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Original Article

In vitro differentiation of human multilineage differentiating stress-enduring (Muse) cells into insulin producing cells

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ABSTRACT

Mesenchymal stem cells (MSCs) is a heterogeneous population. Muse cells is a rare pluripotent subpopulation within MSCs. This study aims to evaluate the pluripotency and the ability of Muse cells to generate insulin producing cells (IPCs) after *in vitro* differentiation protocol compared to the non-Muse cells. Muse cells were isolated by FACSAria III cell sorter from adipose-derived MSCs and were evaluated for its pluripotency. Following *in vitro* differentiation, IPCs derived from Muse and non-Muse cells were evaluated for insulin production. Muse cells comprised $3.2 \pm 0.7\%$ of MSCs, approximately 82% of Muse cells were positive for anti stage-specific embryonic antigen-3 (SSEA-3). Pluripotent markers were highly expressed in Muse versus non-Muse cells. The percentage of generated IPCs by flow cytometric analysis was higher in Muse cells. Under confocal microscopy, Muse cells expressed insulin and c-peptide while it was undetected in non-Muse cells. Our results introduced Muse cells as a new adult pluripotent subpopulation, which is capable to produce higher number of functional IPCs.

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1. Introduction

Diabetes mellitus (DM) is one of the most endocrine and economical disorders worldwide. In 2016 according to the WHO, 422 million patients suffered from diabetes and it was reported to be responsible for 1.5 million deaths in 2012 [1]. Insulin therapy is the gold standard treatment method for diabetic patients, it considered to be the most effective, simplest and oldest method [2]. Nevertheless, hypoglycemia [3], weight gain [4], lipodystrophy and allergic reactions [5], failure to achieve the normal level of HbA1c [6] and possibility to coma and death [7] are the main disadvantages of long-term insulin therapy. The shortage of cadaveric donors, poor of oxygenation, vascularization of the graft and the immune rejection activity are considered as disadvantages of pancreas and islet transplantation [8]. Stem cell therapy is a new alternative treatment method for DM. Human embryonic stem cells (hESCs) is a type of stem cells which have high differentiation capacity in addition to their ability to reduce blood glucose level

after transplantation in streptozotocin-induced diabetic mice [9]. Moreover, induced pluripotent stem cells (iPSCs) also showed their differentiation capacity into IPCs [10]. Bioethical debates for the usage of ESCs and iPSCs are still ongoing due to teratoma formation, embryos destruction and immune rejection for generating hESC [11]. While the defects on genetics and epigenetics level of iPSC [12] and the ability to generate mouse fetus after injecting iPSCs into tetraploid mouse embryos are still debatable [13].

MSCs are multipotent stem cells having multilineage differentiation capacity into the osteocytes, chondrocytes and adipocytes cells [14]. Differentiation of human MSCs into IPCs was previously reported, however the percentage of the generated IPC after *in vitro* differentiation is still modest [15]. The international society for cellular therapy defined MSCs with the plastic adherence potential, multilineage differentiation capacity, positive expression of mesenchymal surface markers (CD90, CD105 and CD73) and negative expression of hematopoietic surface markers (CD14, CD45 and CD34) [16]. Traditional methods for isolating MSCs based on centrifugation steps and plastic adherence potential give rise to a heterogeneous population. Different subpopulations with different surface markers expression, molecular activity, and differentiation capacity had been reported within MSCs [17].

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Muse cells is a rare pluripotent subpopulation derived from adult MSCs which is double positive to the mesenchymal surface markers CD105, CD90 and to the SSEA-3 pluripotent surface marker. It is characterized by the ability to express pluripotent markers (Nanog, Oct3/4, Sox2 and Rex1) [18], and have the capacity to generate cell cluster derived from single Muse cell culture which is very similar to the embryoid body [18]. After *in vivo* transplanting of Muse cells, it revealed the ability of homing into the injured organ with spontaneous differentiation potency to its own cell type [19]. Unlike ESCs and iPSCs, Muse cells are non-tumorigenic pluripotent cells with low level of cell cycle-related factors gene expression [18,20], furthermore, Muse cells till now have no ethical considerations in their usage for clinical application. Muse cells comprise 0.003–0.004% of all mononuclear cells within the bone marrow aspirate [21], 1–5% of human dermal fibroblast [21], 1% of human bone marrow derived MSCs [18] and 1.9–8.8% of human adipose MSCs [22].

Gabr and his colleagues in a previous study compared the percentage of the generated IPCs by applying three different protocols for differentiating whole MSCs, the results showed that only 3% of MSCs were able to differentiate and generate insulin *in vitro* with modest differences between the three protocols [23]. Hence, we aimed to isolate and evaluate Muse cells and differentiated Muse cells into IPCs compared with non-Muse cells.

2. Material and methods

2.1. Retrieval of human MSCs

All required approvals for this study were obtained from the ethical committee of Mansoura University. Liposuction aspirates were obtained from three healthy volunteers during elective cosmetic surgeries after providing informed consent.

