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Mesenchymal Stem Cells from Chronic Pancreatitis Patients Show Comparable Potency Compared to Cells from Healthy Donors

JINGJING WANG,[a] YONG ZHANG,[b] COLLEEN CLOUD,[a] TARA DUKE,[a] STEFANIE OWCZARSKI,[a] SHIKHAR MEHROTRA,[a] DAVID B. ADAMS,[a] KATHERINE MORGAN,[a] GARY GILKESON,[c] HONGJUN WANG [a]

Authored by a member of CORD BLOOD ASSOCIATION

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[a]Department of Surgery, Medical University of South Carolina, Charleston, South Carolina, USA; [b]College of Life Science, Qingdao Agricultural University, Qingdao, People's Republic of China; [c]Department of Medicine, Medical University of South Carolina, Charleston, South Carolina, USA

Correspondence: Hongjun Wang, Ph.D., Department of Surgery, Medical University of South Carolina, BSB 641, 173 Ashley Avenue, Charleston, South Carolina 29425, USA. Telephone: 843-792-1800; e-mail: wangho@musc.edu

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ABSTRACT

Mesenchymal stem cells (MSCs) are proven to be beneficial in islet transplantation, suggesting a potential therapeutic role of them in total pancreatectomy with islet autotransplantation (TP-IAT) for chronic pancreatitis (CP) patients. We investigated whether MSCs derived from CP patients are suitable for use in autologous cell therapy. MSCs from healthy donors (H-MSCs) and CP patients (CP-MSCs) were studied for phenotype, colony formation potential, multilineage differentiation ability, proliferation, senescence, secretory characters, and immunosuppressive functions. The potential protective effect of CP-MSCs was evaluated on hypoxia-induced islet cell death. Cell surface markers were similar between H-MSCs and CP-MSCs, as well as the ability of colony formation, multilineage differentiation, secretion of vascular endothelial growth factor and transforming growth factor (TGF-β), senescence, and inhibition of T cells proliferation in vitro. We found that growth differentiation factor 6 and hepatocyte growth factor (*HGF*) were significantly downregulated, whereas *TGFβ* and matrix metalloproteinase-2 were significantly upregulated in CP-MSCs compared with H-MSCs, among 84 MSC-related genes investigated in this study. MSCs from CP patients secreted less HGF, compared with the H-MSCs. A higher interferon-γ-induced indoleamine 2,3-dioxygenase expression was observed in CP-MSCs compared to H-MSCs. Moreover, CP-MSCs prevented hypoxia-induced β cell deaths to a similar extent as H-MSCs. Regardless of moderate difference in gene expression, CP-MSCs possess similar immunomodulatory and pro-survival functions to H-MSCs, and may be suitable for autologous cell therapy in CP patients undergoing TP-IAT. STEM CELLS TRANSLATIONAL MEDICINE *2019;8:418–429*

SIGNIFICANCE STATEMENT

Mesenchymal stem cells (MSCs) are known for their regenerative and immune modulatory capacities and have shown beneficial effects in hypoxia-induced injury on islets, which is a major stress in islet transplantation after total pancreatectomy for patients with chronic pancreatitis (CP). However, studies show that MSCs derived from patients suffering from various inflammatory diseases exhibit defects that tamper with their beneficial effects. To the authors' knowledge, this is the first study that shows that MSCs derived from CP patients have comparable phenotype and therapeutic capacity as MSCs from healthy donors. This study proves justification for using MSC cotransplant with islets to improve the islet transplantation outcome.

INTRODUCTION

Chronic pancreatitis (CP) is a long-standing inflammatory condition in the pancreas, with a prevalence rate of 41–76 per 100,000 population [1]. CP is characterized by permanent, irreversible, and progressive fibrotic destruction of the pancreatic parenchyma, which can result in chronic pain as well as endocrine and exocrine dysfunction [2]. The debilitating, protracted abdominal pain is typically very difficult to manage and has a multifactorial origin [3]. In severe cases, partial or total pancreatectomy becomes a therapeutic option that can provide better relief in patients with severe pain that could not be alleviated by other treatments [4, 5]. The major drawback of pancreatectomy is that it leads to complete insulin deficiency (type 3c diabetes or "brittle" diabetes). Total pancreatectomy with islet autotransplantation (TP-IAT) is currently a treatment

option to reduce pain and to prevent or minimize the effects of type 3c diabetes [6]. However, the percentage of patients who remain insulin-independent after TP-IAT is relatively low (approximately 30% postoperatively vs. 85% preoperatively, unpublished data). Poor engraftment of transplanted islets has been attributed to several factors, including islet quality, hypoxia, and instant blood-mediated inflammatory reaction [7, 8]. Treatments that can improve islet engraftment after transplantation would be beneficial for CP patients who have islet transplantation.

Mesenchymal stem cells (MSCs) are nonhematopoietic, multipotent, self-renewing stem cells in adults [9]. They can be differentiated into diverse lineages of mesenchymal origin, including osteoblasts, adipocytes, and chondrocytes, and have also shown their potential for differentiating into nonmesodermal origin cells, including pancreatic islet cells [10]. A body of evidence showed that coculture/cotransplant of MSCs with islets improves islets insulin secretory capacity, islet cell survival [11–14], as well as revascularization [12, 15, 16], offering a potential therapeutic method to enhance the outcome of islet transplantation. Meanwhile, MSCs have been shown to secrete angiogenic paracrine factors such as vascular endothelial growth factor (VEGF), hepatocytes growth factor (HGF), transforming growth factor β (TGF-β), and matrix metalloproteinase, which contribute to the establishment of a graft vascular network [13, 14, 17–19]. MSCs also possess a distinctive array of immunosuppressive characteristics, which was observed in many disease models including autoimmune encephalomyelitis [20–22], multiple sclerosis [23, 24], and arthritis [25]. Studies have demonstrated that MSCs can modulate the function of T and B lymphocytes [26, 27], natural killer cells [28], and regulatory T cells [29–31].

A wide range of soluble factors has been involved in MSCs immunomodulatory function, including indoleamine 2,3-dioxygenase (IDO), HGF, TGF-β, and prostaglandin E2 [32–34]. MSCs are currently being tested in more than 30 clinical trials for the treatment of type 1 or type 2 diabetes, diabetes complications and other diseases (clinicaltrials.gov). Our group found that mouse islets cotransplanted with MSCs from CP patients (CP-MSCs) exhibited better survival and function after syngeneic islet transplantation in C57BL/6 mice, with reduced cell death and macrophage infiltration in islet grafts [35]. Furthermore, our recent data from a pilot clinical trial of CP-MSC and autologous islet cotransplantation for patients undergoing total pancreatectomy also indicate potential protective effects of CP-MSCs on patients' islet grafts [36]. As the patients' own stem cells are readily available for autologous transplantation, cotransplant of islets together with MSCs may be a promising means to enhance the outcome of TP-IAT. In addition, MSCs are suggested to be able to inhibit inflammation and damage to pancreatic tissues in acute and CP models in rats [37, 38], indicating the potential usage of MSCs as a regenerative cellular therapy in CP patients. However, the suitability of MSCs derived from CP patients for regenerative cell therapy, compared to MSCs derived from healthy donors, still remains unknown. In the present study, we investigated the phenotype and functionality of CP-MSCs and MSCs from healthy donors (H-MSCs). We found that CP-MSCs are largely comparable with H-MSCs in respect to cell surface markers, colony formation ability, multilineage differentiation potential, secretory function as well as immunosuppressive abilities. Furthermore, we discovered that CP-MSCs were able to protect islets from CP patients against hypoxia-induced cell death in the in vitro cell culture system [39].

