Chorionic and amniotic placental membrane-derived stem cells, from gestational diabetic women, have distinct insulin secreting cell differentiation capacities

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Abstract

Women with gestational diabetes (GDM), and their offspring, are at high risk of developing type 2 diabetes. Chorionic (CMSCs) and amniotic mesenchymal stem cells (AMSCs) derived from placental membranes provide a source of autologous stem cells for potential diabetes therapy. We established an approach for the CMSC/AMSC-based generation of functional insulin-producing cells (IPCs). CMSCs/AMSCs displayed significantly elevated levels of *NANOG* and *OCT4* vs. bone marrow-derived MSCs, indicating a potentially broad differentiation capacity. Exposure of Healthy- and GDM- CMSCs/AMSCs to long-term high glucose culture resulted significant declines in viability accompanied by elevation, markedly so in GDM-CMSCs/AMSCs, of senescence/stress markers. Short-term high glucose culture promoted pancreatic transcription factor expression which when coupled to a 16-day step-wise differentiation protocol; activin A, retinoic acid, EGF, GLP1 and other chemical components, generated functional IPCs from both Healthy- and GDM- CMSCs. Healthy-/GDM-AMSCs displayed betacellulin-sensitive insulin expression which was not secreted upon glucose challenge. The pathophysiological state accompanying GDM may cause irreversible impairment to endogenous AMSCs; however, GDM-CMSCs possess comparable therapeutic potential with Healthy-CMSCs and can be effectively reprogrammed into insulin-secreting cells.

Keywords: cell differentiation, fetal stem cells, gestational diabetes, insulin-secreting cells, regenerative medicine

1 Introduction

Gestational diabetes mellitus (GDM), defined as glucose intolerance first diagnosed during pregnancy accounts for nearly 90% of all pregnancies complicated by diabetes (Zitkus, 2014). Women with a history of GDM have a 7-fold risk of developing type 2 diabetes postpartum compared with healthy pregnancies and approximately 50% of these women are diagnosed with type 2 diabetes 5-10 years following pregnancy (Buchanan et al., 2012). Patients with type 2 diabetes require lifelong anti-diabetic drug or insulin treatment but neither of these either reverse the disease nor correct the beta cell dysfunction (Ashcroft and Rorsman, 2012). Transplantation of cadaveric islet cells is one of the therapeutic strategies for diabetes but a shortage of donors and immune rejection have limited its clinical usage. Nowadays, mesenchymal stem cells (MSCs) which possess a multi-lineage differentiation potential offer an alternative to β-cell replacement therapy (Volarevic et al., 2011).

Studies have reported the generation of insulin-producing cells (IPCs) from adult bone marrow (Xin et al., 2016) and adipose tissue-derived MSCs (Gabr et al., 2017). However, adult MSCs are considered more likely to differentiate toward mesenchymal lineages due to their mesodermal origin, while β-cells are of endodermal origin (Taneera et al., 2006). MSCs from birth-associated tissues, such as placenta and amniotic fluid, on the other hand were found to express endoderm lineage and embryonic markers (Fukuchi et al., 2004; Lesage et al., 2017). IPCs differentiation from placenta tissue-derived MSCs have been described through either one simple differentiation cocktails to multi-step induction protocols (Kadam et al., 2010; Sun and Ji, 2009; Susman et al., 2015); however, these existing approaches lack consistency and produce low levels of insulin protein. The complex and heterogeneous cell population in placenta presents difficulty in the isolation of sole MSCs population and with a high tendency of maternal cells contamination (Sardesai et al., 2017). The amnion-chorion membrane surrounding outside placental surfaces contains abundant MSCs underlying a single layer of epithelial cells, which provides an alternative MSCs source with an easy isolation process (Bacenkova et al., 2011). Amnion and chorion are loosely connected together and both amniotic MSCs (AMSCs) and chorionic MSCs (CMSCs) originate from the extra-embryonic tissues. Given its fetal origin, AMSCs and CMSCs showed multi-lineage differentiation ability and low maternal contamination (Bacenkova et al., 2011). Although healthy placenta-derived MSCs exhibited pancreatic lineage differentiation potential, whether these protocols can be applied to MSCs derived from GDM women have not been explored. Knowing that autologous cell therapy offers distinct advantages over allogeneic approach, GDM women with a high risk of type 2 diabetes may benefit from IPCs derived from their autologous MSCs.

Glucose culture environment (Tang et al., 2004; Tsai et al., 2014). During pancreatic islet development, glucose plays an important regulatory role in β-cell growth, survival, and proliferation (Assmann et al., 2009). In the human body, elevated blood glucose level is a primary activator of β-cell expansion in situations where β-cell compensation is required due to increased metabolic demand (Garcia-Ocaña and Alonso, 2010). With human bone marrow MSCs a culture environment containing 23.3 mM glucose for 15 days was shown to stimulate expression of pancreatic-related genes; *PDX1, PAX4, GLUT2,* and *INS* (Xin et al., 2016). GDM-CMSCs/AMSCs are under prolonged glucose exposure due to hyperglycaemia during pregnancy and some adverse effects of hyperglycaemia on GDM-derived MSCs have been reported; for instance, perivascular stem cells from GDM showed low yield and proliferative rate (An et al., 2017) and MSCs isolated from diabetic placenta were found to exhibit insulin resistance (Mathew and Bhonde, 2017). Knowing that GDM may altered MSCs cellular behaviours, to generate IPCs from MSCs isolated from GDM women, the impairment of hyperglycaemic environment in CMSCs/AMSCs needs to be taken into account as well as the suitability of high glucose concentration in many IPC differentiation protocols, used to mimic the β-cell development environment, for GDM-CMSCs/AMSCs remains to be determined.

