentiation of stem cells to islet-like clusters (ILCs) was adapted from Gershengorn et al. [2004], wherein PANC-1 cells were induced into islet like cell aggregates and later modified by Kadam et al. [2010]. MSCs were re-suspended in serum free (SFM) medium containing 1% BSA and 1× insulin transferrin selenium (ITS). On Day 3 cells were allowed to settle in sterile centrifuge tube

TABLE I. List of Genes Along With the Forward and Reverse Sequences, Annealing Conditions, and Accession Number

Gene symbol	Primer sequences	Ann. temp. (T_a)	Amplicon size (bp)	Accession no.
GAPDH	5′-TCAACAGCAACTCCCACTCTTCCA-3′ 5′-ACCCTGTTGCTGTAGCCGTATTCA-3′	66°C	115	NM_008084.2
Ki-67	5′-GACAGCTTCCAAAGCTCACC-3′ 5′-TGTGTCCTTAGCTGCCTCCT-3′	58°C	230	NM_001081117.2
Sca-1	5′-CAATGTAGCAGTTCC-3′ 5′-CAGGGGCTATAAAGG-3′	44°C	241	NM_010738.2
CD29	5′-GCCAGGGCTGGTTATACAGA-3′ 5′-TCACAATGGCACACAGGTTT-3′	58°C	226	NM_010578.2
CD44	5′-TGGATCCGAATTAGCTGGAC-3′ 5′-AGCTTTTTCTTCTGCCCACA-3′	58°C	189	NM_009851.2
α-SMA	5′-CTGACAGAGGCACCACTGAA-3′ 5′-CATCTCCAGAGTCCAGCACA-3′	58°C	160	NM_007392.2
Vimentin	5′-ATGCTTCTCTGGCACGTCTT-3′ 5′-AGCCACGCTTTCATACTGCT-3′	58°C	206	NM_011701.4
Nestin	5′-ATACAGGACTCTGCTGGAGG-3′ 5′-AGGACACCAGTAGAACTGGG-3′	56°C	421	NM_016701.3
Nkx2.2	5′-TGTGGATTTGAGGGATGTCTGGGT-3′ 5′-GGGACAAGCACCAAGCCAAAGAAT-3′	67°C	93	NM_001077632.1
Nkx6.1	5′-ACCAAGTGGAGAAAGAAGCACGCA-3′ 5′-AGAGTTCGGGTCCAGAGGTTTGTT-3′	65°C	181	NM_144955.2
Pax 6	5′-TCGAAGGGCCAAATGGAGAAGAGA-3′ 5′-ACTGGTACTGAAGCTGCTGCTGAT-3′	64°C	103	NM_001244202.1
Ngn3	5′-AATCGCATGCACAACCTCAACTCG-3′ 5′-AGCGCAGGGTCTCGATCTTTGTAA-3′	65°C	100	NM_009719.6
GATA-4	5′-TCAAATTCCTGCTCGGACTTGGGA-3′ 5′-GTT TGAACAACCCGGAACACCCAT-3′	67°C	163	NM_008092.3
HNF-3β	5′-AAGGGAAATGAGAGGCTGAGTGGA-3′ 5′-ATGACAGATCACTGTGGCCCATCT-3′	62°C	130	NM_010446.2
Pdx1	5′-ACTTAACCTAGGCGTCGCACAAGA-3′ 5′-GGCATCAGAAGCAGCCTCAAAGTT-3′	64°C	135	NM_008814.3
Pan pol	5′-ATGGACTTGCAGCCTCTCTTGTCT-3′ 5′-AGTTTGCAAGGGAGCAGGTTGTTG-3′	65°C	101	NM_008918.1
Insulin (type II)	5′-AGCGTGGCATTGTAGATCAGTGCT-3′ 5′-AGTGGTGGGTCTAGTTGCAGTAGT-3′	62°C	81	NM_008386.3
Somatostatin	5′-AGCCCAACCAGACAGAGAATGATG-3′ 5′-TCAGAGGTCTGGCTAGGACAACAA-3′	65°C	208	NM_009215.1

The amplification program for PCR consisted of an initial denaturation step at 94°C for 5 min, followed by 35 cycles of 30 s denaturing (94°C), 45 s annealing (temperature of the respective gene primer), 45 s elongation (72°C); a final extension at 72°C for 10 min and finally soaking at 4°C.

and reseeded in above medium with addition of 0.3 mM taurine. On Day 7, the cell clusters were fed with SFM containing 1.5% BSA, 100 mM nicotinamide, 3 mM taurine, and 100 mM GLP-1. A progressive cell clustering from Day 2 onwards which led to typical ILCs formation at the end of Day 10 was seen. It is noteworthy that here we used DMEM Knockout as the basal media instead of RPMI-1640.