Materials and Methods

Cell Donor Information

The H-MSCs used in this study were isolated from bone marrow aspirate purchased from Allcell Technology (Chicago, IL) obtained from two male healthy donors (age 25 and 32). CP-MSCs were isolated from bone marrow aspirates collected from six patients with CP aged 26–52. There were four women and two men with disease duration ranging from 5 to 10 years. Risk factors for CP included tobacco use in four patients and alcohol abuse in two. One patient had hereditary pancreatitis. Three patients had clinical evidence of pancreatic exocrine insufficiency and one patient had insulin dependent diabetes mellitus. All patients had morphologic changes of CP on imaging with endoscopic ultrasound, magnetic resonance imaging, or computed tomography (CT) body imaging. Two patients had inflammatory masses in the head of the pancreas, one in the tail of pancreas, one had a dilated main pancreatic duct with intraductal lithiasis, and two with minimal change nondilated duct CP. Umbilical cord-derived MSCs (UC-MSCs) were isolated from umbilical cords of three healthy women with full-term pregnancies and without underlying diseases. Informed consents had been obtained from all donors. Patients gave informed consent for the study under protocols approved by the Medical University of South Carolina Internal Review Board (Pro00028011). This study was approved by the institutional review board of the Medical University of South Carolina and all methods were performed in accordance with the relevant guidelines and regulations.

MSC Isolation and Culture

Up to 10 ml aspirated iliac crest bone marrow were diluted 1:2 with phosphate-buffered saline (PBS) (Life Technologies, Waltham, MA) and layered onto Ficoll (density 1.077 g/ml; GE Healthcare, Uppsala, Sweden) diluted to a density of 1.068 g/ml. After centrifugation at 400*g* for 30 minutes at room temperature, mononuclear cells were collected from the interphase, washed twice with PBS, and plated at a density of 0.25–0.5 million cells per cm^2 in α-MEM (Life Technologies) supplemented with Gentamicin (50 μg/ml) and 10% freshly thawed human platelet lysate (from the Emory University). Cells were incubated at 37°C and 5% CO_2. Nonadherent cells were washed off with PBS after 24–48 hours. Medium was changed twice a week. When cultures reached approximately 80% confluence, cells were detached with CTS TrypLE Select Enzyme (Life Technologies), counted, and replated at 1×10^3 to 5×10^3 cells per cm^2. UC-MSCs were harvested as previously described [40].

Sterility, Endotoxin, and Mycoplasma Tests

Sterility was performed using the BD BACTEC fully automated blood culture system for monitoring bacterial and fungal contamination. For sterility test, a volume of 0.5 CC of the product ("inoculum") was inoculated into aerobic and anaerobic test vessels of the BD BACTEC system and sent to the MUSC clinical Microbiology lab for a 14 days culture. Endotoxin testing was performed using the FDA approved Charles Rivers hand-held EndoSafe PTS Endotoxin Reader according to manufacturer's instruction. MycoAlert Assay system (Lonza, Walkersville, MD) was used to detect mycoplasma. Briefly, a small amount of cells and cell culture media was removed, centrifuged and the supernatant was added to a luminometer cuvette to which MycoAlert

reagent was added and incubated for 5 minutes. The sample was then placed in the luminometer holder for background reading of luminescence (Read A). The MycoAlert substrate was then added and incubated for 10 minutes. The sample was then placed in the luminometer holder and a reading of luminescence taken (Read B). A calculation of the ratio of the readings = Reading B/Reading A is then displayed: Ratio B/A > 1.2 Sample Contaminated; Ratio B/A < 0.9 Clean; Ratio B/A 0.9-1.2 Borderline (Retest sample if possible 24 hours later).

Phenotypes of H-MSCs and CP-MSCs

The established MSCs from both healthy donor and CP patients were characterized for stem cell markers by flow cytometry. The antibodies for analysis were anti-Human CD31, anti-Human CD44, anti-Human CD45, anti-Human CD90, anti-Human CD105, and anti-Human HLA-DR (BD Biosciences, San Jose, CA) which were used at the manufacturer's recommendations.

Colony-Forming Unit-Fibroblast Assay

Cells were seeded in 6 well plates (10–20 cells per cm^2) and cultured in complete culture medium. The medium was replaced every 4 days. After incubation for 14 days, the flasks were washed twice, fixed with 100% methanol and stained with 0.5% crystal violet. Cell clusters consisting of at least 50 fibroblasts were scored as a colony-forming unit-fibroblast (CFU-F) colony.

Bone Marrow-Derived MSC (BM-MSC) Differentiation Assays

To induce healthy and CP patient MSC to differentiate into various cell phenotypes, cells (0.5×10^3 cells per cm^2) were plated in 12-well culture plates and allowed to reach confluence. Osteogenic differentiation medium, consisting of complete culture medium supplemented with 50 μg/ml ascorbic acid, 10 mM β-glycerolphosphate, and 10 nM dexamethasone (all from Sigma), was exchanged every 3 days for 3 weeks. The cells were fixed with 10% neutral buffered formalin for 30 minutes, and then stained with Alizarin Red for 1 hour. For adipocytes differentiation, complete culture medium was supplemented with 50 μg/ml ascorbic acid, 0.5 mM 1-methyl-3-isobutylxanthine, 10 nM dexamethasone, and 10 μg/ml insulin. The cells were fixed with 10% neutral buffered formalin for 30 minutes, and then stained with Oil Red O for 50 minutes. Chondrogenic differentiation was induced using a pellet culture system by culturing the cells pellet in 15-ml conical tubes with media containing DMEM high glucose, 5% FBS, 1% L-glutamine, supplemented with 10% ITS+ Premix tissue culture supplement (Invitrogen, Carlsbad, CA), 100 nM dexamethasone and 10 ng/ml TGF-β (R&D system, NE Minneapolis, MN) for 21 days with medium exchange twice a week. The aggregates were fixed with 10% neutral buffered formalin overnight, frozen in O.C.T and sectioned. Alcian blue was used to stain the extracellular matrix.

Analysis of BM-MSCs Differentiation by Real-Time Quantitative PCR

BM-MSCs differentiation was verified by analyzing the gene expression of adipogenic (peroxisome proliferator-activated receptor [PPAR]γ2 and lipoprotein lipase [LPL]), osteogenic (Runx2 and osteocalcin [OCN]), and chondrogenic (type II collagen a1 chain) markers. DNA-free RNA was extracted using RNeasy Micro kit (Qiagen, Germantown, MD) and revers transcribed using iScript cDNA Synthesis Kit, (Bio-Rad, Hercules, CA). Real-time quantitative PCR was performed in duplicate on a CFX-96 Real-Time PCR system thermal cycler and SYBR green Mastermix (Bio-Rad) was used. Human primers were as described previously [37]. In brief, PCR primer sequences used for these experiments are as following (F and R represent the forward and reverse primers, respectively): PPARγ2 (F) 5′-GACCACTCCCACTCCTTTGA-3′, (R) 5′-CGACATTCAATTGCCATGAG-3′; LPL (F) 5′-TACAGGGCGGCCA CAA GTTTT-3′, (R)5′-ATGGAGAGCAAAGCCCTGCTC-3′; runx2 (F) 5′-TATGAAAAA CCAAGTAGCAAGGTTC, (R) 5′-GTAATCTGACTCTGT CCTTGTGGAT-3′; OCN (F) 5′-GTGCAGAGTCCAGCAAAGGT-3′, (R) 5′-CTAGCCAACTCGTCACAGTC-3′; type II collagen α1 chain (F) TTT CCCAGGTCAAGATGGTC, (R) TCACCTGGTTTTCCACCTTC; IDO (F) 5′-GCCCTTCAAGTGTTTCACCAA-3′, (R) 5′-CCAGCCAGACAAATATATGC GA-3′; β-actin (F) 5′-TCACCCACACTGTGCCCATCTACG-3′, (R) CAGC GGAACCGCTCAT TGCCAATG.

RT2 PCR Array

MSCs (passage 5) were cultured for 48 hours to 80% confluence and total RNA was isolated. Next, 500 ng RNA samples were subjected to cDNA synthesis using RT2 First Strand Kit (Qiagen). Human Mesenchymal Stem Cell RT^2 Profiler PCR Array (Qiagen) was used to analyze the expression of 84 specific genes related to stemness of human MSCs according to manufacturer's instructions on CFX96 Real-Time PCR Detection System (Bio-Rad). Data were analyzed using software supplied by Qiagen. The fold change in gene expression (compared to HBM-MSCs) was calculated using the ΔΔ Ct method. A more than 1.5-fold change in gene expression was considered as the upregulation or downregulation of a specific gene expression.

Enzyme-Linked Immunosorbent Assay

Concentrations of TGF-β, VEGF, and HGF in cell culture supernatant were measured using the human enzyme-linked immunosorbent assay (ELISA) kits (RayBiotech, Norcross, GA) according to manufacturer's instructions.

