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Mesenchymal Cells Appearing in Pancreatic Tissue Culture Are Bone Marrow-Derived Stem Cells With the Capacity to Improve Transplanted Islet Function

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Key Words. Mesenchymal cells • Pancreatic tissue • Islet function

ABSTRACT

Adherent fibroblast-like cells have been reported to appear in cultures of human endocrine or exocrine pancreatic tissue during attempts to differentiate human β cells from pancreatic precursors. A thorough characterization of these mesenchymal cells has not yet been completed, and there are no conclusive data about their origin. We demonstrated that the human mesenchymal cells outgrowing from cultured human pancreatic endocrine or exocrine tissue are pancreatic mesenchymal stem cells (pMSC) that propagate from contaminating pMSC. The origin of pMSC is partly extrapancreatic both in humans and mice, and by using green fluorescent protein (GFP^+) bone marrow transplantation in the mouse model, we were able to demonstrate that these cells derive from the $CD45^+$ component of bone marrow. The pMSC express negligible levels of islet-specific genes both in basal conditions and after serum deprivation or exogenous growth factor exposure, and might not represent optimal candidates for generation of physiologically competent β-cells. On the other hand, when cotransplanted with a minimal pancreatic islet mass, pMSC facilitate the restoration of normoglycemia and the neovascularization of the graft. These results suggest that pMSCs could exert an indirect role of "helper" cells in tissue repair processes. STEM CELLS *2010;28:140–151*

Disclosure of potential conflicts of interest is found at the end of this article.

INTRODUCTION

In vitro and in vivo mesenchymal stem cells (MSC) have the capacity to differentiate into a variety of ectodermal, mesodermal, and endodermal tissues [1, 2]. Because of this plasticity, in recent years they have been proposed for the regeneration of many tissues, including β cells in type 1 diabetes. In fact, it has been reported that MSC in vitro have the potential by genetic modification or defined culture conditions to differentiate into insulin-producing cells, and in some cases, to reverse diabetes in animal models [3–7]. Differentiation to β cells in vivo appears highly unlikely [8–10], but a wide array of experimental evidence indicates that MSC may play an indirect role in protecting remaining β cells from further destruction at onset of disease or in the stimulation of endogenous β cell replacement from tissue-resident stem cells or pre-existing β cells [11–15].

Adherent mesenchymal cells have been reported to appear in cultures of human endocrine or exocrine pancreatic tissue. These cells have been observed during attempts to differentiate human β cells from pancreatic precursors [16–22] and were initially thought to originate from from β cells having undergone an epithelial to mesenchymal transition (EMT) [16–19, 23]. Cell lineage tracing by five groups [24–28] provided compelling evidence against the occurrence of β cells EMT as mechanism of mesenchymal cell differentiation. To this day, a thorough characterization of these pancreatic mesenchymal cells has not yet been performed, and there are no conclusive data about their origin and potential role in regenerative medicine of the pancreas. Here we demonstrate that human mesenchymal cells appearing in cultures of human

Author contributions: V.S.: Conception and design, collection and assembly of data, manuscript writing; R.M., A.M., R.F., C.D., E.L.: Collection and assembly of data; G.F.: Provision of study material, collection and assembly of data; B.A., R.N.: Provision of study material; K.C., E.B.: Conception and design, manuscript writing, final approval of manuscript; L.P.: Conception and design, data analysis and interpretation, manuscript writing.

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pancreatic endocrine or exocrine tissue are mesenchymal stem cells, determine their origin as bone marrow-derived, and show that they aid in the restoration of normoglycemia and support neovascularization when cotransplanted with islets in a mouse model.

Materials and Methods

Isolation of Tissue MSCs from Mouse and Human Pancreas

Primary pancreatic tissues were obtained as previously described from the digest remaining after isolation of islet cells from pancreata of 12-week-old C57 male mice and heart-beating human organ donors [29]. The dense fraction recovered in the pellet, which is normally discarded, was processed to isolate MSCs. Following two washes in phosphate buffered solution (PBS), the equivalent of 0.1 ml of packed pellet was resuspended in Eagle's minimum essential medium, alpha modification (α-MEM) 10% FBS, plated in one T75 tissue culture treated flask (Costar, Cambridge, MA, www.corning.com), and grown at 37°C in a humidified incubator at 5% CO_2. Islets were also washed twice in PBS, seeded at a density of 100 islets/ml, and cultured under the same conditions. After 24 hours, nonadherent material was removed and fresh medium was added to the cells. Medium was changed every 3 days. Cells were harvested by trypsinization and replated at a density of 2,500 cells/cm^2 for up to 30 passages (200 days) under the same culture conditions.

Human bone marrow mesenchymal stem cells (BM-MSCs) were obtained from Cambrex (Cambrex, Walkersville, MD, http://www.cambrex.com) and cultured under the same conditions. When cells showed limited capacity to replicate, cell senescence was assessed with the Senescence β-galactosidase Staining kit (Cell Signaling Technology, Beverly, MA, http://www.cellsignal.com). The pancreatic mesenchymal stem cells (pMSCs) were differentiated to adipocytes, osteoblasts, and chondroblasts with a Cambrex hMSC Differentiation BulletKit according to the manufacturer's instructions. Adipogenic, osteogenic, and chondrogenic differentiation was detected with Oil Red O, Alizarin Red S, and Alcian Blue (Sigma-Aldrich, St. Louis, MO, http://www.sigmaaldrich.com) staining, respectively. To assess pMSC clonogenic capacity, single cells were seeded in 96-well plates and clones counted after 2 weeks in culture. To measure proliferation, cells were cultured for 1, 2, 5, 9, and 12 days in the presence of 10 μM bromodeoxyuridine (BrdU), and double stained for incorporation and surface marker expression using a BrdU Flow Kit (BD Pharmingen, San Diego, CA, http://www.bdbiosciences.com/index_us.shtml). In some experiments, aphidicolin, an inhibitor of eukaryotic nuclear DNA replication, was added to the pMSCs every 24 hours from culture days 2 through 7 at a concentration of 50 μM. Cells were photographed under an inverted microscope (Leica DMIRB equipped with a Leica DC300Fx digital camera).

Endocrine Differentiation

Human pMSCs were grown for proliferation and differentiation experiments with or without the addition of fibroblast growth factor-basic (FGF2, 100 ng/ml), epidermal growth factor (EGF) (100 ng/ml), insulin growth factor-1 (IGF1, 100 ng/ml), stromal cell-derived factor 1α (SDF-1α, 300 ng/ml) (Peprotech, Rocky Hill, NJ, http://www.peprotech.com), betacellulin (BTC, 50 ng/ml, R&D Systems Inc., Minneapolis, MN, http://www.rndsystems.com), ghrelin (30 ng/ml, Santa Cruz Biotechnology, Santa Cruz, CA, http://www.scbt.com), gastrin-1 (10 ng/ml), exendin-4 (glucagon-like peptide one analogue, 10 ng/ml, AnaSpec, San Jose, CA, www.anaspec.com), or activin A (20 ng/ml, Sigma), with different concentrations of Fetal Calf Serum (FCS) (0%, 1%, 10%) in plates treated or untreated for tissue culture. Medium was replaced every 3 days and cells counted after 2 weeks.

Flow Cytometry Analysis of Surface Antigens and Cell Sorting

Cells were directly stained using fluorochrome-conjugated monoclonal antibodies (mAbs) or indirectly stained using mAbs, followed by Cy5-conjugated goat antirat IgG F(ab')$_2$ or FITC-conjugated goat antimouse IgG F(ab')$_2$ antibody (Jackson Immunoresearch Laboratories, West Grove, PA, http://www.jacksonimmuno.com). The following mAbs were used: antimouse Sca1, CD90.2, CD117, CD45.1, CD45.2, CD34, CD44 (BD Pharmingen, San Diego, CA, http://www.bdbiosciences.com/index_us.shtml), and CD133 (Miltenyi Biotec, Bergisch Gladbach, Germany, http://www.miltenyibiotec.com); antihuman AD2 (PE anti-CD73); G44-26 (anti-CD44); Thy1A1 (APC anti-CD90); HIB19 (anti-CD19), SR84 (PE anti-CD49a); IIA1 (PE anti-CD49e); 563 (PE anti-CD34) from BD Biosciences; 241 (anti-Ca19-9) from Biodesign International (Saco, ME, www.biodesign.com); 166,707 (FITC anti-CD105); 105,902 (PE anti-CD166) from R&D System, Inc.; H130 (anti-CD45); TU36 (APC anti-HLA-DR); TuK4 (anti–CD14) and MBC78.2 (anti-CD31) from Caltag Laboratories (Burlingame, CA, www.caltagmedsystems.com); AC133 (anti-CD133/1) from Miltenyi Biotec (Auburn, CA, www.miltenyibiotec.com); LM609 (anti-$\alpha v\beta 3$) and PF16 (anti-$\alpha v\beta 5$) from Chemicon (Temecula, CA, http://www.chemicon.com); A53-B/A2 (anti-cytokeratin19) from Sigma; sc-23788 (anti-EpCam) from Santa Cruz; and 4i112 (anti-HLA B55) from Abcam (Cambridge, U.K., http://www.abcam.com). Cell cycle analysis was performed by propidium iodide (Sigma) staining of permeabilized cells, cell doublets were identified and excluded from analysis. Cell apoptosis analysis was performed with Annexin V (BD Biosciences) staining. Flow cytometry was carried out on a FACSCalibur flow cytometer using Cell Quest software (BD Biosciences, San Jose, CA, USA, www.bdbiosciences. com). Results were expressed as the mean percentage of positive cells and standard deviation from multiple experiments.

