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REGENERATIVE MEDICINE

Human Multipotent Stromal Cell Secreted Effectors Accelerate Islet Regeneration

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ABSTRACT

Human multipotent stromal cells (hMSC) can induce islet regeneration after transplantation via the secretion of proteins that establish an islet regenerative niche. However, the identity of hMSC-secreted signals and the mechanisms by which pancreatic islet regeneration is induced remain unknown. Recently, mammalian pancreatic α-cells have been shown to possess considerable plasticity, and differentiate into β-like cells after near complete β-cell loss or overexpression of key transcriptional regulators. These studies have generated new excitement that islet regeneration during diabetes may be possible if we can identify clinically applicable stimuli to modulate these key regulatory pathways. Herein, we demonstrate that intrapancreatic-injection of concentrated hMSC-conditioned media (CM) stimulated islet regeneration without requiring cell transfer. hMSC CM-injection significantly reduced hyperglycemia, increased circulating serum insulin concentration, and improved glucose tolerance in streptozotocin-treated mice. The rate and extent of endogenous β-cell mass recovery was dependent on total protein dose administered and was further augmented by the activation of Wnt-signaling using GSK3-inhibition during CM generation. Intrapancreatic hMSC CM-injection immediately set in motion a cascade of regenerative events that included the emergence of proliferating insulin^{+} clusters adjacent to ducts, NKX6.1 expression in glucagon^{+} cells at days 1–4 suggesting the acquisition of β-cell phenotype by α-cells, and accelerated β-cell maturation with increased MAFA-expression for >1 month postinjection. Discovery and validation of islet regenerative hMSC-secreted protein may lead to the development of cell-free regenerative therapies able to tip the balance in favor of β-cell regeneration versus destruction during diabetes. STEM CELLS *2019;37:516–528*

SIGNIFICANCE STATEMENT

Cell-based therapies using human multipotent stromal cells (hMSC) to dampen autoimmunity while inducing islet regeneration represents a promising approach to treat diabetes. However, poor survival and retention of transplanted hMSC in the damaged pancreas complicates clinical translation. Here, it is demonstrated that hMSC secrete an array of islet-regenerative proteins and intrapancreatic delivery of hMSC-conditioned media (CM) could stimulate islet regeneration without the need for cell transfer. Wnt-pathway stimulated hMSC CM-injection set in motion a cascade of events consistent with the emergence of functional islets. Discovery of hMSC-secreted, islet-regenerative effectors may lead to the development of cell-free therapies to combat diabetes.

INTRODUCTION

Both type 1 (T1D) and type 2 diabetes (T2D) are characterized by insulin deficiency due to β-cell failure. Thus, curative strategies for diabetes treatment will require renewal of functional β-cell mass [1]. Even with modern insulin therapy, dysregulated glucose homeostasis results in devastating complications, and current therapeutic approaches to replace β-cells include whole pancreas transplantation or intraportal delivery of isolated islets [2]. Unfortunately, implementation of these strategies is limited due to an extreme shortage of donor tissue. In addition, islet transplantation therapy is associated with eventual graft failure despite aggressive, life-long immunosuppression with detrimental side effects [3, 4].

In recent years, considerable progress has been made in development of strategies to replace β-cells via the production of β-like cells from pluripotent stem cells. In principle, pluripotent stem cells possess the potential to generate an unlimited number of β-cells for diabetes therapy [5]. Through the recapitulation of sequential stages of pancreas development, using growth factor-mediated differentiation, the production of cells

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that secrete insulin in response to elevated glucose, and revert hyperglycemia after transplantation into streptozotocin (STZ)-treated mice has been achieved [6–10]. Although these studies represent impressive advances in β-cell replacement, the cells generated secrete insulin at variable efficiencies and deeper understanding of the mechanisms that govern somatic β-cell genesis are still required.

Two central mechanisms controlling β-cell regeneration have been proposed. First, pre-existing β-cells can undergo proliferation to generate greater β-cell mass [8, 9]. Second, β-cell regeneration can be initiated from within islets or the ductal epithelial niche through the activation of facultative endocrine precursors via β-cell neogenesis [10–12]. In addition, α-β-cell conversion, accompanied by epithelial to mesenchymal transition (EMT), can occur within the pancreas [13], recapitulating a developmental pattern elegantly demonstrated using lineage-tracing studies in mice [14]. Currently, the stimuli that control these islet regenerative processes are unknown and remain the key to harnessing β-cell neogenesis in situ as a therapy for diabetes.

In our previous studies on islet regeneration, transplantation of human bone marrow-derived multipotent stromal cells (hMSC) induced the emergence of small, recipient-derived islet-like structures associated with the ductal epithelial niche [15–17]. Detailed proteomic analysis of secretory factors deposited by hMSC revealed Wnt-signaling as a central pathway in islet regenerative hMSC [18]. Indeed, active Wnt-signaling in hMSC generated conditioned media (CM) that increased β-cell survival and proliferation in cultured human islets. Others have suggested that tail vein injection of MSC CM could increase β-cell proliferation in STZ-treated rats and intraductal delivery of the MSC-secreted protein periostin, resulted in islet regeneration and glucose homeostasis in STZ-treated mice. [19, 20]. Collectively, these findings suggest that activation of islet regenerative programs can be achieved via protein-based effectors derived from hMSC. Therefore, preclinical studies delivering hMSC CM into the mouse pancreas are required to assess the induction of β-cell regeneration in vivo.

Herein, we investigated the direct delivery of hMSC CM into the pancreas of STZ-treated mice and show for the first time that islet regeneration can be stimulated after CM delivery without cellular transfer. The regenerative response was augmented by increased protein dose and activation of Wnt-signaling during CM generation heightened the islet regenerative potency of CM. We also show that restoration of glucose homeostasis involved paracrine activation of multiple regenerative pathways consistent with the emergence of islets associated with ducts, and stimulation of α-β-like cell transition programs, followed by functional β-cell maturation.

Materials and Methods

Human Subjects

Human bone marrow was obtained from healthy donors after informed consent at the London Health Science Centre (London, Canada). All studies were approved by the Human Research Ethics Board at the Western University (REB#12934).

Generation of hMSC CM

hMSC cultures (~80% confluent), from 2 independent donors (*n* = 2) were washed twice with phosphate-buffered saline to remove residual growth factors and plated in AmnioMax media without supplement to collect proteins secreted by hMSC for 24 hours (Invitrogen, Carlsbad CA, USA). CM was collected at passage 4 the morning of injection (D10), and concentrated (~40×) using 3 kDa filter spin columns (Millipore, Burlington MA, USA, UFC300324). Briefly, hMSC CM was centrifuged at 4,600*g* for 60 minutes, after which the filtrate was discarded and proteins remaining above the 3 kDa filter were quantified and normalized to 0.2 or 0.4 μg/μl. hMSC CM was used immediately for all in vivo experiments. To generate Wnt-pathway modulated CM, Wnt-pathway stimulation (WNT+) was induced during media conditioning by 10 μM CHIR99021 (AbMole Biosciences, Houston Tx, USA), a selective inhibitor of GSK3, preventing the phosphorylation and degradation of β-catenin, the central regulator of canonical Wnt-signaling. Wnt-pathway inhibition (WNT−) was induced during media conditioning by 20 μM IWR-1 (Sigma, St. Louis MO, USA), a molecule that stabilizes turnover of Axin2, and inhibits the accumulation of β-catenin. Importantly, the 3 kDa spin columns were used to concentrate proteins (40×) in all CM samples generated, and to remove residual small molecule inhibitors (<3 kDa) in the WNT+ and WNT-samples flow through effluent.

RNA Isolation and qPCR

RNA was purified in triplicate from CHIR99201 or IWR-1-treated hMSC using RNeasy kit (Qiagen, germantown MD, USA) following manufacturer's instructions. cDNA was synthesized using High Capacity cDNA Reverse Transcription Kit (Invitrogen, Carlsbad CA, USA). Reverse trasncription-polymerase chain reaction (RT-PCR) was performed using SYBR Green with *BCL9* and *c-MYC* primers (Genecopoeia, Rockville MD, USA). cDNA was amplified and measured using the CFX384 RT-PCR Detection System (Bio-Rad, Hercules CA, USA).

Mouse Preparation, CM Injection, and Monitoring

The Animal Care Committee at Western University approved all procedures (AUP#2015-033). To induce hyperglycemia in non-obese diabetic severe combined immune deficiency (NOD/SCID) mice, STZ (Sigma, germantown MD, USA) was administered intraperitoneally (35 mg/kg per day) daily for 5 days. For intrapancreatic (iPan) injections, mice were anesthetized; the pancreas and spleen exposed; a single dose of CM was microinjected (20 μl) into the splenic portion of the pancreas containing 4 μg or 8 μg total protein on day 10 (D10) as previously described [21]. Control mice were injected with unconditioned media (basal media) and normoglycemic mice received citric acid buffer (CAB) vehicle instead of STZ. Mice were monitored weekly for non-fasted blood glucose by tail vein puncture using a FreeStyle glucometer (Abbott, Chicago IL, USA). Mice were intraperitoneally injected with 100 μl of 2.5 mg/ml EdU 24 hours prior to euthanasia. Experiments were performed in groups of 10–12 mice and included hMSC CM injections alongside various controls.