Western Blot

H-MSCs and CP-MSCs lysates (30 μg) were separated by SDS-PAGE, transferred to PVDF membranes, and incubated with primary antibodies against human matrix metalloproteinase-2 (MMP2) (Abcam) and GAPDH (Cell signaling, Danvers, MA). Horseradish peroxidase-conjugated secondary antibodies were from Cell Signaling Technology. Signals were visualized using an ECL detection kit (Thermo Scientific). Relative protein expression was quantified using the ImageJ (NIH) or Image Lab Software (Bio-Rad, Cambridge UK).

Senescence-Associated β-Galactosidase Staining

Expression of pH-dependent senescence associated β-galactosidase (SA-β-gal) in MSCs was measured as described previously [41]. Briefly, MSCs from three donors in each group were grown in a microwell plate (3 wells/individual donors), washed and incubated in 3% PFA for 5 minutes at room temperature. After washing two times with PBS, β-Gal staining solution (final concentration 1 mg/ml, pH 6.0) was added to each well and incubated at 37°C overnight. Cells were examined under a light microscope. Percentage of senescent cells was determined by counting SA-β-gal positive cells in at least three fields from triplicate wells for each donor.

Immunosuppression Assay

Cell trace violet-labeled peripheral blood mononuclear cells (PBMCs) were cultured at 1 million cells per cm^2 in 96-well plate with H-MSCs, CP-MSCs or UC-MSCs at ratio MSC:PBMC of 1:2, and 1:5 in R10 (RPMI, 10% FBS, 100 U/ml of penicillin/streptomycin) and the ratio 1:2 was decided to be the optimal cell ratio for this experiment. Stimulation of the T lymphocytes was induced by adding 0.2 μg of anti-human CD3 and CD28 antibodies (UCSF Antibody Core) to the coculture. T cell proliferation was determined 48 hours later by flow cytometry analysis of fluorescence intensity.

IDO Assay

BM-MSCs (passage 4) were seeded in a 24-well plate (1×10^5 cells per cm^2), cultured overnight and then stimulated with human interferon-γ (IFNγ) (5 ng/ml) for 6 hours. Cells were collected and RNA was extracted using RNeasy Micro kit (Qiagen). Expression level of *IDO* mRNA relative to β-actin was evaluated by quantitative SYBR green real-time PCR. Delta CT method was applied to calculate the fold induction of *IDO* over the unstimulated control.

Human Islet and MSC Coculture

BM-MSCs (passage 4–5) were seeded in 6-well plates (2.2×10^4 cells per cm^2) and cultured in complete medium for 24 hours. Then, the medium was changed into RPMI-1640 (Corning, Corning, NY) supplemented with 10% FBS and 100 U/ml of penicillin/streptomycin, followed by the addition of 100 human islets directly onto the MSC monolayers. After 24 hours of coculture, for hypoxia groups, the islet: MSC system was exposed to hypoxia (1% oxygen) for 24 hours.

TdT-Mediated dUTP Nick End Labeling Assay

After coculture, islets were collected and embedded in O.C.T (TissueTek, Torrance, CA) blocks and 8 μm frozen sections were obtained by a cryostat. Sections were fixed with cold acetone for 5 minutes. A TdT-mediated dUTP nick end labeling (TUNEL) kit (Roche, Indianapolis, IN) was used according to the manufacturer's instruction, followed by the incubation of primary antibody overnight (anti-insulin, 1:100, Invitrogen). An Alexa-Fluor 568 labeled anti-guinea pig was used to detect insulin-positive cells. Images were collected using confocal microscopy (Leica SP5) and fluorescent microscopy (Zeiss Axio Imager M2).

Statistical Analysis

Data are expressed as mean ± SD. Differences between groups were compared for statistical significance by one-way ANOVA with Tukey's multiple comparison or Student's *t* test; $p < .05$ denoted significance.

RESULTS

Quality and Sterility of MSCs from Healthy Donors and CP-Patients

MSCs from healthy controls and patients with CP appeared as typical monolayers of spindle-shaped fibroblast-like cells during ex vivo expansion (Fig. 1A) with similar proliferative rate based on cell counting during passaging the cells up to passage 6 (data not shown). MSCs from both types of donors passed sterility tests for bacterial and fungal contamination in our center. Also, test samples from both H-MSCs and CP-MSCs exhibited endotoxin levels within our internal endotoxin limit (less than 0.5 EU/ml). Microbiological tests such as mycoplasma testing on both types of MSCs preps indicated "clean" samples as calculated ratios B/A for every batch of samples showed values less than 0.9. These results indicate that CP-MSCs prepared are free of contamination and can readily be developed as a potential cellular therapy.

Surface Marker Expression and Colony Formation Potential of CP-MSCs in Comparison to H-MSCs

In accordance with the proposed criteria for the definition of MSCs [42], both H-MSCs and CP-MSCs expressed the surface markers CD44, CD90, and CD105 (Fig. 1B). The absence of endothelial and hematopoietic cells was confirmed by the lack of $CD31^+$, $CD45^+$ cells, and HLA-DR antigen (Fig. 1C). To assess the colony forming abilities of CP-MSCs, we performed the CFU-F assay. The frequency of CFU-Fs was slightly lower, but not significantly different in CP-MSCs (23.5 ± 3.5) compared with H-MSCs (26.0 ± 4.0, Fig. 1D). These results demonstrate that MSCs from healthy donors and CP patients did not differ in their phenotypic characteristics.

Multilineage Differentiation Potential of CP-MSCs in Comparison to H-MSCs

At passage 3, multilineage potential of CP-MSCs and H-MSCs was examined. Cell type–specific staining showed that MSCs from both sources successfully differentiated into adipocytes, osteoblasts, and chondrocytes, when they were induced in vitro by adipogenic, osteogenic, and chondrogenic media, respectively (Fig. 2A–2F). Quantitative PCR analysis also detected upregulations of the cell type–specific marker genes expressions during differentiation (Fig. 2I–2K). We found that there was no significant difference in respect to the induction levels of marker genes between MSCs from healthy donors and CP patients at 14 days and 21 days after differentiation, with regard to the adipogenic markers, PPARγ, and LPL (Fig. 2G, 2H) as well as osteogenic markers Runx2 and OCN (Fig. 2I, 2J). The elevation of chondrogenic marker type II collagen a1 chain (Col2a1) was only detected at 21 days in both cells after exposure to chondrogenic media (Fig. 2K). These results verified our hypothesis that the cells isolated from CP patient BM possess MSC phenotypes to the similar extent as those from healthy donors.

To further evaluate the characteristic of CP-MSC in respect of cell proliferative activity and senescence, the expression levels of a panel of genes critical for cell proliferation (cyclin A, cyclin E) or cellular senescence ($p16^{Ink4a}$, p19, p21 p27, and Bmi) were measured in MSCs at passage 6 from three different donors in each group. No significant difference was observed for either proliferation-related markers (cyclin A, cyclin E) or senescence-related markers ($p16^{Ink4a}$, p19, p21 p27, and Bmi). In addition, SA-β-gal staining was performed on a later passage (passage 9) of MSCs. We found that the CP-MSCs expressed similar amount of SA-β-gal compared to H-MSCs (Fig. 3C, 3D), indicating that CP-MSCs are comparable to H-MSCs with regard to cell proliferation and senescence.