To establish a therapeutic use potential of autologous placental membrane-derived MSCs for diabetes treatment, we aimed to establish a feasible approach for the generation of IPCs from CMSCs/AMSCs and determine the role, if any, of GDM in this capacity. In this study we have shown that a short-term high glucose exposure followed by our optimised protocol, CMSCs derived from GDM and healthy women were reprogrammed into functionally indistinguishable IPCs.

2 Materials and Methods

2.1 Isolation and characterization of AMSCs/CMSCs from human placenta

All placentas were collected from Royal Stoke University Hospital, UK, after obtaining Research Ethics Committee and Health Research Authority approvals (Reference 15/WM/0342). All women provided written informed consent. Placentas were collected

after caesarean sections and cells isolated within an hour. Amniotic membrane was manually peeled from the underlying chorionic membrane and a removal of maternal decidual tissue from chorion by forceps was required. Amnion and chorion were washed with PBS to remove blood clots, incubated with 0.05% trypsin/EDTA solution at 37°C for 1 h to release epithelial cells, supernatant was discarded, membranes were washed with PBS and followed by digestion with 1 mg/ml collagenase type IV and 25 µg/ml DNase I (ThermoScientific, USA) at 37°C for 1-1.5 h. Once the membranes were completely dissolved, cells were centrifuged at 200g for 5 minutes and cultured in DMEM containing 1g/L glucose, 1% L-glutamine, 10% fetal bovine serum (FBS), and 1% non-essential amino acids (NEAA).

Characterization of MSCs was assessed by immunophenotypic analysis and trilineage differentiation at passage 2. A panel of cell surface markers defined by the International Society for Cellular Therapy was used to characterized MSCs (Dominici et al., 2006), including positive expression for CD73, CD90, CD105 and negative expression for CD14, CD19, CD34, CD45, and HLA-DR (Table S1). Surface markers were examined using flow cytometry (Beckman Coulter Cytomics FC 500 and CXP software, USA) and data were processed with Flowing Software 2.

To induce osteogenesis, cells were cultured in growth medium supplemented with 50 µM ascorbic acid, 10 mM β-glycerol phosphate, and 0.1 µM dexamethasone while for adipogenesis, growth medium was supplemented with 0.5 µM dexamethasone, 0.5 mM isobutylxanthine, 10 µg/ml insulin, and 100 µM indomethacin. To induce chondrogenic differentiation, $1x10^5$ cells were suspended in 8 µl of medium and dropped in the centre of the well as a micromass. After 1-hour incubation to allow cells to adhere with the culture surface, micromasses were then replenished with chondrogenic differentiation media, consisting of DMEM with 1% FBS, 1% L-glutamine, 1% NEAA and supplemented with 1% ITS, 0.1 µM dexamethasone, 50 µM ascorbic acid, 40 µg/mL L-proline, 1% sodium pyruvate, and 10 ng/mL transforming growth factor 3 (TGF-3; Peprotech, UK). To evaluate the trilineage differentiation after 21-day induction, the mineral deposition of differentiated osteoblasts was detected by Alizarin red; lipid accumulation in adipocytes was stained with Oil-Red-O; and proteoglycan-rich matrix accumulation in chondrocytes was detected by Alcian Blue. All the chemicals used were obtained from Sigma.

Commercially sourced bone marrow mononuclear cells were obtained from Lonza and human embryonic cell line SHEF-2 was obtained under approval from UK Stem Cell Bank. The isolation and characterization methods were described previously (Agrawal et al., 2016; Akram et al., 2014).

2.2 Cell Viability Assay

The ratio of Live:Dead cells was identified by staining with calcein-acetoxymethyl (calcein-AM) indicating intracellular esterase activity and ethidium homodimer-1 (EthD-1) indicating membrane integrity. Cells were incubated with calcein-AM (1 µM) and EthD-1 (4 µM) in PBS for 20 minutes before being gently rinsed to remove residual fluorescent dyes (ThermoScientific, USA). Live:Dead cell quantification was performed via fluorescence microscope observation.

2.3 Beta-Galactosidase Senescence Assay

Senescence was confirmed by the catalytic activity of SA-β-gal which catalyses the hydrolysis of Xgal (5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside) and results in the accumulation of blue precipitate. The Cellular Senescence Assay Kit (Merck, USA) was applied according to manufacturer's instructions. Cells were first fixed using the formaldehyde based solution supplied with the kit and incubated with freshly prepared X-gal solution before image collection via a bright-field microscope. The percentage of β-gal-positive cells was determined by counting the number of blue-stained cells within each independent field.