INSULIN ELECTRO-CHEMILUMINESCENCE IMMUNOASSAY (ECLIA)

For the glucose-stimulated insulin release assay about 1,000 ICAs were handpicked in an eppendorf tube on days 3, 7, 10, washed twice with Krebs Ringer's Bicarbonate-HEPES buffer (KRBH-120 mM NaCl, 5 mM KCl, 2.5 mM $CaCl_2$, 1.1 mM $MgCl_2$, and 25 mM $NaHCO_3$, pH 7.4) without glucose and incubated with 200 μl KRBH buffer containing 5.5 mM glucose for 1 h at 37°C. The secreted insulin (extracellular) was measured by ECLIA with Roche Elecsys Cobas diagnostic kits (Catalogue number: 12017547) on COBAS 6000 MODULAR SYSTEM, with e 601 module for immunoassays. The Elecsys Insulin assay employs the sandwich principle with a biotinylated insulin-specific antibody, and an insulin-specific antibody labeled with a ruthenium complex. Results are determined via a calibration curve which is instrument-specifically generated by 2-point calibration and a master curve provided via the reagent barcode. The samples were analyzed in duplicates as per the manufacturer's instructions.

RESULTS

ISOLATION, GROWTH CHARACTERISTICS SURFACE MARKER EXPRESSION, AND MULTIPOTENCY OF MSC-LIKE CELLS ISOLATED FROM MOUSE PANCREAS

We show that MSC-like cells can be derived independently from the mouse pancreas. Following a small modification of our earlier report on large scale isolation of murine islets [Shewade et al., 1999], we successfully isolated plastic adherent fibroblastoid-like cells in the culture flasks within 48 h by using DMEM Knockout media (Fig. 1A). After carefully removing the floating islets and the acinar cells present in the supernatant, the attached cells were allowed to grow as adherent cultures. They resembled MSCs in morphology and attained confluency by days 3–4 (Fig. 1B–D). The cells could be consistently maintained in an undifferentiated state/phenotype for more than 10 passages. However they showed a trend to become more flattened in morphology towards the later passages such as P9–P10 (Fig. 1E).

The cells showed a population doubling time of 20 h which was consistent till 6–7 passages (Fig. 2A); beyond P7 the proliferation rate declined and the doubling time increased considerably (data not shown). So cells from P3 to P6 were used for the characterization and differentiation experiments to maintain uniformity of results. PI staining (at P5) revealed that 54.5% of the cells were in G0/G1 phase whereas almost 22% were actively dividing (S + G2/M) (Fig. 2B). The