pMSC and Angiogenesis

Multiplex quantitative analysis using the suspension array system (*Bio-plex* from Bio-Rad, Hercules, CA, http://www.bio-rad.com) through *Luminex* xMAP bead technology was used to detect angiogenetic factors released by 10^5/ml pMSC during an overnight culture. In vitro angiogenesis was assessed as formation of capillary-like structures of endothelial cells cocultured in transwell with pMSC. The experimental procedure followed the manufacturer's protocol provided with the in vitro angiogenesis kit (AngioKit, TCS Cellworks, Buckingham, U.K., www.tcscellworks.co.uk). Briefly, pMSC were cocultured with or without suramin (a VEGF blocker) in a transwell system with endothelial cells in the very early stages of tubule formation, and medium was replaced at days 1, 4, 7, and 9. At day 11, endothelial cells were fixed and stained using an anti-CD31 antibody. To assess the formation of the capillary network, the number of tubules, of junctions between tubules and the total length of tubules per field were quantified by image analysis at $\times 5$ magnification with AngioSys software (TCS Cellworks).

BM Transplantation

BM transplantation experiments were performed using a green fluorescent protein (GFP) transgenic strain as a donor and a co-isogenic strain, expressing the allelic form of CD45 antigen (CD45.1), as the recipient. Murine BM cells were harvested from 7-week-old male transgenic C57BL/6-TgN(ACTbEGFP)1Osb mice and from C57BL/6 mice (Jackson Laboratories, Bar Harbor, ME, http://www.jax.org) by flushing femurs and tibiae. We injected 3×10^6 donor BM GFP$^+$ cells/mouse into the tail vein of 7-week-old C57BL/6-CD45.1 recipient mice (B/6.SJL-CD45^a-Pep^{3b}, Jackson Laboratories), lethally irradiated with a dose of 975 cGy. In a separate set of experiments, CD45$^+$ and CD45$^-$ cells were sorted from GFP$^+$ and GFP$^-$ BM, and mice were transplanted with BM composed of CD45$^+$GFP$^+$/CD45$^-$GFP$^-$ or CD45$^+$GFP$^-$/CD45$^-$GFP$^+$ cells. Two $\times$ 10^6 total cells of sorted populations (CD45$^+$/CD45$^-$ ratio is 1:1) were transplanted. Sort purities were determined by reanalysis of each cell

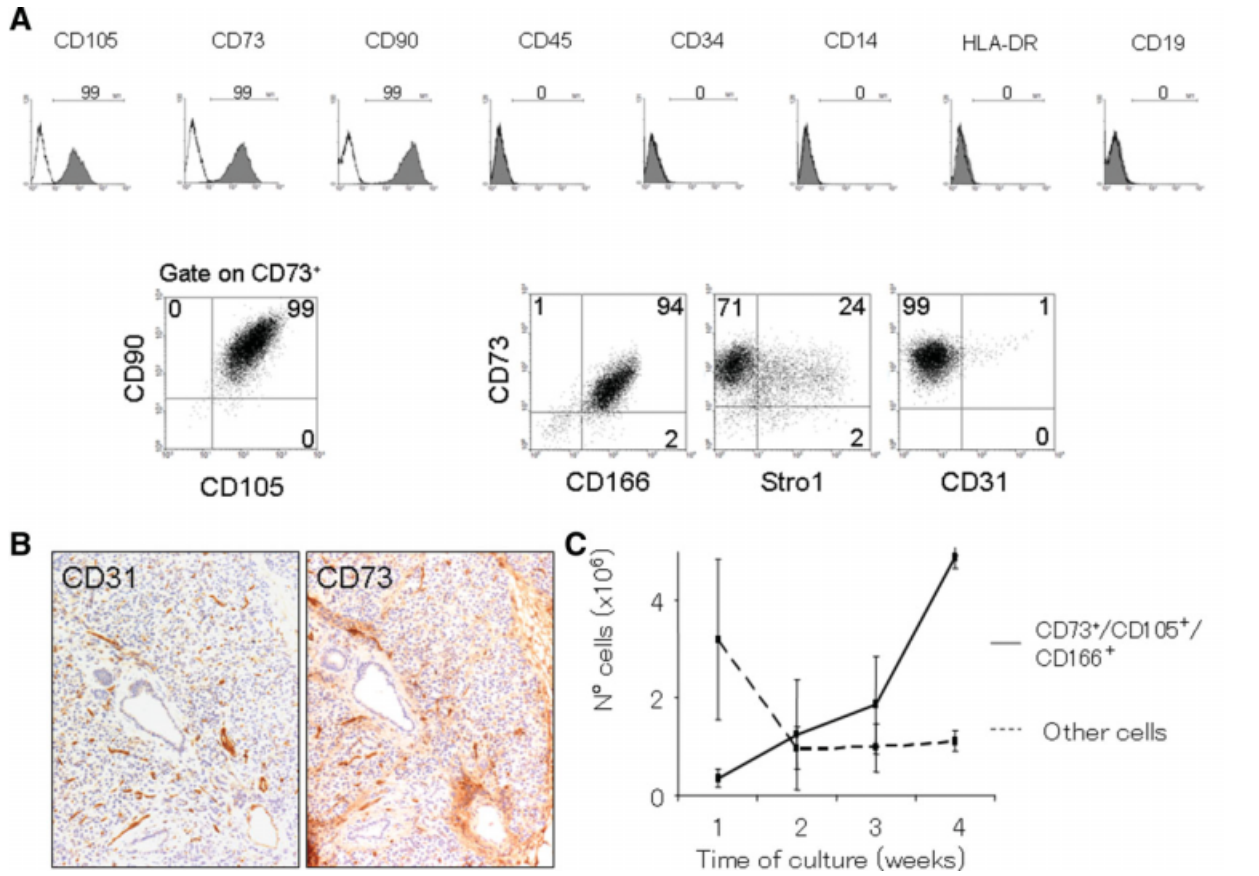

Figure 1. Pancreatic mesenchymal stem cells (pMSCs). (**A**): Cell surface antigen expression profile of pMSCs analyzed by flow cytometry, one experiment represented of 12 performed. (**B**): Immunohistochemistry staining for CD31 and CD73 (brown) on normal human pancreas frozen sections; nuclei counterstained with hematoxylin (blue); ×10 magnification. (**C**): CD73/CD105/CD166 triple positive cells Abbreviations: Nº, number. (mean + SD, $n = 3$) in a population of pMSCs expanded from pancreatic digest for 1 month, counted weekly, and analyzed by flow cytometry.

population sorted. The purity for CD45 expression was >98%. Mice were killed 8 weeks after BM transplantation and GFP cell engraftment in BM was evaluated by fluorescence-activated cell sorting (FACS) analysis. Donor chimerism was >90%, as $CD45.2^+/(CD45.2^+ + CD45.1^+)$ cells in the bone marrow. When $CD45^+/GFP^+$ cells were transplanted, the proportion of GFP^+ cells was >20%.

We then sorted $CD45^{bright}$ and $CD45^{dim}$ cells from GFP^+ and GFP^- BM, and transplanted mice with cells composed of $CD45^{bright}GFP^+/CD45^{dim}GFP^-$ or $CD45^{bright}GFP^-/CD45^{dim}GFP^+$ subpopulations. GFP^- cells were derived from the coisogenic C57BL/6-CD45.1 mice. The purity of the sorted populations was 94% and 92%, respectively, for $CD45^{dim}$ and $CD45^{bright}$. Mice were killed 2 weeks after BM transplantation and GFP cell engraftment in BM and in pancreas was evaluated by fluorescence-activated cell sorting analysis. When $CD45^{bright}/GFP^+$ cells were transplanted, the proportion of GFP^+ cells ranged from 45 to 53% in BM and was 5.0% in pancreas. When $CD45^{dim}/GFP^+$ cells were transplanted, the proportion of GFP^+ cells ranged from 7 to 22% in BM and was 6.3% in pancreas.

Mice were killed 2 to 8 weeks after BM transplantation, hematopoietic tissues (BM, spleen, and thymus) were collected for flow cytometry analysis, and pancreata were isolated for immunohystochemistry and in vitro culture. All experiments were approved by the Institute's Animal Care and Use Committee.