2.4 Differentiation of CMSCs and AMSCs to insulin producing cells

CMSCs and AMSCs at passage 3 were pre-cultured in high glucose (25mM) DMEM consisting of 1% L-glutamine, 10% FBS, and 1% NEAA for 10 days. Cells were then seeded at a density of 1×10^6 cells/well in a six-well plate and IPC differentiation performed through a three-stage protocol (Figure 4a). For stage 1, inducing definitive endoderm, cells were cultured in serum-free 17 mM glucose DMEM/F12 containing 1% BSA (Invitrogen, USA), 50 µM β-mercaptoethanol (Sigma,USA), 1mM sodium butyrate (Sigma,USA), and 50 ng/ ml activin A for 3 days before the addition of 2 µM retinoic acid to the media for an additional 3 days, in replacement/instead of Activin A. At stage 2, cell media was switched to 17 mM glucose DMEM/F12 containing 1% BSA, 20 ng/ml EGF and 0.3mM taurine (Sigma,USA) for 3 days to induce pancreatic specialisation. At stage 3, 10 mM nicotinamide, 50 nM glucagon-like peptide (GLP)-1, and 1% NEAA were added to stage 2 media and continued in culture for another 7 days to induce IPC maturation. For AMSCs differentiation, GLP1 was replaced with exendin-4 (Sigma, USA) or betacellulin at stage 3. All media and supplement were from Lonza and growth factors were from Peprotech, UK.

2.5 Quantitative real-time PCR

Total RNA was extracted by Trizol Reagent (Invitrogen, USA) and then reversed transcribed using High-Capacity cDNA Reverse Transcription Kit (ThermoScientific, USA) in a thermal cycler (MJ Research PTC-200), following the manufacturer's instructions. For real-time PCR, all samples were run in triplicates using QuantiFast SYBR Green PCR Kit (Qiagen, Germany) and the reactions were carried out in Agilent MX3005P real time thermal cycler. An arbitrary threshold was set to be above background fluorescent levels within the exponential phase of amplification curve and the threshold cycle (Ct) reflected the cycle number at which threshold was passed. The housekeeping gene *GAPDH* was used as the reference gene and the relative expression level of gene of interest was calculated using ΔCt ($\Delta Ct = Ct_{(target)} - Ct_{(reference)}$) or ΔΔCt ($\Delta\Delta Ct = \Delta Ct_{(experimental)} - \Delta Ct_{(control)}$) method as appropriate. The fold change was calculated by $2^{-\Delta\Delta Ct}$ method. Primer sequences are described in Table S2.

2.6 Confocal immunofluorescence

Cells were fixed in 4% paraformaldehyde, blocked and permeabilised with 1% BSA and 0.1% TritonX-100 in PBS for 1 h at room temperature, then incubated overnight at 4 °C with primary antibodies. After PBS washes cells were incubated with secondary antibodies at room temperature for 1 h following by counterstaining with 4,6-diamidino-2-phenylindole (DAPI). Antibodies and dilutions were: anti-insulin (1:100, Santa Cruz sc-9168, USA), anti-PDX-1 conjugated to Alexa Fluor 488 (1:100, Santa Cruz sc-390792 AF488, USA), anti-glucagon conjugated to Alexa Fluor 488 (1:100, Santa Cruz sc-57171 AF488, USA) and Alexa Fluor 594 (1:200, Invitrogen, USA). Representative images were taken using an Olympus IX83 confocal microscope and FluoView FV1000 software was used to capture and process the images. The colocalisation of insulin and glucagon was analysed by calculating the Pearson correlation coefficient (R) using Image J Coloc2 plugin. The R value ranges from +1 (total positive correlation, colocalisation) to -1 (total negative correlation, anti-colocalisation) and 0 indicates no association

2.7 Glucose challenge and insulin release

Cells were washed with PBS and pre-incubated for 2 hours in phenol red-free DMEM without glucose (FisherScientific, USA). To stimulate insulin secretion, cells were incubated in phenol red-free DMEM containing 5.5 mM glucose (low glucose) at 1 ml per well in 6-well plate for 1 h and supernatant collected. Cells were subsequently incubated with phenol red-free DMEM containing 25 mM glucose (high glucose) for 1 h, supernatant collected, and low glucose stimulation repeated. Finally, cells were incubated in phenol red-free DMEM containing 30 mM KCl (depolarization challenge) without glucose for 30 min, then supernatant collected. Due to the suspension cluster formed during differentiation process, at each media change, supernatant was carefully removed without disturbing the IPC clusters and centrifuged at 1000 rpm for 3 min. Supernatant was transferred to a new Eppendorf and stored at -80 °C for ELISA analysis, while the pellet was resuspended with 1 ml glucose stimulation media and added to the culture plate for incubation together with undisturbed/attached differentiated cells. Following the final step of KCL challenge, differentiated cells were collected and IPC clusters were dissociated into single cells using Accumax (ThermoScientific, USA) for cell counting. To measure intracellular insulin content, protein was extracted by radioimmunoprecipitation assay buffer (ThermoScientific, USA). Insulin release and content were measured by Human Insulin ELISA Kit (Abcam, UK). Insulin secretion was normalised to total cell numbers and intracellular insulin content was normalised to total protein content measured by BCA kit (ThermoScientific, USA).