2.2. Isolation and expansion of MSCs

Human liposuction aspirates were digested using 0.075% collagenase type I (Sigma Aldrich, USA) in phosphate buffered saline (PBS) (Gibco, USA) and were shaken in water bath at 37 °C for 30 min. An equal volume of α MEM supplemented with 10% hyclone fetal bovine serum (FBS) (Fisher Scientific, USA) was added to inactivate the collagenase. To isolate the mononuclear cells from the remaining fat, a centrifugation step was performed at 800g for 10 min [24]. Cells were then cultured in α MEM media supplemented and 10% FBS, then were transferred to tissue culture flasks (BD Bioscience, USA) coated with Laminin 521 (Biolamina, Sweden). After 3 days, only MSCs was able to adhere and survive, while the nonadherent cells were discarded. MSCs were then cultured to 80% confluence before passaging by trypsin and the cells were then seeded at a ratio of 1:2. This step was repeated again for second passage. At this point, the cells were spindle-shaped and displayed a fibroblast-like appearance.

2.3. Characterization of the isolated MSCs

2.3.1. Phenotyping

At passage three, 1×10^6 cells of MSCs were resuspended in 1 mL PBS. Aliquots of 100 μ L were incubated for 30 min in 20 μ L of antibodies against CD14, CD45 (FITC) or CD73, CD34 phycoerythrin (PE) or in 5 μ L of CD105 PE or CD90 (FITC) (BD Biosciences), then washed with 1 mL of stain buffer (BD Pharmingen, USA), and resuspended in 500 μ L of stain buffer. The labeled cells were analyzed using an argon ion laser at a wavelength of 488 nm by BD FACSCalibur (BD Biosciences). 1×10^4 events were analyzed using CellQuest software (BD Biosciences).

2.3.2. Multilineage differentiation potential

MSCs at passage 3 were induced to differentiate into adipocytes, chondrocytes and osteocytes using differentiation protocol as previously described [14]. Oil-Red solution was used to evaluate adipocytes, Alcian blue was used to evaluate chondrocytes and Alizarin-Red was used to stain osteocytes

2.4. Isolation and expansion of Muse cells

Isolation protocol of Muse cells by FACS Aria III was previously introduced by Kurado [21]. 1×10^6 cells in a single tube were centrifuged at 210g for 5 min, and resuspended in FACS buffer. 5 μ L of anti-SSEA-3 antibody (STEMCELL Technologies, Canada) was incubated on ice for 25 min in dark. The cells were then resuspended in 200 μ L FACS buffer. SSEA-3 positive fraction (Muse cells) was isolated by SORP FACS Aria III (BD Biosciences). Once the isolation step was performed, samples from the two fractions were then analyzed for its purity by flow cytometer. The isolated two fractions (Muse cells and non-Muse cells) were expanded in the same conditions as MSCs.

2.5. Differentiation of Muse cells into IPCs

Differentiation was performed according to a protocol previously reported by Tayamma and his team [25]. Initially, the cells were cultured for 3 days in serum-free DMEM supplemented with Trichostatin-A (TSA) at a concentration of 55 nmol (Sigma). Then, the cells were cultured for 7 days in high-glucose (25 mmol) medium containing a 1:1 ratio of DMEM:DMEMF12 (Sigma) supplemented with 10% FBS and 10 nmol of glucagon-like peptide-1 (GLP-1) (Sigma).

2.6. Gene expression by RT-qPCR

Total RNA was extracted according to RNeasy Plus Mini Kit protocol (Qiagen, Germany). Three μ g of total RNA was converted into cDNA using RT² First Strand kit (Qiagen). Gene expression was evaluated for pluripotent genes (Nanog, Sox2, and Oct-4), nestin and PDX-1 genes at the end of expansion phase (Table 1). Custom gene arrays CAPH13024D were designed and supplied in 96-well plates for pancreatic endocrine genes (Qiagen) including; insulin, glucagon, and somatostatin, transcription factors (PDX-1, RFX6, and Neurod-1), glucose transporter (Glut-2), and pancreatic enzyme (glucokinase). Human islets were included to serve as a positive control for pancreatic endocrine genes and GAPDH as an internal control. MSCs gene expression for pluripotent markers served as negative control for mathematical calculations. Amplifications were performed in a 25 μ L reaction volume in each well that contains 12.5 μ L 2X SYBR Green Rox Master Mix (Qiagen), 1 μ L of cDNA template, and 11.5 μ L of nuclease-free water. The plate array was inserted in CFX96 real-time system (Bio-Rad, USA) and programmed according to manufacturer instructions. A mathematical model introduced by Pfaffl [26] was used for the relative quantification of target genes.

