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IGF-1 and somatocrinin trigger islet differentiation in human amniotic membrane derived mesenchymal stem cells

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ABSTRACT

Aim: To induce differentiation of human amniotic membrane derived mesenchymal stem cells (hAMMSCs) into insulin producing cells (IPCs) by treating with somatocrinin or growth hormone releasing hormone (GHRH) and Insulin-like growth factor-1 (IGF-1).
Main method: In this investigation, we cultivated and characterized hAMMSCs and then treated with IGF-1 and somatocrinin to find out whether this combination gives better yield of insulin producing cells. We showed that hAMMSCs can give rise to IPCs on exposure to serum-free defined media containing specific growth factors and differentiating agents in presence of IGF-1 and somatocrinin.
Key finding: A combination of IGF-1 and somatocrinin lead to differentiation of large number of IPCs from hAMMSCs. These IPCs were found to be positive for dithizone indicating their insulin secretory mechanism. Moreover these cells were also found to be positive for C-peptide. IPCs released insulin in response to glucose challenge. Gene expression analysis exhibited significant up-regulation of pancreatic transcription factor GLUT2 and Insulin.
Significance: Our data thus demonstrates for the first time that somatocrinin and IGF-1 synergistically enhance the differentiation of hAMMSCs into IPCs.

1. Introduction

Diabetes mellitus is becoming one of the main threats to human health in the 21st century [1]. The amniotic membrane has been reported to contain a population of multipotent stem cells exhibiting characteristics of mesenchymal stem cells including surface markers such as CD73, CD105 and CD90 and negative for hematopoietic markers CD34 and CD45 [2,3].

Differentiation of these hAMMSCs into insulin producing cells could be a glimmer. It is known that insulin is the only gene that is express generally in the yolk sac amniotic membrane. The yolk sac amniotic membrane and the pancreas of both human and mouse are the only tissues that produce insulin [1,4]. This suggests that yolk sac amniotic membrane imprinting of insulin is an ancestral trait.

Somatocrinin or Growth hormone-releasing hormone (GHRH) is a peptide consisting of 44 amino acid produced by the hypothalamus. It is carried to the anterior pituitary through the portal vessels and stimulates the release of growth hormone (GH) after binding to the GHRH receptor (GHRH-R) on cell membranes [5–8]. Production and secretion of downstream factors such as insulin growth factor 1 (IGF-1) are controlled by growth hormone-releasing hormone (GHRH) [6]. GHRH and its receptors are expressed in the hypothalamus and pituitary, also expressed in peripheral tissues. Thus, in addition to modulating growth hormone release, GHRH indirectly regulates the proliferation of cells in multiple other tissues through a GHRH/GH/IGF-1 axis [2,8]. GHRH directly regulate cell growth by binding to the GHRH receptor on target cells through paracrine and endocrine mechanisms. Because of this, GHRH has attracted wide attention in recent years as global regulators

Abbreviations: DTZ, dithizone; GHRH, growth hormone releasing hormone; GSIS, glucose stimulated insulin secretion; hAMMSCs, human amniotic membrane derived mesenchymal stem cells; IGF-1, Insulin-like growth factor-1; IPCs, insulin producing cells

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of cell growth with therapeutic potential including tissue regeneration [8].

Human amniotic membrane-derived mesenchymal stem cells (hAMMSCs) recognized by their plastic adherence, expression of specific cell surface antigen, and multilineage differentiation potential [2,9]. Because of their multipotency and immuno-privileged properties, MSCs have been widely used to promote tissue regeneration [3,10]. However, the full regenerative potential of MSCs for clinical application is limited by poor differentiation of MSCs in the adverse microenvironment of a diabetic pancreas [11]. Therefore, we describe the combined role of somatocrinin (GHRH) and IGF-1 in the differentiation of hAMMSCs in to IPCs.

2. Materials and methods

2.1. Ethical considerations

Human placentae of full term Cesarean section deliveries were collected after informed written consent of the patient (n = 10). The study was conducted after obtaining clearance from the Institutional Ethics Committee.

2.2. Sample collection

Amniotic membrane from placentae were separated aseptically and collected in sterile container containing medium (phosphate buffered saline (PBS) with penicillin 200 U/ml and streptomycin 200 µg/ml) and transported at ambient temperature to the laboratory. All amniotic membrane samples collected were negative for infectious test. Processing of amniotic membrane tissue was made in the first 6 h following collection to avoid any loss in cell viability.

Table 1
List of primers.