Pancreatic Islets and pMSC Transplantation

Next, 1×10^6 human pMSCs were transplanted under the kidney capsule of athymic mice, made diabetic by treatment with STZ (225 mg/kg). Islet function was monitored for 2 months by twice-weekly glucose measurements and once-weekly measurements of human c-peptide production (RIA commercial kit, Medical System, Genoa, Italy, www.medicalsystem.it). In separate experiments, C57 Bl6 mice were made diabetic by treatment with STZ (225 mg/kg). Transplantation was performed in mice with severe diabetes, defined on the basis of blood glucose levels >400 mg/dl on at least 2 consecutive days. Experiments were also performed in which 1×10^6 mouse pMSCs were transplanted retroductally into the pancreas or via the portal vein into the liver of diabetic mice as previously described [30, 31]. Reversal of diabetes mellitus was defined on the basis of blood glucose levels <200 mg/dl on at least 2 consecutive days. Islet function was monitored for 15 weeks by twice-weekly glucose measurements. Experiments were performed to reach $n = 10$ for each condition.

Additional experiments were performed using a model of minimal islet mass transplantation in which 200 mouse islets were transplanted under the kidney capsule as previously described [32], or either 2.5×10^5 pMSCs or control murine mesangioblasts were cotransplanted with the islets. Mesangioblasts [33] were kindly provided by Dr. Sampaolesi M (Stem Cell Research Institute, Dibit, H. San Raffaele, Milan, http://www.sanraffaele.org/EN_home/Research/Departments-Institutes_e_Research_Programs/Stem_Cells_Research_Institute_(SCRI)/index.html).

Immunofluorescence and Immunohistochemistry

Reverse Transcription Polymerase Chain Reaction

See supporting methods.

Statistical Analysis

Statistical analyses were performed using SPSS 13.0 (Chicago, IL) for Windows.

RESULTS

Mesenchymal Cells Appearing in Cultures of Human Pancreatic Tissue Are Pancreatic Mesenchymal Stem Cells

Islets and adherent cells from islet-depleted digests (<1% endocrine tissue) of human adult pancreas were cultured in α-MEM without growth and/or differentiation factors supplemented with 10% FCS. Under these conditions, part of the cells adhered to the plate and within 21 days formed a homogenous monolayer of cells with mesenchymal morphology similar to that described in other culture protocols [16–21, 23]. Phenotypically, these cells expressed the same markers of bone marrow mesenchymal stem cells (BM-MSCs): $CD73^+$ (Ecto-5′-nucleotidase), $CD105^+$ (Endoglin), $CD166^+$ (ALCAM), $CD90^+$ (Thy1), $CD44^+$ (H-CAM), $CD49e^+$ (VLA-5), $CD49a^+$ (VLA-1), $CD45^-$, $CD14^-$, $CD19^-$, $HLA\text{-}DR^-$, $CD34^-$ and $CD31^-$ (PECAM-1) (Fig. 1A and 1S).

We next assessed the growth properties and clonogenic potential of pancreatic cells. Cells were grown continuously for 40-50 population doublings (PD), depending on the donor, over a period of 6 months (doubling time: 84 ± 16 hours) and the phenotype persisted without alteration. After 6 months of continuous replication, we saw the onset of significant cell senescence manifest as greatly reduced replication rate, enlarged cell size, and the expression of pH-dependent β galactosidase activity (data not shown). Clonogenicity was assessed by single cell dilution in 96-well plates: cloning efficiency was 43.7 ± 14%. These clones maintained the phenotype of the original culture (Fig. 2S). Overall, pMSCs exhibited self renewal and high clonogenic potential.

To confirm that the pancreas-derived cells were mesenchymal stem cells, we induced their differentiation into bone, fat, and cartilage under standard differentiation conditions. Consistent with mesenchymal stem cell behavior, original primary culture and clonally-derived pMSC showed the ability to undergo adipogenic, osteogenic, and chondrogenic differentation (Fig. 2S). Results were reproducible with pMSCs from multiple male and female donors aged 21-65 years ($n = 12$). Further experiments were performed on islet depleted digest-derived pMSC.

Human pMSCs Propagate From pMSCs Residing in the Pancreas

To determine whether pMSCs in culture derive from pancreatic cells undergoing an EMT or from a mesenchymal population resident in the pancreas, we checked for the presence of

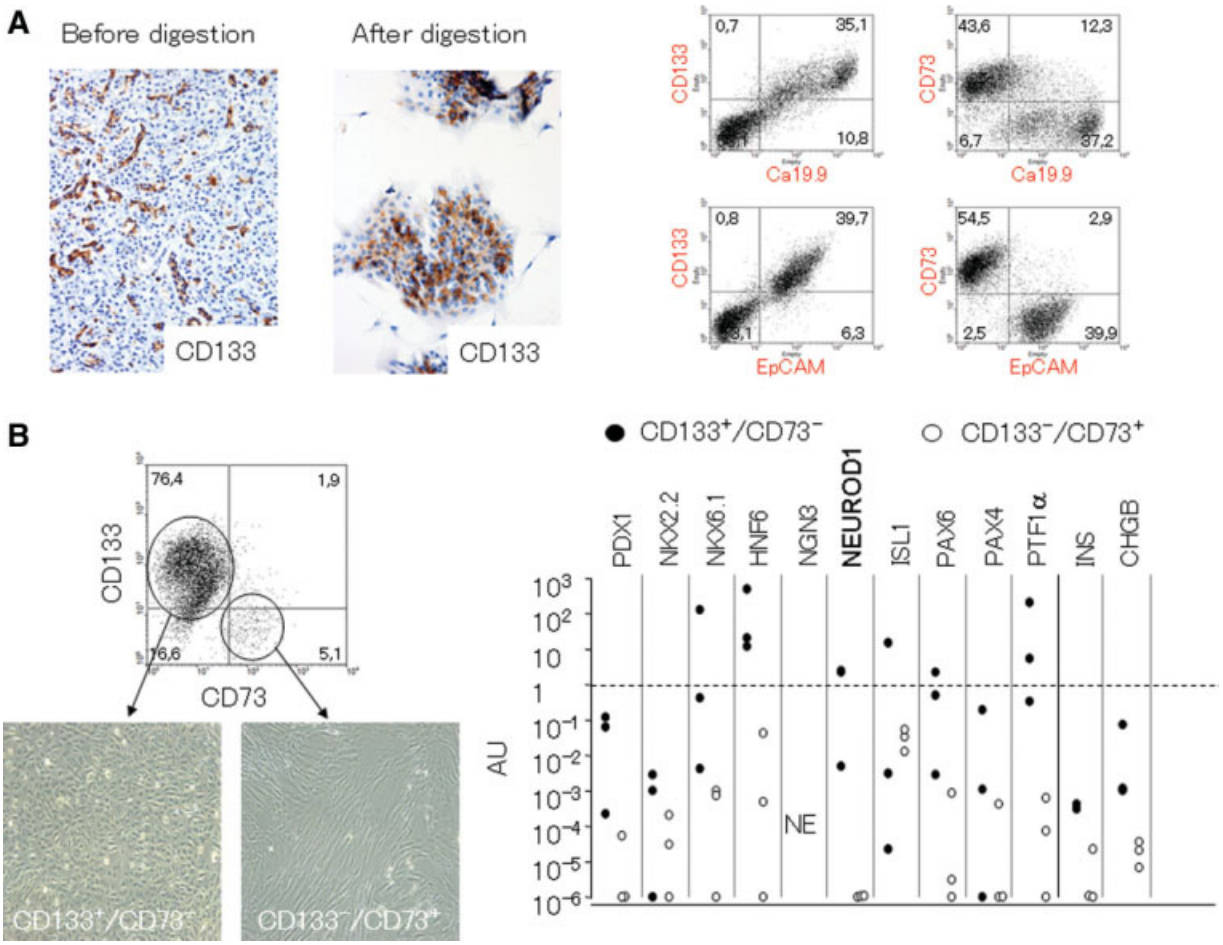

Figure 2. $CD133^+$duct cells are the predominant component of early pancreatic culture. (**A**): Left: CD133 staining (brown) by immunohistochemistry in pancreas before digestion and after 2 days of culture in chamber slides, ×40 magnification. Right: expression of EpCAM, and Ca19.9 in $CD73^+$ and $CD133^+$ cells by flow cytometry after 2 weeks of pancreas digest culture. (**B**): Left: representative morphology of mesenchymal $CD73^+/CD133^-$ and epithelial $CD73^-/CD133^+$cells after sorting and 1 week of culture. Right: quantitative RT-PCR analysis of mRNA levels of transcriptional factors involved in pancreas development in $CD73^+/CD133^-$ (black dots) and $CD73^-/CD133^+$cells (white dots). Abbreviations: NE, not expressed.