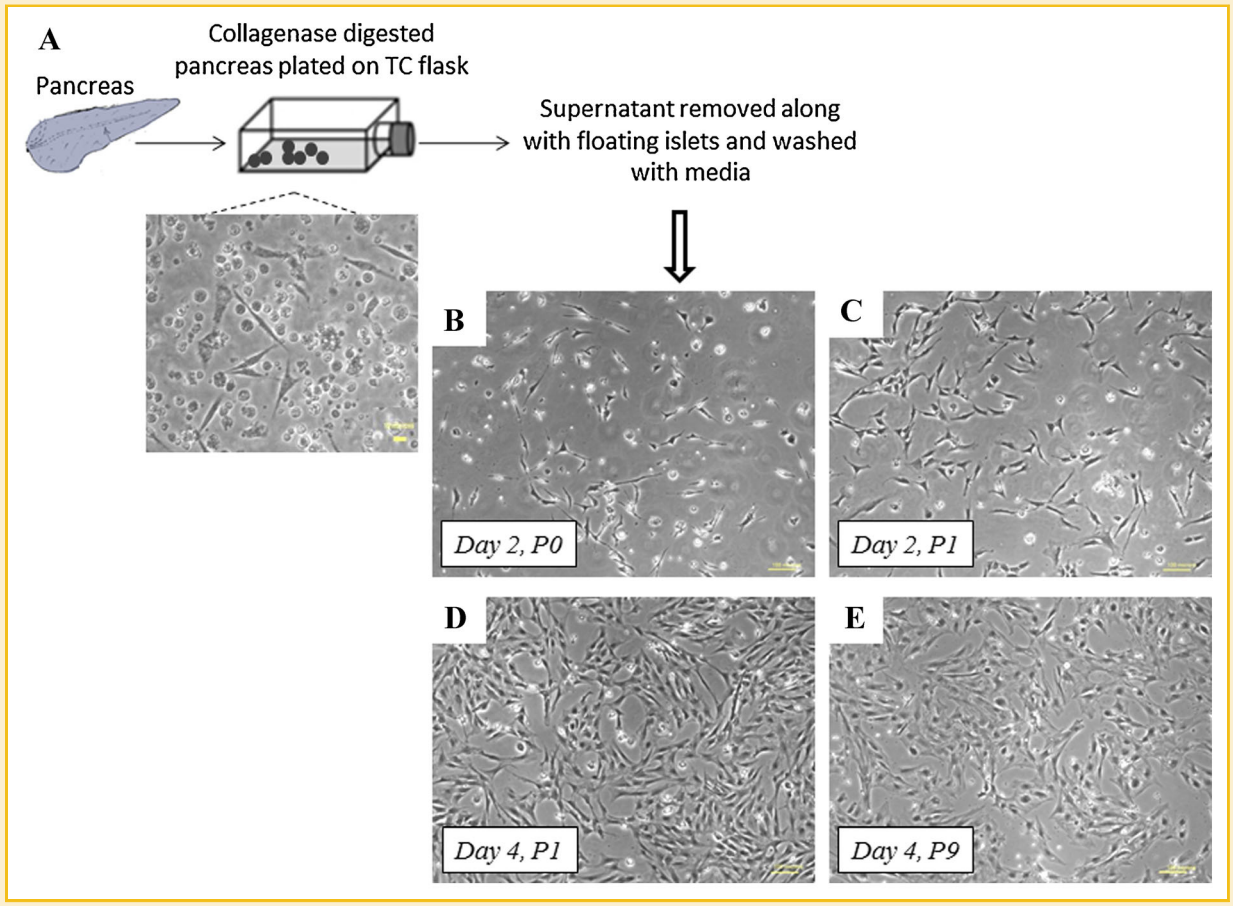

Fig. 1. Isolation of MSC-like cells from mouse pancreas. A: Schematic representation of the isolation procedure in brief: Collagenase digested pancreas plated in Knockout DMEM led to appearance of adherent cells which were then propagated after removal of the supernatant and floating islets. Scale bar: 10 μm. B–E: Morphology of the isolated cells at different passages: P0—Day 2, P1—Day 2, P1—Day 4 (confluent culture) and P9—Day 4 (flattened morphology) respectively. Scale bar: 100 μm.

MSCs were characterized using flow cytometry based positive reaction for known mesenchymal lineage markers like Sca-1$^+$ (95.6%), CD90$^+$ (86.6%), CD44$^+$ (74.2%), CD73$^+$ (66.7%); and negative for hematopoietic marker CD45$^-$ (2.9%) (Fig. 2C). The surface antigen expression profile conformed to the criteria defined for mouse multipotent MSCs [Peister et al., 2004]. Further, the MSC-like cells upon induced differentiation gave rise to osteocytes and adipocytes, as evidenced by positive staining with Alizarin Red S and Oil Red O respectively (Fig. 2D), albeit to a lesser extent when compared to mouse bone marrow derived MSC (data not shown). Moreover, neuronal differentiation was readily achieved when the cells were treated with high concentration of bFGF (100 ng/ml) in low serum conditions for 12 days (Fig. 2D). Hence we conclude that the cells isolated from pancreas are multipotent demonstrating the capacity to differentiate into multiple cell types surpassing lineage barriers.

MESENCHYMAL MARKERS ARE EXPRESSED AT THE mRNA TRANSCRIPT LEVEL EVEN AFTER DIFFERENTIATION

Immunocytochemical analysis of fixed adherent cells showed positive expression of cytoskeletal proteins like nestin, vimentin, and α-SMA (Fig. 3A–C). The cells were also positive for Pdx1 and GLUT2 (Fig. 3D–G). The MSC-like cells were allowed to differentiate into islets as described earlier (see the Isolation of Total RNA, Preparation of cDNA and RT-PCR Section) and samples were collected on days 0, 3, 7, and 10 of differentiation for RT-PCR studies. The proliferation marker Ki-67 was found to be expressed at Day 0 but showed a gradual downregulation beginning from Day 3 to Day 10 (Fig. 3H) as expected. However the mesenchymal markers like Sca-1, CD29, CD44, α-SMA, vimentin and the intermediate filament protein nestin were expressed almost uniformly throughout the course of differentiation (Fig. 3H). Conspicuously, this is in accordance with the data on intra-islet precursors reported by Kayali et al. [2007]. It was shown that expanded islet cells redifferentiated into islet-like clusters which were closer in phenotype to mesenchymal cells than to the endocrine cells from which they were derived [Kayali et al., 2007].