2.7. Flow cytometric analysis for generated IPCs

IPCs were fixed in 4% formaldehyde for 10 min at 37 °C, permeabilized by using chilled 90% methanol for 30 min and blocked in incubation buffer for 10 min at RT. Cells were then incubated with the conjugated antibody for 1 h at RT. The cells were washed with incubation buffer, then centrifuged and resuspended in 0.5 mL PBS. The labeled cells were evaluated using a 15 mW argon ion laser at a wavelength of 488 nm by BD FACSCalibur flow cytometer. 1×10^4 of cells was analyzed using CellQuest software (Becton, Dickinson). Mouse pancreatic islets served as a positive control.

Table 1
List of human gene-specific primers for RT-qPCR.

Gene	Forward primer	Reverse primer	PCR product	Accession number
SOX2	GGATAAGTACACGCTGCCCG	CTGTCCATGCGCTGGTTCAC	111	NM_003106.3
NANOG	GAAGGCCTCAGCACCTACCT	GGTTGCTCCACATTGGAAGGTT	95	NM_024865.3
NES	GGGCCTACAGAGCCAGATCG	CAGGAGGTCCTGTACGTGG	103	NM_006617.1
Oct-4	TGCCAAGCTCCTGAAGCAGA	CGTTTGGCTGAATACCTTCCCAAA	100	NM_002701.5
PDX-1	GCTGGCTGCATGTTGAACT	CGCTTCTTGTCCTCCTCCTT	93	NM_000209.3
GAPDH	TCTTTTGGCTGCCAGCC	ACATGTAACCATGTAGTTGAGGTC	178	NM_002046.5

SOX2 (SRY-box2), **NANOG** (Nanog homeobox), **POU5F1** (POU class 5 homeobox 1, also known as OCT3; OCT4; OTF3; OTF-3) and **GAPDH** (glyceraldehyde-3-phosphate dehydrogenase).

2.8. Immunofluorescence

Cultured cells on eight-chamber slides (Nunc, Thermo Scientific, USA) were fixed with 4% paraformaldehyde for 10 min at RT, permeabilized by 100% chilled methanol for 10 min and blocked with 5% normal goat serum for 1 h at RT. Cells were incubated overnight with the primary antibodies at 4 °C. These included mice monoclonal anti-insulin, rabbit polyclonal anti-c-peptide (cell signaling technology, USA). Subsequently, the cells were washed with PBS and incubated with the secondary antibodies for 2 h at RT anti-mouse IgG (H + L) Alexa flour 488 conjugate and anti-rabbit IgG (H + L). Alexa flour 555 conjugate (Cell Signaling Technology). Anti-SSEA-3 antibody, clone MC-631 (Merk Millipore, USA) was used to detect the expression of SSEA-3 in Muse cells. The nuclei

was counterstained with DAPI (Invitrogen, UK). Negative controls were obtained by omitting treatment with the primary antibody. Confocal images were captured using Leica TCS SP8 microscope (Leica Microsystems, Mannheim, Germany).

2.9. Determination of in vitro insulin and C-peptide release in response to increasing glucose concentrations

1×10^6 cells were incubated for 3 h in glucose-free Krebs-Ringer bicarbonate buffer (KRB). Then the cells were incubated for 1 h in 3.0 mL of KRB containing 5.5, 12, and 25 mM glucose concentrations. Finally, the KRB solution was collected after incubation period and frozen at -70 °C until assayed using an ELISA kit with a minimum detection limit of 1.76 μ U/mL (IMMUNOSPEC, USA).