Gene	Primer sequence	Product size
ß-ACTIN	5′-TCACCCACACTGTGCCCATCTACGA-3′ 5′-CAGCGGAACCGCTCATTGCCAATGG-3′	295
GLUT2	5′-GGTTTGTAACTTATGCCTAAG-3′ 5′-GCCTAGTTATGCATTGCAG-3′	211
INSULIN	5′-AGCCTTTGTGAACCAACACC-3′ 5′-GCTGGTAGAGGGAGCAGATG-3′	245

2.3. Isolation and culture of human amniotic membrane derived mesenchymal stem cells (hAMMSCs)

Initially, the amniotic membrane tissue was washed with PBS. Blood clots and vessels were removed mechanically and the tissue was minced into small pieces and washed again with PBS. The minced tissues were then digested with 0.25% Trypsin- EDTA (Gibco-Invitrogen) for 45 min at 37 °C for removal of the trophoblasts. The sample was then filtered through a 250 µm metal sieve. The remnants were collected and digested with collagenase- I (Gibco-invitrogen) 0.1% (1 h at 37 °C). Digested tissue was passed through a 250 µm metal sieve and 100 µm cell nylon membranes, to eliminate undigested tissue fragments. Cells were collected, following centrifugation of the filtrate at 300*g* for 5 min at 20 °C, and were washed with 10 ml 1× PBS (Sigma Aldrich). Cell suspension were loaded on Ficoll at a ratio of 1:2 and centrifuged at 300*g* for 15 min at 20 °C. Mononuclear cells were collected and washed with 1× PBS and finally centrifuged at 300 *g* for 5 min at 20 °C. The cell pellet was suspended in a complete culture medium consisting of DMEM (low glucose 1 g/l content, L-DMEM) supplemented with 20% fetal bovine serum (FBS) plus 100 U/ml penicillin, 100 µg/ml streptomycin, 1 mM L-glutamine and seeded into two 75 cm^2 flasks.

Cultures were incubated at 37 °C in a humidified 5% CO2 incubator. Non-adherent cells were removed (after 24 h). Medium was replaced with fresh medium every three days, and adherent cells were trypsinized with 0.25% Trypsin-EDTA (Gibco-Invitrogen) upon reaching 80% confluence after 7 days of culture. All the experiments were carried out using cells at passage 4–6.

2.4. Characterization of hAMMSCs

The cells were dislodged using 0.25% Trypsin-EDTA (Gibco-Invitrogen), and re-suspended in DMEM. Then, the cells were fixed in chilled 70% ethanol, and incubated in mouse anti-human FITC/PE conjugated antibodies against CD34, CD45, CD73, CD90, and CD105 (1:100 dilution) for 1 h on ice (all antibodies were purchased from sigma Aldrich, USA). Finally, the cells were counted using a flow cytometer laser 488 nm, and the data were analyzed using BD CellQuest™ Pro software.

2.5. Differentiation of hAMMSCs into insulin producing cells

hAMMSCs were differentiated into IPCs in presence of IGF-1, somatocrinin and combination of both. Briefly, hAMMSCs harvested and

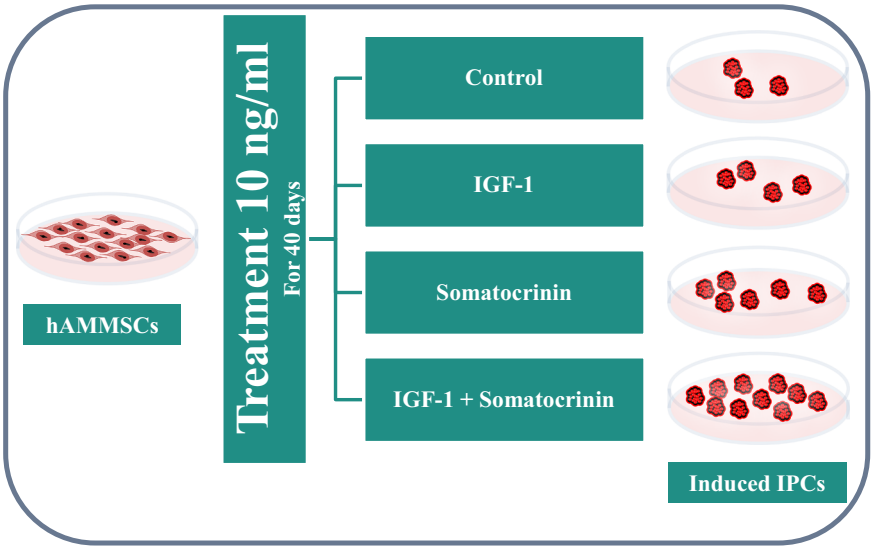

Fig. 1. Schematic representation of different induction treatment used to generate IPCs.

