Gene 671 (2018) 50–57

Contents lists available at ScienceDirect

Gene

journal homepage: www.elsevier.com/locate/gene

Research paper

PCL/PVA nanofibrous scaffold improve insulin-producing cells generation from human induced pluripotent stem cells

Mohammad Foad Abazari[a,1], Fatemeh Soleimanifar[b,1], Maryam Nouri Aleagha[a], Sepehr Torabinejad[a], Navid Nasiri[c], Gholamreza Khamisipour[d], Javad Amini Mahabadi[e], Hossein Mahboudi[f], Seyed Ehsan Enderami[g], Ehsan Saburi[g], Javad Hashemi[h], Mousa Kehtari[i,*]

[a] Department of Genetics, Tehran Medical Sciences Branch, Islamic Azad University, Tehran, Iran
[b] Dietary Supplements and Probiotic Research Center, Alborz University of Medical Sciences, Karaj, Iran
[c] Department of Biology, Central Tehran Branch, Islamic Azad University, Tehran, Iran
[d] Department of Hematology, Faculty of Allied Medicine, Bushehr University of Medical Sciences, Bushehr, Iran
[e] Gametogenesis Research Center, Kashan University of Medical Sciences, Kashan, Iran
[f] Department of Biotechnology, School of Advanced Technologies in Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran
[g] Stem Cell Technology Research Center, Tehran, Iran
[h] Department of Molecular Sciences, School of Medicine, North Khorasan University of Medical Sciences, Bojnourd, Iran
[i] School of Biology, College of Sciences, University of Tehran, Tehran, Iran

ARTICLE INFO

Keywords:
Human induced pluripotent stem cells
Pancreas-specific genes
Insulin-producing cells
PCL/PVA scaffold

ABSTRACT

Pancreatic differentiation of stem cells will aid treatment of patients with type I diabetes mellitus (T1DM). Synthetic biopolymers utilization provided extracellular matrix (ECM) and desired attributes *in vitro* to enhance conditions for stem cells proliferation, attachment and differentiation. A mixture of polycaprolactone and polyvinyl alcohol (PCL/PVA)-based scaffold, could establish an *in vitro* three-dimensional (3D) culture model. The objective of this study was investigation of the human induced pluripotent stem cells (hiPSCs) differentiation capacity to insulin-producing cells (IPCs) in 3D culture were compared with conventional culture (2D) groups evaluated at the mRNA and protein levels by quantitative PCR and immunofluorescence assay, respectively. The functionality of differentiated IPCs was assessed by C-peptide and insulin release in response to glucose stimulation test. Real-Time PCR results showed that iPSCs-IPCs expressed pancreas-specific transcription factors (Insulin, Pdx1, Glucagon, Glut2 and Ngn3). The expressions of these transcription factors in PCL/PVA scaffold were higher than 2D groups. In addition to IPCs specific markers were detected by immunochemistry. These cells in both groups secreted insulin and C-peptide in a glucose challenge test by ELISA showing *in vitro* maturation. The results of current study demonstrated that enhanced differentiation of IPCs from hiPSCs could be result of PCL/PVA nanofibrous scaffolds. In conclusion, this research could provide a new approach to beta-like cells replacement therapies and pancreatic tissue engineering for T1DM in the future.

1. Introduction

Diabetes mellitus is a group of metabolic diseases in which, for different reasons, glucose levels are higher than typical levels. According to statistics indicated by the American Diabetes Association diabetes is the seventh leading reason for death in the United States and unfortunately, its prevalence is expanding (Rathmann and Giani, 2004; Association AD, 2016). Type I diabetes mellitus (T1DM) which comprises 5–10% of diabetes, results from immune system destruction of pancreatic beta-like cells and pancreas's inability to produce enough insulin (Abdulazeez, 2015).