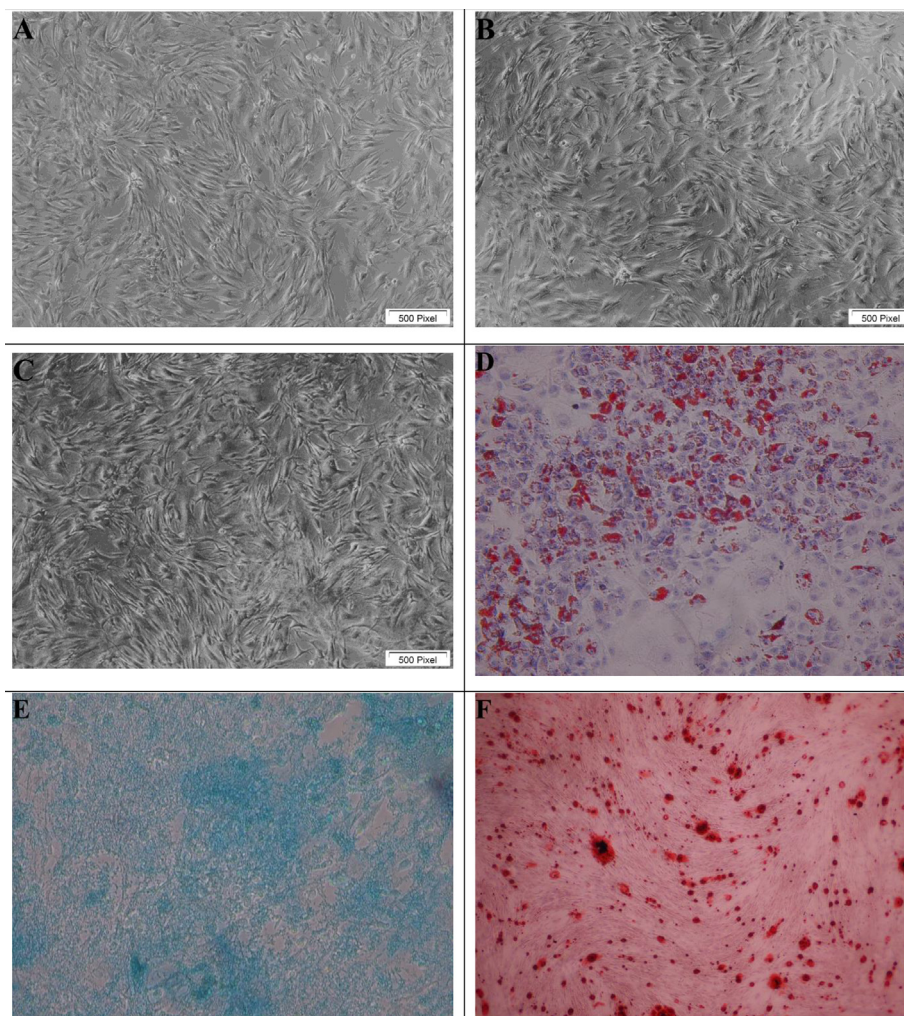


Fig. 1. Morphological features of MSCs, Muse and non-Muse cells during expansion. A. Cultured MSCs. B. Cultured Muse cells. C. Cultured non-Muse cells. D. Adipocyte cells stained with Oil-Red. E. Chondrocyte cells stained with Alcian-blue. F. Osteocyte cells stained with Alizarin-Red.

Table 2
Flow cytometric quantitation of the isolated MSCs.

Sample	CD73	CD105	CD90	CD14	CD34	CD45
1	97.46%	95.72%	97.53%	0.13%	0.13%	0.11%
2	95.55%	92.75%	97.96%	0.14%	0.12%	0.16%
3	92.62%	92.63%	96.49%	0.26%	0.15%	0.13%
4	94.49%	94.73%	97.26%	0.12%	0.22%	0.06%
5	91.42%	94.32%	96.26%	0.16%	0.27%	0.11%
6	90.86%	94.45%	95.34%	0.07%	0.3%	0.12%
Mean \pm SD	93.2 \pm 2.4	94.1 \pm 1.2	96.8 \pm 0.96	0.15 \pm 0.06	0.2 \pm 0.08	0.12 \pm 0.03

2.10. Statistical analysis

Statistical analyses were carried out using the program SPSS 16. Data from three donors are present as mean and standard error (SE) and the error bar in the bar graph represented SE. Data were examined to determine whether they were normally distributed with the One-Sample Kolmogorov-Smirnov test and were found to be normally distributed, comparison of measurement data between the two groups were performed by independent sample *t*-test. Statistical tests were two-tailed and a *p* value of less than 0.05 was considered statistically significant [27].

3. Results

3.1. General characteristics of MSCs and Muse cells

Muse cells and MSCs showed the plastic adherence potential and exhibited a spindle shape fibroblast-like morphology (Fig. 1a–c). MSCs were highly positive to mesenchymal surface markers expression (CD90, CD105, and CD73), while were negligible for the expression of hematopoietic surface markers expression (CD14, CD34 and CD45) (Table 2). Multilineage differentiation capacity was confirmed after induction with appropriate growth