Expression Profile of MSCs-Related Genes in H-MSCs and CP-MSCs

To further compare MSCs from CP patients with the ones from healthy donors, an RT^2 Profiler PCR Array was used to screen a panel of 84 genes associated with pluripotency differentiation and

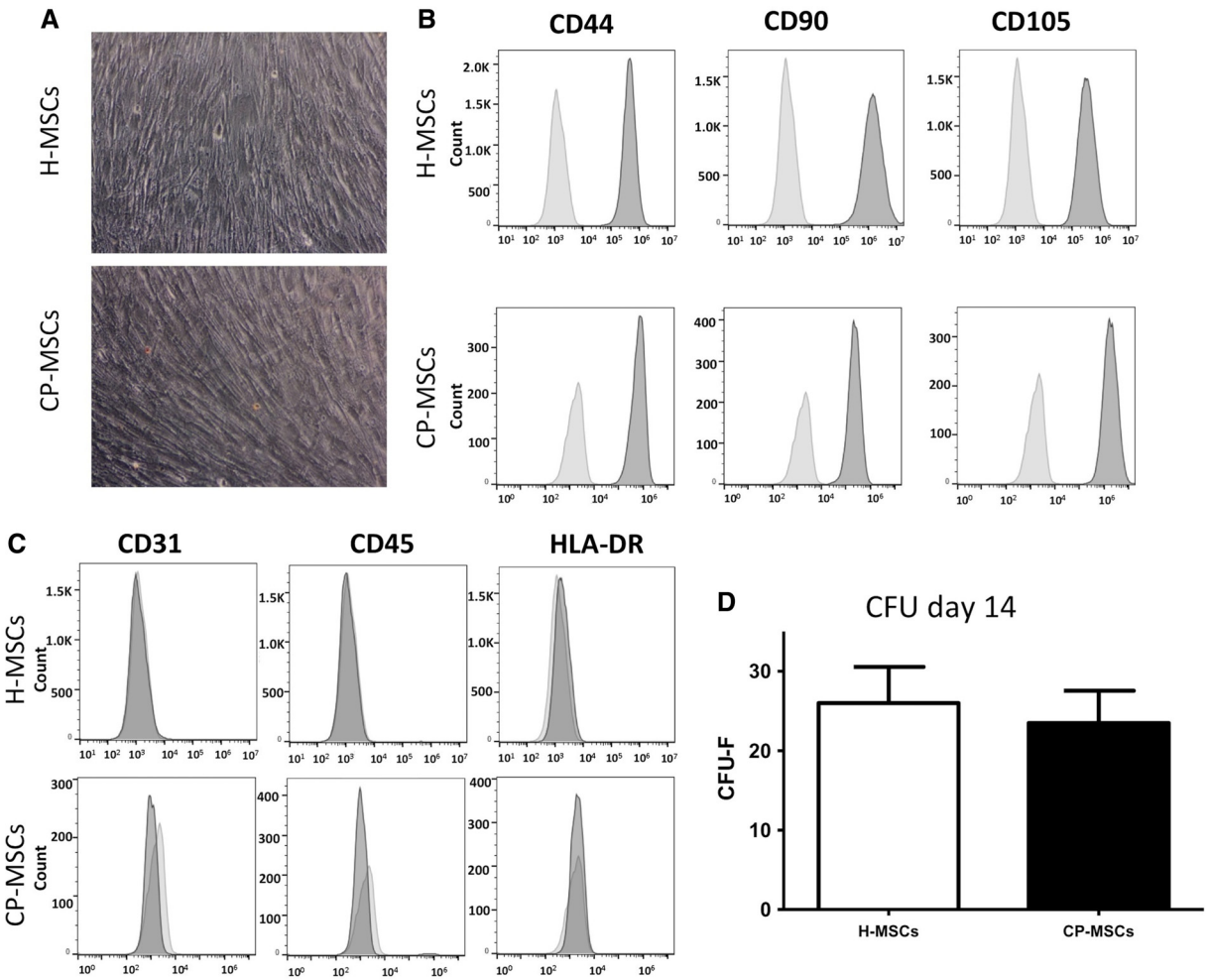

Figure 1. Phenotype and colony forming ability of H-MSCs and CP-MSCs. **(A):** Images of representative cultures of H-MSCs (upper panels) and CP-MSCs (lower panels) that were taken using a phase contrast microscope. Light gray: isotype control; dark gray: expression of indicated molecules on H-MSCs or CP-MSCs. **(B):** Representative FACS analysis of MSC defining surface positive marker panel (CD44, CD90, CD105). **(C):** FACS analysis of CD31, CD45 and HLA-DR on H-MSCs and CP-MSCs. **(D):** Total CFU-F in H-MSCs and CP-MSCs with crystal violet at 14 days after culture. Abbreviations: CFU-F, colony forming units-fibroblast; CP-MSC, mesenchymal stem cells from chronic pancreatitis patients; H-MSC, mesenchymal stem cells from healthy donors.

self-renewal. Among all 84 genes measured, CP-MSCs expressed four genes significantly differentially compared with H-MSCs (Fig. 4A). Among these genes, growth differentiation factor 6 (*GDF6*) and *HGF* were significantly decreased. Meanwhile, *MMP2* and *TGF-β3* were significantly increased (Fig. 4B). As MMP2 plays an important role in the differentiation, angiogenesis, proliferation, and migration of MSCs, Western blot was performed to further investigate the protein levels of MMP2 in H-MSCs and CP-MSCs. We found that CP-MSC exhibited similar amount of MMP2 at protein level compared to H-MSCs (Fig. 4C, 4D). This is consistent with our finding that no significant difference was observed in respect to multilineage differentiation potential between H-MSCs and CP-MSCs.

Growth Factors Secreted By H-MSCs and CP-MSCs

A growing body of evidence suggests that much of the disease-modulating activity of MSCs is due to paracrine factors secreted by the cells [43–45]. Specific factors secreted from H-MSCs and CP-MSCs at two different passages (passage 2 and passage 4) were measured with ELISA after 6 hours of culture. We found that MSCs from CP patient produced similar amount of VEGF, a critical factor for angiogenesis, as those from healthy donors, at both passages (Fig. 5A). TGF-β is an important soluble immunomodulatory molecule that is constitutively secreted by MSC. The addition of anti-TGF-β antibody attenuated the suppressive effect of MSC on proliferation of peripheral blood lymphocytes [46]. We found that TGF-β secretion was slightly higher in CP-MSCs compared with H-MSCs at an earlier passage (P2), but no difference was observed at a later passage (P4) between the two groups (Fig. 5B). HGF was proven to be neuroprotective in a glutamate induced excitotoxicity model [47] and anti-apoptotic in a rodent model of acute kidney failure [48]. Interestingly, in the current study, we discovered that HGF production was significantly less in CP-MSCs compared with H-MSCs at both passages examined in this study (Fig. 5C), which is consistent with our observation of decreased *HGF* gene expression in CP-MSCs (Fig. 4B).

Immunosuppressive Potential of H-MSCs and CP-MSCs

The immunosuppressive properties of MSCs toward T cells were compared between H-MSCs and CP-MSCs. Two ratios