Intraperitoneal Glucose Tolerance Test

On D42, fasted mice (4–6 hours) were injected intraperitoneally with glucose (2.0 g/kg). Blood glucose levels were measured by repeated tail vein punctures for up to 2 hours.

Serum Insulin and Glucagon ELISA

On D14 or D42, blood was collected for serum insulin and glucagon quantification in duplicate by ultra-sensitive ELISA (Alpco, Salem NH, USA) following manufacturer's specifications.

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Immunohistochemistry and Immunofluorescent Analyses

Pancreata were frozen in optimal cutting temperature media and sectioned (10 μm) such that each slide contained 3 sections >150 μm apart. Sections were fixed in formalin, blocked with mouse serum, incubated with mouse insulin antibody and detected with peroxidase-labeled anti-mouse antibody (Vector Labs, Burlingame CA , USA). Criteria for islet enumeration required a minimum of 10 insulin$^+$ cells. Islet size and number were quantified using light microscopy counting all islets within 3 sections per mouse. Islet circumference was calculated using AxioVision software. β-Cell mass was calculated by: β-cell area/ total area × pancreas weight. Pancreas sections were also stained with immunofluorescent antibodies to detect insulin, glucagon, NKX2.2, NKX6.1, ck19, vimentin, and MAFA. Concentrations for all antibodies are shown in Supporting Information Table S1. Insulin was costained with EdU to calculate β-cell proliferation by counting insulin$^+$ EdU$^+$ cells/insulin$^+$ cells for all islets in 3 sections per mouse. For the detection of EdU incorporation, pancreas tissue sections were treated as described above and sections were incubated with the EdU detection cocktail as per manufacturer's instructions (ThermoFisher Scientific, Carlsbad CA, USA, C10337). Glucagon was costained with NKX6.1, MAFA, or insulin to capture α-β-cell-like transitions. Cytokeratin-19 (ck19) was used to label ductal structures not associated with, or in direct contact with, insulin$^+$ islets. Finally, NKX2.2 and NKX6.1 and MAFA were costained with insulin to assess β-cell identity and maturation, and were quantified by counting the number of NKX2.2, NKX6.1, or MAFA positive nuclei/number of nuclei within all islets present. All histological quantification was performed by manual counting in a blinded fashion by 2 individuals. Concentration of all antibodies used and product information can be found in Supporting Information Table S1.

Confocal Microscopy Analyses

Confocal microscopy was performed using 20 μm pancreas sections. Tissue was fixed with formalin and permeabilized with Triton X-100, and sections were blocked in heat inactivated fetal bovine serum. Pancreas sections were stained with insulin and glucagon for immunofluorescent analysis (Supporting Information Table S1). Images were acquired using a LSM 510 confocal laser scanning microscope (Zeiss, San Diega CA, USA) and optimization was performed by Zeiss ZEN 2009 software. Pancreas cryosections were immunostained for insulin and glucagon were analyzed for coexpression of endocrine hormones. Minimum thresholds for each dual-channel image were set using islets from a CAB-treated mouse. Image-Pro Premier software (Media Cybernetics, Rockville MD, USA) applied thresholds to images and Manders coefficients were generated using Image-Pro Premier to assess the degree of colocalization between insulin and glucagon. All confocal images were acquired using a ×20 objective using standard Z-stack optimization for slice thickness (Zeiss Zen Software).

Statistical Analyses

Values are represented as mean ± SD unless otherwise stated and considered significant if $p < .05$ by one-way ANOVA with Tukey's post hoc test compared with unconditioned media. Data were analyzed using Prism V6.01.

RESULTS

Intrapancreatic Injection of hMSC CM Reduced Hyperglycemia

We assessed whether iPan-injection of hMSC CM could be used to stimulate islet regeneration. hMSC were grown to ~80% confluence, switched to basal media without supplement, and CM was collected fresh after 24 hours incubation, concentrated (40×) and protein content was quantified. STZ-treated, hyperglycaemic (15–25 mmol/l) NOD/SCID mice, showing substantial β-cell ablation compared with CAB-treated mice (Supporting Information Fig. S1A, S1B), were iPan-injected on day 10 (D10) with hMSC CM containing either 4 μg or 8 μg total protein and blood glucose levels were monitored weekly for 42 days (Fig. 1A). Compared with STZ-treated mice injected with unconditioned media or mice injected with low-dose (4 μg) hMSC CM that remained severely hyperglycemic from D10 to D42, mice injected with high-dose (8 μg) hMSC CM showed reduced hyperglycemia within 4 days after injection (Fig. 1B). Injection of 8 μg hMSC CM consistently reduced area under the curve (AUC) for systemic glucose over 42 days (Fig. 1C). At D42, all mice injected with unconditioned media (n = 10) showed fivefold reduced serum insulin compared with normoglycemic CAB-treated controls (Fig. 1D). However, mice injected with 8 μg hMSC CM (n = 7) showed significantly increased serum insulin compared with mice injected with unconditioned media or 4 μg hMSC CM. Additional mice were euthanized at early time points (D11 and D14) to further assess the kinetics of hyperglycemia reduction (Supporting Information Fig. S2). Compared with mice injected with unconditioned media or 4 μg hMSC CM that showed consistently elevated glycemia, mice injected with 8 μg hMSC CM showed consistently decreased blood glucose by D14 (Supporting Information Fig. S2A). Mice that received 8 μg hMSC CM also showed significantly increased serum insulin at D14 compared with mice injected with unconditioned media (Supporting Information Fig. S2B). Intraperitoneal glucose tolerance tests were also performed on D42 to assess whether mice could adequately respond to glucose challenge. Compared with normoglycemic (4.4 ± 1.0 mmol/l) CAB-mice that showed return to baseline glucose levels within 60 minutes, mice injected with unconditioned media or 4 μg hMSC CM showed little response to glucose bolus and glycemia never returned to starting concentration (Fig. 1E). In contrast, mice injected with 8 μg hMSC CM showed glucose levels that peaked at 30 minutes (23.6 ± 2.2 mmol/l) and gradually reduced to nonfasted resting concentration (16.6 ± 2.9 mmol/l) by 90 minutes. The AUC for mice injected with 8 μg hMSC CM was significantly reduced compared with mice injected with unconditioned media or 4 μg hMSC CM (Fig. 1F). Despite a transient decrease in weight at D10–D14 that recovered by D42 (Supporting Information Fig. S3A), there was no permanent weight loss observed for any treated mouse group (Fig. 1G), suggesting the observed reductions in hyperglycemia were not due to restricted feeding or concurrent weight loss. Using hMSC derived from 2 independent donors, mice transplanted with high-dose (8 μg) hMSC CM consistently demonstrated significant recovery of glycemic control.

Activation of Wnt-Signaling Improved Islet Regenerative Capacity

We have recently established active Wnt-signaling as a characteristic of islet regenerative hMSC [18]. To investigate whether pharmacological modulation of Wnt-signaling during CM generation

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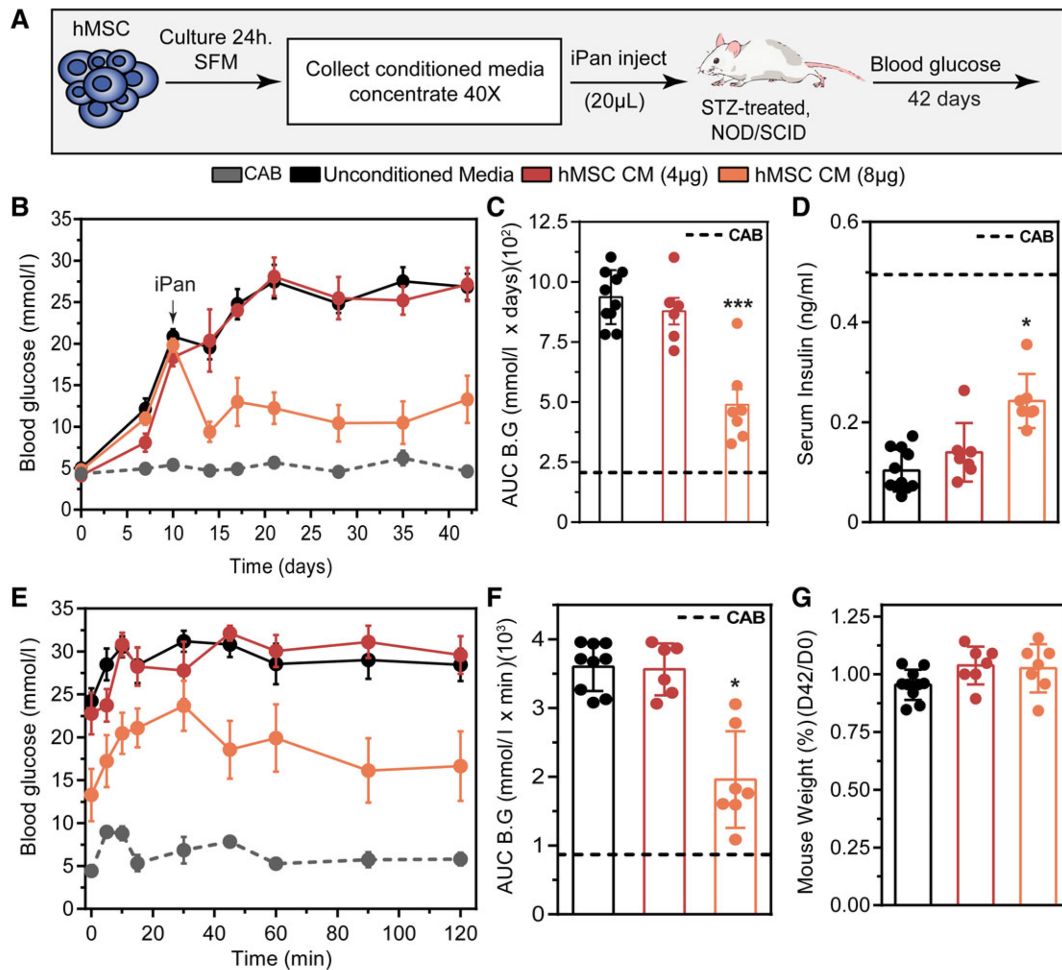