PANCREATIC MARKERS UNDERGO UPREGULATION DURING IN VITRO ISLET DIFFERENTIATION

We show here that the undifferentiated MSC-like cells readily differentiated into islet-like clusters in presence of induction media

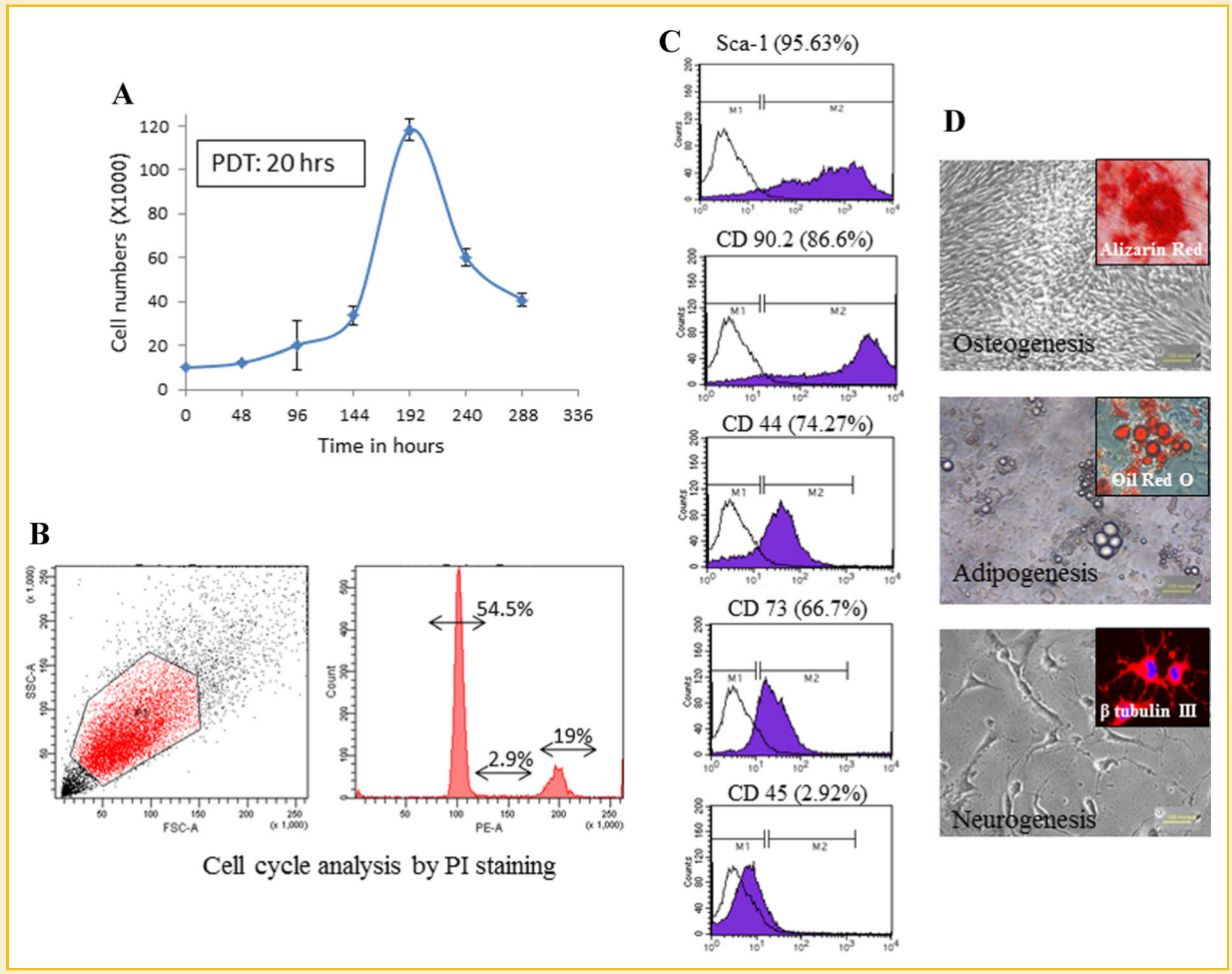

Fig. 2. Growth characteristics and surface marker expression of the isolated MSC-like cells. A: Population doubling time (PDT) determined in triplicates by trypan blue cell counting method at P3, PDT: 20 h. B: Cell cycle analysis by PI staining: G0/G1 (54.5%), G2/M (19%), and S (2.9%) phase populations are shown. C: Surface marker expression by flow cytometry using PE-labeled rat anti-mouse IgG antibodies. Non-specific fluorescence was negated with isotype controls; positive expression was seen for Sca-1 (95.63%), CD90.2 (86.61%), CD44 (74.27%), CD73 (66.7%) and the cells were negative for the hematopoietic marker CD45 (2.92%). D: In vitro osteogenesis (inset—as evidenced by calcium deposition stained with Alizarin Red S), in vitro adipogenesis (inset—demonstrated by Oil Red O staining) and neurogenesis (inset—immunofluorescent staining with β-tubulin III antibody). Scale bar: 100 μm.

(Fig. 4A–C). The 10-day-old islets stained strongly positive for insulin as shown by immunocytochemistry as compared to the undifferentiated MSC-like cells. Primary islets were used as positive control (Fig. 4D,E). Remarkably, even at the undifferentiated level, the isolated cells expressed the key transcription factors as well as the hormones of pancreatic lineage. A prominent upregulation of Nkx2.2, Nkx6.1, Pax6, Ngn3, GATA4, Pdx1, insulin2, somatostatin, and pancreatic polypeptide