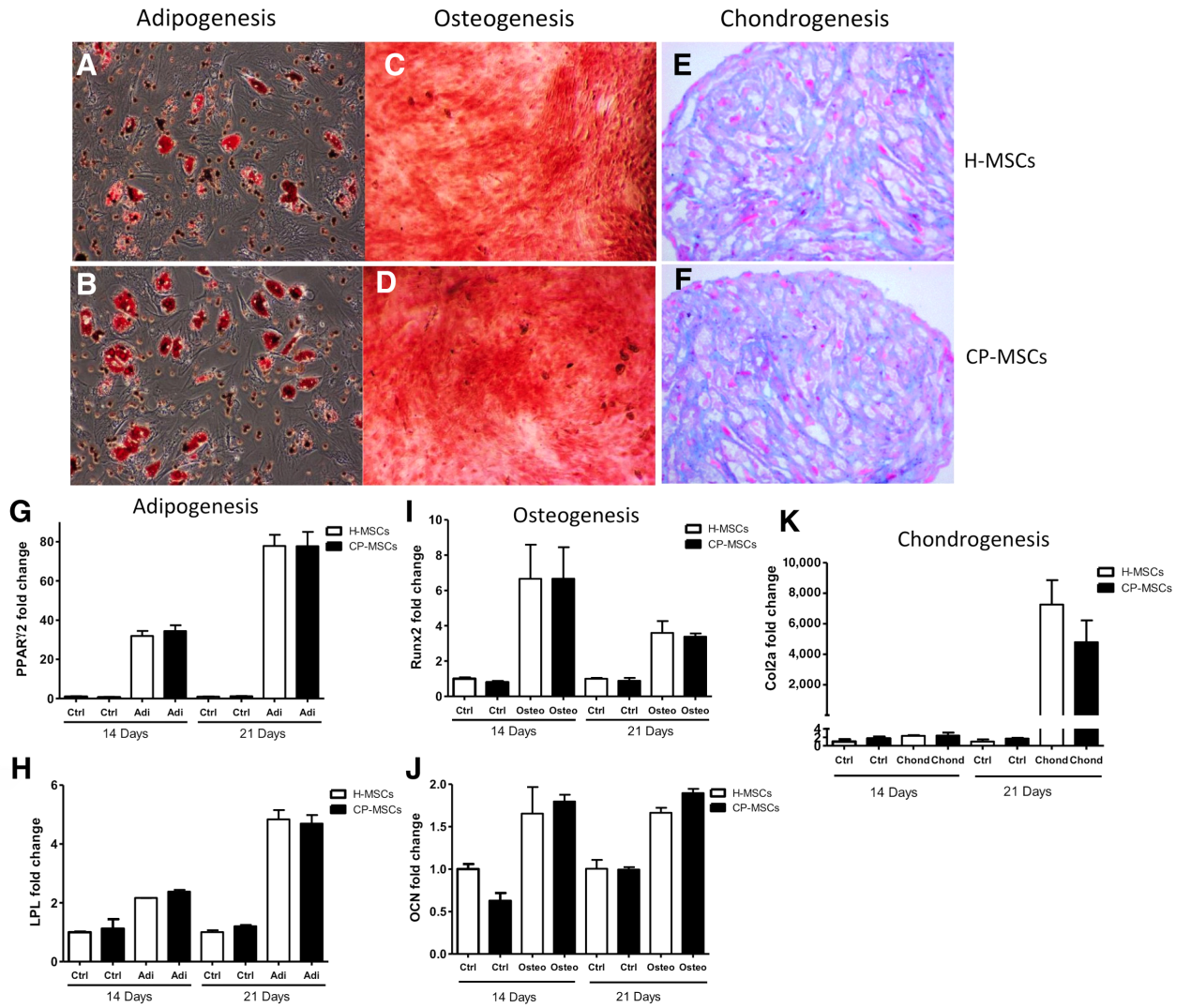

Figure 2. Multilineage differentiation of H-MSCs and CP-MSCs: **(A, B)** adipogenic differentiation indicated by oil red staining; **(C, D)** osteogenic differentiation indicated by alizarin red staining; **(E, F)** chondrogenic differentiation indicated by alcian blue staining. Molecular markers of gene expression during each differentiation by real-time PCR, **(G)** PPARγ, **(H)** LPL, **(I)** Runx2, **(J)** OCN, and **(K)** Col2a. Bars represent mean ± SD; data are representative of at least three independent experiments. Abbreviations: Col2a, collagen a1; CP-MSC, mesenchymal stem cells from chronic pancreatitis patients; H-MSC, mesenchymal stem cells from healthy donors; LPL, lipoprotein lipase; PPARγ, peroxisome proliferator-activated receptor 2; OCN, osteocalcin.

(5:1 and 2:1, MSCs:PBMCs) and three incubation time periods (24, 48, and 72 hours) were tested for immunosuppressive activity and we observed that both H-MSCs and CP-MSCs inhibited T cell proliferation most effectively at the ratio of 5:1 with the incubation time of 48 hours (Fig. 6A). We proved that MSCs from CP patient efficiently suppressed both $CD4^+$ and $CD8^+$ T cells proliferation, to a similar extent as the H-MSCs. Furthermore, we also investigated the IFNγ-induced expression levels of *IDO* in H-MSCs and CP-MSCs, because IDO enzymatic activity of MSC is the main mechanism of T cell suppression, and the level of IDO correlated with the suppressive potential of a given MSC donor [33]. Both H-MSCs and CP-MSCs exhibited marked increase in *IDO* mRNA expression 6 hours after incubation with IFNγ (Fig. 6B). Interestingly, we found that CP-MSCs expressed significantly more *IDO* mRNA in response to cytokine stimulation compared with H-MSCs. In addition, both H-MSCs and CP-MSCs showed higher levels of *IDO* expression compared with UC-MSCs from four donors, which is used as another control in this study (Fig. 6B). These results strongly suggest that CP-MSCs have preserved immunosuppressive potentials that are to the same extent as healthy controls.

CP-MSCs Protected Human Islets from Hypoxia-Induced Cell Death

Hypoxia-induced injury is a key factor associated with islet graft dysfunction. Lu et al. showed that MSCs protected islets from hypoxia/reoxygenation-induced injury [49]. In the present study, experiments were performed to examine if CP-MSCs preserved this protective effect on human islets (Fig. 7A–7E). Human islets were cocultured with H-MSCs or CP-MSCs for 24 hours, followed by the exposure of hypoxia condition (1% oxygen) for 24 hours. Pancreatic β cells were identified by immunostaining with insulin antibody. We found that in the presence of hypoxia, H-MSCs significantly reduced $TUNEL^+$ β cells compared with the islet only group, (2.5 ± 2.23% vs. 8.3 ± 3.47%) (Fig. 7G). Importantly, CP-MSCs decreased hypoxia-induced β cells death to a similar level

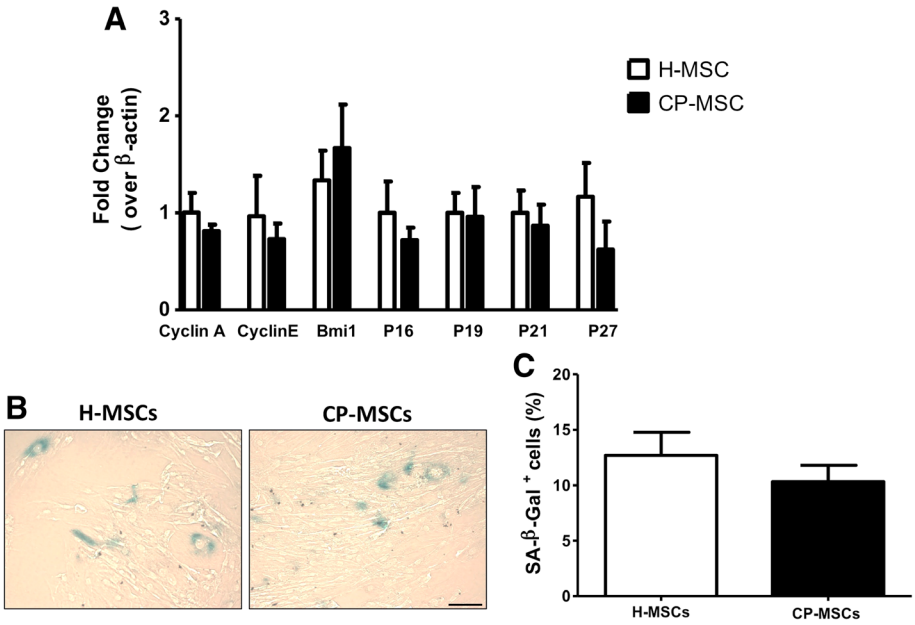

Figure 3. Proliferation and senescence of H-MSCs and CP-MSCs. **(A):** Expression of cell cycle- and senescence-related genes at mRNA level in H-MSCs and CP-MSCs at passage 6. **(B):** Representative images of SA-β-gal staining on H-MSCs and CP-MSCs at passage 9. Scale bar = 100 μM. **(C):** Percentages of senescent cells quantified by SA-β-gal + cells among all the cells. Bars represent mean ± SEM. Abbreviations: CP-MSC, mesenchymal stem cells from chronic pancreatitis patients; H-MSC, mesenchymal stem cells from healthy donors; SA-β-gal, senescence associated β-galactosidase.