Recently, much research has sought to apply stem cells to differentiate beta-like cells and focus on the generation and development of these alternative sources (Shen et al., 2013; Piran et al., 2017; Enderami et al., 2017a). Meanwhile, induced pluripotent stem cells (iPSCs), because of their high capability of self-renewability and differentiation as

Abbreviations: T1DM, type I diabetes mellitus; ECM, extracellular matrix; 3D, three-dimensional; 2D, two-dimensional; PCL/PVA, polycaprolactone/polyvinyl alcohol; hiPSCs, human induced pluripotent stem cells; IPCs, insulin producing cells; Pdx1, pancreatic and duodenal homeobox 1; Glut2, glucose transporter 2; Ngn3, neurogenin 3; Gcg, glucagon; β_2m, β_2 microglobulin

* Corresponding author at: School of Biology, College of Sciences, University of Tehran, P.O. Box: 1997775555, Tehran, Iran.
E-mail address: ehsan.enderami@strc.ac.ir (M. Kehtari).
[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.gene.2018.05.115
Received 18 February 2018; Received in revised form 28 May 2018; Accepted 30 May 2018
Available online 31 May 2018
0378-1119/

well as low immunogenicity, are a suitable cell source for use in elective therapies (Takahashi et al., 2007). Human iPSCs can be expanded *in vitro* and by utilizing growth factors and transcription elements that recapitulate expansion of pancreatic cells, can be differentiated to insulin-producing cells (IPCs) (Enderami et al., 2017a; Enderami et al., 2017b; Mansour et al., 2018). Therefore, many studies have stated that iPSCs can effectively differentiate into beta-like cells in the 2D culture (Enderami et al., 2017a; Lahmy et al., 2014; Kondo et al., 2017). In recent years, much research have tried to apply cell-based therapy for T1DM (Aghazadeh and Nostro, 2017). To get the cells required for cell therapy; these studies have used different strategies, such as the use of differentiation medium and gene transfer for the differentiation of stem cells into beta-like cells (Chen et al., 2009; Raikwar and Zavazava, 2012; Zhang et al., 2009; Wei et al., 2013). These procedures are costly and time consuming. In order to circumvent these problems, a novel approach must be improved.

Several studies have demonstrated that the use of three-dimensional (3D) environments can be a strategy to overcome the limitations of current beta-like cells production methods (Aloysious and Nair, 2014; Amer et al., 2014). Recent advances in tissue engineering and regenerative medicine have empowered the control of cells through the scaffolds made of biodegradable and biocompatible polymers. In the field of biomaterials, these materials rapidly adjust to the human body and do not stimulate the immune system (Ellis et al., 2017; Greggio et al., 2013; Ravi et al., 2015).These platforms give a 3D environment that improves the differentiation of stem cell types into beta-like cells (Enderami et al., 2017b). It appears that these 3D nanofiber scaffolds, by imitating *in vivo* condition, support the cell-extracellular matrix (ECM) interactions and in addition the cell-cell contacts (Mahboudi et al., 2018). Many synthetic polymers have been used to fabricate nanofibers. In the present study, we plan to utilize an adaptable, biocompatible, biodegradable and hydrophilic polymer as a suitable substrate for differentiating iPSCs into beta-like cells. Polycaprolactone and polyvinyl alcohol (PCL/PVA)-based scaffold may indicate biological properties and give suitable synthetic ECM for the improvement pancreatic beta-like cells differentiation and islet organization. PCL is biocompatible and biodegradable polyester with hydrophobicity characteristic and high mechanical stability which has been intensively studied as a good candidate for fabrication of 3D culture (Xue et al., 2014; Zarekhalili et al., 2017). However, cell affinity of PCL is poor. Thus, this synthetic polymer does not have favorable property, and PVA is a biodegradable polymer with low toxicity, inexpensive and hydrophilic polymer with thermal stability and good mechanical. This biopolymer has been used for tissue engineering and biomedical applications (Zarekhalili et al., 2017; Maheshwari et al., 2013). Specially, to our knowledge, there is no study to evaluate the pancreatic differentiation of iPSCs on PCL/PVA scaffold. With this background, the aim of our study is to evaluate whether iPSCs seeded on PCL/PVA scaffold compared to tissue culture plates (TCPS) may enhance differentiation toward pancreatic lineages. To this purpose, we studied the effects of 3D culture on viability and expression of pancreatic-specific genes including insulin, glucagon, pdx1, ngn3 and glut2. The results showed that PCL/PVA scaffold compared 2D culture can provide a good pancreatic differentiation condition for iPSCs and generate the IPCs.