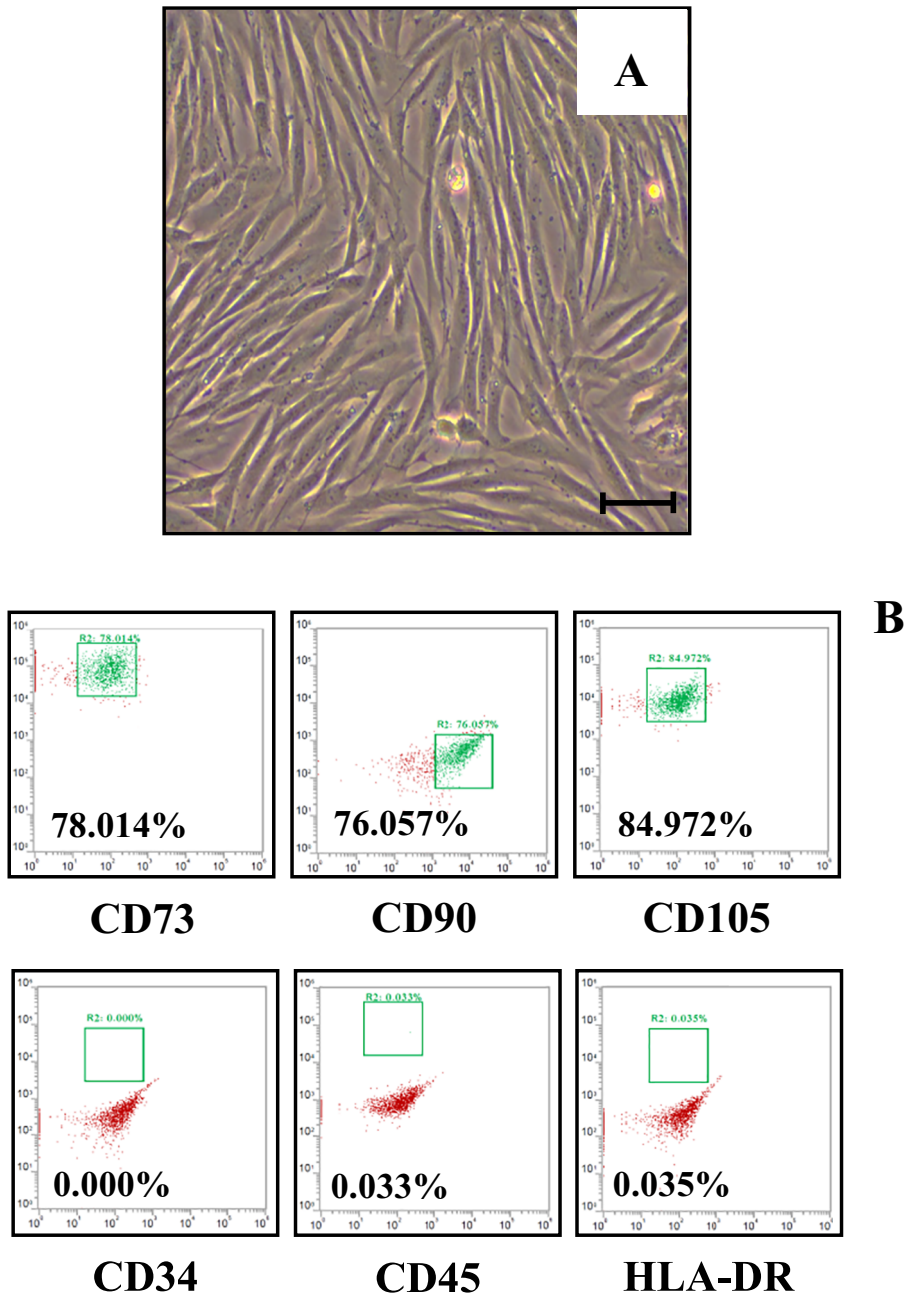

Fig. 2. Isolation and characterization of hAMMSCs. **(A)** hAMMSCs at passage 4. Scale bars = 100 μm, **(B)** Flow cytometry analysis for MSC-specific cell surface antigens in hAMMSCs.

5×10^3 cells were seeded into 6-well plates and incubated for 24 h. The medium was then replaced with the appropriate differentiation medium containing DMEM, 1 mM nicotinamide, 1× Insulin-Transferrin-Selenium (ITS), supplemented with either 10 ng/ml IGF-1 or 10 ng/ml GHRH or a combination of both. All differentiation studies were conducted at passage 4 of the hAMMSCs cells. Cells were fed with appropriate medium after every five days. After 40 days of induction, cells were analyzed for IPC properties and clusters were counted.