was detected on differentiation Day 7 (Fig. 4F). However these markers were significantly down regulated at Day 10 possibly due to physiological apoptosis and degeneration of the in vitro generated islets which is not surprising. The only exception was HNF3-β which started appearing a bit late at Day 7 and was upregulated at Day 10 (Fig. 4F). The band intensities of the various markers were measured at Day 0 and Day 7 by densitometry and normalized to GAPDH controls (Marker value/GAPDH value). Nkx6.1, Nkx2.2, Pax 6, and somatostatin showed two- to threefold increase at Day 7 versus Day 0, whereas the expression of Ngn3, Pdx1, GATA4, and insulin2 was 1.5-fold higher at Day 7. Pancreatic polypeptide and HNF3-β were absent at Day 0 and had an intensity value of 60.6 and 25 at Day 7 respectively (Fig. 4G). Gene transcripts expressed in primary mouse islets were used as reference standards.

DIFFERENTIATION INTO ISLET-LIKE AGGREGATES AND INSULIN SECRETION

The differentiated islets were subjected to a glucose challenge at a basal level of 5.5 mM and an induction level of 16.5 mM for the estimation of secreted insulin. Insulin levels were undetectable by ECLIA in the undifferentiated cells (5×10^6 cells) but gradually increasing levels of secreted insulin were detected at the basal glucose concentration on Day 3, Day 7, and Day 10 with values of 54.72, 383.8, and 498 uIU/ml, respectively (Fig. 4H). Primary mouse islets were used as control in this experiment. When the islets were plated in serum containing media, they readily reverted back to an adherent fibroblast-like morphology within 4–5 days (data not shown).