2.8 Statistical analysis

Statistical significance was determined by ANOVA and Tukey post-hoc tests, with a p-value below 0.05 defined as statistically significant (*$p \leq 0.05$, **$p \leq 0.01$, and ***$p \leq 0.001$). The analysis was performed using GraphPad Prism software and data presented as mean ± SEM.

3 Results

3.1 Derivation and characterization of CMSCs/AMSCs from healthy and GDM placenta

Chorionic and amniotic MSCs were isolated from placental membranes of 7 healthy (H-CMSCs/AMSCs) and 7 GDM (G-CMSCs/AMSCs) women and characterized through tri-lineage differentiation and MSC marker examination. H-/G-CMSCs/AMSCs exhibited osteogenic, adipogenic, and chondrogenic potential (Figure 1a) and expressed elevated MSC surface markers, CD73, CD90, and CD105, and scanty levels of CD14, CD19, CD34, CD45 and HLA-DR (Figure 1b and Table S3). The pluripotent marker status of CMSCs/AMSCs was analysed by establishing expression levels of *NANOG, SOX2*, and *OCT4*. Human embryonic stem cells (ESCs) levels of *NANOG* and *OCT4* expression were significantly higher than either placental MSCs (H-/G-CMSCs/AMSCs) or bone marrow MSCs (BM-MSCs). *NANOG* expression in H-/G- CMSCs and AMSCs was comparable and significantly higher than BM-MSCs (Figure 1c). Similarly, H-/G- CMSCs and AMSCs had significantly increased *OCT4* and *SOX2* expression than BM-MSCs (Figure 1d). Notably, placental MSCs expressed high levels of *SOX2,* particularly in H-/G- CMSCs which showed significantly elevated *SOX2* expression when compared to H-/G- AMSCs, ESCs, and BM-MSCs (Figure 1e).

3.2 Exposing CMSCs and AMSCs to high glucose concentration induces morphological change and decreases cell viability

As glucose concentration is crucial for β-cell growth and widely used in the IPC generation process, we investigated the effect of high glucose on H-/G- CMSCs/AMSCs as a first step in determining a suitable environment for IPC differentiation. CMSCs and AMSCs were cultured in high glucose (HG) DMEM (25 mM) and low glucose (LG) DMEM (5.5 mM) media containing 10% FBS. H-CMSCs cultured under HG conditions began to form condensations after day 20 where the number and dimension of cell condensations continued to increase until day 30 (Figure 2a). Similar, though less pronounced, changes were also observed in G-CMSCs under the same conditions. H-/G- AMSCs demonstrated less obvious morphological changes in HG culture. Some small cell condensations were detected in H-AMSCs whereas no distinguishable difference in morphology of G-AMSCs was observed during the 30-day culture period in either HG or LG conditions (Figure 2b).

Cell viability was affected by long-term HG exposure. During 30-day HG culture, H-CMSCs and G-CMSCs displayed a gradual decrease in cell viability (Figure 2c). Viable H-CMSCs percentage declined from 97.7% on day 20 to 90.6% at 30 while viable G-CMSCs reduced from 93.5% and 87.8% on day 20 and 30, respectively (Figure 2e). Likewise, H-AMSCs and G-AMSCs exposed to HG resulted in lower viable cell numbers when compared with LG control (Figure 2d). Significant reductions in H-/G- AMSC viability was observed from day 20-30 from 93.2-80.6% in H-AMSCs and 92.5-77.8% in G-AMSCs (Figure 2e). Although HG culture contributed to significantly reduced cell viability, H-/G- CMSC and AMSC viability was maintained at 99-100% in 10-day HG culture.

3.3 Prolonged high glucose culture induces premature senescence in GDM-CMSCs/AMSCs

Senescence-associated-beta-galactosidase (SA-β-Gal) activity staining was used to indicate the occurrence of senescence. Significantly elevated SA-β-Gal was first detected after 20 days of HG culture in G-CMSCs rising to approximately 30% of cells by day 30. Elevation of SA-β-Gal was only seen in H-CMSCs at day 30 with around 15% of the population staining positively (Figure 3a-b). H-AMSCs and G-AMSCs both displayed elevated SA-β-Gal from day 10 onwards with markedly elevated and significant increases seen thereafter (Figure 3a-b). A greater number of senescent cells were detected in G-AMSCs than in H-AMSCs from day 20 onwards reaching 75% by day 30 vs. 55% with H-AMSCs (Figure 3b).

Prolonged HG culture contributed to higher levels of SA-β-Gal staining in GDM- than Healthy- CMSCs/AMSCs. However, this did not occur in LG culture with SA-β-Gal activity in H-/G- CMSCs remaining unchanged during the culture period while an increase in SA-β-Gal activity was observed in H-/G- AMSCs on day 30 (Figure S1 and S2).