2. Materials and methods

2.1. Expansion of induced pluripotent stem cells

Human iPSC lines were given by Prof. Masoud Soleimani of Stem Cells Technology Research Center that previously characterized and reported (Ardeshirylajimi et al., 2014). The use of human iPSC was approved by the Ethics Committee of Shahid Beheshti University of Medical Sciences (Tehran, Iran). Human iPSCs were cultured and expanded on a mouse embryonic fibroblast (MEF) feeder cell layer inactivated with mitomycin-C (Sigma) in iPSCs medium (DMEM/F12 supplemented with 20% knockout serum replacement, 2 mM L-glutamine, 0.1 mmol/l nonessential amino acids, 0.1 mM β-mercaptoethanol and 10 ng/ml of recombinant human basic fibroblast growth factor (bFGF), all from Invitrogen) in a 5% CO_2 and 95% humidity. Every 3–5 days, human iPSC colonies were passaged enzymatically using 0.1% Collagenase type IV (Invitrogen), and transferred onto inactivated MEF for expansion (Enderami et al., 2017a).

2.2. Scaffold fabrication

Nanofibrous PCL/PVA scaffolds were fabricated and Produced by an electerospinning method. Amount 0.8 g of PCL polymer was broken down in 9 ml of chloroform and 1 ml of dimethylformamide and 1.2 g of PVA polymer were dissolved in 10 ml of high temp water. Next, solutions were loaded into two 10 ml plastic syringe and placed across the collector at a fixed distance of 18 cm. Electrospinning process was done at a flow rate of 1 ml/h, under a positive voltage of 23 kV amongst needle and collector (Enderami et al., 2018).

2.3. Surface modification

After nanofibrous scaffold fabrication, to optimize the hydrophilicity, surface plasma treatment of PCL/PVA scaffolds was done under optimized states previously (Ardeshirylajimi et al., 2013), in brief, of 40 kHz low frequency at 30 w with a cylindrical quartz reactor (Diener Electronics, Germany) by bring pure oxygen into the reaction chamber at 0.4 mbar pressure and then the glow discharge was performed for 5 min.

2.4. Scanning electron microscopy

The cell-polymer constructs were fixed in 2.5% glutaraldehyde (45 min). The fixed samples were flushed two times with PBS for 5 min; after that they dehydrated in ethanol arrangement (50%, 60%, 70%, 80%, 95% and 100% v/v) for 5 min each and dried. The dried nanofibrous scaffolds mounted onto aluminum sample holders with Araldite TM glue and covered with palladium gold in a sputter-coater (Hitachi, Tokyo, Japan). Then, the samples were observed utilizing a scanning electron microscope (SEM; Hitachi S-4500, Japan).

2.5. Cell seeding on scaffolds

In order to sterilization, the PCL/PVA scaffolds were punched to the coveted size and sterilized under ultraviolet light for 30 min. Then scaffolds were flushed with PBS and were put overnight in the base medium supplemented with 10% serum at 37 °C. Before differentiation, in order to embroid body (EB) generation, human iPSCs colonies were detached enzymatically using 0.1% Collagenase type IV (Invitrogen) and transferred to non-adherent culture plate in EB medium (iPSC medium without bFGF) for 5 days. Shaped EBs plated on PLLA/PVA scaffolds at a density of 5.0×10^4 cells per each scaffold in MEF-adapted medium for 2 days.

2.6. Experimental design

Four groups including 2 control and 2 experimental groups were considered. The hiPSCs were seeded onto the PCL/PVA scaffolds in iPSCs media and utilized as the 3D control group. The hiPSCs were cultured in tissue culture flask under iPSCs media as the 2D control group. The cultured hiPSCs in IPCs differentiation media were utilized as the 2D experimental group. The hiPSCs were seeded onto the PCL/PVA scaffolds in IPCs differentiation media and considered as the 3D experimental group. A three-step differentiation protocol was utilized to induce hiPSCs into IPCs. At Step 1: the hiPSCs were seeded (37 °C, 5% CO_2) for 7 days in the medium I containing serum-free high glucose DMEM (25 mmol/l), 0.5 mmol/l beta-mercaptoethanol (Invitrogen) and

50 ng/ml activin A (Sigma). Next, in step 2: the hiPSCs were cultured in the differentiation medium II containing 20 ng/ml basic fibroblast growth factor (bFGF; Sigma), 20 ng/ml epidermal growth factor (EGF; Sigma), 1% nonessential amino acids (Invitrogen), 2 mmol/L L-glutamine (Sigma), and 10 ng/ml exendine-4 (Sigma), 2% B27 (Invitrogen) for 7 days. Then, in Step 3: the hiPSCs were treated for an additional 7 days in differentiation medium III containing 2% B27, 10 ng/ml betacellulin (Sigma), 10 mmol/l nicotinamide (Sigma), and 10 ng/ml exendine-4. The differential media was replaced with new medium every 2 days.