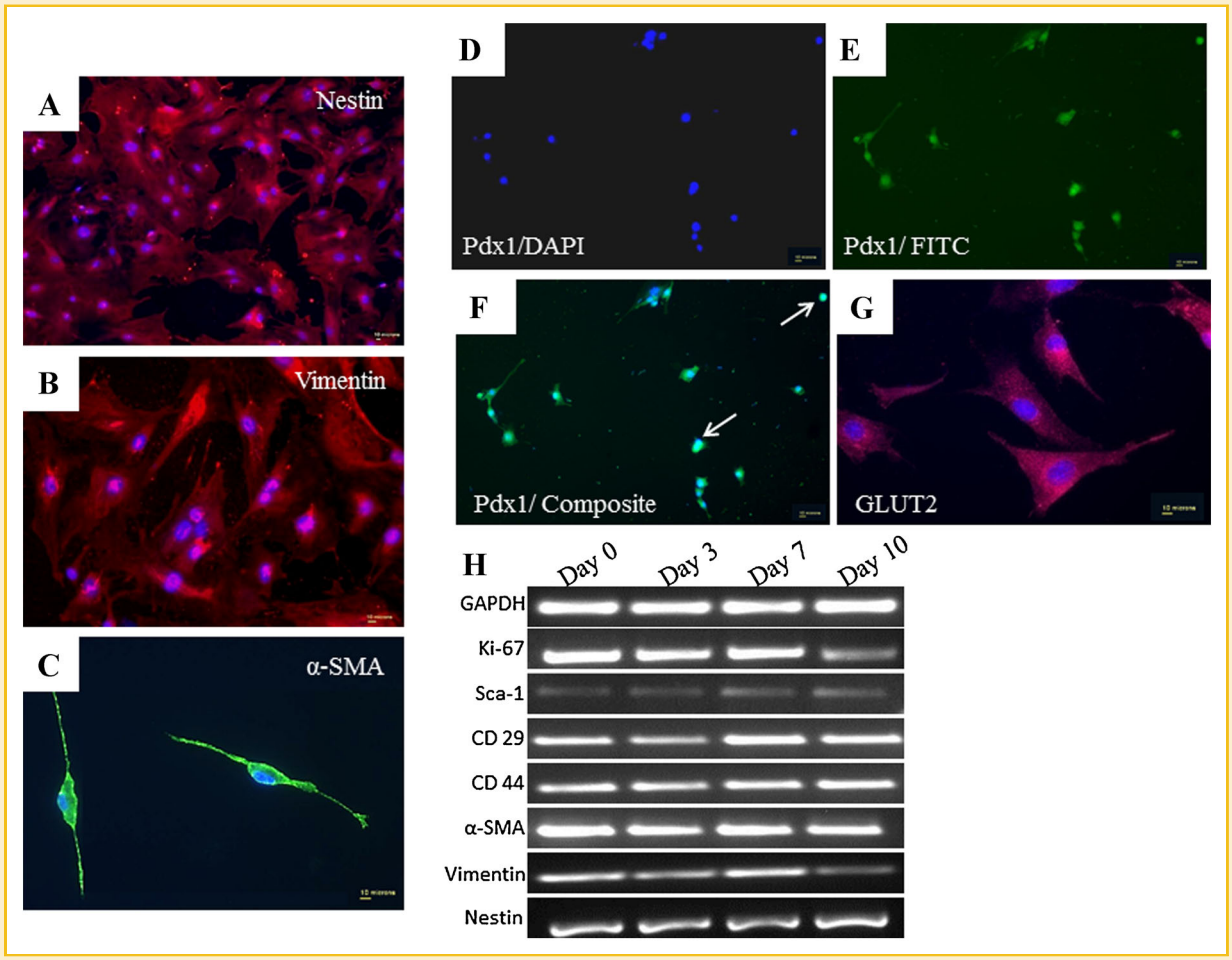

Fig. 3. Mesenchymal markers are expressed at the mRNA transcript level even after differentiation. Immunocytochemistry of the MSC-like cells: (A) nestin (Alexa Fluor 594 + DAPI), (B) vimentin (Alexa Fluor 594 + DAPI), (C) α-SMA (FITC + DAPI), and (D–F) Pdx-1 (FITC + DAPI), arrows show nuclear localization, (G) GLUT2 (Alexa Fluor 594 + DAPI); scale bar: 10 μm. H: Reverse transcriptase PCR analysis of cells harvested at Day 0, Day 3, Day 7, and Day 10 of differentiation in serum-free induction media for mesenchymal markers showed their presence through the course of differentiation almost uniformly (samples normalized to GAPDH). PCR products ran on a 1.5% agarose gel stained with ethidium bromide (EtBr).

DISCUSSION

In the present study, we isolated MSC-like cells from the pancreatic tissue of adult mice using a simple isolation procedure with collagenase digestion and culture in Knockout DMEM media. The floating population of cells majorly composed of islets and acinar cells which were removed after 48 h so that the adherent monolayer cultures could further be maintained and expanded. During the last few years, different approaches for the neogenesis of β-cells have been described employing embryonic stem cells, mesenchymal stem cells, non-pancreatic cell types and even adult stem cells residing in the pancreas [Peck et al., 2001]. In the postnatal pancreas there is no evidence of stem or progenitor cell contribution to either endocrine or exocrine compartment, the growth of which can be accounted for by proliferation of existing differentiated cells [Dor et al., 2004; Desai et al., 2007; Strobel et al., 2007]. Till date, three main theories are proposed to explain the origins of newly formed islets: (i) transdifferentiation of acinar or ductal cells into islets, (ii) islet neogenesis, a process reminiscent of islet formation during embryonic development, and (iii) replication of preexisting islet cells. Nonetheless the origin of new adult β-cells remains controversial and these theories are still questioned [Granger and Kushner, 2009]. The process of neogenesis from islet precursors may be activated following damage to the pancreas or to the β-cells themselves [Murtaugh and Kopinke, 2008].