3.4 High glucose culture promotes pancreatic lineage transcription factor expression

Short-term HG culture was sufficient to induce expression of endocrine lineage markers; *PDX1* and *NEUROG3* (Figure 3c-d). *PDX1* expression in H-CMSCs was significantly elevated at day 10 and gradually increased until day 30. *NEUROG3* expression in H-CMSCs was also significantly elevated at day 10 and thereafter. G-CMSCs similarly displayed upregulated *PDX1* and *NEUROG3* expression at day 10 but which both then declined with continued culture. *PDX1* and *NEUROG3* in H-/G- AMSCs were both significantly increased after initial HG culture with both undergoing reductions across the remaining time course. Neither H-/G-CMSCs or H-/G-AMSCs displayed substantially increased *INS* expression over the 30-day HG culture period where H-CMSCs provided a notable exception with their 5-fold increase in *INS* expression (Figure 3e). Taken together this is suggestive that while HG culture was inductive for pancreatic lineage differentiation, the conversion of H-/G- CMSC and AMSCs into mature insulin producing cells (IPCs) required additional factors.

3.5 A three-stage differentiation protocol induces morphological changes in H-/G- CMSCs and AMSCs

HG culture promoted formation of H-/G- CMSC condensations and while H-CMSCs displayed elevated pancreatic β-cell marker, G-CMSCs did not. Further, neither H-AMSCs nor G-AMSCs formed condensations or displayed upregulated β-cell markers. Following on from our previous observations we primed H-/G- CMSCs and AMSCs in 25mM glucose media for 10 days and then H-/G-CMSCs/AMSCs were induced to differentiate into mature IPCs through the optimised 3-stage differentiation protocol (Figure 4a).

H-/G- CMSCs condensations formed by the end of stage 1 which developed further into larger condensations or spheroid clusters during stage 2. In stage 3 increased dimension and mass of spheroid clusters was accompanied by detachment from the substrate and continued culture as organoids (Figure 4b). In contrast, H-/G- AMSCs cultured under the same differentiation condition displayed evidence of condensation formation during stage 1 and 2, with increased size in stage 3. However, H-/G- AMSCs remained adherent without any detached spheroid formation (Figure 4b).

3.6 H-/G-CMSC-IPCs exhibit elevated pancreatic lineage and mature β-cell marker expression

Gene expression was examined at the end of the 3-stage differentiation process and increased fold change of each gene calculated by direct comparison to undifferentiated H-/G- CMSCs/AMSCs. Endocrine progenitor marker, *NEUROG3* and *ILS1*, expression was noted in H-/G-CMSC-IPCs showing comparably upregulated levels while the upregulation in H-/G-AMSC-IPCs was significantly higher than in H-/G-CMSC-IPCs (Figure 4c). Pancreatic lineage differentiation and β-cell development transcriptional markers, *PDX1* and *PAX6*, were also induced in H-/G-CMSC-IPCs with comparable levels of *PDX1* but higher *PAX6* expression in H-CMSC-IPCs. Notably, the induction of *PDX1* in H-/G-AMSC-IPCs displayed significantly enhanced levels above H-/G-CMSC-IPCs (Figure 4d). The expression of endocrine and pancreatic lineage genes indicated that both Healthy- and GDM- CMSC/AMSCs were induced to differentiate into pancreatic lineages.

To determine H-/G- CMSC and AMSC development into mature IPCs, expression of β-cell specific markers, *INS* and *GLUT2* were examined (Figure 4e). Significantly elevated expression of *INS* and *GLUT2* were observed in H-/G- CMSC-IPCs (vs. H-/G- AMSC-IPCs) with approximately 30-fold and 10-15-fold induction, respectively. Direct comparison of IPCs generated from GDM- and Healthy- CMSCs established that, with the exception of *PAX6* which was lower in G-CMSC-IPCs, both expressed comparable level of progenitor, pancreatic transcription factor, and mature β-cell genes. On the other hand, H-/G-AMSC-IPCs expressed significantly lower levels of mature β-cell marker expression. The expression of both *INS* and *GLUT2* displayed no significant changes vs. undifferentiated H-/G- AMSCs (Figure 4e). Given the level of endocrine progenitor markers expression (*NEUROG3, ISL1,* and *PDX1*) and β-cell markers (*INS* and *GLUT2*) in H-/G- AMSC-IPCs vs. CMSC-IPCs it remains possible that the IPCs derived from H-/G- AMSCs may reflect an immature β-cell state.

The basal induction of mature β-cell marker expression in H-/G- AMSC-IPCs was inconsistent with endocrine and pancreatic lineage gene expression. To explore induction further we supplemented the culture medium with additional molecules to improve their maturation; GLP-1, Exendin-4, and Betacellulin. GLP-1 was incorporated at Stage III of our original protocol to promote IPC maturation while exendin-4 and betacellulin have been widely used to promote the maturation and differentiation of MSCs into functional pancreatic cells (Kumar et al., 2014; Li et al., 2010; Wong, 2011).

The expression of *INS* showed an approximate 5-fold upregulation in H-/G-AMSC-IPCs under GLP1 or exendin-4 supplementation when compared to undifferentiated AMSCs. Neither GLP-1 (100nM) nor exendin-4 (20nM) supplementation induced significant increases of *GLUT2* expression in H-/G- AMSC-IPCs. In contrast, betacellulin, at 20nM, induced *INS* and *GLUT2* expression in both H-AMSC-IPCs and G-AMSC-IPCs, which showed significantly upregulated levels than GLP-1 or exendin-4 supplementation (Figure 4f).