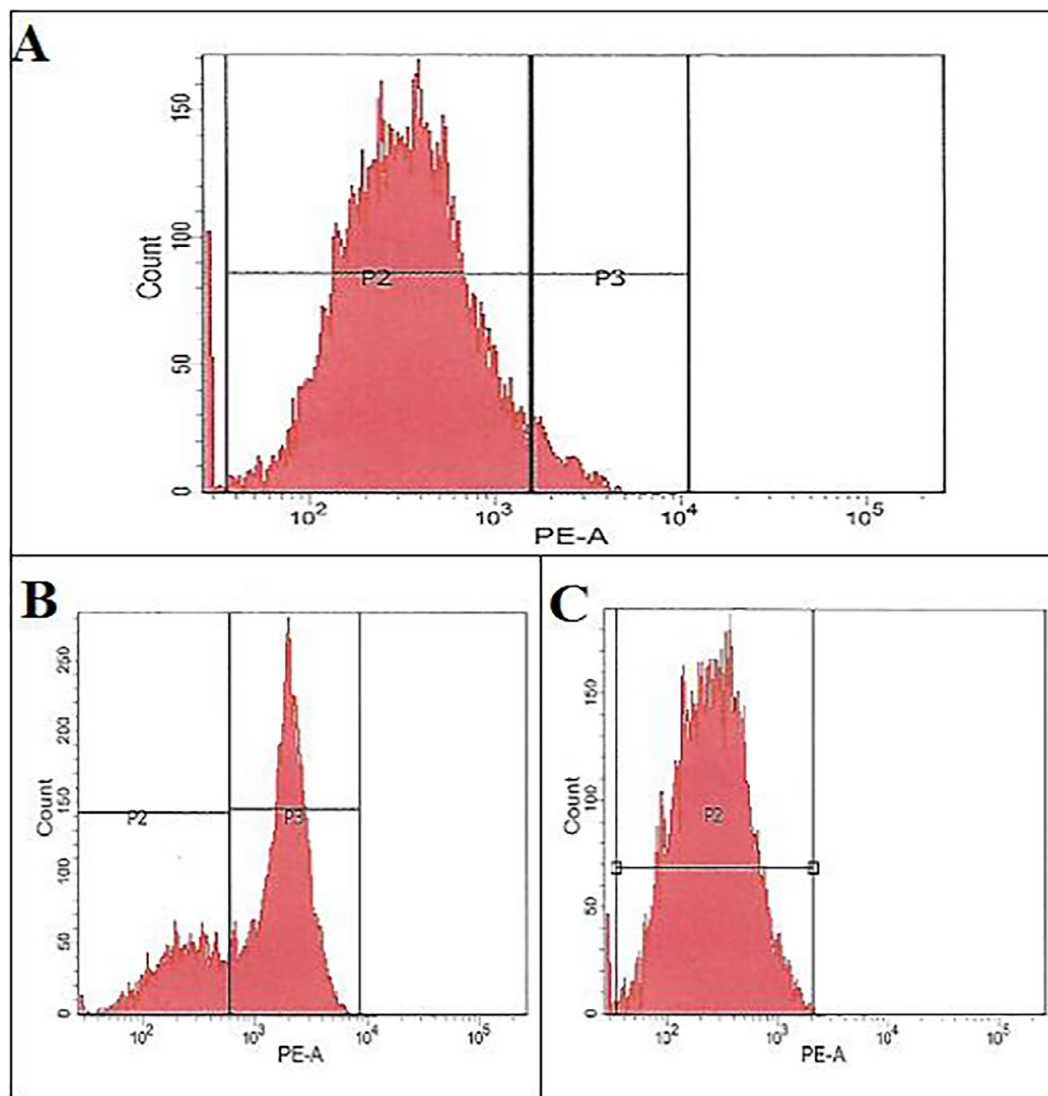


Fig. 2. Verification of Muse cells after FACSaria III separation. A. Percentage of Muse cells among MSCs. B. Percentage of Muse cells within the positive fraction. C. Percentage of non-Muse cells within the negative fraction.

factors (Fig. 1d–f). These results indicated that a pure population of MSCs was successfully isolated from fat tissue.

3.2. FACS separation of Muse cells

Muse cells were isolated by FACSARIA III instrument after staining MSCs with an anti-SSEA-3 antibody. The mean percentage of Muse cells within the whole MSCs population was $3.2 \pm 0.7\%$, while flow cytometric analysis of the isolated fractions (Muse cells and non-Muse cells) was performed directly after cell sorting and the results indicated that $82.2 \pm 10.6\%$ of Muse cells fraction were positive to anti-SSEA-3 while only $1.8 \pm 0.9\%$ of non-Muse cells were positive to SSEA-3 ($P < 0.05$) (Fig. 2).

3.3. Immunofluorescence

Muse and non-Muse cells were stained with anti-SSEA-3 antibody after FACS separation, the expression of SSEA-3 was undetected in non-Muse cells and confirmed in Muse cells by confocal microscope (Fig. 3a, b). While, immunofluorescence study of the

generated IPCs derived from Muse and non-Muse cells showed the presence of insulin granules within the cytoplasm. About $(4.1 \pm 2.7\%)$ of Muse cells were positive to insulin staining, while it was undetected in non-Muse cells. Only in Muse cells, immunostaining for c-peptide was also positive. Co-expression of insulin and c-peptide within the same cells was detected following electronic merging (Fig. 3c–f).

3.4. Flow cytometric analysis of IPCs

At the end of differentiation, flow cytometric analysis indicated that the proportion of IPCs generated from Muse cells was $10.1 \pm 1.7\%$ while $3.9 \pm 2.1\%$ was generated from non-Muse cells ($P < 0.05$) (Fig. 4).