Figure 1. Intrapancreatic-injection of human multipotent stromal cell (hMSC) conditioned media (CM) improved glucose control. **(A):** hMSC CM was generated in serum-free media (24 hours), concentrated (40×) by filter centrifugation, iPan-injected into streptozotocin (STZ)-treated hyperglycemic mice on D10, and blood glucose was monitored for 42 days. **(B, C):** Mice injected with 8 µg hMSC CM (*n* = 7, orange) showed reduced hyperglycemia from D14 to D42 compared with mice injected with unconditioned media (*n* = 10, black) or with 4 µg hMSC CM (*n* = 7, red). Blood glucose levels without STZ-treatment is shown for citric acid buffer (CAB)-injected normoglycemic mice (*n* = 4, dotted gray). **(D):** Compared with mice injected with unconditioned media or with 4 µg hMSC CM, mice injected with 8 µg hMSC CM showed increased serum insulin at D42. **(E, F):** After intraperitoneal injection of 2.0 g/kg glucose bolus on D42, mice injected with 8 µg hMSC CM showed improved glucose tolerance compared with mice injected with unconditioned media or with 4 µg hMSC CM. Glucose tolerance is also shown for CAB-injected normoglycemic mice. **(G):** Mouse weights were not significantly changed at D42 for all treatment groups. Blood glucose data are represented as mean ± SEM remaining data are represented as mean ± SD (*, $p < .05$; **, $p < .01$; ***, $p < .001$). Dotted lines represent measurements observed in CAB-injected mice.

could further improve glycemic control after iPan CM-injection, we sought to activate or inhibit basal Wnt-signaling in hMSC using small molecules CHIR99201 or IWR-1, respectively [22, 23]. The optimal concentration for Wnt-pathway activation was determined using qPCR on 2 downstream Wnt-target genes, *BCL9* (Supporting Information Fig. S4A) and *MYC* (Supporting Information Fig. S4B). Expression of both targets was significantly increased upon treatment with 10 µM CHIR99201. We also quantified total intracellular β-catenin accumulation by flow cytometry [24]. Treatment with 10 µM CHIR99201 increased β-catenin levels ~1.9-fold (Supporting Information Fig. S4C). Optimal inhibition of Wnt-signaling was titrated similarly, showing significantly decreased *BCL9* and *MYC* expression, and decreased β-catenin levels at 20 µM IWR-1 (Supporting Information Fig. S4D–S4F).

Wnt-activated (WNT+) or inhibited (WNT−) CM was generated by culturing hMSC for 24 hours in serum-free basal media supplemented with 10 µM CHIR99201 or 20 µM IWR-1, and CM was collected and concentrated as described above. Hyperglycemic mice were injected with WNT+ CM at 4 µg or 8 µg total protein or with 8 µg WNT− CM on D10. Compared with mice injected with unconditioned media (*n* = 10) that remained hyperglycemic until D42, mice injected with 4 µg WNT+ CM (*n* = 10) or 8 µg WNT+ CM (*n* = 7) showed significantly reduced systemic glycemia from D14 to D42 (Fig. 2A). In contrast, 8 µg WNT− CM (*n* = 6) did not reduce hyperglycemia post injection (29.7 ± 2.2 mmol/l). Injection of WNT+ CM significantly reduced AUC for blood glucose compared with mice injected with unconditioned or WNT− CM (Fig. 2B). Mice injected with WNT+ CM also showed 3.2-fold (4 µg) and 4.7-fold (8 µg) increased serum insulin at D42, compared with mice injected with unconditioned media or WNT− CM (Fig. 2C). Notably, mice injected with 8 µg WNT+ CM demonstrated circulating serum insulin equivalent to CAB controls (*n* = 4). In addition, mice injected with WNT+ CM demonstrated rapid recovery from hyperglycemia within 4 days of

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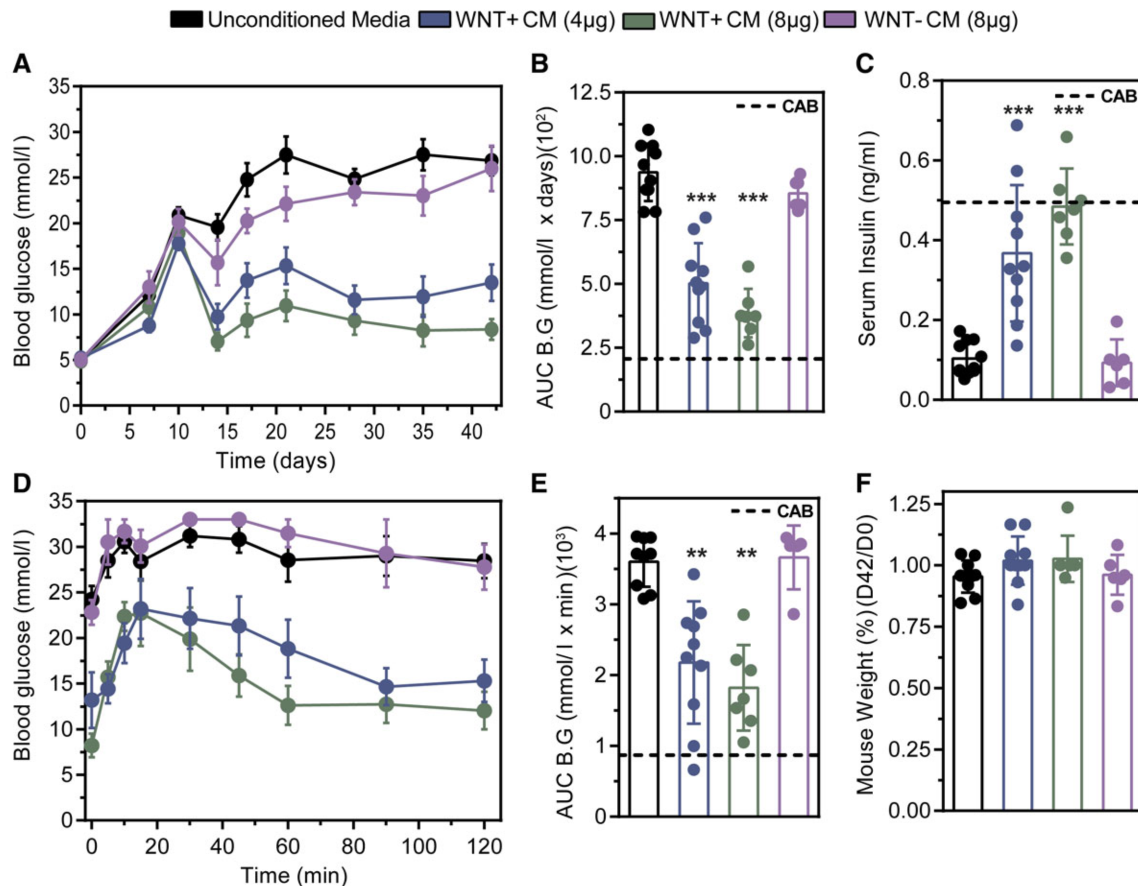

Figure 2. Intrapancreatic-injection of Wnt-pathway activated human multipotent stromal cell (hMSC) conditioned media (CM) further augmented glucose control. **(A, B):** Hyperglycemic mice were iPan-injected on D10 with 4 μg WNT+ CM (*n* = 10, blue) or 8 μg WNT+ CM (*n* = 7, green) showed reduced hyperglycemia from D14 to D42 compared with mice injected with unconditioned media (*n* = 10, black) or with 8 μg WNT– CM (*n* = 6, purple). **(C):** Mice injected with 4 or 8 μg WNT+ CM showed increased circulating serum insulin at D42, compared with mice injected with unconditioned media or with 8 μg WNT– CM. **(D, E):** After a 2.0 g/kg intraperitoneal bolus of glucose, mice injected with 4 or 8 μg WNT+ CM demonstrated improved glucose tolerance compared with mice injected with unconditioned media or with 8 μg WNT– CM. **(F):** Mouse weights were not significantly changed at D42 for all treatment groups. Blood glucose data are represented as mean ± SEM remaining data are represented as mean ± SD (*, *p* < .05; **, *p* < .01; ***, *p* < .001). Dotted lines represent measurements observed in citric acid buffer-injected mice (*n* = 4).