2.7. Cell proliferation assay

MTT assay was utilized to investigate the proliferation ability and viability of iPSCs on PCL/PVA scaffold for 3 days. Briefly, sterilized nanofibrous membranes were seeded in a 48-well cell culture plate, cultured at a cell density of 4×10^3 cells/cm^2 and incubated at 5% CO_2, 37 °C. At 24, 48 and 72 h after seeding hiPSCs, 50 μl MTT reagent (3-[4, 5-dimethyl-thiazolyl-2]-2, 5-diphenoltetrazolium bromide) with a concentration of 5 mg/ml in DMEM was added to each well. After 3 h incubation, the formazan crystals were solubilized with dimethylsulfoxide (DMSO). Then, Plates were shaken to dissolve the precipitate. The sample absorbance was evaluated by absorbance measurement at 570 nm utilizing a BioTek Synergy HT micro-plate reader (BioTek Instruments Inc., USA). This assay was performed in triplicate independently.

2.8. RNA extraction and quantitative Real-Time PCR

Total RNA was isolated from control cells and differentiated hiPSCs at the end of the induction procedure with the RNeasy Mini Kit (Qiagen) following the manufacturer's instruction. Next, total RNA was reverse transcribed with the random hexamer primer to complementary DNA (cDNA) at 42 °C for 60 min and 70 °C for 5 min in a total reaction volume of 15 μl using M-MuLV Reverse Transcriptase kit (Fermentas). Quantitative Real-Time PCR was performed according to the manufacturer's protocol and with the ABI Light Cycler (ABI step one) in a total reaction volume of 10 μl in 96-well optical reaction plates using a SYBR premix ExTaq (Takara, Korea). The primer sequences were designed by AlleleID software (Primer Biosoft), which is shown in Table 1. All reactions were completed at 95 °C for 2 min, followed by 40 amplification cycles in triplicate, and the data were normalized against the Ct value of the endogenous β2m. The relative quantification ($2^{-\Delta\Delta Ct}$) method was utilized to analyze the data.

2.9. Immunofluorescence assay

The differentiated iPSCs in both 2D and 3D groups and control groups were fixed with 4% (w/v) paraformaldehyde (Sigma) at 4 °C for 30 min in end stages of the differentiation process. All samples were permeabilized with 0.2% triton X-100 (Sigma) and blocked with 10% goat serum (Sigma) for 1 h at RT. For maturation stage primary antibody against insulin (1:200; #ab181547) and for definitive endoderm stage, primary antibody against human Pdx1 (1:2000; #ab47267) were diluted with 1% BSA in PBS. Then, all samples incubated overnight with these primary antibodies at 4 °C. Next, samples were incubated with Fluorescein isothiocyanate-labeled secondary antibodies for 90 min at RT in darkness. Finally, the nuclei were stained with 0.1 μg/ml DAPI (Sigma) for 5 min. Images were captured using a phase contrast fluorescent microscope (Nikon.US). The immunocytochemistry images were imported to ImageJ software (Version 1.45s, NIH, USA) for creation of quantity data.