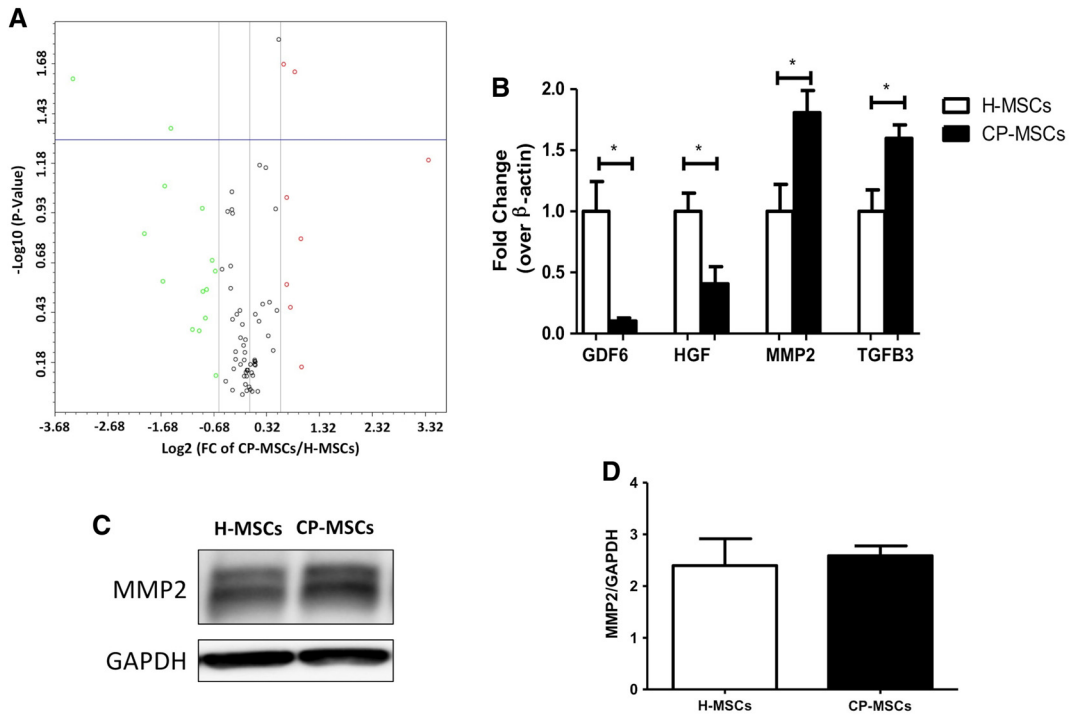

Figure 4. Expression profile of MSCs-related genes in H-MSCs and CP-MSCs. **(A):** Volcano plot of 84 human MSCs-related genes in CP-MSCs versus H-MSCs. Fold change of more than 1.5 was used as a threshold limit, with green circle indicating <1.5 and red circle indicating >1.5. The blue horizontal line indicates that the *p* value of the Student's *t* test threshold is .05. **(B):** Folds change of genes that are differentially expressed in H-MSCs and CP-MSCs. **(C):** Representative image on Western blot shows the expression level of MMP2 in H-MSCs and CP-MSCs at passage 6. **(D):** Quantification of band intensities of MMP2 expressions in HMSCs and CP-MSCs. Bars represent mean ± SEM. *n* = 3. Abbreviations: CP-MSC, mesenchymal stem cells from chronic pancreatitis patients; GDF6, growth differentiation factor 6; HGF, hepatocyte growth factor; H-MSC, mesenchymal stem cells from healthy donors; MMP2, matrix metalloproteinase-2.

as H-MSCs (2.7 ± 3.57% vs. 2.5 ± 2.23%). No apparent cells death was detected in any of the normoxia groups (Fig. 7A–7C). These results indicated that CP-MSCs possess similar ability as H-MSCs to prevent hypoxia-induced injury in islet β cells, further proved the feasibility of the usage of autologous MSCs cotransplant with islets in patients undergoing TP-IAT.

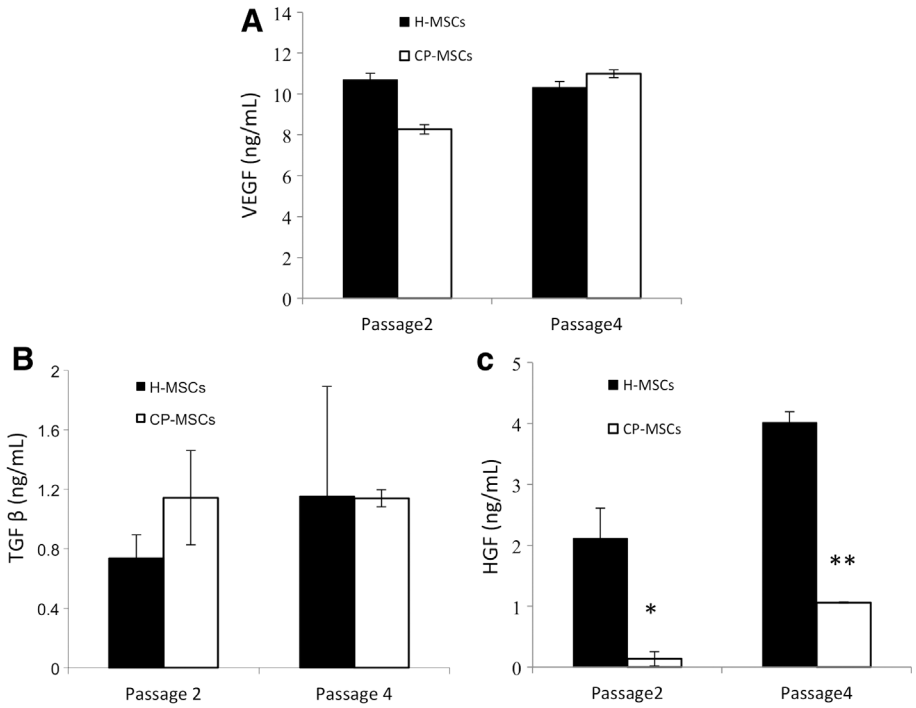

Figure 5. Secretion levels of growth factors by H-MSCs and CP-MSCs. **(A)** VEGF, **(B)** TGF-β, **(C)** HGF secretion in H-MSCs and CP-MSCs at passage 2 and passage 4 measured by ELISA (n = 3). Values represent mean ± SD, and n = 3 for both H-MSCs and CP-MSCs. * and ** indicates $p < .05$ and $p < .01$ versus H-MSCs, respectively. Two-tail Student's t test. Abbreviations: CP-MSC, mesenchymal stem cells from chronic pancreatitis patients; HGF, hepatocyte growth factor; H-MSC, mesenchymal stem cells from healthy donors; VEGF, vascular endothelial growth factor.

Discussion

Unlike insulin-dependent type 1 diabetes, glycemic control after total pancreactectomy is notoriously difficult with standard pharmacologic insulin therapy due to the absence of glucagon-dependent counter-regulation from islet α cells. For these patients, TP-IAT has the distinct advantage of allowing patients the ability to avoid this surgically induced "brittle diabetes." However, only approximately 30% of patients at 3 years after TP surgery were insulin independent [50]. Therefore, therapies that can improve the outcomes of autologous islet transplantation could substantially benefit patients undergoing TP-IAT. A growing body of evidence suggests that cotransplant of islets with MSCs results in enhanced islet graft survival and function in allogeneic mouse islet transplantation models, strongly indicating a potential protective role of MSCs on islet grafts in TP-IAT. In this study, we demonstrated that CP-MSCs possessed similar properties as H-MSCs and UC-MSCs, in respect to cell surface markers, colony formation ability, multilineage differentiation potential, secretory function as well as immunosuppressive abilities. We found that four genes were differentially expressed in CP-MSCs comparing with H-MSCs, among the 84 human MSC-related genes studied. Also, TUNEL assays suggest that CP-MSCs protected islets from CP patients against hypoxia-induced cell death. Consistent with this result, our recent data from a pilot clinical trial of CP-MSC and islet cotransplantation showed that MSC group required lower amounts of insulin during the peritransplantation period and had lower 12-month fasting blood glucose levels [36], suggesting a beneficial effect of CP-MSCs in TP-IAT. These findings strongly indicate that CP-MSCs can be developed as a regenerative cellular therapy for CP patients.