transplantation (Supporting Information Fig. S2D, S2E). Lastly, mice that received WNT+ CM demonstrated increased circulating serum insulin at D14 compared with mice injected with unconditioned media or WNT– CM (Supporting Information Fig. S2F). For glucose tolerance tests, mice injected with 4 μg WNT+ CM showed blood glucose peaked at 15 minutes (23.2 ± 2.8 mmol/l) and gradually decreased to nonfasted resting concentration (13.5 ± 1.6 mmol/l) at 90 minutes (Fig. 2D). Furthermore, mice injected with 8 μg WNT+ CM showed blood glucose peaked at 10 minutes (22.3 ± 1.8 mmol/l) and more rapidly returned to nonfasted resting concentration (11.5 ± 2.1 mmol/l) within 60 minutes. In contrast, mice injected with unconditioned media or 8 μg WNT– CM showed little response to glucose bolus injection. Overall, AUC for mice injected with WNT+ CM were significantly reduced compared with mice injected with unconditioned media or WNT– CM (Fig. 2E). There was no significant weight loss observed for any treated mouse groups (Fig. 2F; Supporting Information Fig. S3A). For direct comparisons of all iPan-injected mouse groups from Figures 1 and 2, summaries of mouse weights, resting blood glucose, blood glucose AUC, and serum insulin concentrations at D42 are shown in Supporting Information Figure S3A–S3D. Taken together, mice injected with 4 μg WNT+ CM demonstrated significant, albeit partial recovery of endocrine function, whereas mice injected with 8 μg WNT+ CM demonstrated further improvement in glycemic control. Thus, pharmacological activation of Wnt-signaling ex vivo consistently generated hMSC CM with enhanced glucose lowering capacity after iPan-injection and inhibiting basal Wnt-signaling levels within hMSC during CM generation reversed this beneficial effect.

Heat Denaturing Abrogated the Glucose Lowering Function of hMSC CM

To determine whether the glucose lowering capacity of hMSC CM was reversed by the destruction of CM content (primarily protein) prior to injection in vivo, WNT+ CM was denatured by heating at 90°C for 20 minutes (Supporting Information Fig. S5A, S5B). Next, mice were injected with an equivalent dose (4 μg) of heat denatured CM (dWNT+) or native WNT+ CM. As previously shown, injection of 4 μg WNT+ CM significantly reduced systemic blood glucose over 42 days compared with mice injected with unconditioned media. In contrast, injection of 4 μg dWNT+ CM did not reduce blood glucose levels (Supporting Information Fig. S5C) or AUC measurements (Supporting Information Fig. S5D). Serum insulin content in mice injected with dWNT+ CM was also 6.8-fold

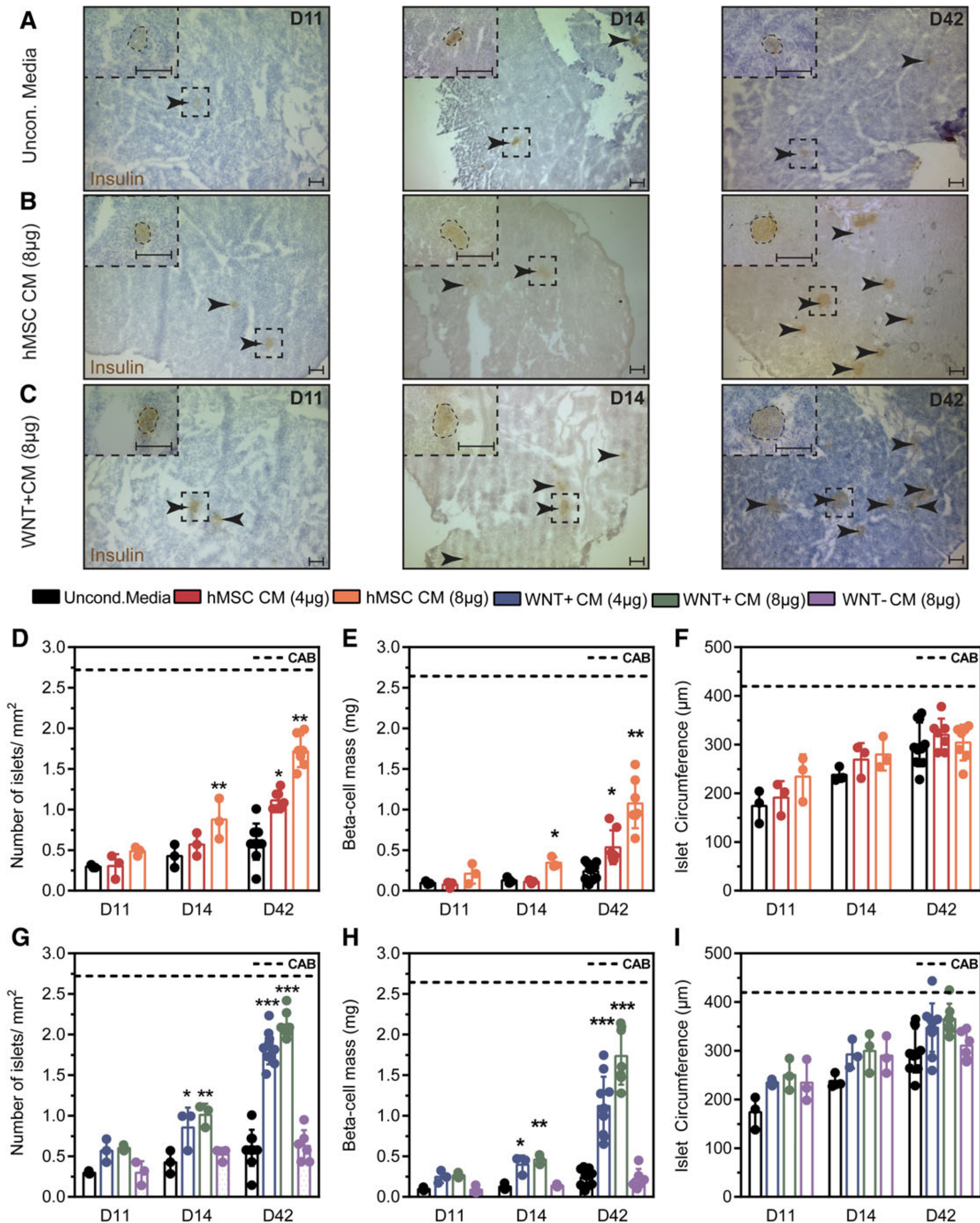

Figure 3. Intrapancreatic-injection of human multipotent stromal cell (hMSC) conditioned media (CM) increased islet number and β-cell mass. **(A–C):** Representative photomicrographs of insulin expressing islets at D11, D14, and D42 in mice injected with unconditioned media, 8 μg hMSC CM, or 8 μg WNT+ CM. Arrow heads denote islets, inlets show ×2.5 magnified view of islets outlined with dotted box. Scale bars = 200 μm. **(D–F):** Compared with mice injected with unconditioned media (*n* = 10, black) or mice injected with 4 μg hMSC CM (*n* = 7, red), mice injected with 8 μg hMSC CM (*n* = 7, orange) showed significantly increased islet number and β-cell mass at D14 and D42, with no difference in islet size. **(G–I):** Compared with mice injected with unconditioned media (black) or with 8 μg WNT− CM (*n* = 10, purple), mice injected with 4 μg of WNT+ CM (*n* = 7, blue) or 8 μg of WNT+ CM (*n* = 6, green) showed increased islet number and β-cell mass at D14 and D42, with no difference in islet size. Data are represented as mean ± SD (*, $p < .05$; **, $p < .01$; ***, $p < .001$). Dotted lines represent measurements observed in citric acid buffer-injected mice (*n* = 4).

lower than WNT+ CM (Supporting Information Fig. S5E). This reversal in regenerative function following heat denaturing suggested that WNT+ CM protein denaturation could abrogate islet regenerative function. Furthermore, investigation on whether other components within the CM, such as microRNAs, have any role in islet regenerative function is a topic for future investigation.

hMSC CM-Injection Increased β-Cell Mass

Next, pancreas sections from all mice euthanized at D11, D14, and D42, were stained for murine insulin (Fig. 3A–3C) to investigate the dynamics of islet recovery after hMSC CM injection. Compared with mice injected with unconditioned media, mice that received 8 μg hMSC CM showed significantly increased

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islet number (Fig. 3D), and quantified total β-cell mass (Fig. 3E) at D14 and D42, but no difference in islet size (Fig. 3F). Similarly, mice injected with 4 μg or 8 μg WNT+ CM also demonstrated significantly increased islet number (Fig. 3G), and β-cell mass (Fig. 3H) at D14 and D42, but no difference in islet size (Fig. 3I). When basal Wnt-signaling within hMSC was inhibited, islet number and β-cell mass were similar to unconditioned media injected controls (Fig. 3G–3I). Islet recovery was maximal after transplantation of 8 μg WNT+ CM with β-cell mass increasing consistently over time. Although β-cell mass in WNT+ CM injected mice remained lower than in CAB-treated controls at D42, these data confirmed that hMSC CM can induce regeneration of β-cell mass without the transfer of cell and activating Wnt-signaling during CM generation augments β-cell mass regeneration.