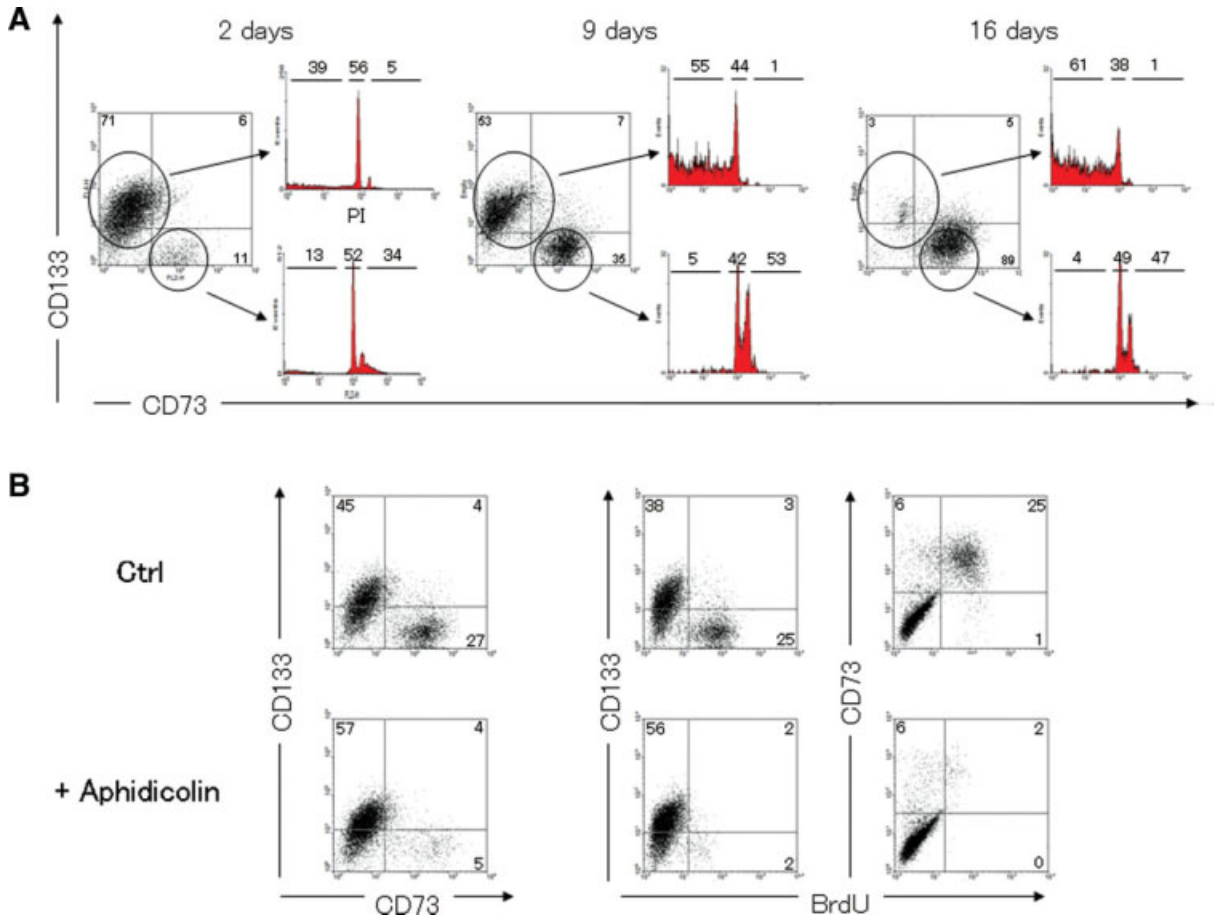

Figure 3. Pancreatic mesenchymal stem cells do not derive from CD133$^+$duct cell EMT. (**A**): CD73/CD133 double staining and cell cycle staining by propidium iodide of CD133$^+$ sorted cells after 2, 9, and 16 days of culture. One representative experiment of four performed. (**B**): Flow cytometry analysis of CD73/CD133, CD73/bromodeoxyuridine and CD133/bromodeoxyuridine on a population of CD133$^+$ sorted cells cultured for 7 days after isolation in the presence or absence of aphidicolin (proliferation blocker). One representative experiment of three performed. Abbreviations: BrdU, bromodeoxyuridine; Ctrl, control; EMT, epithelial to mesenchymal transition.

pMSCs in normal undigested human pancreas. Mesenchymal CD73$^+$ cells with long cytoplasmic processes were detected in the periacinar, perivascular, and periductal space of the healthy pancreas (Fig. 1B).

When pancreatic digest fractions were cultured for 24 hours, triple staining of the adhered cells identified a small percentage of CD73$^+$/CD105$^+$/CD166$^+$ fibroblast-like cells (7.5 ± 2.1% of the total adhered cells), whereasmost of the adherent cells were CD73$^-$ and had an epithelial morphology. The frequency of CD73$^+$/CD105$^+$/CD166$^+$ cells in culture increased over time until they completely colonized the culture (2nd week, 49.8 ± 17.5%; 3rd week, 64.8 ± 5.6%; 4th week, 80.2 ± 4,4%) (Fig. 1C). Measurement of BrdU incorporation at different times (1, 2, 5, 9, and 12 days) showed that CD73$^+$/BrdU$^+$ cells were present at all times, whereas practically no proliferating cells were present in the CD73$^-$ fraction. Cell cycle evaluation by propidium iodide staining confirmed the data obtained by BrdU incorporation (data not shown).

Thus, detection of pMSCs in pancreatic tissue before digestion and at the onset of cell culture, in addition to their extensive proliferation observed in vitro, confirms that almost all of the CD73$^+$ pMSCs derived from the proliferation of an already present population residing originally in the pancreas.

Short-Lived CD133$^+$ Duct Cells Account for Endoderm-Related Transcription Factor EXPRESsion and Epithelial Morphology in Early Primary Cultures of Pancreatic Digests

In early primary cultures of pancreatic digests and islets, CD73$^+$ pMSCs represented only a small fraction of adherent cells. In fact, during the first week, most of the adherent cells were CD73$^-$ and had an epithelial morphology. Consistent with a duct cell identity, these CD73$^-$ cells were positive for the adult pancreatic duct cell marker CD133 [34] and epithelial markers, including EpCAM and Ca19.9 (Fig. 2A). The mRNA levels of transcription factors involved in pancreas development were assessed by quantitative RT-PCR in CD73$^+$ pMSCs and CD133$^+$ duct cells purified (>95%) by cell sorting (Fig. 2B). This revealed that all factors detected in human pancreatic islets were absent or profoundly lower in CD73$^+$ pMSCs (generally three log lower), with the exception of Isl-1. The CD133$^+$ duct cells thus heterogeneously express transcription factors involved in pancreas development at higher levels than CD73$^+$ pMSCs.

CD73$^+$ sorted pMSCs and CD133$^+$ sorted duct cells were each cultured for 1 month. The CD73$^+$ pMSCs maintained a homogenous monolayer of cells with mesenchymal

morphology, identical to that described above. The $CD133^+$ duct cells showed the expected epithelial morphology, but in these culture conditions were never seen to proliferate and were highly apoptotic, as demonstrated by the subG1 fraction of cell cycle (Fig. 3A) and by annexin V staining (data not shown). Strikingly, although the number of $CD133^+$ cells in these cultures progressively decreased, the percentage of $CD73^+$ mesenchymal cells increased (Fig. 3A). By the end of the culture period, the $CD133^+$ sorted cells were completely replaced by $CD73^+$ pMSCs. Consistent with an increase in $CD73^+$ cells, mesenchymal marker (Snail1, nestin, vimentin) expression increased during culturing, whereas epithelial marker (CK19, HNF1β) expression decreased (Fig. 3SA). Similarly, many pancreatic endoderm-related trascription factors decreased, with the exception of Isl-1, which is also expressed in pancreatic mesenchyme. Blocking proliferation by adding aphidicolin prevented appearance of the $CD73^+/CD133^-$ population in $CD133^+$ sorted duct cell cultures, confirming that the switch from $CD133^+/CD73^-$ to $CD133^-/CD73^+$ is exclusively dependent on cell proliferation rather than duct cell EMT (Fig. 3B). Additionally, single cell cloning of $CD133^+$ cells never resulted in a $CD73^+$ proliferating clone, whereas single cell cloning of $CD73^+$ cells gave rise to $CD73^+$ proliferating clones with an efficiency of about 40%. We conclude that after pancreas digestion it is possible to produce primary cultures containing two cell populations: 1) a short-lived epithelial population originating from duct cells, and 2) a persistent mesenchymal stem cell population that quickly expands and replaces the epithelial cells.