Autologous cell therapy is a preferred choice for cellular therapy because of no immune rejection response involved. Although it has been suggested that MSCs can engraft in allogeneic recipients, this immune privilege is not universal. Indeed, Eliopoulos et al. demonstrated that class I and II MHC-mismatched MSCs elicited a robust and specific cellular immune response in allogeneic hosts with normal immune systems [51]. Furthermore, the immunogenicity might be augmented immediately post-thaw [52], which would considerably compromise the therapeutic effects of unmatched random donor MSCs if they were cryopreserved. Therefore, autologous MSCs from CP patients freshly isolated and expanded in vitro seems to be a more suitable option for cotransplantation in TP-IAT. However, concerns arise that MSCs derived from patients suffering from autoimmune or inflammatory disorders such as CP may have defects in phenotype, colony formation ability, differentiation potential and immunosuppressive functions, and thus may not be equivalent to MSCs from healthy donors. Evidence has suggested that MSCs from patients with systemic lupus erythematous exhibit slower proliferation rate, downregulated cytokines production [53], as well as impaired capacity of osteogenic differentiation [54]. Also, MSCs from patients with systemic sclerosis exhibited intrinsic deregulation of vascular smooth muscle and myofibroblast differentiation in response to key-mediators including TGF-β [55]. These results prompted us to investigate the phenotype and function of CP-MSCs and compare them with H-MSCs. We found that culture-expanded BM-MSCs obtained from healthy donors and patients with CP showed no significant difference in their expression of cell surface markers, ability to form colonies, as well as multilineage differentiation capacity. Furthermore, one of the key

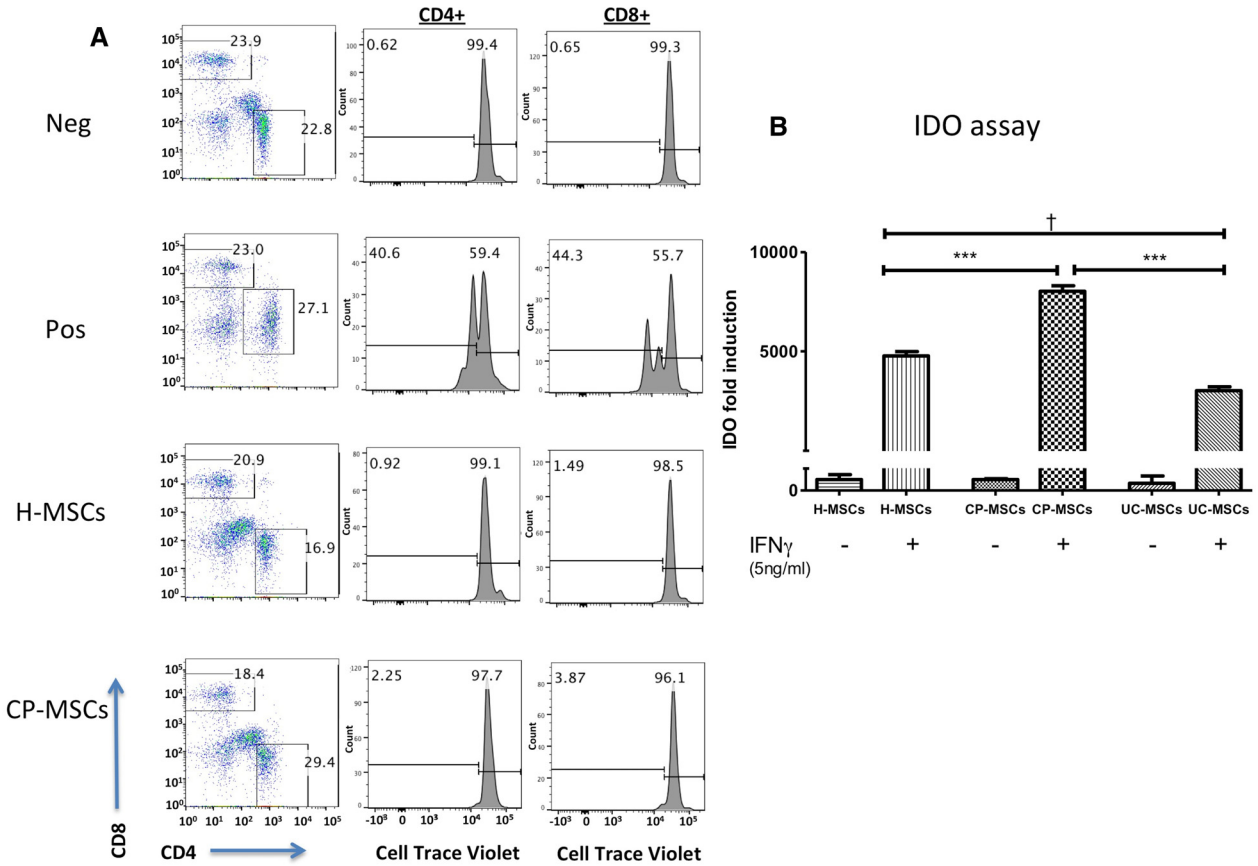

Figure 6. Immunosuppressive potential of H-MSCs and CP-MSCs. **(A):** Represent figure of cell proliferation measured by flow cytometry. $CD4^+$ (middle panels) and $CD8^+$ (right panels) T cell proliferation assay was performed using Cell Trace violet-labeled human PBMCs. Cells were activated by anti-CD3 and anti-CD28 antibodies. MSC:PBMC ratio was 5:1. Neg: negative control. Pos: positive control. **(B):** mRNA expression of *IDO* after IFN-γ stimulation in H-MSCs and CP-MSCs. Expression level of *IDO* mRNA relative to β-actin was evaluated by quantitative SYBR green real-time PCR. Delta CT method was applied to calculate the fold induction of *IDO* over the unstimulated control. Bars represent mean ± SD. n = 3–5, *** indicates $p < .001$ versus CP-MSCs stimulated with IFNγ, † indicates $p < .001$ versus UC-MSCs stimulated with IFNγ. One-way ANOVA with Tukey's multiple comparison. Abbreviations: CP-MSC, mesenchymal stem cells from chronic pancreatitis patients; H-MSC, mesenchymal stem cells from healthy donors; IDO, indoleamine 2,3-dioxygenase; IFN-γ, interferon-γ; PBMC, peripheral blood mononuclear cell; UC-MSCs, umbilical cord-derived MSCs.

therapeutic characters of MSCs is their ability to inhibit T-cell proliferation in vitro. We demonstrated in this study that CP-MSCs possess similar suppressive effects on 48 hours after culturing with antibody-activated PBMCs as those of both H-MSCs and UC-MSCs. UC-MSCs were used as a functional control in our immunosuppressive experiments because they are considered as a noncontroversial yet promising alternative source of MSC in regenerative cell therapy for patients undergo islet transplantation.

In human MSCs, IDO is considered the main actor in inhibiting T-cell growth and function through the degradation of tryptophan, an amino acid essential for lymphocyte proliferation. The depletion of tryptophan by IDO results in the formation of toxic metabolites such as kynurenine, leading to T cell inhibition [56]. The expression of IDO was shown to be induced following exposure to IFN-γ [56, 57], and the level of expression of IDO correlated with the suppressive potential of a given MSC donor in patients undergoing elective hip replacement surgery [33]. Interestingly, we observed a higher *IDO* mRNA expression level in CP-MSCs in response to IFNγ exposure compared to that of H-MSCs and UC-MSCs. A possible reason for this is that CP-MSCs may be more sensitive to cytokine stimulation due to the prolonged disease status. Regardless of this discrepancy, it is worth noting that CP-MSCs exhibited similar ability of suppressing T cell proliferation to H-MSCs.

Another major benefit of MSCs in terms of homing and repairing damaged tissues is their ability to secrete angiogenic and other paracrine growth factors. We found that CP-MSCs secret two of the key growth factors (VEGF and TGF-β) that are typically produced by MSCs, to a similar level as H-MSCs. VEGF is of special interest because evidence showed that it is required for the vessel recruitment and revascularization of transplanted islets [58]. Also, it was demonstrated in mouse syngeneic transplantation model that MSCs expressed high levels of VEGF, which was accompanied by increased vascular density in islet grafts in cotransplantation of MSCs with pancreatic islets [17]. Our result that CP-MSCs are able to produce similar amount of VEGF both at mRNA level and protein level indicates a potential proangiogenic benefit of CP-MSCs. However, we found a less amount of HGF secreted by CP-MSCs compared with H-MSCs, as well as at the mRNA level. HGF was indicated to be both anti-apoptotic [13] and seemed to be able to enhance angiogenesis synergistically with VEGF [59]. Also, HGF was reported to be able to significantly