hMSC CM-Injection Stimulated β-Cell Proliferation at Early Time Points

β-Cell regeneration after STZ treatment can occur via the induction of β-cell proliferation and by islet formation from ductal or islet derived precursor cells via putative neogenic mechanisms [10–12]. EdU incorporation into insulin-expressing islets (injected 24 hours before euthanasia) was assessed at D11, D14, and D42 to first determine whether β-cell proliferation was stimulated after CM injection (Fig. 4A–4C). In mice injected with unconditioned media, proliferating insulin$^+$ β-cells were rare (≈0.5%), indicating a slow turnover of β-cells even after STZ-treatment. The frequency of proliferating β-cells was increased at D11 after the injection of hMSC CM or WNT+ CM but quickly returned to baseline (CAB-treated mice) levels at D14 and D42 (Fig. 4D, 4E). Mice that received 8 μg hMSC CM showed proliferation rates ~twofold higher compared with unconditioned media at D11 (Fig. 4D), and mice that received WNT+ CM (8 μg) showed ~fourfold increased islet cell proliferation at D11 that returned to baseline at D14 and D42 (Fig. 4E). All conditions showed β-cell proliferation rates equivalent to CAB-treated control mice at D42, suggesting the proliferative stimulus was short-lived and not sustained beyond D14. Collectively, injection of CM increased the frequency of proliferating β-cells indicating hMSC secrete potential β-cell proliferative effectors into CM. However, the stimulated proliferation was modest (<2%) and transient, suggesting alternative mechanisms were likely occurring in order to explain the large and sustained increase in β-cell mass after CM delivery.

hMSC CM-Injection Stimulated Islet Formation Associated with Pancreatic Ducts

We have previously shown that islet regeneration following hMSC injection was predominantly associated with ductal structures [16, 17]. Others have also suggested EMT occurs in ductal regions, accompanied by endocrine cell specification, to compensate for β-cell loss after STZ-treatment or partial pancreatectomy [13, 14, 25]. To investigate whether islet regeneration was initiated in ductal regions after hMSC CM injection, costaining for insulin and ck19, to mark ductal epithelial cells, was performed (Fig. 5A–5C). Compared with CAB-treated mice with direct islet-duct association at ~30%, mice that received 8 μg hMSC CM showed significantly increased islet-ductal association at D11 and D42 (Fig. 5D), and mice injected with 4 μg or 8 μg WNT+ CM showed significantly increased islet-ductal association at D11, D14, and D42 (Fig. 5E). Staining for vimentin$^+$ mesenchymal cells associated with ck19$^+$ ducts was also performed to assess whether evidence of EMT occurred following hMSC CM injection (Supporting Information Fig. S6). Interestingly, all mice that received STZ-treatment (Supporting Information Fig. S6A) showed vimentin$^+$ cell hyperplasia in ductal regions, which was not evident in CAB-treated mice (Supporting Information Fig. S6B), suggesting potential activation of EMT programs after STZ-damage. However, insulin$^+$ clusters within areas of vimentin$^+$ cell hyperplasia and adjacent to ducts was most pronounced at D11 in mice injected with WNT+ CM (Supporting Information Fig. S6C, S6D). Furthermore, emergence of glucagon$^+$ cells within vimentin$^+$ areas surrounding ducts was also observed in mice injected with WNT+ CM (Supporting Information Fig. S6E, S6F). Taken together, these data suggested WNT+ CM-induced α-and-β-cell emergence adjacent to the ductal epithelial niche coincided with mesenchymal cell hyperplasia suggestive of potential EMT induction.

hMSC CM-Induced Islet Regeneration Showed Characteristics Consistent with α-β-Cell Transition

Recently, the Collombat group elegantly demonstrated α-β-cell transition can increase β-cell mass in the murine and human pancreas [13, 14]. To assess whether hMSC CM-injection generated islets with features consistent with the induction of α–βcell transition, we first quantified glucagon expression within regenerating islets after hMSC CM injection (Fig. 6). The frequency of glucagon$^+$ cells within islets was elevated under all treatment conditions (≈45%) at D11 compared with CAB-treated mice (≈25%), indicating STZ-mediated β-cell ablation increased relative abundance of α-cells within islets (Fig. 6A, 6B). However, mice injected with 8 μg hMSC CM showed reduced glucagon$^+$ cell frequency within islets at D42 compared with unconditioned media controls, and glucagon$^+$ cell frequency was equal to CAB-treated controls (Fig. 6C). Importantly, mice injected with 4 μg or 8 μg WNT+ CM showed accelerated return of basal glucagon$^+$ cell frequency at D14 and D42 (Fig. 6D), indicating intraislet α-cell frequency decreased as β-cell number increased. Importantly, α-cell-mediated secretion of glucagon was unaltered at D42 under all conditions (Fig. 6E, 6F). Finally, at D11 and D14 we consistently observed islets with colocalized staining of insulin and glucagon (Fig. 6G, 6H), and the ratio and distribution of glucagon$^+$ insulin$^+$ cells was similar to CAB controls at D42. To investigate hormone coexpression, colocalization analysis was performed using fluorescent image analyses with Manders coefficients [26, 27]. Compared with mice injected with unconditioned media that showed very low colocalization (Fig. 6G), mice injected with 8 μg WNT+ CM showed increased colocalization at D14 (Fig. 6H), and insulin/glucagon colocalization was not observed in CAB-treated controls (Fig. 6I). Although lineage tracing will be required to definitively demonstrate α-β-cell transition, areas of insulin and glucagon colocalization were relatively abundant at D11 and D14 in mice injected with WNT+ CM, and comparatively rare or absent in mice injected with unconditioned media or CAB. Thus, iPan-injection of hMSC CM induced islet regeneration with attributes consistent with previously reported induction of α-β-cell transition.

hMSC CM-Injection Accelerated β-Cell Maturation within Regenerating Islets

To assess the recovery of β-cell identity following CM injection, we investigated the expression of NKX2.2 and NKX6.1 in regenerated islets at D11, D14, and D42. Representative photomicrographs showed nuclear localization of NKX2.2 (Supporting