Bone Marrow Is a Source of pMSC

We addressed the origin of pMSCs in both human and mouse. We first verified that cells with pMSC characteristics could be derived from mouse adult pancreatic tissue, and were able to obtain a population of bona fide murine $Sca1^+$, CD90.2 $(Thy1.2)^+$, $CD44^+$, $CD34^-$, $CD133^-$ $CD45^-$ and $CD117^-$ pMSCs after 6 weeks of culture (Fig. 4S). We subsequently studied mouse pancreas after a GFP^+ bone marrow transplantation (BMT) to determine if pMSCs originate from bone marrow. Total bone marrow cells from donor GFP^+ transgenic mice (C57BL/6-TgN (ACTbEGFP) 1Osb) were transplanted into lethally irradiated coisogenic recipient C57BL/6-CD45.1 mice. After 12 weeks, all recipients had a high level of bone marrow chimerism, scored as $CD45.2^+/(CD45.2^+ +$

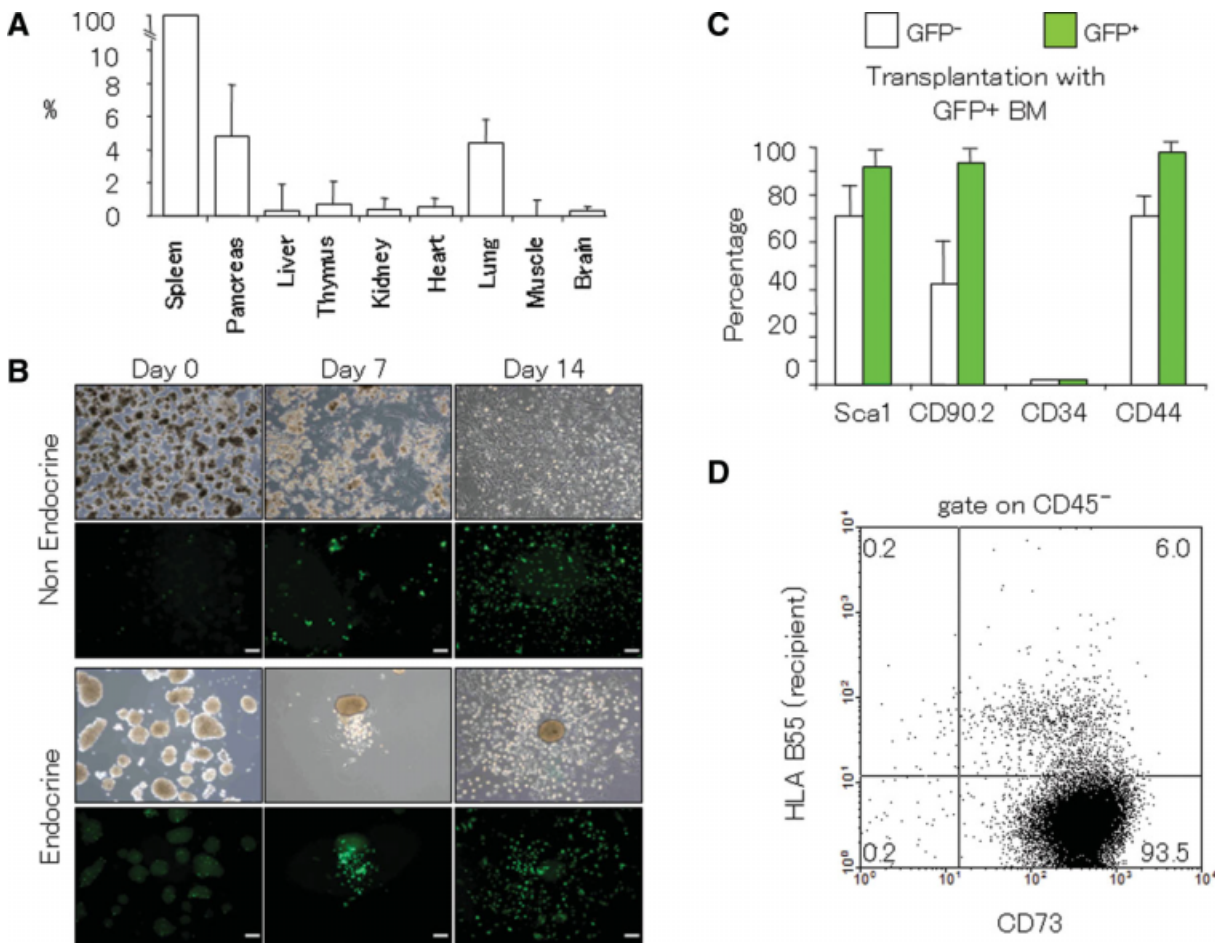

Figure 4. Mouse pancreatic mesenchymal stem cells (pMSCs) derive from bone marrow. (**A**): Mean percentage + SD of green fluorescent protein (GFP^+) cells counted on 10 sections for each organ in mice 1 month after GFP^+ bone marrow transplantation (BMT). (**B**): Cells derived from endocrine and nonendocrine pancreatic tissues of mice transplanted with GFP^+ bone marrow, immediately after isolation and after 7 and 14 days in culture. Above: phase contrast image. Below: fluorescent image of the same field showing GFP^+ scale bar: 100 μm. (**C**): Surface marker expression profile by flow cytometry of GFP^- (white bars) and GFP^+ (green bars) pMSCs derived from pancreas of mice transplanted with GFP^+BM reported as mean percentage + SD of positive cells from three experiments. The analysis was performed 6 weeks after pancreas digestion. (**D**): Pancreatic mesenchymal stem cells cultured from explanted human pancreas stained for the expression of CD45, CD73, and recipient HLA (HLA B 55) by flow cytometry. Abbreviations: BM, human bone marrow cells; GFP, green fluorescent protein.

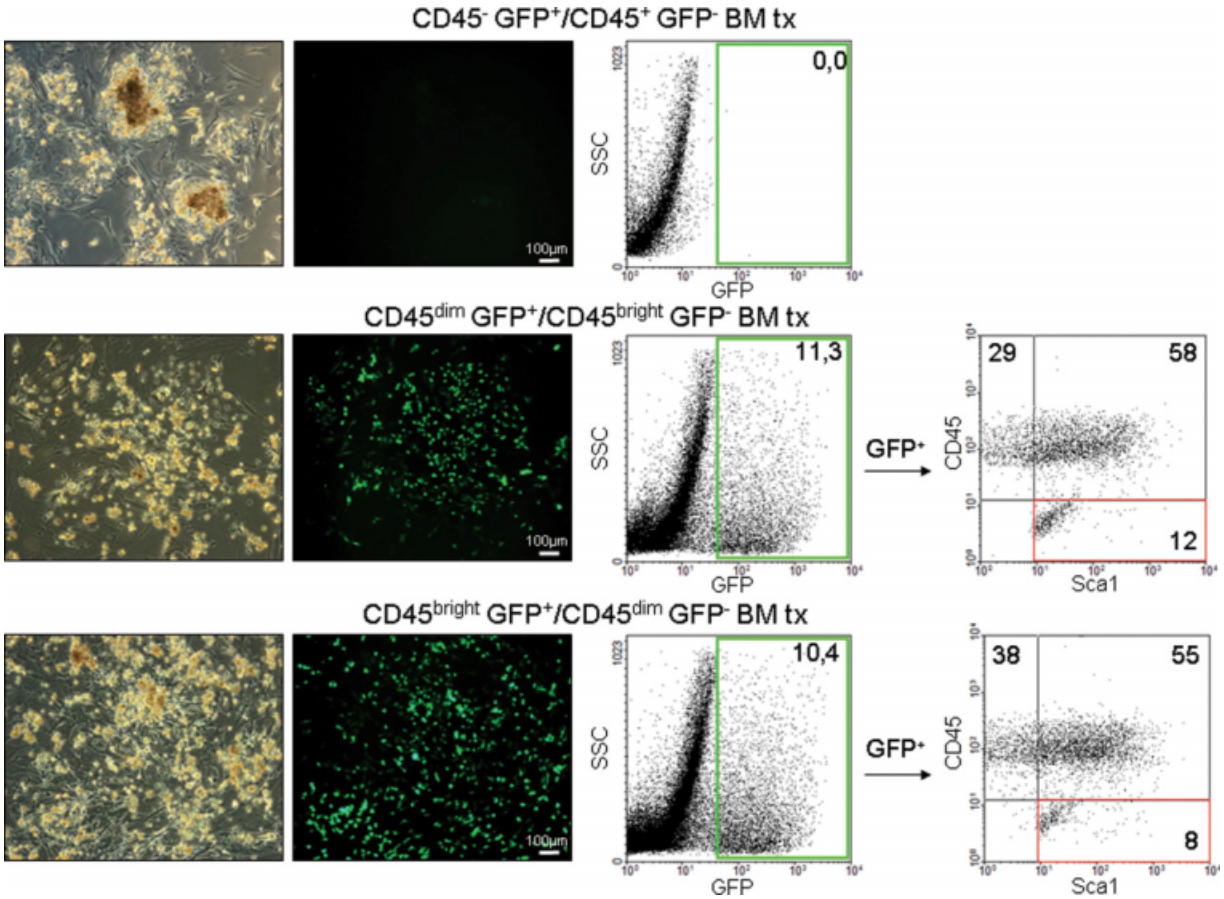

Figure 5. Pancreatic mesenchymal stem cells derive from the CD45$^+$ component of bone marrow. Cells derived from nonendocrine pancreatic tissues of mice transplanted with CD45$^-$/ green fluorescent protein (GFP$^+$) (upper panels), CD45^{dim}/GFP$^+$ (medium panels), and CD45^{bright}/GFP$^+$ (lower panels) bone marrow cells. Left: representative phase contrast image and fluorescent image of the same field showing GFP$^+$cells of nonendocrine pancreatic tissue after 1 week of culture. Right: flow cytometry analysis; reported are representative dot plots of total cells (GFP$^+$ versus side scatter) and of GFP+ cells (Sca1$^+$ versus CD45$^{+)}$ after 1 week of culture.