3.7 GDM and Healthy CMSC-IPCs secrete insulin upon glucose challenge

Insulin was co-expressed with transcription factor PDX1 in both Healthy- and GDM- CMSC-IPC spheroids (Figure 5a) derived from the protocol described in Figure 4A. H-/G-AMSC-IPCs were differentiated through the optimised method where GLP-1 was replaced with 20nM betacellulin in the last differentiation stage. Suspension spheroid cultures were not observed with H-/G- AMSC-IPCs with little progression beyond cell condensations at the end of differentiation process. However, irrespective of above, co-expression of insulin and PDX1 was noted in H-/G- AMSC-IPCs (Figure 5a and S3).

To establish functionality of IPC, we performed insulin ELISA to investigate the secretion of insulin following glucose stimulated insulin secretion. IPCs were incubated in glucose free media prior to sequentially challenging with low (5.5mM), high (25mM), low glucose, and depolarization with KCL, followed by measurement of insulin release into supernatants (Figure 5b). Undifferentiated H-/G- CMSCs/AMSCs released low or undetectable basal levels of insulin (data not shown). H-/G- CMSC-IPCs released insulin in response to glucose content changes, where a significant increase in insulin release was observed when switching from low to high glucose challenge. H-/G-AMSC-IPCs derived from the betacellulin-induced differentiation method displayed no significant change in released insulin levels in response to high glucose challenge, noting that around 50% of H-AMSC donor samples displayed evidence of glucose sensitivity.

Finally, we examined the intracellular insulin protein content to further understand whether the poor response to glucose stimulation in H-/G- AMSC-IPCs was caused by low insulin synthesis during differentiation (Figure 5c). Surprisingly, no significant difference in intracellular insulin level was observed across all IPCs suggesting a broad comparability in insulin synthesis ability.

3.8 The expression of glucagon and insulin in IPCs cell clusters

Despites the majority being insulin-positive cells, human pancreatic islets are also composed of glucagon-producing cells and somatostatin-producing cells to regulate glucose metabolism in the body, especially the balance between insulin and glucagon (Da Silva Xavier, 2018). IPCs differentiated from both H-/G- CMSCs and AMSCs expressed significantly increased levels of the insulin gene up to 20-40-fold upregulation and also displayed approximately 5-8-fold increase in glucagon expression compared to undifferentiated H-/G-CMSCs/AMSCs (Figure 6a). Somatostatin was not detected in IPCs derived from either H-/G-CMSC or AMSC (data not shown). Immunofluorescence staining identified the expression of insulin and glucagon, indicating a mixed population of insulin-positive and glucagon-positive cells in H-/G-CMSC/AMSC-IPC clusters (Figure 6b). The co-localisation of insulin and glucagon was analysed by the Pearson correlation coefficient (R), with the R value of -0.48, -0.27, -0.11 in H-CMSC-IPCs, H-AMSC-IPCs, G-AMSC-IPCs, respectively, and 0.04 in G-CMSC-IPCs (Figure S4).

4 Discussion

Coupled to the growing interest in autologous and allogeneic cell therapy, an understanding of how disease state impairs the regenerative capacity of endogenous MSCs is crucial. In this study we have provided three key observations; the first being the description of an optimised protocol for generating IPCs from placental chorion-derived MSCs, the second being that amnion-derived MSC are refractory to IPC generation in our differentiation approach, and the third being that the above primary characteristics of CMSCs are GDM-independent. GDM-derived CMSCs were capable of differentiating into IPCs with upregulated expression of β-cell markers and functionally indistinguishable from healthy CMSC-IPCs. For H-/G- AMSCs, with 20nM betacellulin supplementation, H-AMSCs from three healthy individuals were induced into functional IPCs with the ability to secrete insulin; however, this differentiation protocol was unsuccessful for G-AMSCs. This suggests that the pathophysiological state of GDM caused irreversible impairment in the differentiation capacity of AMSCs, but did not affect CMSCs differentiation potential towards pancreatic lineage.

Glucose is an influential factor for β-cells development, where exposure to concentrations of 20-30 mM promoted enhanced β-cells replication (Cao et al., 2004). Starvation of BM-MCSs in serum-free LG media prior to 25 mM glucose culture resulted in the formation of small spheroid clusters and upregulated expression of GLUT2 and insulin (Oh et al., 2004). We did not observe 7-day HG culture-induced spheroid cluster formation; however, we did note upregulated pancreatic transcription factor expression following on from short-term HG exposure. Long-term HG culture led to profound increases in cell death and levels of senescence in GDM samples when compared to healthy counterparts. Direct comparison of our findings to other studies is not possible though noteworthy comparisons are provided via elevated caspase-3 and caspase-8 pro-apoptotic activity in adipose-derived MSCs from type 2 diabetes patients and the premature senescence, accompanied by p16 and p21 upregulation, observed with human bone marrow MSCs following 28-day HG culture (Chang et al., 2015; Cramer et al., 2010).