3.5. Gene expression by RT-qPCR

Relative quantitative gene expression of PDX-1, pluripotent markers; Nanog, Oct-4 and Sox2, and nestin, was performed to confirm the pluripotency of Muse cells, the expression was multiplied and highly expressed in Muse cells by 5.4-fold in Nestin,

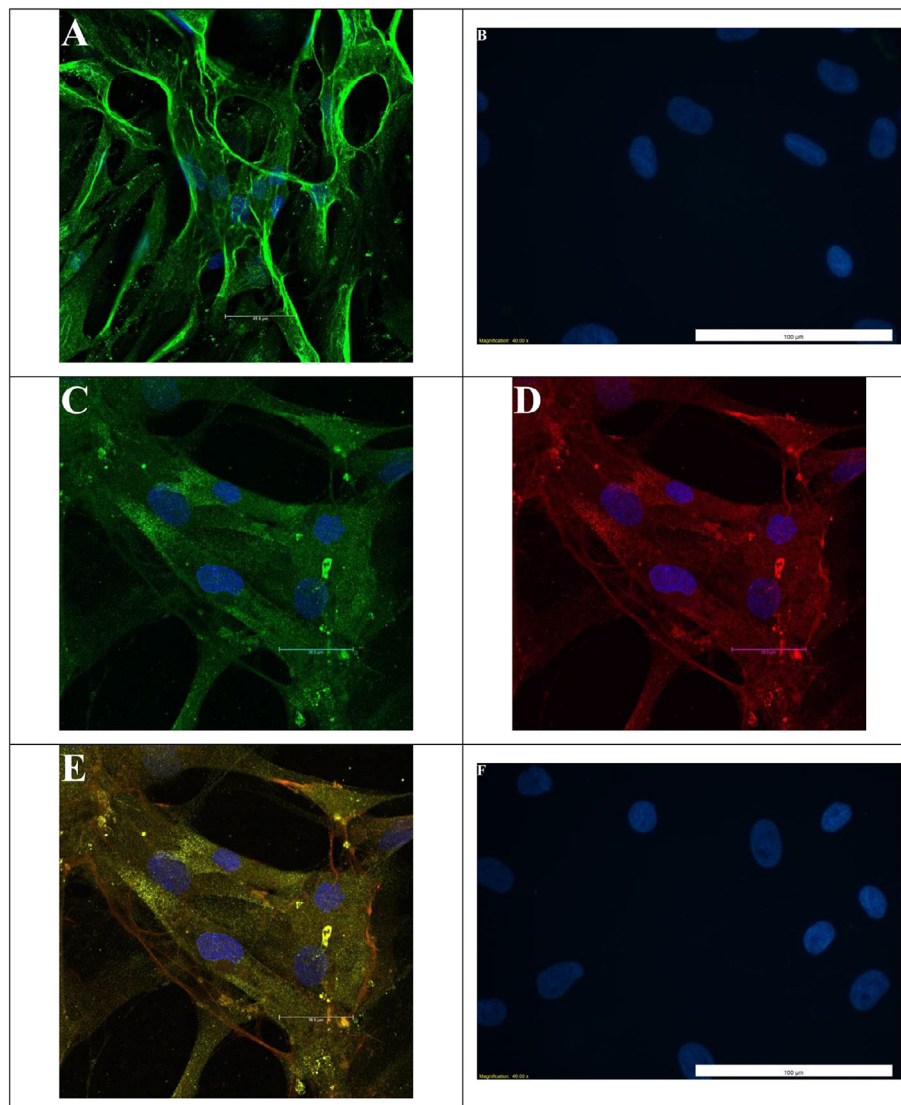


Fig. 3. Immunofluorescence staining of Muse and non-Muse cells with counterstaining for DAPI (Blue). A. Muse cells stained with anti-SSEA-3 (Green). B. Non-Muse cells stained with anti-SSEA-3. C. IPCs derived from Muse cells stained with anti-insulin (Green). D. IPCs derived from Muse cells stained with anti-c-peptide (Red). E. Co-expression of insulin and c-peptide by the same IPCs derived from Muse cells by electron merge (Yellow). F. Non-Muse cells stained with anti-insulin.

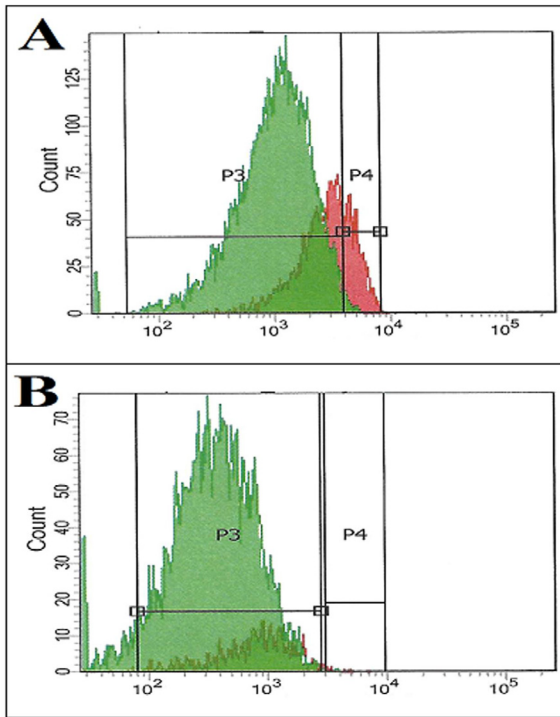


Fig 4. Flow cytometric analysis of IPCs generated from Muse and non-Muse cells after *in vitro* differentiation.