For standard control group, hAMMSCs were differentiated into IPCs by using a previously described and widely accepted protocol [14,15,26–28]. Briefly, differentiation was carried out in three stages; firstly the cells were cultured in α-MEM with 17.5 mM glucose, 1% BSA, 1× insulin-transferrin-selenium (ITS) for 2 days. On the third day, the medium was changed to α-MEM with 17.5 mM glucose, 1% BSA, ITS, and 0.3 mM taurine. The cell aggregates were cultured in this medium for another 4 days, and shifted on seventh day to the medium containing α-MEM with 17.5 mM glucose, 1.5% BSA, ITS, 3 mM taurine, 100 nM glucagon- like peptide (GLP)-1, and 1 mM nicotinamide. The cell aggregates were fed with fresh medium every 2 days for further 4 days.

2.6. Characterization of IPCs by dithizone dye (DTZ) staining

IPCs were washed three times with PBS and fixed in 4% paraformaldehyde solution for 30 min. Again washing with PBS twice, the IPCs were subjected to1 mg/ml DTZ stain.

The dye was completely removed by washing the cells several times with PBS without Zinc (Zn). Positive aggregated cells were observed by phase contrast microscopy (EVOS-Core, Life technologies).

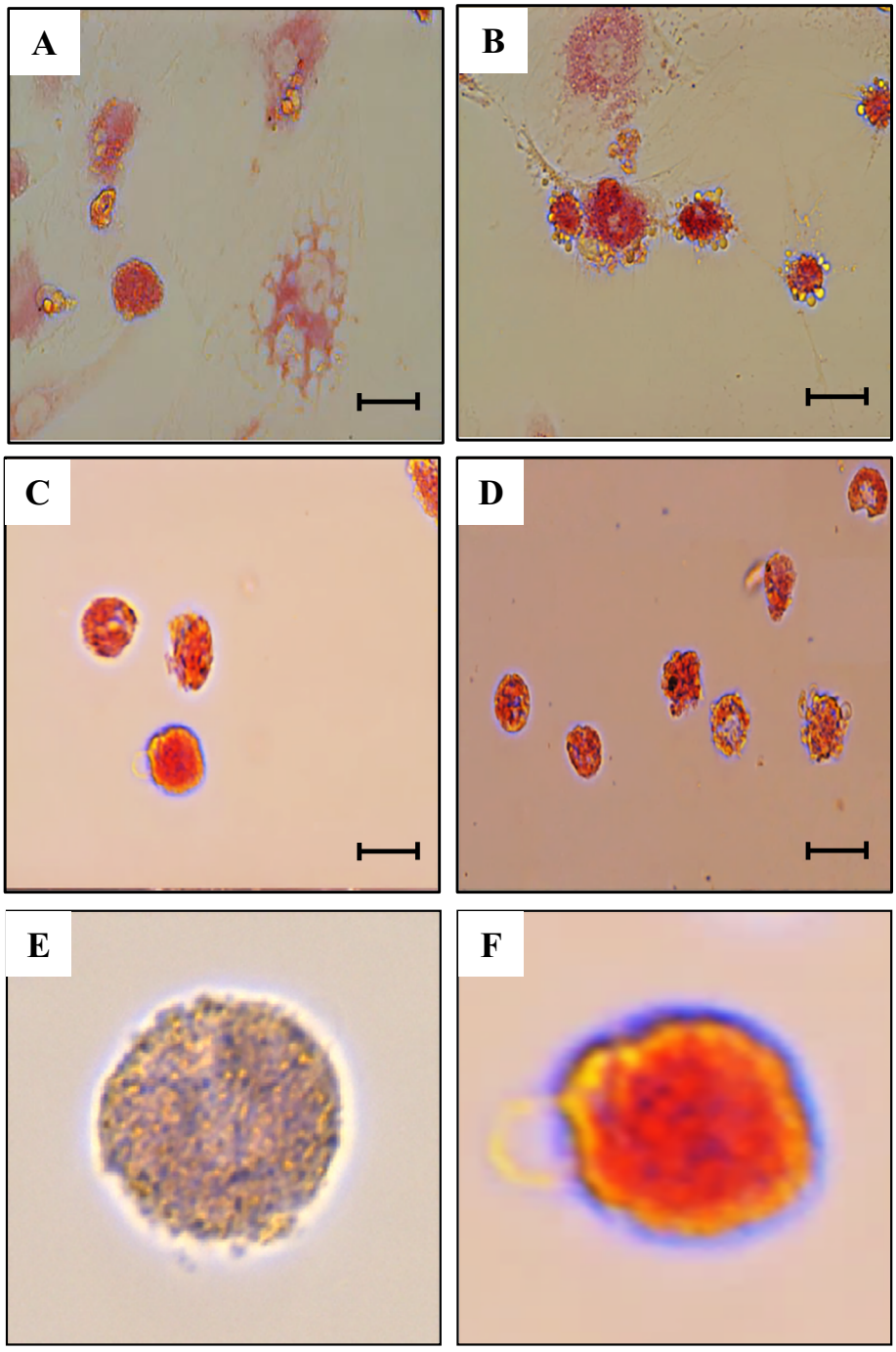

Fig. 3. Functional staining of IPCs by dithizone. **(A)** Control, **(B)** IGF-1, **(C)** Somatocrinin, **(D)** IGF-1 + Somatocrinin. Scale bar = 100 µm, **(E & F)** Enlarged single cluster observed before and after staining.