Short-term HG culture provided a balance point with low levels of cell death, senescence, and elevated β cell-linked transcripts for priming of placental membrane MSCs followed by induction of mature IPC differentiation via our three-stage differentiation process. Our protocol was modified from previously published protocol by Chandra *et al.* (Chandra et al., 2009) who generated IPCs from adipose-derived MSCs. The protocol has also been applied to umbilical cord-derived MSCs (Wang et al., 2011). Notably, several steps were modified in our study. Firstly, the ITS (insulin-transferrin-selenium) used in Chandra's differentiation media was withdrawn in our protocol. We found that BSA and 2-mercaptoethanol were sufficient to support CMSC/AMSCs survival in serum-free condition. In fact, insulin contained in ITS may enhance the chance of taking up insulin from differentiation media instead of supporting insulin synthesis in IPCs. Secondly, after stimulating with activin A, an inducer for definitive endoderm formation (McLean et al., 2007), the additional RA stimulation combined with activin A improved morphological changes towards spheroid cluster formation. RA signalling induces the generation of endocrine progenitors and functions as an effective inducer for *PDX1* while the disruption of RA signalling leads to pancreatic agenesis (Ostrom et al., 2008). Thirdly, Chandra established a 10-day maturation protocol for IPCs; however, for H-/G- CMSCs/AMSCs increased incubation time was required especially for the last

maturation stage and the addition of EGF and nicotinamide to enhance the growth and differentiation of IPCs (Otonkoski et al., 1993; Zarrouki et al., 2014)

GLP-1, which is widely used in IPC differentiation protocols, has important physiological functions in fetal β-cell development, insulin synthesis, and secretion (Yue et al., 2006). H-/G-CMSCs displayed good responsiveness to our IPC induction protocol while H-/G-AMSCs showed low expression of mature β-cell markers under GLP-1 supplementation. We explored supplementation with either exendin-4 or betacellulin as a substitute for GLP-1 in the last IPC maturation stage. Exendin-4, a GLP-1 agonist improves glucose tolerance in diabetes patients and enhances insulin secretion (Papaetis et al., 2015). Exendin-4 supplementation promoted β-cell gene transcription in mouse embryonic stem cell-derived IPCs (Zhao et al., 2016). In this instance we saw no significant, or dose-dependent, induction of either *INS or GLUT2* transcription in H-/G-AMSC-IPCs following exendin-4 supplementation. In contrast, we noted significant increases in both *INS* and *GLUT2* in H-/G- AMSCs following betacellulin supplementation. Betacellulin is an epidermal growth factor (EGF) family member with demonstrable regulation of pancreatic regeneration through activation of EGF receptors (Oh et al., 2011). GLP-1 and exendin-4 interact with the high affinity receptor, GLP-1R which lacks kinase activity and depends on EGFR to activate its downstream pathways (Voisin et al., 2002). In turn, signal transduction of GLP-1R via EGFR require the proteolytic processing of membrane-anchored betacellulin or other EGF-like ligands (Buteau et al., 2003). Therefore, the direct treatment of betacellulin likely had a more direct effect on EGFR regulation of insulin expression.

Irrespective of above, successful induction of insulin expression was not reflected in significant increases in insulin secretion from H-/G-AMSC-IPCs. Moreover, H-/G-AMSC-IPCs failed to progress into suspension islet- like clusters during differentiation. To explore the underlying gene and molecular regulation in IPC development in order to improve H-/G-AMSCs differentiation capability, reverse engineering approach using gene sequencing to identify regulatory signalling and networks will elucidate the distinct response in AMSCs to our differentiation protocol and further understand the IPC differentiation process.

Our differentiation protocol successfully generated *in vitro* functional IPCs from GDM and healthy CMSCs and the critical problem of sufficient insulin release and survival of IPCs after transplantation will be investigated in our future study using diabetic mouse. Nevertheless, the *in vitro* glucose challenge-induced insulin secretion, along with the significant upregulation of pancreatic-associated gene and protein expression in CMSC-derived IPCs reflects the promising potential towards the future generation of pancreatic islets for diabetes therapy. Moreover, our finding of functionally indistinguishable GDM-CMSC-derived IPCs from Healthy-CMSC-derived IPCs, provides a potential candidate for use as an autologous cell source for the diabetes treatment of GDM women.

Conflict of interest

The authors declare that they have no conflict of interest.

Author contributions

All authors contributed to experimental design. L.C. acquired, analysed and interpreted the data and wrote the manuscript. N.R.F. and P.W. supervised the study, interpreted the data, and revised the manuscript. P.W. coordinated the placenta sample collections from individuals. All authors approved the final version of the manuscript. P.W. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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Figure Legends

Figure 1. Characterization of H-/G- CMSCs and AMSCs

(a) Tri-lineage differentiation capacity was assessed by osteogenesis, adipogenesis, chodrogenesis and verified by Alizarin red, Oil-Red-O, and Alcian blue stain, respectively. **(b)** Immunophenotyping of MSCs surface markers by flow cytometry. Grey solid histograms represent IgG negative control, n=7 in each group, representative examples are shown. **(c-e)** Gene expression levels of pluripotent markers – *NANOG, OCT4*, and *SOX2* in H-/G- CMSCs and AMSCs compared with embryonic stem cell line (SHEF2) and bone marrow MSCs (BMNCs). Relative expression levels were calculated byΔCt method. *$p< 0.05$, **$p< 0.01$, ***$p< 0.001$, ns, not significant.