CD45.1$^+$)×100 (mean 89 ± 10%). Analysis of histologic sections after BMT showed that GFP$^+$ bone marrow-derived cells localized in different nonlymphatic tissues with preferential homing in two organs: pancreas (GFP$^+$ cell: 4.82 ± 4% of total) and lung (GFP$^+$ cell: 4.43 ± 2.3% of total) (Fig. 4A). We derived pMSCs from either isolated pancreatic islets or the nonendocrine component. In 2 weeks, these pMSCs adhered to plastic, proliferated, exhibited a fibroblast-like morphology, and colonized the plates (Fig. 4B). After 3 to 6 weeks in culture, 18.5 ± 4% of the islet-derived pMSCs were GFP$^+$. These GFP$^+$ pMSCs had the same proliferative capacity as GFP$^-$ pMSCs, and were Sca1$^+$, CD90.2 (Thy1.2)$^+$, CD44$^+$, CD34$^-$ (Fig. 4C). After 3 weeks under standard osteogenic culture conditions, they produced calcium that stained positive for alizarin red S (data not shown), demonstrating unequivocally that the isolated GFP$^+$ cells were pMSCs derived from BM.

In man, because of the impracticability to recover pancreas after BM transplantation, we studied the CD73$^+$ pMSC population in pancreas removed after allotransplantation. Eight months after transplantation, a 29-year-old female patient with type 1 diabetes (HLA A: 24,26; B: 55,58; DR*03) experienced thrombotic thrombocytopenic purpura, and consequently the transplanted pancreas (HLA A: 1,24; B: 44,40; DR*: 16,03) was removed. We successfully isolated pMSCs (CD73$^+$, CD105$^+$, CD166$^+$, CD31$^-$, CD45$^-$, CD34$^-$). About 6% of pMSCs expressed recipient HLA B55, indicating that the original donor population had been partially replaced by an extrapancreatic source from the recipient (Fig. 4D).

To better characterize the pMSC BM origin, we performed experiments in which irradiated recipient mice received transplantation of BM with GFP$^+$ trackable subpopulations segregated by CD45 expression (CD45$^-$/GFP$^+$, CD45$^+$/GFP$^+$, CD45^{dim}/GFP$^+$ or CD45^{bright}/GFP$^+$ sorted subpopulations; see Materials and Methods) (Fig. 5 and 5S).

GFP$^+$ pMSC (CD45$^-$/Sca1$^+$/GFP$^+$) were obtained after transplantation of CD45$^+$GFP$^+$/CD45$^-$GFP$^-$ BM, but not after transplantation of CD45$^+$GFP$^-$/CD45$^-$GFP$^+$ BM. Since BM-MSC were reported to derive from the CD45^{dim} subpopulation [35], we tested, in a second set of experiments, whether the CD45 expression level (CD45dim vs. CD45bright) identifies pMSC precursors in BM. CD45^{dim}GFP$^+$/CD45^{bright}GFP$^-$ BM or CD45^{dim}GFP$^-$/CD45^{bright}GFP$^+$ BM were infused in irradiated C57/BL6 recipient mice (n = 6). CD45 intensity does not discriminate pMSC precursors since GFP+ pMSC were derived in both conditions (Fig. 5).

pMSC Expansion and Capacity to Differentiate as β Cells

We cultured human pMSCs in the presence of factors reported to influence β cell differentiation and proliferation, using either tissue or nontissue culture dishes and different serum concentrations (Fig. 6). Proliferation of pMSC (Fig. 6A) was stimulated 3.1-fold by fibroblast growth factor-2 (FGF-2;

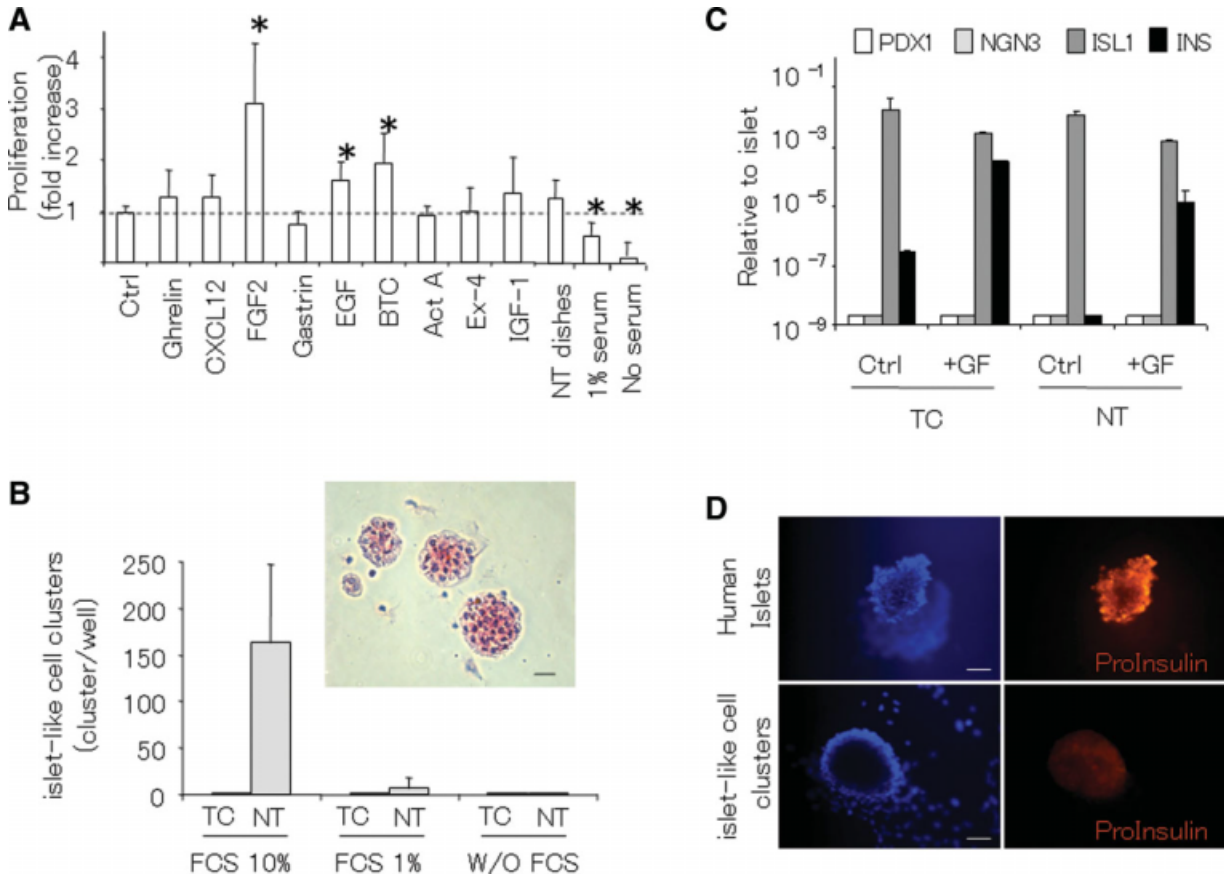

Figure 6. Pancreatic mesenchymal stem cells (pMSC) proliferation and islet-like cell cluster (ICC) formation under different culture conditions. (**A**): Pancreatic mesenchymal stem cells were cultured in α-MEM with 10% fetal calf serum (FCS) (control) or in medium supplemented with growth factors, with 1% or 0% FCS, in not treated (NT) culture dishes as indicated on the *x*-axis. Cell proliferation is expressed as cell number fold increase (mean ± SD of six experiments). (**B**): ICC number after 2 weeks of culture of pMSC (30,000 cells/well) in 10%, 1%, 0% FCS in tissue culture (TC) and NT 6-well plates, mean ± SD of four experiments and a representative image of ICCs stained with hematoxylin-eosin, scale bar = 100 μm. (**C**): Pdx1, Ngn3, Isl1 and Ins expression (mean ± SD of four experiments) measured by reverse transcription polymerase chain reaction after 2 weeks of culture of pMSCs with GF (FGF2, BTC, EGF, IGF1, ghrelin) in TC and NT dishes. Levels of mRNA are expressed as relative values with respect to islets expression, set as 1. (**D**): Immunofluorescence staining for proinsulin (red) in ICC and human islets. Nuclei are stained with DAPI in blue. Scale bar: 50 μm. Abbreviations: Act A, activin A; BTC, betacellulin; Ctrl, control; EGF, epidermal growth factor; Ex-4, Exendin-4; FCS, fetal calf serum; FGF2, fibroblast growth factor-basic; ICC, islet-like cell cluster; IGF1, insulin growth factor-1; NT, not treated; TC, tissue culture.

$p < .05$ versus control, $n = 4$), 1.9-fold by betacellulin (BTC; $p < .05$ versus control, $n = 4$) and 1.6-fold by epidermal growth factor (EGF; $p < .05$ vs. ctrl; $n = 4$), and was unaffected by ghrelin, CXCL12/SDF1α, gastrin, activin A, exendin-4, and insulin-like growth factor one (IGF-1). Serum reduction inhibited pMSC proliferation, whereasserum deprivation induced pMSC death.