22-fold in Nanog, 35-fold in Sox2, 12-fold in Oct-4 and 4-fold in PDX-1 (Fig. 5a). At the end of differentiation protocol, the expression of endocrine genes was multiplied in Muse cells versus to non-Muse cells, by 5.5-fold in insulin, 5.4-fold in glucagon, 4.5-fold in PDX-1 and 6.9-fold in somatostatin, relatively to human islet gene expression as shown in (Fig. 5b).

3.6. *In vitro* human insulin and C-peptide release in response to a glucose challenge

Insulin and c-peptide release from generated IPCs derived from Muse and non-Muse were gradually increased in response to increasing glucose concentrations ($P < 0.05$). The amounts of insulin and c-peptide release were significantly higher in Muse cells compared to non-Muse cells (Fig. 6).

4. Discussion

The ability to isolate and manipulate multipotent stem cell from non-embryonic origin has been considered as a revolutionary success in regenerative medicine for many diseases especially diabetes mellitus, which had led the scientific community to investigate and apply several studies to identify and evaluate MSCs characteristics. Previous studies had revealed the heterogeneity of MSCs population with several surface markers and functions which identifies several sub-populations [28,29].

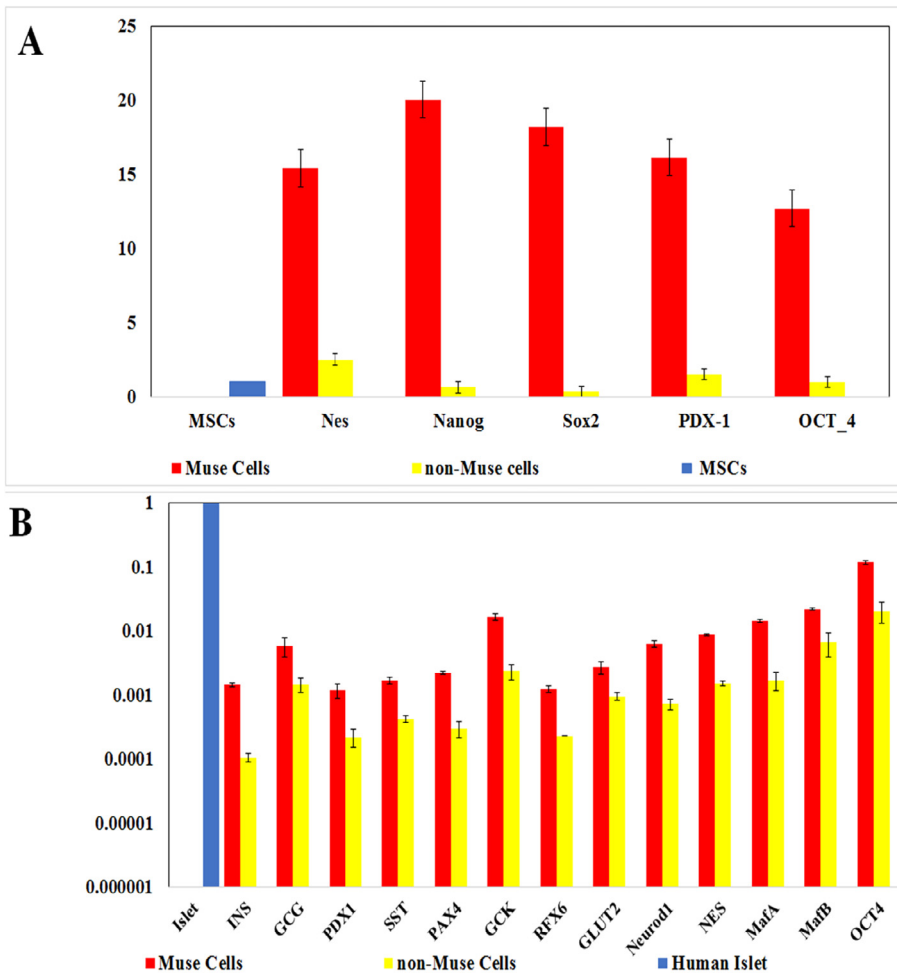


Fig. 5. RT-qPCR of Muse and non-Muse cells. A. Relative gene expression of pluripotent markers, nestin and PDX-1 of the undifferentiated Muse and non-Muse cells relative to MSCs. B. Endocrine gene expression of IPCs derived from Muse and non-Muse cells relative to human islets gene expression.