2.7. C-peptide measurements

For stage-specific C-peptide assay during IPCs development, conditioned medium was collected from all experimental groups such as control, IGF-1, somatocrinin and combination of both somatocrinin and IGF-1. The medium was quantified using ultra-sensitive C-peptide human ELISA kit (Sigma RAB1389).

2.8. Glucose stimulated insulin secretion (GSIS)

To assess the insulin secreting function of ICAs, Glucose Stimulated Insulin Secretion (GSIS) was carried out according to the previously published protocol. The medium size islets ranging from 100 to 150 µm, 4–6 in number were handpicked and added to each well of 24 well plates (Nunc A/S) in triplicates. They were then washed with 250 µl of PBS thrice. The islets were then glucose starved in Krebs-Ringer bicarbonate buffer (pH 7.4) supplemented with 10 mM HEPES (now called KRBH without glucose) at 37 °C in CO2 incubator for an hour. This represents a stabilization and glucose starvation phase. The supernatant was discarded and the islets were subjected to low high glucose (16.75 mmol/L) as control and few additives like IGF-1, somatocrinin and a combination of IGF-1 and somatocrinin for an hour. The supernatant was then collected and stored at −20 °C. Further, the insulin secretion was assessed with commercially available mouse/

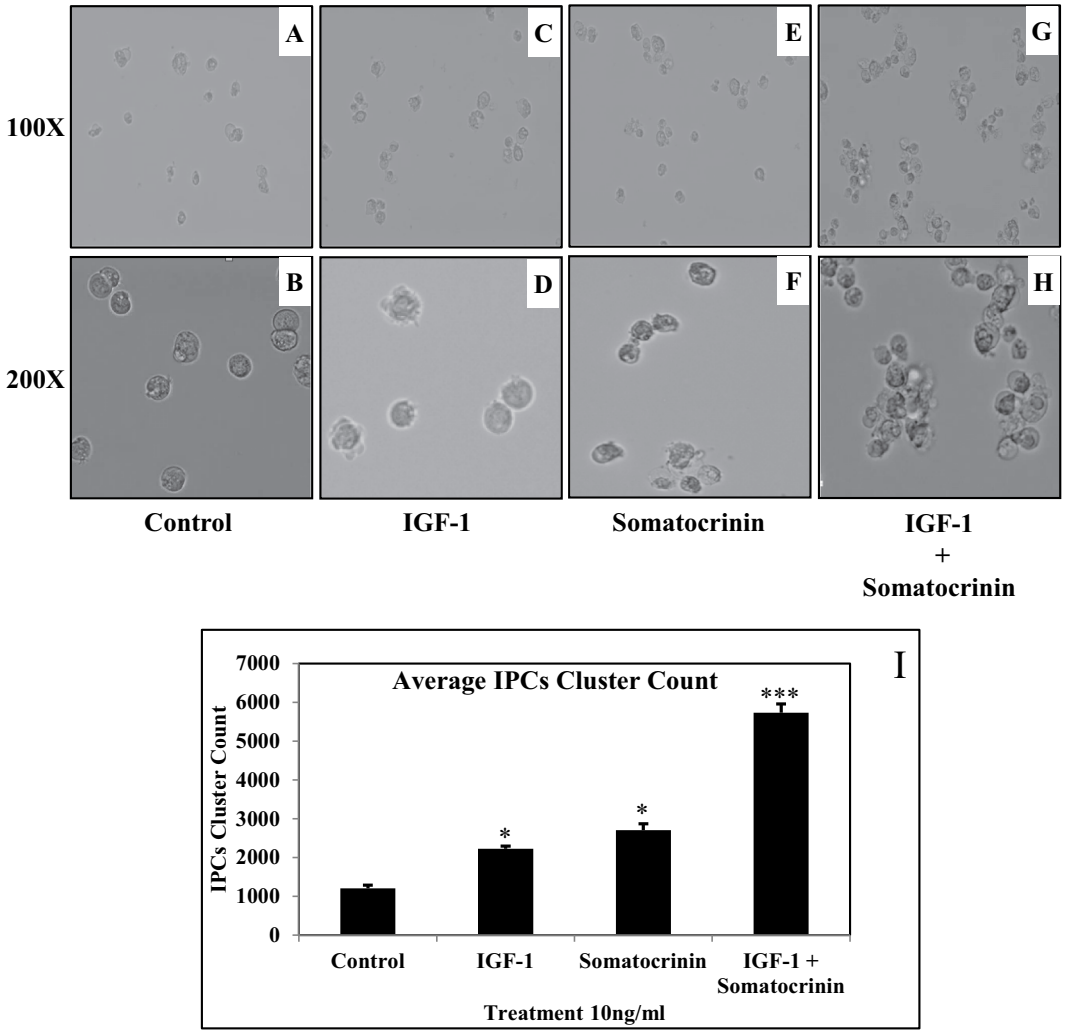

Fig. 4. IGF-1 and Somatocrinin (GHRH) trigger insulin producing cells differentiation in hAMMSCs. **(A–B)** Control, **(C–D)** IGF-1, **(E–F)** somatocrinin and **(G–H)** IGF-1 + somatocrinin. **(I)** Graphical representation of IPC cluster count (average IPC cluster number) ($p < 0.05$ for IGF-1; $p < 0.05$ for somatocrinin; $p < 0.01$ for combination).

human insulin ELISA kit (Mercodia, AB, Sweden) [12].

2.9. RNA isolation and quantitative reverse transcription-PCR

The ICAs were pelleted down and the total RNA was extracted using Trizol method (Takara). RNA (1 μg) was reversely transcribed using cDNA synthesis kit (Revertaid, Thermo Scientific) according to the manufacturer's guidelines. Quantitative analysis of GLUT2 and INSULIN was carried out using SYBR Green PCR master mix (Applied Biosystem) on 7500 Real-Time PCR system (Applied Biosystem). Expressions of target genes were normalized to ß-actin using the ΔΔCt method. The cycle threshold (CT) values for each gene were corrected using the mean CT value. RT-PCR data were quantified using the $2^{-\Delta\Delta Ct}$ method and presented as relative gene expression normalized to the average CT for the β-actin gene. The primers used are shown in the Table 1. (See Fig. 1.)

2.10. Statistical analysis