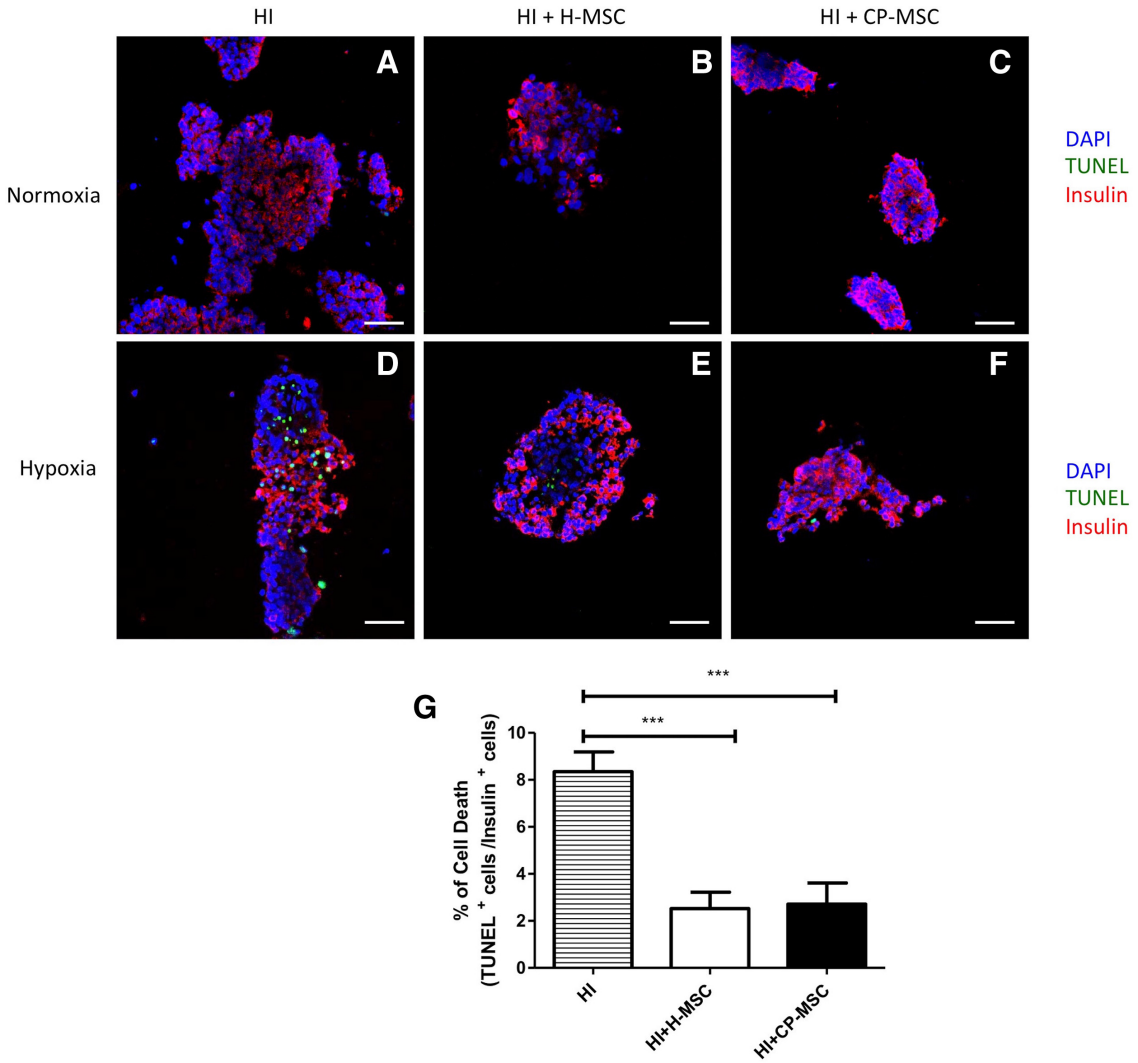

Figure 7. CP-patient islets coculture with or without CP-MSCs 48 hours under normal or hypoxic conditions (1% oxygen). Representative pictures of immunofluorescent staining of islets cultured alone or with H-MSCs or CP-MSCs under normal **(A–C)** or hypoxia conditions **(D–F)** stained for insulin (red) and TUNEL$^+$ (green) cells. At least 20 islets have been counted in each group. Scale bar = 100 μM. **(G):** Percentages of TUNEL$^+$ cells among insulin$^+$ cells in human islets cultured alone (HI), with H-MSCs or with CP-MSCs. ***, $p < .001$, One-way ANOVA with Turkey's multiple comparison. Abbreviations: CP-MSC, mesenchymal stem cells from chronic pancreatitis patients; H-MSC, mesenchymal stem cells from healthy donors; TUNEL, TdT-mediated dUTP nick end labeling.

improve the insulin content and secretion [60], as well as β cell proliferation [61] and regeneration [62]. Furthermore, kidney capsule islet transplantation models both in mouse and nonhuman primate suggested that HGF-overexpression enhanced the islet graft function by 2–3 folds [63, 64]. However, few studies demonstrated a direct beneficial effect of MSC-secreted HGF in islet transplantation. Cavallari et al. found that preconditioned adipose-derived MSCs with increased secretion of several angiogenic factors, including HGF, facilitated islet revascularization with improved outcome after intrahepatic cotransplantation of islets with these MSCs in rats [65]. Others found that cotransplantation of endothelial progenitor cells (EPCs) with islets led to better graft revascularization and greater β-cell proliferation, and higher HGF production from EPCs was associated with higher β-cell proliferation in the islet-EPC group [66]. Therefore, further in vivo studies on islet graft revascularization and function are needed to determine if a less amount of HGF would reduce the therapeutic effect of MSCs in TP-IAT. Furthermore, TGFβ negatively regulates HGF expression in tubular epithelial cells [67], fibroblasts [68], and mammary carcinoma cells [69]. On the other hand, HGF secreted by MSC suppresses TGFβ expression in glomerular mesangial cells [70]. Consistent with these results, a higher *TGFβ* mRNA level was observed along with significantly lower HGF expression in CP-MSCs. However, further studies are required to elucidate the potential connection between these two growth factors in CP-MSCs.

One of the major stresses leading to islet cell death after TP-IAT is the low oxygen supply. Previous studies reported protective effects of H-MSCs in various hypoxia conditions including hypoxia/reoxygenation-induced injury on islets [49], hypoxic–ischemic injury of the preterm brain [71], and ischemic acute kidney injury [72]. Importantly, beneficial effects of CP-MSCs on human islets against hypoxia stimulation were detected in our study. In the ex vivo coculture system of human islets and CP-MSCs, both increased viability and preserved insulin content were observed compared to the hypoxia control. One of the

most common adverse events of TP-IAT is portal vein thrombosis (PVT) [73]. Whether MSCs cotransplanted with islets may increase the risk of PVT is unknown. In our pilot clinical trial of CP-MSC and islet cotransplantation, PVT was observed in two out of three patients in the cotransplant group, although in one of the patient the diagnosis might have been caused by incomplete contrast timing of the CT image. However, the chance of PVT is positively correlated with total islet pellet weight [36, 73], and both of the two patients who developed PVT received islet pellets with higher weights than the average islet pellet weight of historical patients with PVT. Therefore, neither of them were determined by the Data Safety Monitoring Committee to be directly related to MSC infusion.

In the present study, no significant difference in either cell phenotype or the potential of differentiation was found between H-MSCs and CP-MSC. MSCs from both sources suppressed T cell proliferation ex vivo, as well as exhibited IFNγ-induced *IDO* expression. The levels of VEGF and TGF-β secreted by CP-MSCs were similar to that produced by H-MSCs. Furthermore, CP-MSCs protected CP patients' islets from hypoxia-induced cell death.

Conclusion

Although compared to H-MSCs there are some changes of gene expression in CP-MSCs, the latter seem to have similar characteristics as H-MSCs with regard to immunosuppressive and anti-apoptotic functions. In conclusion, we propose that autologous MSCs may be suitable for regenerative cell therapy in CP patients undergoing TP-IAT.

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Author Contributions

J.W.: performed experiments and wrote the manuscript; Y.Z.: performed experiments; C.C., T.D., and S.M.: performed cell culture and analysis in the GMP facility; S.O., D.A., and K.A.: consented patient and collected patient samples; G.G.: participated in research design; H.W.: designed the experiments and revised the manuscript.

Disclosure of Potential Conflicts of Interest

The authors indicated no potential conflicts of interest.

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