Figure 2. Prolonged high glucose culture promotes cell condensations but reduces cell viability

(a-b) H-/G- CMSCs/AMSCs were cultured in 5.5 mM glucose DMEM until passage 2 and then exposed to 25 mM glucose (HG) DMEM for investing glucose effect or continually maintained in 5.5 mM glucose (LG) DMEM. During 30 days of HG culture, H-/G- CMSCs were induced to form cell condensations while H-/G- AMSCs showed less obviously morphological changes. H-/G- CMSCs/AMSCs in LG condition showed steady growth in the same time period and retained an adherent fibroblast-like morphology. Representative images are taken from one of three independent experiments (Scale bar, 200 μm). **(c-d)** Fluorescent images indicate cell viability; calcein (green, alive cells) and ethidium homodimer-1 (red, dead cells). Long term HG culture caused decreased cell viability in both H-/G- CMSCs and AMSCs (Scale bar, 400 μm). **(e)** The mean percentage of live and dead cells calculated from 3 independent experiments. *$p< 0.05$, **$p< 0.01$, ***$p< 0.001$

Figure 3. The effect of high glucose on cellular senescence and the induction of pancreatic lineage genes.

(a) Representative images of cellular senescence were examined by the positive staining of SA-β-Gal expression. Premature senescence induced by HG culture occurred earlier in GDM- than Healthy- CMSCs/AMSCs (Scale bar, 200μm). **(b)** Quantification of SA-β-Gal-positive cells. The SA-β-Gal-positive cells of 100 random cells was counted from at least three images of each sample (n=3) using phase-contrast microscopy. **(c-e)** Gene expression was analysed by real-time PCR at day 10, 20, and 30 of HG culture. The gene expression was normalised to *GAPDH* and then fold change was calculated by comparing to the expression level on day 0 using $2^{-\Delta\Delta Ct}$ method. There was no significant increase in *PDX1, NEUROG3,* and *INS* expression under LG day 0 vs. LG day 30 culture in each group. (n=4). Data represent mean ± SEM of 4 independent experiments. *$p< 0.05$, **$p< 0.01$, ***$p< 0.001$

Figure 4. Optimised IPC differentiation induces morphological changes and pancreatic beta-cell gene expression

(a) Differentiation scheme for generating IPCs from CMSCs and AMSCs including the growth factors, small molecules, incubation time at each stage. **(b)** H-/G- CMSCs formed suspension spheroid structures during three-stage IPC differentiation process while H-/G- AMSCs formed adherent condensations. Control images were undifferentiated H-/G- CMSCs/AMSCs cultured in complete growth media (5.5 mM glucose DMEM containing 10% FBS) which retained a fibroblast-like morphology. The image is representative of six independent experiments (Scale bar, 200 μm). **(c-e)** IPCs generated from H-/G- CMSCs and AMSCs express pancreatic lineage genes analysed by real-time PCR. The relative expression level of **(c)** endocrine progenitor markers, *NEUROG3* and *ILS1* **(d)** pancreatic lineage transcription factors, *PDX1* and *PAX6* **(e)** mature β-cell makers – *INS* and *GLUT2*. Gene expression was normalised to *GAPDH* and y-axis indicates fold increase by comparison with undifferentiated H-/G- CMSCs/AMSCs using $2^{-\Delta\Delta Ct}$ method. Data represent mean ± SEM. *$p<0.05$, **$p<0.01$, ***$p<0.001$ **(f)** The improvement of *INS* and *GLUT2* expression in H-/G-AMSCs-IPCs by GLP1, exendin-4, or betacellulin supplementation. Gene expression was normalised to *GAPDH* and y-axis indicates fold increase by comparison with undifferentiated H-/G-AMSCs using $2^{-\Delta\Delta Ct}$ method. Data represent mean ± SEM. #$p<0.001$ indicates significantly increased gene expression under betacellulin compared with GLP-1 or Exendin-4 supplementation.

Figure 5. Detection of insulin protein expression and IPC hormone release upon glucose challenge

(a) Confocal images of H-/G- CMSCs and AMSCs derived IPCs show co-expression of inuslin (red) and PDX1 (green). DAPI as nuclear counterstain in blue. Scale bar, 100 μm. **(b)** ELISA measurement of functional IPCs insulin releasing capacity assessed by glucose challenge. IPCs were subsequently incubated with low glucose, switched to high glucose, and then low glucose. After glucose challenges, cells were depolarized with 30mM KCl. **(c)** Insulin content was measured by ELISA and normalised to total protein content. ***$p < 0.001$ indicates statistical significance compared to undifferentiated MSCs. ns, no significant difference between each group.

Figure 6. The expression of insulin and glucagon

(a) IPCs generated from H-/G- CMSCs and AMSCs expressed insulin and glucagon gene analysed by real-time PCR. Gene expression was normalised to *GAPDH* and y-axis indicates fold increase by comparison with undifferentiated H-/G- CMSCs/AMSCs using $2^{-\Delta\Delta Ct}$ method. Data represent mean ± SEM. ***$p<0.001$ (b) Confocal images show the expression of insulin-positive and glucagon-positive cells population in H-/G- CMSCs and AMSCs derived IPCs. Scale bar, 100μm.