The experiments were repeated thrice and the samples were run in triplicates (n = 10). All the data values are presented as mean ± standard deviation (SD). Experimental groups were compared using one-way analysis of variance (ANOVA) test. For all analyses *p*-value < 0.05 was considered significant (Levels of significance: *$p < 0.05$ and ***$p < 0.01$).

3. Results

3.1. Morphology

Human amniotic membrane MSCs (hAMMSCs) exhibited fibroblast-like morphology after isolation and adhered to surface of the flask (Fig. 2A).

3.2. hAMMSCs express stem cell specific surface antigens

The immunophenotypes of hAMMSCs were analyzed by flow cytometry. hAMMSCs were found to be positive for mesenchymal stem cell markers CD90, CD73, and CD105, and negative for CD34 and CD45. Additionally, the absence of HLA-DR (MHC class II surface antigen) was consistent with the general description of MSCs (Fig. 2B). These findings show that hAMMSCs possess both the capacity for self-renewal and immune-modulatory properties.

3.3. IGF-1 and GHRH induce differentiation of hAMMSCs to insulin producing cells (IPCs)

IGF-1 and somatocrinin (GHRH) increase the rate of differentiation

of hAMMSCs insulin producing cells. Mesenchymal stem cells have potential to trans-differentiate into endodermal lineage cells.

In order to generate IPCs from hAMMSc introducing combination of IGF-1 and somatocrinin, we applied the differentiation protocol using either of the two factors and combination of both under study. Interestingly, hAMMSCs induced by the combination of IGF-1 and somatocrinin exhibited almost the same morphological changes previously observed with the protocol [6,13], where the cells lost their fibroblast-like shape and tended to form aggregates by the end of the differentiationprotocol. After 40 days of induction, the mature IPCs stained red for islet specific DTZ stain (Fig. 3).

Differentiation of hAMMSCs to IPCs using IGF-1, somatocrinin and combination of somatocrinin and IGF-1 yield more number of IPC clusters compared to that of standard control ($p < 0.05$ for IGF-1; $p < 0.05$ for somatocrinin; $p < 0.01$ for combination) (Fig. 4A–I).

3.4. Treatment with IGF-1 and somatocrinin (GHRH) promote comparatively high C-peptide and insulin release in hAMMSC differentiated to IPCs

C-peptide secretion in the secretome collected from the IPCs induced using combined treatment of IGF-1 and somatocrinin was found to be 0.27 pmol/ml; 2 fold higher than the rest of the groups ($p < 0.01$) (Fig. 5A).

The IPCs were stimulated with high glucose and it was found that the IPCs generated could respond to glucose challenge. Compared to high glucose the IPCs significantly enhanced the insulin secretion to 2ulU/ml/ml ($p < 0.05$) with IGF-1, 1.96 ulU/ml with somatocrinin, while the combination of IGF-1 and somatocrinin showed maximum insulin secretion to 3.5ulU/Ml ($p < 0.01$) (Fig. 5B).