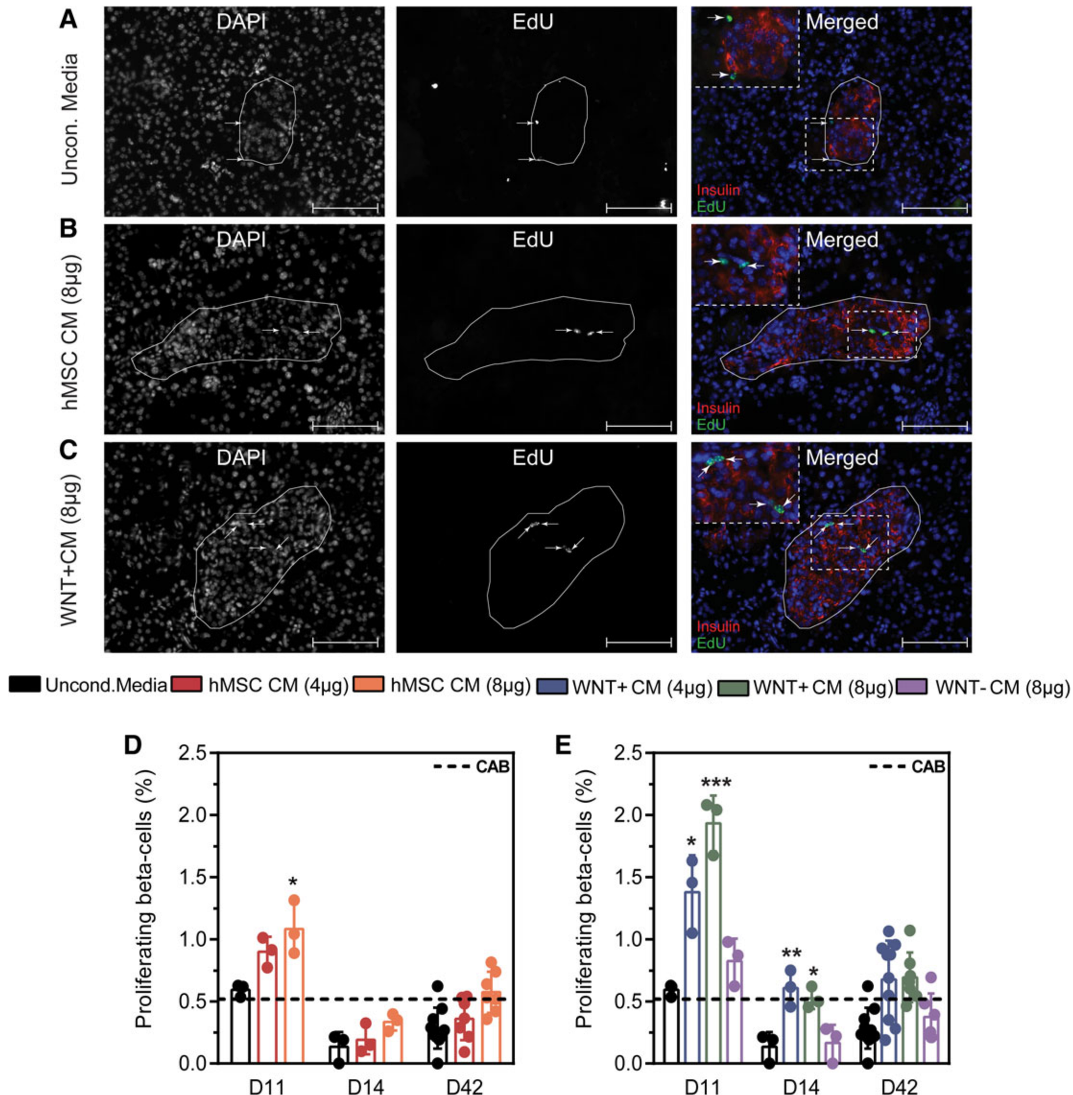

Figure 4. Injection of Wnt-activated human multipotent stromal cell (hMSC) conditioned media (CM) increased β-cell proliferation. **(A–C):** Representative photomicrographs of EdU+ (green) insulin+ (red) cells within islets at D11 in mice injected with unconditioned media, 8 µg hMSC CM, or 8 µg WNT+ CM. Arrows denote EdU+ nuclei in islets outlined with a white line. Insets show ×4 magnified view of proliferating β-cells with DAPI staining in blue. Scale bars = 100 µm. **(D):** Compared with mice injected with unconditioned media (n = 10, black) or mice injected with 4 µg hMSC CM (n = 7, red), mice injected with 8 µg hMSC CM (n = 7, orange) showed an increased frequency of proliferating β-cells at D11 with no difference at D14 or D42. **(E):** Compared with mice injected with unconditioned media (n = 10, black) or 8 µg WNT− CM (n = 6, purple), mice injected with 4 µg of WNT+ CM (n = 10, blue) or 8 µg WNT+ CM (n = 7, green) showed increased frequency of proliferating β-cells at D11 and D14 with no difference at D42. Data are represented as mean ± SD (*, $p < .05$; **, $p < .01$; ***, $p < .001$). Dotted lines represent measurements observed in citric acid buffer-injected mice (n = 4).

Information Fig. S7A–S7E); indicated endocrine cell specification occurred within regenerating islets at similar frequencies under all conditions and time points, including normoglycemic CAB-treated mice. In contrast, NKX6.1 expression, marking β-cell lineage commitment and β-cell identity within regenerating islets, varied between transplanted groups (Fig. 7A–7C). Compared with mice injected with unconditioned media, mice injected with 8 µg hMSC CM, or 4 µg or 8 µg WNT+ CM showed significantly increased frequencies of nuclear localized NKX6.1 at D11, D14, and D42 (Fig. 6D, 6E). Mice injected with 4 µg hMSC CM or WNT− CM showed NKX6.1 expression similar to mice injected with unconditioned media. Overall, these data confirm that injection of hMSC CM or WNT+ CM correlated with accelerated recovery of β-cell identity within regenerating islets. We also investigated the expression of MAFA in regenerated islets at D11, D14, and D42 to assess β-cell maturation. MAFA expression is required for insulin production and is a marker for mature, insulin-secreting β-cells. Representative photomicrographs showed nuclear localization of MAFA (Fig. 7F–7H) also varied after CM-injection. Mice injected with 8 µg hMSC CM or WNT+ CM showed significantly increased frequency of nuclear MAFA at D14 and D42 compared with mice injected with unconditioned media, 4 µg hMSC CM, or WNT− CM (Fig. 7I, 7J). Interestingly, mice that received CM injection did not reach NKX6.1 and MAFA expression levels observed in CAB-treated mice (~58%), suggesting β-cell identity and maturation was accelerated after hMSC CM-injection but not all β-cells reached full maturation by D42.

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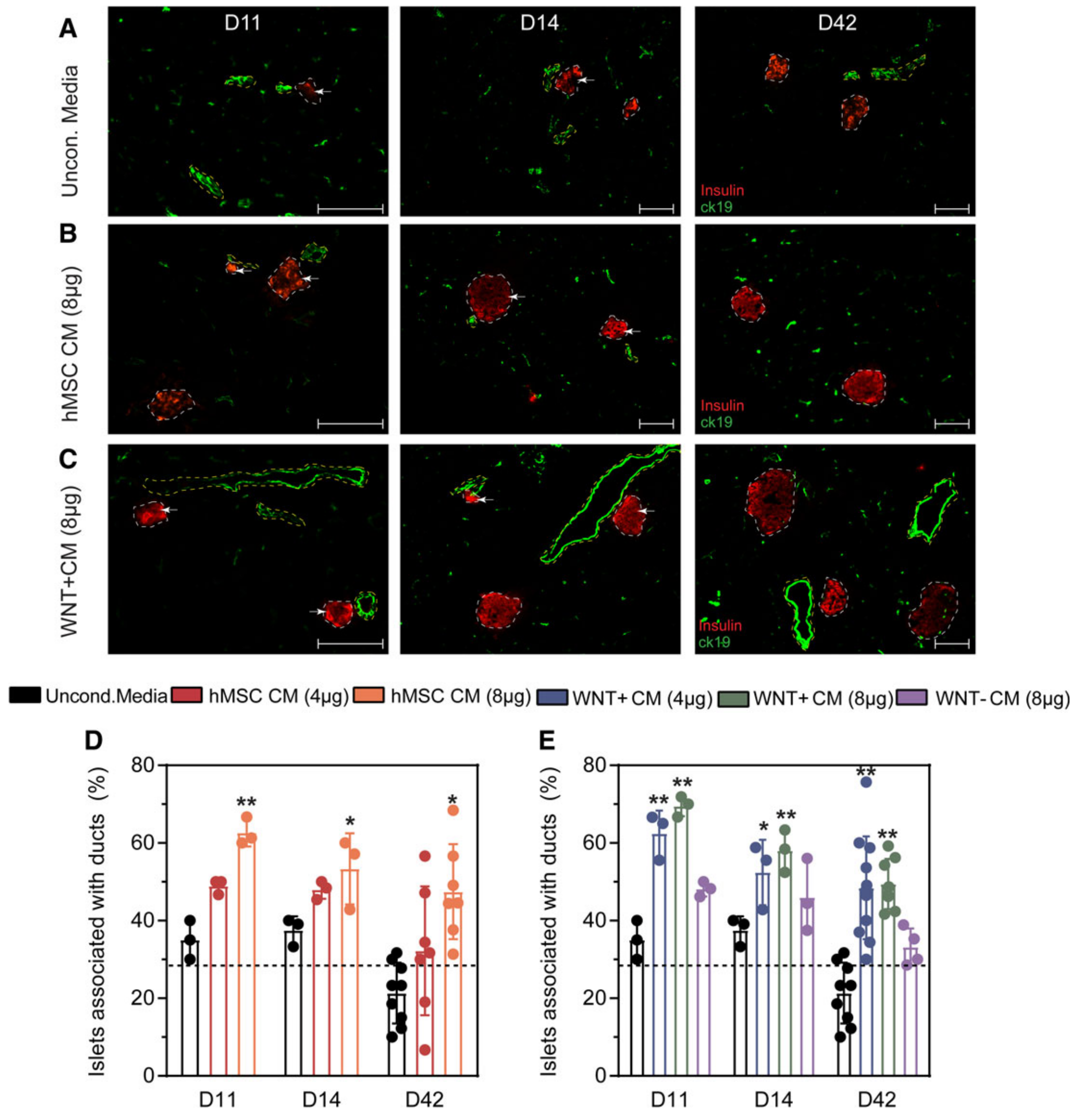

Figure 5. Intrapancreatic-injection of human multipotent stromal cell (hMSC) conditioned media (CM) increased islet association with ducts. **(A–C):** Representative photomicrographs of ck19[+] ducts (green) associated with insulin[+] islets (red) at D11, D14, and D42 in mice injected with unconditioned media, 8 µg hMSC CM, or 8 µg WNT+ CM. Arrows indicate associated ducts. Islets are outlined with a white line and ducts are outlined with a yellow line. Scale bar = 200 µm at D11 and 100 µm at D14 and D42. **(D, E):** Compared with mice injected with unconditioned media (n = 10, black), 4 µg hMSC CM (n = 7, red) or mice injected with 8 µg WNT− CM (n = 6 purple), mice injected with 8 µg hMSC CM (n = 7, orange), 4 µg of WNT+ CM (n = 10, blue) or 8 µg of WNT+ CM (n = 7, green) showed increased frequency of islet associated with ducts at D11 and D42. Data are represented as mean ± SD (*, $p < .05$; **, $p < .01$). Dotted line represents measurements in citric acid buffer-injected mice.