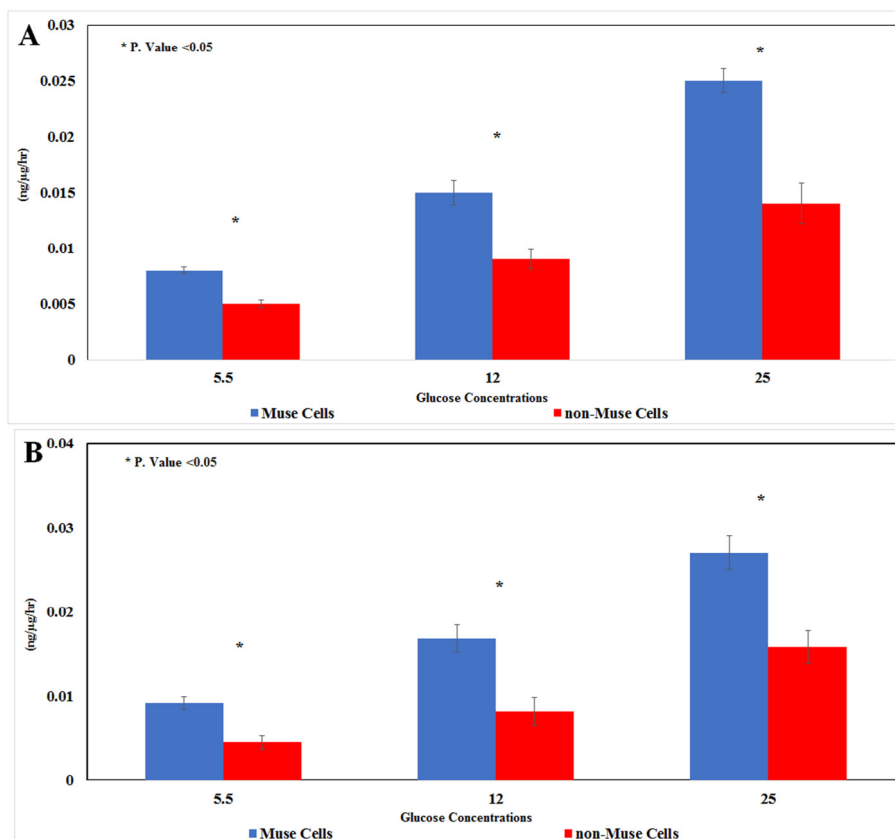


Fig. 6. Human insulin and c-peptide release by IPCs derived from Muse and non-Muse cells by ELISA technique in response to glucose concentration challenge. A. Insulin release. B. C-peptide release.

Adipose tissue is a rich and easily obtained source for multipotent MSCs and considered to be a feasible source for autologous stem cell transplantation [24]. Along with the higher percentage of Muse cells existence in adipose tissue 3% compared to bone marrow 1–2% and dermal fibroblast 2–3% [21].

Muse cells can be easily isolated by several methods including environmental stress; hypoxia, long-term trypsinization (LTT) and nonserum culture [18] and FACS separation [21], while during our work, we have found that FACS separation is the most feasible and simple method for isolating pure population of Muse cells.

Previous data showed that SSEA-3 surface marker is not the only pluripotent marker expressed by MSCs. SSEA-4 is another pluripotent surface marker expressed by MSCs [30], CD49f was reported to have a critical role in maintaining pluripotency and also expressed in MSCs [31], also very small embryonic-like stem (VSEL) cells is another pluripotent sub-population within MSCs [32]. Thus, theoretically these sub-populations are supposed to be within non-Muse cells according to FACS separation method.

Laminin 521 is one of the isoforms of the laminin family which are extracellular matrix proteins. Human laminin 521 is expressed in the inner cell mass (ICM) of the embryo, it has a role in enhancing the proliferation and the adhesion of MSCs and protects pancreatic human islets *in vitro* [33].

Glucose is an essential growth factor for differentiating stem cells into IPCs. Therefore the differentiating medium should contain glucose. Thus, several investigators suggested that the cells may have the ability to absorb glucose from the medium which enhances the cells to release insulin [34]. Accordingly, to avoid the argument of insulin origin, intracellular immunofluorescence staining was positive in Muse cells for insulin, c-peptide and the co-expression of insulin and c-peptide by the same cell emphasized the capacity of proinsulin synthesis.

The poor insulin release in response to glucose challenge as shown in the present study was previously reported in both differentiated IPCs generated from ESCs [35] and IPCs derived from adult stem cells [23]. Also, our results showed the poor of insulin and c-peptide release, while there was a stepwise up-regulation in the release in Muse cells compared to non-Muse cells after exposure to different concentrations of glucose in Muse rich cells.

SSEA-3 is a globoseries glycosphingolipid epitope surface marker, which is a well-known stemness marker for ESCs [36,37]. A previous report demonstrated that there is no correlation between the expression of SSEA-3 and SSEA-4 and the expression of pluripotent markers after the depletion of these two surface markers in ESCs [38].

5. Conclusion

Muse and non-Muse are capable to generate functional IPCs, while the percentage was higher by Muse cells. To this end, Muse cells offers a new cell type in translational medicine as an adult pluripotent sub-population. Culture methods and differentiation protocol of Muse cells need further studies in order to be clinically meaningful. This study is regarded to be the premiere study which evaluated the ability of Muse cells to differentiate and produce insulin and c-peptide *in vitro*. Muse cells have not shown to be the only sub-population within MSCs which have the differentiation potency into IPCs.

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