Culture in nontissue culture dishes did not significantly modify proliferation, but did induce the formation of islet-like cell clusters (ICC) (Fig. 6B). In fact, within only 24 to 48 hours, the cells started to form aggregates of increasing size, measuring between 50 to 250 μm in diameter. Over time, these cell clusters became stable and persisted for more than 3 weeks. We assessed mRNA levels of Pdx1, Ngn3, insulin, and Isl1 after ICC formation or exposure to a growth factor combination that included FGF-2 (100 ng/ml), BTC (50 ng/ml), EGF (100 ng/ml), IGF-1 (100 ng/ml), and ghrelin (30 ng/ml) (Fig. 6C). None of these conditions modified Pdx1, Ngn3 or Isl1 expression. Exposure to growth factors increased insulin expression, but it remained 10,000-fold less than that of mature islets. ICC consistently stained negative for proinsulin (Fig. 6D). To further test their capacity to generate β cells, single pMSCs or islet-like pMSC clusters were grafted into diabetic nude mice. Two months after transplantating 1×10^6 pMSCs under the kidney capsule of STZ-diabetic athymic mice, despite the documented survival of pMSC (Fig. 3S), we saw no evidence of improved glycemic control and likewise detected no human C-peptide in mice receiving pMSCs. Also, transplantation of 1×10^6 mouse pMSC retroductally into the pancreas or via the portal vein into the liver of diabetic mice brought no beneficial effect on the disease (data not shown). These data suggest that pMSCs do not have the capacity to differentiate in β cells.

Pancreatic Mesenchymal Stem Cells Release Proangiogenetic Factors and Induce Neoangiogenesis In Vitro

We evaluated the release of growth related oncogene-α (GROα), macrophage colony-stimulating factor (M-CSF), macrophage migration inibitory factor (MIF), stem cell growth factor (SCGFβ), stromal cell-derived factor-1 (SDF1α), Interleukin 6 (IL6), Interleukin 8 (IL8), granulocyte-macrophage colony stimulating factor (GM-CSF), monocyte chemotactic protein-1 (MCP1), vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), basic fibroblast growth factor (bFGF), and platelet-derived growth factor

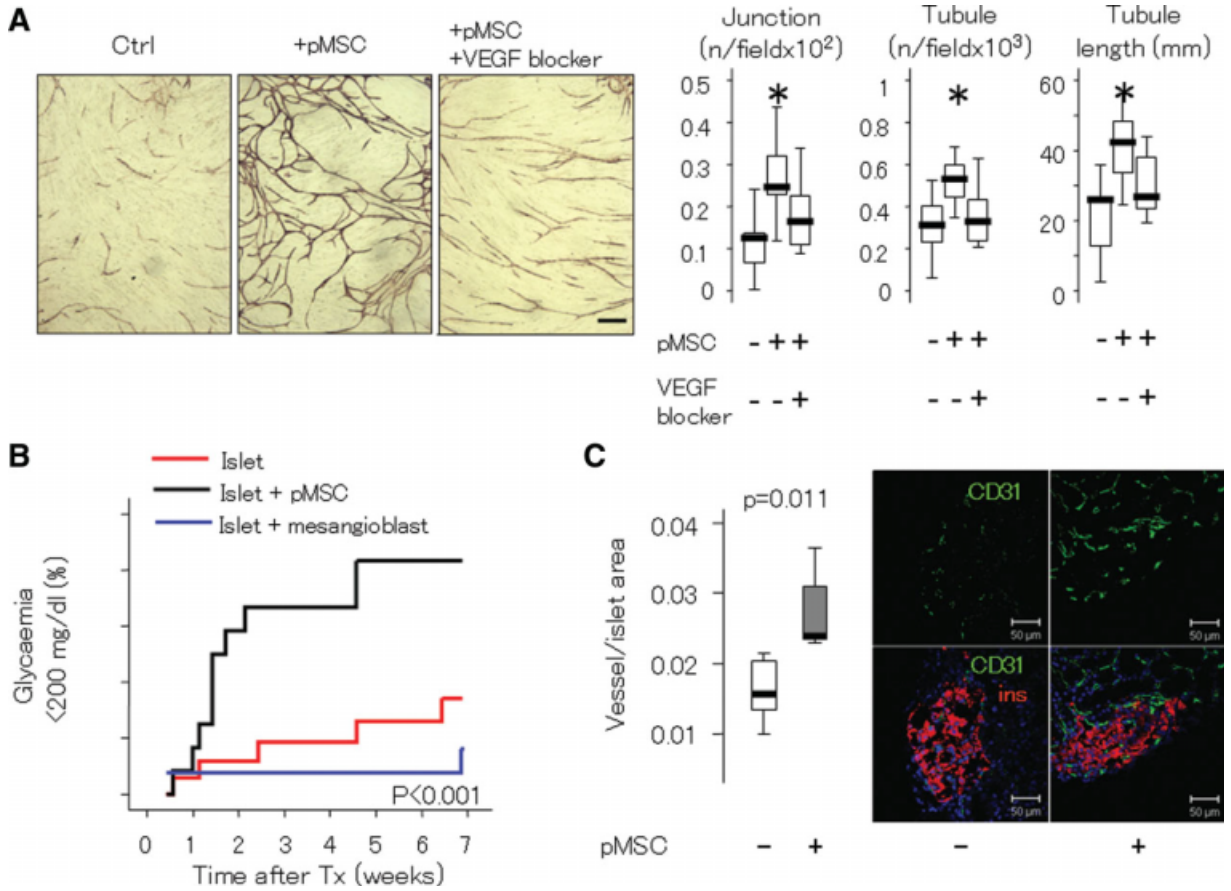

Figure 7. Pancreatic mesenchymal stem cells (pMSCs) aid islet cells in a model of minimal islet mass transplantation. (**A**): In vitro angiogenesis assay: angiogenesis was evaluated as the capacity to form a netwotk of anastomosing tubules from endothelial cells. Upper panel: representative images of tubules formation in the absence (Ctrl) o in the presence of pMSC. To block VEGF, suramin (1 mM) was added to the culture. Scale bar: 100 μm. Lower panel: tubule number, junction and length evaluation. ($n = 10$). Data are presented as box plots. *<0.05, Mann-Whitney U test. (**B**): C57BL/6 recipients were transplanted with 200 autologous islets under the kidney capsule alternatively without ($n = 17$, red line) or with 2.5×10^5 pMSC ($n = 12$, black line) or 2.5×10^5 mesangioblasts ($n = 13$, blue line). Kaplan-Meier analysis for reaching normoglycemia (<200 mg/dl on consecutive days) is shown. Differences between groups were tested using the log-rank statistic ($p < .001$). In post hoc analysis: islets versus islets + pMSC, $p = .006$; islets versus islets + mesangioblasts, $p = .362$; islets + pMSC versus islets + mesangioblasts, $p = .002$. (**C**): Quantitative evaluation of $CD31^+$ cell area in islet graft with islets alone ($n = 7$) or islets plus pMSC ($n = 7$) 3 weeks after transplantation. Left panel: The vessel/islet area is shown for transplanted mice. $CD31^+$ area was determined and divided by the $insulin^+$ area in 35 ± 11 islet sections in islets alone and 36 ± 24 islet sections in islets plus pMSC transplantation, respectively. Data are represented as box plots. Statistical analysis was performed by Mann-Whitney U test. Right panel: representative immunofluorescence images showing CD31 (green), insulin (red), and DAPI cell nuclei staining (blue) in islet graft with islets alone or islets plus pMSC. Abbreviations: Ctrl, control; pMSCs, pancreatic mesenchymal stem cells; VEGF, vascular endothelial growth factor.

(PDGF) by pMSCs (10^5/ml) cultured for 12 hours. High concentrations of VEGF (4.4 ± 0.6 ng/ml), PDGF (1.0 ± 0.8 ng/ml), GROα (3.4 ± 2.3 ng/ml), M-CSF (0.7 ± 0.6 ng/ml), MIF (1.9 ± 0.2 ng/ml), SCGFβ (39.3 ± 4.2 ng/ml), SDF1α (1.0 ± 0.2), IL6 (19.5 ± 16.6 ng/ml), IL8 (10.8 ± 10.5 ng/ml), MCP1 (71.8 ± 12.1 ng/ml), but not of GM-CSF (0.1 ± 0.2 ng/ml), HGF (0.1 ± 0.1 ng/ml) and bFGF (0.1 ± 0.2 ng/ml) were detected.

The angiogenetic capacity of pMSC was then analyzed in an in vitro system: pMSC stimulated tubule formation and anastomosis. This was prevented by the addition of suramin (VEGF blocker). These data demonstrate that pMSCs are able to induce a VEGF mediated angiogenesis (Fig. 7A).