2.10. Insulin and C-peptide release and insulin content assay

The differentiated iPSCs in both 2D and 3D groups and control groups were washed 2 times with PBS and were pre-incubated in Krebs–Ringer bicarbonate (KRB) buffer containing 25 mM NaHCO3, 1.1 mM MgCl2, 2.5 mM CaCl2, 5 mM KCl, 120 mM NaCl, and 0.1% BSA (all from Sigma) in glucose-free for 2 h. Then, differentiated cells and control groups were incubated for 2 h in KRB buffer with different concentrations of glucose. The supernatant was collected and frozen at −70 °C until the time of analyzation. To estimation of insulin and C-peptide secretion, the Ultrasensitive ELISA kit (#10-1132-01, #10-1141- 01, Mercodia, Uppsala, Sweden) was used according to the manufacturer's instructions. In addition to measurement of intracellular insulin, the cells were washed three times with PBS, extracted in 0.2 ml acid alcohol (10% glacial acetic acid in absolute ethanol) at 4 °C overnight, and then cells were subsequently sonicated three times 15 s each at 40–60 w, and centrifuged (13.000 rpm, 15 min, 4 °C). Then, the supernatant was collected and stocked at −70 °C until being assayed. Total protein concentration was determined by the BCA protein assay system and 50 μg of protein was used for detection of intracellular insulin in each well of ELISA kit. This assay was done for six times.

2.11. Statistical analysis

All experiments were done for several times, and the obtained data were reported as Mean ± SEM (standard error mean). Results were analyzed by one-way ANOVA, and Bonferroni's post-hoc test was utilized for comparison of 2D and 3D groups by Graphpad Prism 6 software (Graphpad Software Inc., La Jolla, CA, USA). In between the compared groups, a *P-value* of < 0.05 was considered statistically significant.

3. Results

3.1. Morphological changes of hiPSCs differentiation into IPCs

To produce IPCs from hiPSCs, we used a formerly reported stepwise

Table 1
Specifications of primer sequences used for quantitative real-time PCR.

Human genes	Primer sequence	Annealing temperature (°C)	Product size (bp)
Pdx1	Forward: 5′-ATGGATGAAGTCTACCAAAGC-3′ Reverse: 5′-CGTGAGATGTACTTGTTGAATAG-3′	60	159
Glut2	Forward: 5′-TCACTGCTGTCTCTGTATTCC-3′ Reverse: 5′-TGCTCACATAACTCATCCAAG-3′	60	147
Ngn3	Forward: 5′-AGAGAGCGTGACAGAGGC-3′ Reverse: 5′-GCGTCATCCTTTCTACCG-3′	60	182
Gcg	Forward: 5′-ACCAGAAGACAGCAGAAATG-3′ Reverse: 5′-GAATGTGCCCTGTGAATG-3′	59	191
Insulin	Forward: 5′-GCTTCTTCTACACACCCAAG-3′ Reverse: 5′-GGTAGAGGGAGCAGATGC-3′	60	171
β2m	Forward: 5′-ATGCCTGCCGTGTGAAC-3′ Reverse: 5′-ATCTTCAAACCTCCATGATG-3′	60	91

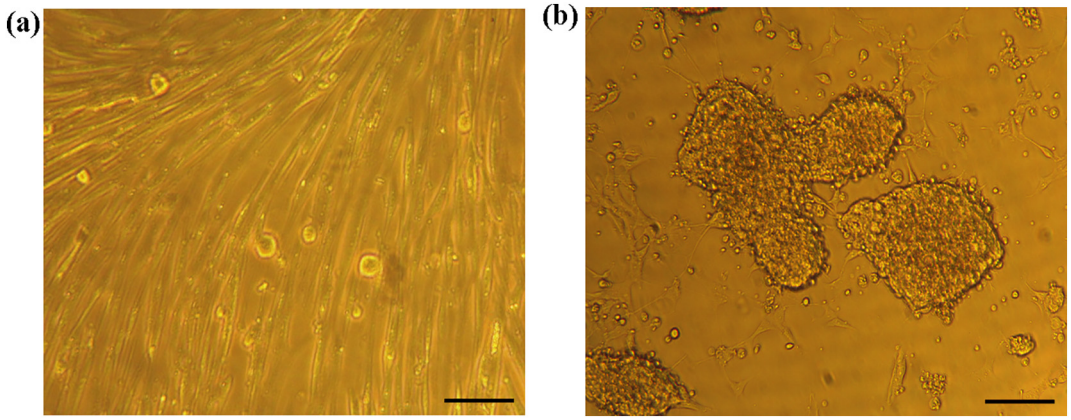

Fig. 1. Morphological changes in 2D culture. (a) hiPSCs in DMEM (control group). (b) hiPSCs in IPC differentiation media. Scale bars are 100 μm.