3.5. IGF-1 and somatocrinin (GHRH) induced IPCs exhibit expression of pancreatic markers

The IPCs expressed pancreatic markers. The expression of transcripts like INSULIN and GLUT2 were significantly up-regulated at day 40 of differentiation. GLUT2 showed 4-fold ($p < 0.01$) increase whereas INSULIN showed 3 fold ($p < 0.01$) increase in the expression as compared to those of control in the presence of IGF-1 and somatocrinin (Fig. 6A & B).

4. Discussion

The hAMMSCs showed adherence to plastic and the fibroblast-like morphology (Fig. 2B). Immunophenotypic characterization of hAMMSCs displayed the presence of MSC-specific surface markers (CD90, CD73, and CD105), also absence of hematopoietic markers CD34, CD45, and HLA-DR. Mesenchymal stem cells have been shown to have potential to form insulin-producing cells [14–16]. Our data differs from these reports wherein we have used somatocrinin (GHRH) and insulin-like growth factor-1 (IGF-1) for enhanced islet-like cell differentiation. (Figs. 3 & 4). To our knowledge, this is the first report to induce IPCs differentiation in hAMMSCs employing IGF-1 and somatocrinin.

The role of somatocrinin is attributed to glucose homeostasis through mechanisms promoting the proliferation and survival of the pancreatic islet cells [13]. A previous study reported that agonists of somatocrinin promoted growth, function, expression of cellular insulin, IGF-1, and GHRH receptor, and also stimulated insulin secretion in response to glucose challenge inrat pancreatic β-cell line (INS-1) and islets [17–19]. The main focus of our study is to evaluate role of somatocrinin in combination with IGF-1 for obtaining higher yield of functional insulin producing cells. Triggering of functional insulin-producing cells in vitro could prove as a promising source for cell-based therapy. Insulin is stored in the IPCs secretory granules and subsequently converted, in equimolar concentrations, to C-peptide and insulin. C-peptide and insulin are secreted into the circulation following beta cell stimulation [20]. In our investigations, treatment of hAMMSCs with somatocrinin and IGF-1 significantly increased C-peptide expression in IPCs (Fig. 5A) indicating intact insulin producing machinery.

GHRH as well as IGF1 have been shown to increase β-cell proliferation in transplanted human and fetal rat islets [25,26]. An earlier study implicated the potential role of a GHRH agonist in islet cell proliferation and survival. The detection of the GHRH receptor on β-cells in rat and human islets supports the view that GHRH may exert a direct signal transduction within the pancreas independent and/or in addition to the effects mediated by the GH/IGF1 pathways [21–23]. In our earlier study, we reported that pretreatment of hAMMSCs with somatocrinin stimulated cell proliferation, enhanced differentiation into osteoblast, chondrocyte in combination with IGF-1 [24]. Since IGF-1 is a prominent trophic factor in islet development and function; it may be required for β-cell differentiation in vitro [25]. We found that addition of IGF-1 and somatocrinin stimulate cell aggregation and cluster formation. These newly generated IPCs showed insulin secretion upon glucose stimulation (Fig. 5B).

IGF-1 is the primary mediator of the effects of GHRH [26]. IGF-1 plays an important role in islet cell growth, insulin secretion, and maintenance of insulin sensitivity via JAK/STAT pathway [27]. JAK/STAT signaling pathway and its regulators have been implicated in pancreatic beta cell neogenesis during embryonic development [28]. So it is likely that JAK/STAT pathway indirectly regulating the synergistic effect of IGF-1 and somatocrinin in the induction of IPCs from hAMMSCs. However the underlying molecular mechanisms are yet to be elucidated.

In our differentiation protocol we did not use GLP-1, which has been incorporated in many of the protocol for IPCs differentiation [29–31]. This could be the reason for prolonged time period of differentiation for getting mature IPCs (Fig. 6A & B). However, deletion of GLP-1 has made