Pancreatic Mesenchymal Stem Cells Aid Islet Transplantation and Increase Neovascularization

Since pMSCs originate from an extrapancreatic source and the BM-MSC have been shown to provide help to islets in a transplant model, we asked whether pMSC recruitment is beneficial for islet survival or function. To address this issue, we used a syngeneic murine model of marginal islet mass transplantation. Implantation of a marginal β cell mass (200 islets) is characterized by achievement of normoglycemia in only a proportion of recipients or a delay in reversal of hyperglycemia as compared with transplantation of 500 islets. Manipulations that lead to a higher percentage of successful grafts or to a reduction of the lag to normoglycemia are to be interpreted as beneficial in favoring islet survival or engraftment [36, 37]. STZ-induced severely diabetic mice (mean nonfasting glycemia before transplantation: 546 ± 64 mg/dL) were transplanted under the kidney capsule with 200 syngenic islets alternatively with ($n = 12$) or without ($n = 17$) 2.5×10^5 murine pMSC. The probability and the median time to reach euglycemia (<200 mg/dl) in our marginal islet mass transplantation were: 83% and 10 days for islet + pMSC transplants (CI95%, 6.6-13) and 29% and >50 days for islet alone transplants ($p = .004$, Pearson chi-square test; $p = .007$, log-

rank test in Kaplan Meyer analysis). Glycemic control was not improved when the pMSCS were substituted by a similar number of other murine cells (that is, mesangioblasts) in the cotransplant ($n = 13$), demonstrating a specific role for pMSCs (Fig. 7B). In another set of experiments, MSC derived from pancreas ($n = 5$) and from bone marrow ($n = 5$) were transplanted together with islets in the model of minimal islet mass transplantation and both resulted effective in normalizing glycemia (Fig. 6S). Moreover, transplantation of murine pMSC alone and mesangioblast alone did not induce any change in glycemia (data not shown). To exclude the possibility that better glycemic control was caused by pMSC differentiation into insulin secreting cells, we cotransplanted islets from wild-type mice along with pMSCs derived from GFP^+ transgenic mice. Two to 3 weeks after transplantation, the host mice were killed and the graft analyzed. No insulin/GFP double positive cells were present (data not shown). In a second set of experiments with seven mice in each group, we analyzed $CD31^+$ endothelial cells in the transplant 3 weeks after transplantation and the vessel density was significantly higher in islets plus pMSC than in islets alone (Fig. 7C), indicating that one of the benefits provided by pMSC is increased neovascularization of the graft.

Discussion

In this study we show that the mesenchymal cells appearing from pancreatic tissue culture are not derived from an epithelial to mesenchymal transition, but are rather the expansion of a pool of resident MSCs, located mainly in the periacinar, perivascular, and periductal space that have a bone marrow-derived origin and that preserve, at least in vitro, a stable phenotype, a high proliferation rate, and multilineage differentiation potential. We classified cells emerging from pancreatic culture as mesenchymal stem cells on the basis of the minimal criteria for defining MSC established by the International Society for Cellular Therapy [38]: plastic-adherence in standard culture conditions; expression of CD105, CD73 and CD90; lack of expression of CD45, CD34, CD14, CD19, and HLA-DR surface molecules; capacity to differentiate to osteoblasts, adipocytes and chondroblasts in vitro. CD73 expression in pMSCs proved useful to follow these cells during processing and culture. These pMSC seem to have an advantage in terms of proliferation and survival compared with other cells of pancreatic origin in culture, and become the predominant cell type in cultures without a requirement of epithelial to mesenchymal switch. In agreement with our data in man, four groups recently used direct lineage tracing via transgenic cre/loxP-based systems [25–28] to provide compelling evidence against β cell EMT in the mouse. Importantly, one of these studies not only demonstrated that "fibroblast-like cells" from mouse islets are not derived from β cells, but also excluded an endoderm pancreatic origin and thus discounted the idea that pMSCs originate from a pancreatic exocrine or endocrine EMT. In man, a recent approach of viral-mediated lineage tracing of human β cells showed that they can dedifferentiate and expand in vitro, but their lifespan and rate of division (doubling time 7 days, 16 population doublings) are much lower than those of pMSC, so it is likely that they are two distinct populations of pancreatic cells [24].

In man, we found that part of the $CD73^+$ pMSC population in allogeneic pancreas removed after transplantation expressed recipient HLA, suggesting that an extrapancreatic source "refilled" the graft. In mouse recipients of a GFP^+ bone marrow transplant, we conclusively demonstrated that pMSCs originate from BM. Even if we cannot exclude the existence of other sources of pMSC, this evidence strongly suggests the existence of a cross talk between BM and pancreas, evidence also supported by the fact that the pancreas is a preferential site of GFP^+ cells localization after GFP^+ BM transplantation in mice. Regarding the mechanisms involved in pMSC recruitment, we previously reported that human pancreatic islets can attract BM-MSCs in vitro, and this attraction is principally mediated by two chemokines: CX3CL1 and CXCL12 [21]. Currently, we are unable to determine whether pMSC migration from the bone marrow occurs in the absence of pancreatic injury, or if pMSCs constitute a renewable pool of cells that migrate early in development to create a pancreatic stem cell niche that replaces dying cells. It is possible that the resident pMSCs are derived from the vessels within the pancreas as self-renewing "vascular" stem cells resident pool. On the other hand, the BM could reinforce the pancreatic pMSCs population only in stress situations, as in the radiation-based bone marrow transplant setting or the organ damage in the inflammatory events at donor death, and do not participate in the pMSCs turn-over during healthy status.

Interestingly, the bone-marrow transplant model in mice suggests that the pool of MSCs in the pancreas is renewed from the $CD45^+$ BM compartment. This could appear in contrast with the established CD45 negative MSC phenotype, but this phenomenon has already been described [35, 39–43]. MSCs require prolonged culturing of dispersed cell populations on plastic, and the expression of CD45 by MSC could be drastically downregulated during in vitro culture conditions [35, 39]. Obviously the claim that all the MSC are derived from a $CD45^+$ population goes far beyond the purposes of our work. We limit our observation to the MSC cells obtained by the pancreas that, although coming from the bone marrow, may have a different lineage derivation from that of BM MSC.

Since MSCs have been reported to cross the mesodermal lineage and give rise to endoderm both in vitro and in vivo [1, 44–48], it is possible that pMSC could be directed to become β cells and thus serve as an abundant source of insulin-producing cells for transplantation. By adopting methodology that was previously used to differentiate β cells from mesenchymal precursors [1, 44–48], we tested the ability of pMSCs to generate functional islets. The pMSCs formed islet-like cell clusters, but they expressed negligible levels of islet-specific genes under basal conditions, serum deprivation, or exogenous growth factor exposure. Moreover, they were unable to significantly improve glucose metabolism in vivo when transplanted into diabetic nude mice. In agreement with our data, it was recently reported that islet-like clusters obtained from pancreatic MSCs are closer in microarray phenotype to mesenchymal cells than endocrine cells [49]. This confirms that generation of functional islets by culture manipulation of expanded pMSCs is not yet achievable.

In pilot experiments, we found that transplanting pMSCs together with a minimal islet mass enhanced islet function. We excluded that improved glycemic control was caused by pMSC differentiation into insulin-secreting cells, and proposed that pMSCs act as "helper" cells for islets. A beneficial effect of bone marrow-derived MSCs in experimental and preclinical models was recently attributed to the production of endocrine or paracrine factors, not cell differentiation. For example, intravenous administration of MSCs stimulated revascularization of ischemic tissues despite differentiation of only a small number of cells into cardiomyocytes and endothelial cells [50]. Furthermore, Prockop and colleagues [14] showed that stromal cells from human bone marrow home to and promote repair of pancreatic islets in diabetic mice.

Generally this happens without evidence of long-term engraftment, suggesting that the favorable effects of MSCs reflect the impact of transitory paracrine effects or secreted factors rather than engraftment, differentiation, or cell fusion [51–53]. Also in our model, the higher vessel density in islets cotransplanted with pMSC proposes that MSC have an indirect beneficial effect on the graft. This is supported by the recent findings that MSC enhanced endothelial cells proliferation, sprout formation, and in-growth in an in vitro system of composite endothelial cells-MSC-pancreatic islets [54]. The increase in endothelial cells in islet grafts could be consistent not only with an increased blood flow, but also with a $CD31^+$ cells mediated paracrine effect in sustaining β cell survival. Further studies are ongoing to confirm how pMSCs favor neovasculogenesis and to explore whether the protective effect of pMSC on transplanted tissue is mediated by other potential mechanisms such as prevention of islet cells from apoptosis, stimulation of islet cell proliferation, or the release of growth factors required by islets for maintenance of efficient insulin secretion.

In conclusion, pMSCs appearing in cultures of human pancreatic endocrine and exocrine tissue propagate from a population of mesenchymal stem cells, likely of bone marrow origin, resident in the pancreas. These cells express negligible levels of islet-specific genes, but are able to facilitate glycemia regulation when cotransplanted with a minimal islet mass into diabetic mice. This suggests that pMSCs are not optimal candidates to generate physiologically competent β cells, but considering the large number of pMSCs obtainable from digested pancreas, they could be useful as islet "helper" cells. The better characterization of bone marrow pancreas interaction and the identification of the mechanisms by which these cells aid islets will likely have implications for therapeutic cell transplantation and diabetes treatment.

Acknowledgments

This work was supported by EU (DIAPREPP Project, HEALTH-F2-2008-202013), Telethon Italy and the Juvenile Diabetes Research Foundation (JT01Y01), Italian Telethon Foundation (TIGET grant) by Award from Fondazione Cariplo (2007-5165) and by award from Fondazione Siemens Italia (Milan, Italy). Valeria Sordi is enrolled as a Ph.D. student at the Ludwig-Maximilians University of Munich, Germany.

Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

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