The isolated cells conformed to the general characteristics of MSCs in being plastic adherent and showing positive expression of specific cell surface markers on flow cytometry-based analyses. Moreover, gene transcripts of mesenchymal markers like Sca-1, CD44, CD29, vimentin, and α-SMA were also observed. The expanded monolayer cells differentiated into osteocytes, adipocytes, and neural cells on treatment with appropriate induction media. In addition they demonstrated the potential to readily differentiate into insulin producing ICAs. Although the exact origin of these unique population of cells remains elusive, but one could rule out the β-cell dedifferentiation theory as several studies have irrevocably disproved

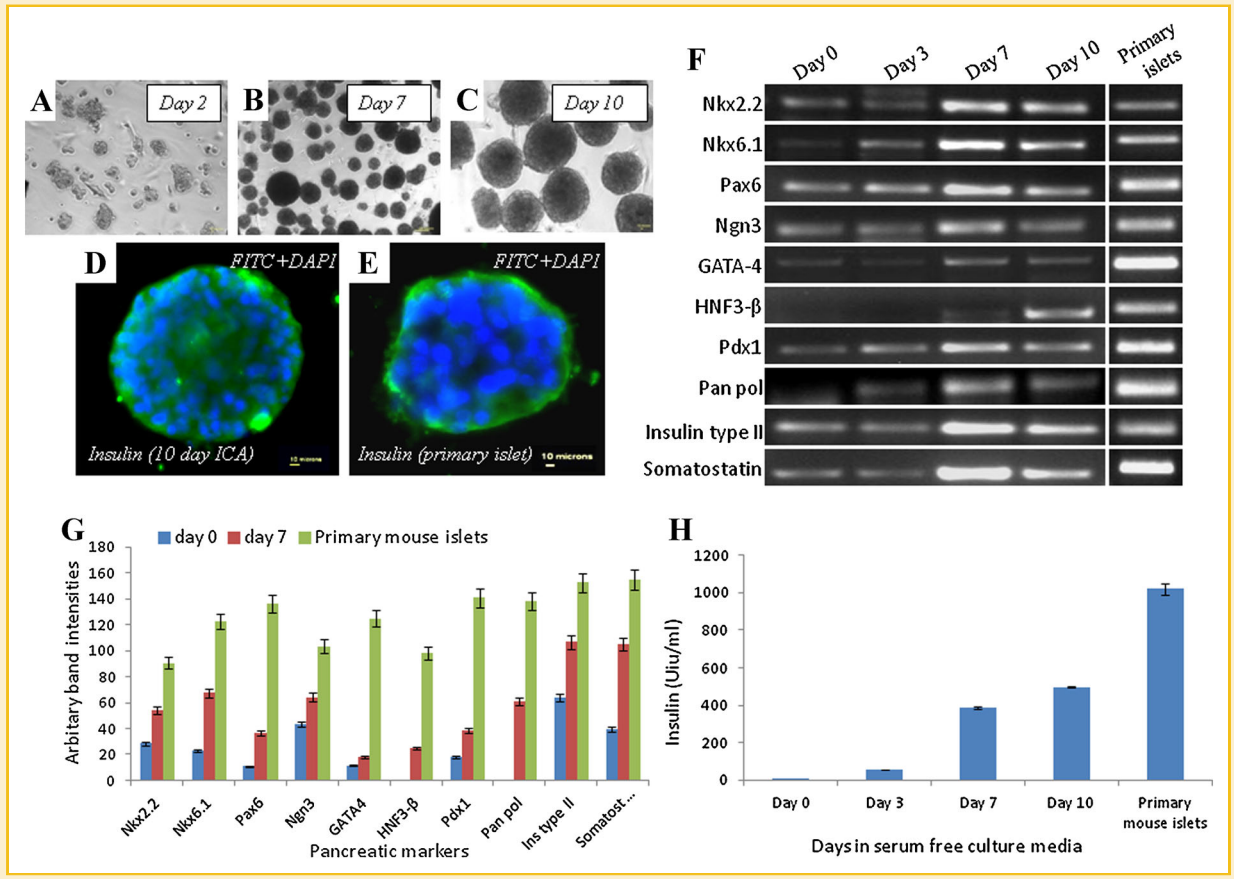

Fig. 4. Expression profile of early and late-stage pancreatic markers and functional studies of the in vitro generated islet-like cell aggregates (ICAs). A–C: Morphology of the islet-like aggregates at Day 2, Day 7, and Day 10 respectively. D: Ten-day aggregates stained positive for insulin (FITC + DAPI). E: Primary islet stained positive for insulin (FITC + DAPI). Scale bar: 10 μm. F: Reverse transcriptase PCR analysis of cells at Day 0, Day 3, Day 7, and Day 10 of differentiation in serum-free induction media for pancreatic and islet-specific markers showed their upregulation at Day 7 of differentiation (samples normalized to GAPDH). HNF-3β started appearing at Day 7 and was upregulated at Day 10. PCR products ran on a 1.5% agarose gel stained with EtBr. Gene transcripts of ICAs were compared with that of mouse pancreatic islets. G: Arbitrary band intensities estimated by densitometry at Day 7 versus Day 0 and normalized to GAPDH (Marker value/GAPDH value). Densitometry values of transcripts in mouse primary islets were considered as reference standards; (H) Insulin secretion of the islet-like aggregates at Day 0, Day 3, Day 7, and Day 10. Insulin levels were undetectable by ECLIA in the undifferentiated cells (5×10^6 cells) but increasing levels of extracellular insulin were detected at the basal glucose concentration (5.5 mM) at Day 3, Day 7, and Day 10. Freshly isolated mouse primary islets were used as control in this experiment.

the occurrence of mesenchymal transition of mouse islet β-cells in vitro [Atouf et al., 2007; Chase et al., 2007; Morton et al., 2007; Weinberg et al., 2007]. Only one study so far has been reported on non-islet pancreatic cells (pancreatic discard) for islet generation, that too from human samples [Todorov et al., 2006]. Todorov's group found the pancreatic discard (2006) to contain cells expressing acinar markers, a lesser percentage of cells positive for ductal markers and less than 1% endocrine components. However to date, no adult pancreatic stem cell has been completely characterized.