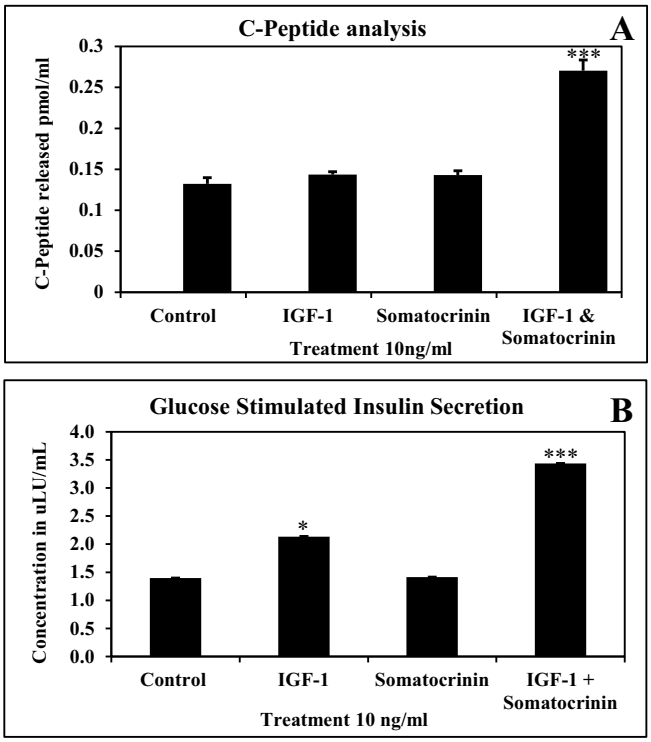

Fig. 5. Graphical representation C-peptide analysis and glucose stimulated insulin secretion in IPCs. **(A)** C-peptide level is 2-fold higher in IPCs generated using combination of IGF-1 and somatocrinin ($p < 0.01$), **(B)** Comparative analysis of GSIS in Control, IGF-1, Somatocrinin and IGF-1 + somatocrinin induced IPCs from hAMMSCs ($p < 0.05$ for IGF-1; $p < 0.01$ for IGF-1 and somatocrinin).

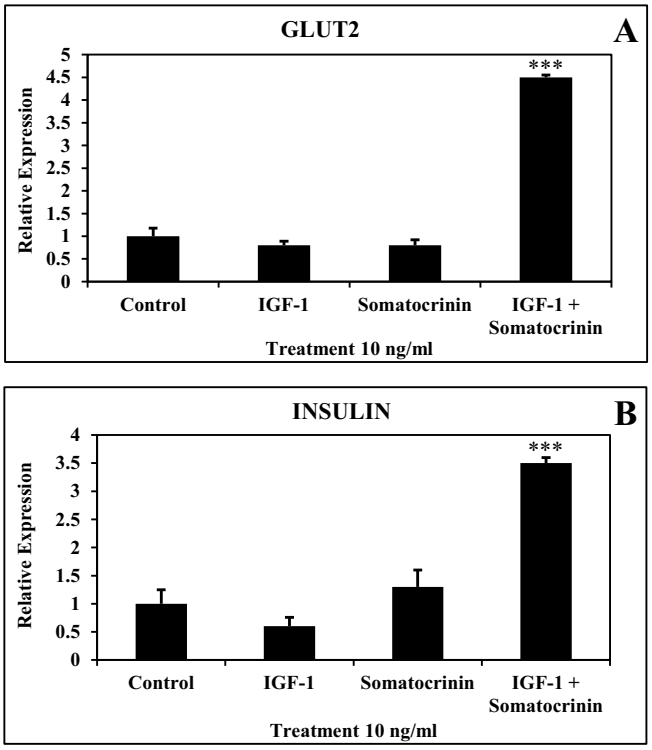

Fig. 6. Comparative expression of pancreatic markers in Control, IGF-1, somatocrinin and IGF-1 + somatocrinin induced IPCs. **(A)** GLUT2 showed 2-fold upregulation in IGF-1 + somatocrinin induced IPCs than rest of the groups ($p < 0.01$), **(B)** INSULIN showed 2-fold upregulation in IGF-1 + somatocrinin induced IPCs than rest of the groups ($p < 0.01$).

our protocol economical. This novel report implicates direct crosstalk between somatocrinin and IGF-1into pancreatic islet-like differentiation. Up-regulation in the expression of GLUT2 and INSULIN was detected in IPCs after 40 days induction, which has been shown to be indicative of fully differentiated and functional IPCs.

Various studies have emphasized on MSCs from different types of tissues to formulate a successful method for differentiation into insulin producing cells [14–17]. However there is a scope to explore novel differentiation promoting agents to produce IPCs. Cell aggregation has an important role in enhancing function and viability of stem cells-derived IPCs in vitro mimicking the ultrastructure and morphology of the native pancreas islets [32]. We have obtained well defined functional IPCs ranging from 100 to 300 μm manifesting the exclusiveness of our approach. However underlying key molecules affected by the IGF-1 and somatocrinin treatment during IPC generation needs to be evaluated.

5. Conclusion

In conclusion, our data showed for the first time that hAMMSCs treated with combination of IGF-1 and somatocrinin enhanced production of IPCs clusters. The functionality of IPCs was confirmed at both transcriptional and translational level as evidenced by gene expression, GSIS and C-peptide analysis.

Acknowledgment

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Conflict of interest

Authors declare that there is no conflict of interest.

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