Discussion

Induction of islet regeneration in situ combined with dampening of autoimmunity represents an attractive strategy for T1D therapy. Our study demonstrates protein content secreted by human BM-derived hMSC can accelerate islet regeneration after intrapancreatic injection, without the need for cell transfer. Importantly, regenerated β-cells effectively secreted insulin in response to elevated blood glucose and reversed chemically induced hyperglycemia in vivo. The extent of hyperglycemic recovery was dependent on the dose of CM administered and regenerative capacity was augmented by activation of Wnt-signaling during CM generation. Impressively, mice injected with Wnt-activated CM showed efficient recovery of endocrine function, and accelerated recovery of β-cell mass resulted from stimulation of multiple regenerative mechanisms including islet formation associated with ductal regions, the induction of β-cell proliferation at early time points, islet-regenerative characteristics consistent with the promotion of α to β-cell transition, and accelerated recovery and expression of β-cell transcription factors such as NKX6.1 and MAFA. These processes occurred within a 4-day therapeutic window after a single administration of CM generated from independent hMSC samples. Although circulating serum insulin and β-cell mass showed near complete recovery, β-cell maturation remained incomplete and glucose tolerance was less efficient compared with CAB-injected controls. Nonetheless, targeted protein-based therapies have the potential to bolster functional β-cell mass and represent a novel strategy for the treatment of diabetes.

After transplantation, hMSC can encounter harsh and unfamiliar microenvironments coupled with detachment-induced

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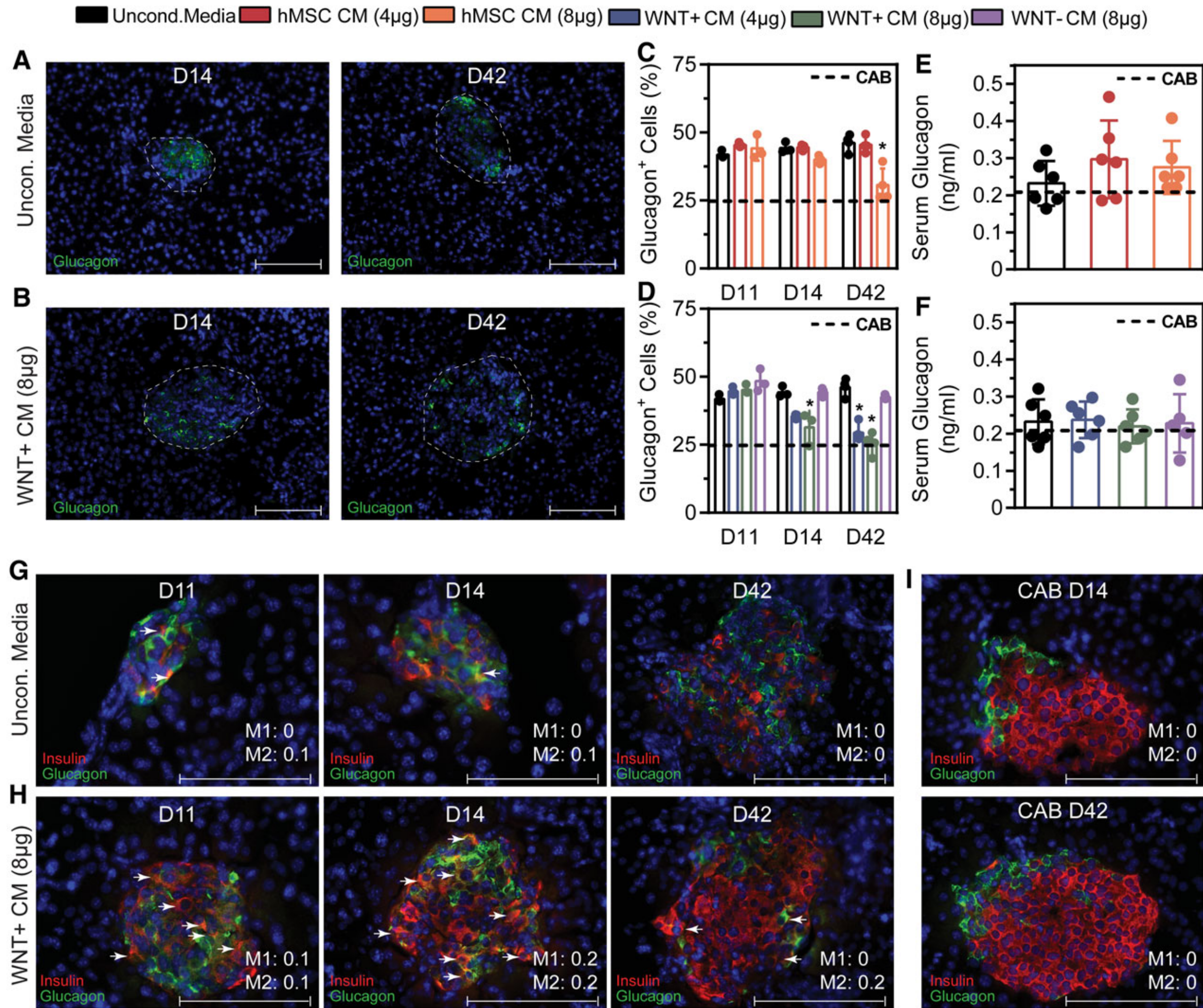

Figure 6. Intrapancreatic-injection of human multipotent stromal cell (hMSC) conditioned media (CM) or WNT+ CM induced islet regenerative characteristics consistent α-β-cell transition. **(A, B):** Representative photomicrographs of glucagon+ (green) cells within islets at D14 and D42 in mice injected with unconditioned media or 8 μg WNT+ CM. Islets are outlined with a white dotted line with DAPI staining in blue. Scale bars = 100 μm. **(C):** Compared with mice injected with unconditioned media (*n* = 10, black) or mice injected with 4 μg hMSC CM (*n* = 7, red), mice injected with 8 μg hMSC CM (*n* = 7, orange) showed a decreased frequency of glucagon+ cells at D42. **(D):** Compared with mice injected with unconditioned media (*n* = 10, black) or 8 μg WNT– CM (*n* = 6 purple), mice injected with 4 μg of WNT+ CM (*n* = 10, blue) or 8 μg WNT+ CM (*n* = 7, green) showed decreased frequency of glucagon+ cells at D14 and D42. **(E, F):** Serum glucagon levels at D14 were not altered in all treatment groups. **(G–I):** Representative photomicrographs of colocalized glucagon (green) and insulin staining (red) within islets at D11, D14, and D42 in mice injected with unconditioned media, 8 μg WNT+ CM or citric acid buffer (CAB)-treated controls. Arrow heads indicated areas of colocalization with DAPI staining in blue. Scale bars = 100 μm. Mice injected with 8 μg WNT+ CM showed increased colocalized staining within islets at D14 compared with mice injected with unconditioned media or CAB. Colocalization analysis shows increased Manders coefficients for mice injected with 8 μg WNT+ CM at D11 and D14. Data are represented as mean ± SD dotted line represents measurements in CAB-injected mice.

apoptosis [28], that limit engraftment within the injured pancreas [29] and can generate highly variable responses after transplantation. Although direct cell contact has been previously implicated in the protection of islets after exposure to inflammatory cytokines in vitro [30, 31], recent studies have shown that preculturing islets with a cocktail of hMSC-secreted G-protein coupled receptor ligands increased islet survival and glucose-stimulated insulin secretion in vitro, and improved the outcome of islet transplantation in vitro [32]. In addition, administration of an hMSC CM regenerative cocktail may represent a cell-free approach to improve clinical islet transplantation outcomes while avoiding many safety, regulatory and logistical hurdles required to incorporate hMSC into clinical islet transplantation protocols. Moreover, CM injection consistently and efficiently accelerated the emergence of functional islets in situ. Increased β-cell mass was only observed when sufficient protein dose was administered (8 μg), and the extent of glycemic recovery was augmented by CM generation under Wnt-activated conditions, suggesting that CM-delivery triggered regenerative programs dependent on both protein concentration and content. Indeed, a single, low-dose (4 μg) of WNT+ CM increased islet number, serum insulin and β-cell mass resulting in hyperglycemia recovery similar to 8 μg hMSC CM without Wnt-pathway stimulation.

Previous exploration of components within hMSC CM implicated protein as the likely mediator of islet regeneration [20]. However, hMSC CM generation can also include cargo