The MSC-like cells expressed β-cell specific transcription factors and islet hormones at the transcriptional levels but not at the translational (protein) level, indicating the selective propensity towards islet lineage only. The endocrine transcription factors like Nkx6.1, Nkx2.2, Pdx1, and Pax6 showed a profound amplification upon serum withdrawal followed by induction especially on Day 7 of differentiation. The gene transcript levels of key hormones like insulin (type II), somatostatin and pancreatic polypeptide were also clearly enhanced on Day 7 exhibiting a similar trend. The cascade of transcription factors we studied here along with many others are specifically involved in normal pancreatic development and maturation of endocrine lineages [Oliver-Krasinski and Stoffers, 2008], thereby rationalizing our proposition. The induction of these cells at a high seeding density in glass petri dishes with serum free medium containing a cocktail of ITS, nicotinamide, and taurine showed a progressive cell clustering from Day 2 onwards which led to typical ICA formation by Day 7–Day 10. These clusters stained positive for the islet-specific DTZ stain (data not shown), which is known to selectively stain pancreatic beta cells because of their high zinc content.

An increased amount of insulin was secreted by the aggregates over time in culture upon exposure to a glucose concentration of 5.5 mM; the day10 clusters showing a maximum of 498 uIU/ml. However the insulin (type II) gene expression levels were higher at Day 7 than Day 10 in differentiation. The correlation of mRNA and protein levels may not be accurate at all times; several post-transcriptional and post-translational modifications may account for

the observed differences between the transcriptome and translatome [Maier et al., 2009]. We reason that a similar phenomenon could be responsible for the discrepancy we observed between the gene expression pattern and secreted insulin levels by the ICAs. Furthermore, there is enough evidence suggesting that insulin exocytosis is not an all-or-none process [MacDonald et al., 2005]. Hence it is difficult to draw a direct correlation between the insulin data at the mRNA and protein levels.

Nevertheless this study differs from other published literature so far in that the MSC-like cells have not been isolated from the intra-islet precursors of mouse pancreas. The monolayer cultures of MSC-like cells could be maintained for several passages in their original state, exhibiting the potency to readily differentiate into insulin producing aggregates on exposure to differentiation cocktail. Importantly this unique protocol can be easily scaled up for various experimental purposes so as to generate large number of ICAs for prospective diabetes research and therapeutic applications. Cellular therapy using MSCs has been proposed as an improved and feasible alternative to whole-organ transplantation for the management of diverse diseases. We strongly believe that these unconventional MSC-like cells could be used as an alternative source of stem cells for treating diabetes. In addition to known pancreatic progenitor population residing within pancreatic ducts, the MSC-like cells reported in our study can act as a potential therapeutic target for diabetic subjects to induce islet neogenesis endogenously leading to restoration of beta cell mass and control of hyperglycemia.

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