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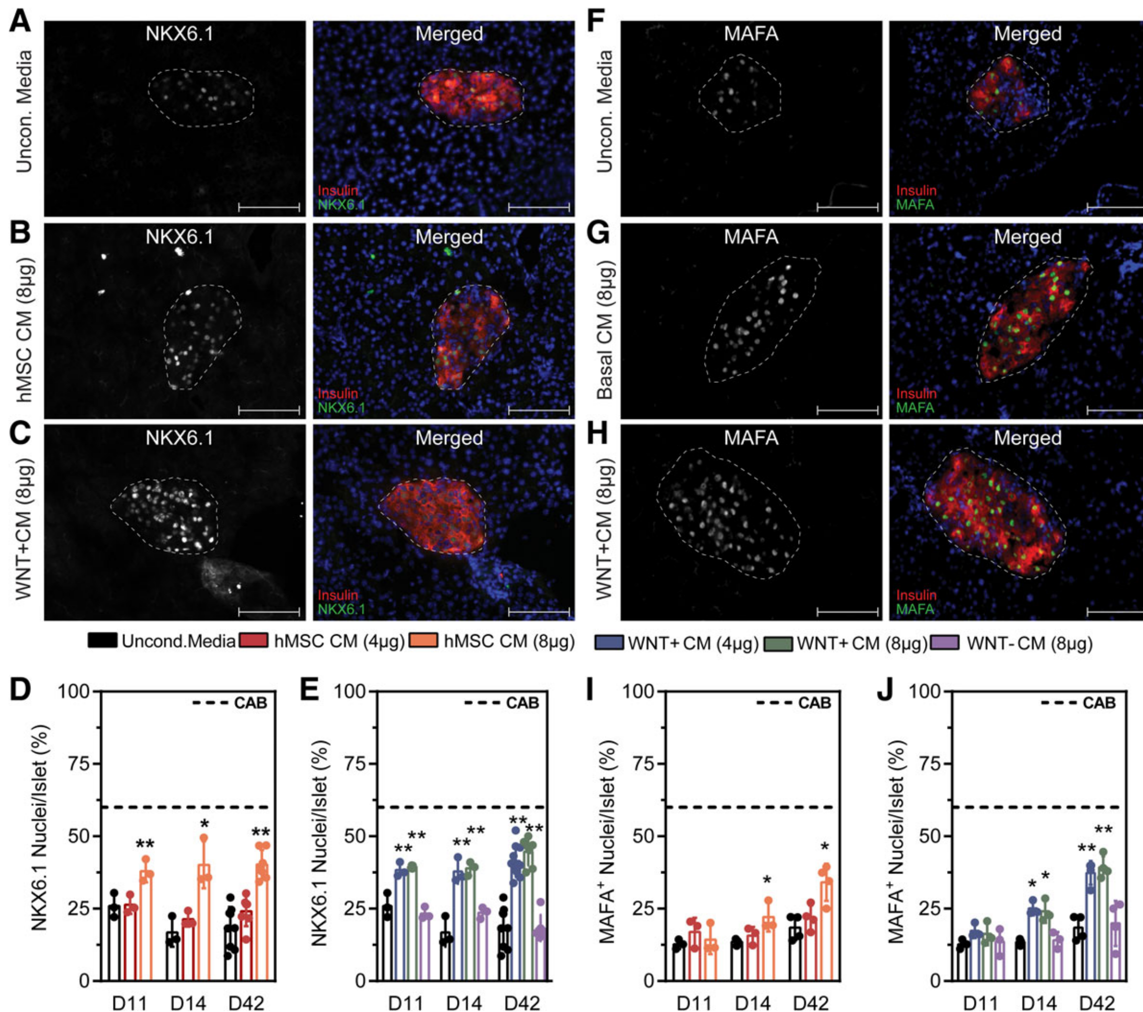

Figure 7. Intrapancreatic-injection of human multipotent stromal cell (hMSC) conditioned media (CM) accelerated β-cell maturation. **(A–C):** Representative photomicrographs of NKX6.1+ cells (green) within insulin+ islets (red) at D42 in mice injected with unconditioned media, 8 μg hMSC CM, or 8 μg WNT+ CM. Islets are outlined with a white line. DAPI staining is shown in blue. Scale bars = 100 μm. **(D, E):** Compared with mice injected with unconditioned media (n = 10, black), or 4 μg hMSC CM (n = 7, red) or mice injected with 8 μg WNT– CM (n = 6, purple), mice injected with 8 μg hMSC CM (n = 7, orange), 4 μg WNT+ CM (n = 10, blue) or 8 μg of WNT+ CM (n = 7, green) showed increased frequency of NKX6.1+ nuclei per islet at D11, D14, and D42. **(F–H):** Representative photomicrographs of MAFA+ β-cells (green) within insulin+ islets (red) at D42 in mice injected with unconditioned media, 8 μg hMSC CM, or 8 μg WNT+ CM. Islets are outlined with a white line. DAPI staining is shown in blue. Scale bar = 100 μm. **(I, J):** Compared with mice injected with unconditioned media (n = 10, black), 4 μg hMSC CM (n = 7, red) or 8 μg WNT– CM (n = 6, purple), mice injected with 8 μg hMSC CM (n = 7, orange), 4 μg WNT+ CM (n = 10, blue) or 8 μg WNT+ CM (n = 7, green) showed increased frequency of MAFA+ nuclei per islet at D14 and D42. Data are represented as mean ± SD (*, $p < .05$; **, $p < .01$). Dotted lines represent measurements in citric acid buffer-injected mice.

compartmentalized in extracellular vesicles, and miRNA content analyses as well as bioactive lipid and metabolite profiling represent additional avenues for future investigation. Recently, γ-aminobutyric acid (GABA) was shown to induce islet hyperplasia via α-β-cell conversion [13, 33]. Although the impact of GABA was not directly assessed in our study, hMSC can produce GABA [34], that may contribute to regeneration. However, in experiments by the Collombat group, daily administration of GABA was >1 month in duration before islet hyperplasia was observed. In our experiments, mice received a single dose of CM and islet recovery occurred primarily within 4 days, making it unlikely that GABA was solely responsible for recovery of β-cell mass. Furthermore, hMSC influence cellular processes through release of extracellular vesicles shuttling both protein and miRNA [35]. Although miRNA is known to regulate β-cell function during islet development [36], microarray analyses on extracellular vesicles harvested from hMSC revealed high expression of miR-21, miR-146a, and miR-181, linked to downstream products important in the immune response [37], but did not identify any known miRNA previously associated with β-cell homeostasis or insulin-secretion. Nonetheless, both protein and microRNA content released during the generation of hMSC CM could impact islet regeneration.

The endogenous mechanisms of β-cell regeneration induced by hMSC CM were multifactorial. in vitro cultivation of ductal tissue preparations has provided evidence that ductal cells may differentiate into insulin expressing β-cells [38, 39]. Although we did not observe direct evidence for ductal cell conversion to endocrine-specific cells expressing NKX6.1 or 2.2, insulin or glucagon, increased representation of ck19+ cells adjacent to

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regenerating islets accompanied by vimentin$^+$ cell hyperplasia in ductal regions suggested the ductal epithelial niche as a site of regenerative activity following CM injection. Modest activation of β-cell proliferation was also observed at early time points in mice that received Wnt-activated CM, and Wnt-ligands have been documented to initiate β-cell proliferation in vitro and vivo [18, 40]. However, the short burst of modest β-cell proliferation in our study suggested that alternative mechanisms for β-cell regeneration were required to account for the large increase in β-cell mass observed following injection of hMSC CM. Although lineage tracing needs to be performed to conclusively demonstrate α-β-cell transition, several observations support this transition as a potential mechanism for recovery of β-cell mass after CM injection. First, as β-cell mass increased after WNT+ CM injection, the high frequency of glucagon$^+$ cells (~50%) initially observed after STZ-treatment was gradually diminished. Second, using MAFA as an indication of mature β-cells, WNT+ CM also accelerated functional maturation of β-cells only at later time points whereas the endocrine cell marker NKX2.2 remained constant across all conditions. Third, detection of cells that showing colocalization of glucagon and NKX6.1 and both glucagon and insulin, which have also been detected during differentiation of hESC and in studies with nonhuman primates [13, 41–43] supported the possibility that α-cells may transition into β-cells following hMSC CM injection.

Additional preclinical testing in relevant models of autoimmune diabetes, and exploration of alternate modes of delivery such as intraductal injection [20], need to be addressed before translating these findings to patients with diabetes. In STZ-treated NOD/SCID mice, endogenous islet regeneration can occur unabated in the absence of inflammation or autoimmunity. Thus, iPan CM injection into NOD mice, combined with strategies to dampen autoimmunity, requires investigation to determine if regeneration can occur in the face of ongoing autoimmunity. A promising benefit of hMSC-derived CM delivery is that CM may also contain proteins that suppress immune responses [18]. Transplantation of hMSC into NOD mice has been shown to delay diabetes progression by induction of regulatory T-cell function [44]. Finally, transplantation of BM-derived hMSC into patients with diabetes has been shown to preserve residual β-cell function for >2 years [45]. Thus, transfer of a therapeutic cocktail with the potential for immunomodulation combined with the islet regenerative properties shown herein, represents an attractive, protein-based strategy to induce islet regeneration and dampen autoimmunity during T1D.

Conclusion

This study demonstrates for the first time that: (a) hMSC CM can induce efficient regeneration of murine islets without transferring cells; (b) the magnitude of endocrine recovery was depended on protein concentration and content enhanced by Wnt-pathway stimulation; (c) islet regenerative characteristics were suggestive of possible α-β-cell conversion and, (d) regeneration of β-cell mass involved modest β-cell proliferation and accelerated maturation. Based on these findings, we propose that intrapancreatic delivery of Wnt-activated hMSC CM or a mixture of recombinant proteins represents a novel and promising therapeutic approach for T1D treatment.

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Author Contributions

M.K.: conception and design, collection and assembly of data, data analyses and interpretation, manuscript writing; R.M.E.: collection and assembly of data, data analyses and interpretation; G.I.B.: conception and design, collection and assembly of data; A.X.: provision of study material or patients; G.A.L.: conception and design, financial support, collection and assembly of data, data analyses and interpretation; D.A.H.: conception and design, financial support, collection and assembly of data, data analyses and interpretation, manuscript writing, final approval of manuscript.

Disclosure of Potential Conflicts of Interest

The authors indicated no potential conflicts of interest.

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