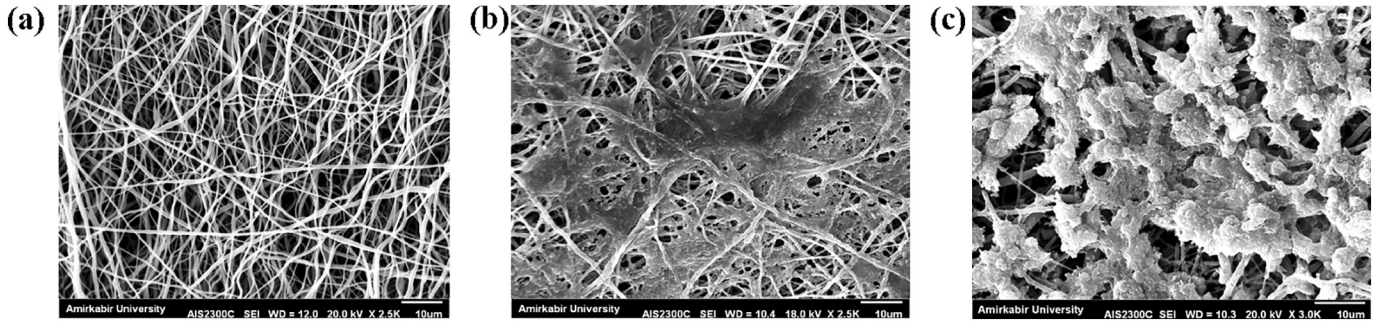

Fig. 2. Morphological changes in 3D culture. (a) Unseeded scaffold. Interconnected pores are observed. (b) hiPSCs-seeded scaffold in DMEM (control group). The cells show spindle-shape morphology. (c) hiPSCs-seeded scaffold in IPC differentiation media. The cells form spherical clusters of round cells. Scale bars are a, b and c: 10 μm.

differentiation process that followed human pancreatic beta-like cells formative principles with a few modifications, such as obtaining IPCs in a quick time of differentiation. Under the scanning electron microscope and inverted microscope, the differentiation of hiPSCs into beta-like cells and cell morphologies were recorded at the end of differentiation stages (Figs. 1, 2). SEM images displayed that the PCL/PVA nanofibrous scaffold was very porous and contained a various interconnected pores (Fig. 2a). After the hiPSCs were seeded in plates and on the scaffolds and following serial addition of differentiation medium I, II and III for 21 days, hiPSCs altered to sheets of cells and occupied the pores of the PCL/PVA scaffolds in both control and experimental 3D culture. In the control group, hiPSCs took a stretched and flat shape (Fig. 2b); while the differentiated cells in the experimental group were bunch formed structure which is a usual morphological feature of pancreatic islet (Fig. 1b, 2c). Finally, these bunch formed structures were used for further assessment to test the efficiency of differentiation protocol in various groups.

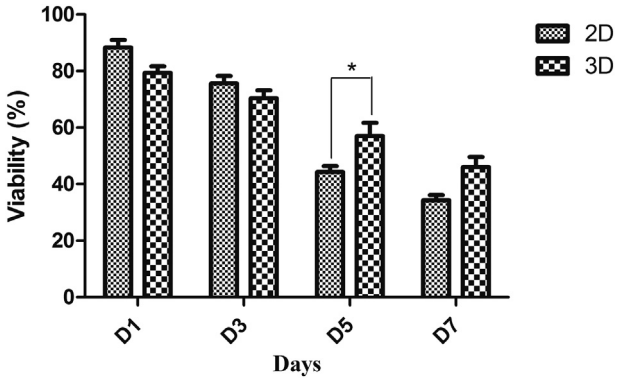

Fig. 3. MTT cell proliferation assay of hiPSCs on PCL/PVA scaffold (3D) and tissue culture polystyrene (2D) during 1, 3, 5 and 7 days of culture. *P-value < 0.05.

3.2. Cell viability assessment

MTT test showed biocompatibility of PCL/PVA scaffolds and its effectiveness in supporting cell viability. As shown in Fig. 3, the proliferation rate of the hiPSCs on nanofibrous was significantly higher than the 2D culture group. Cell viability on scaffolds show that increased compared with the sample of 2D culture on 5th and 7th days; however, on 1th and 3rd days of the culture, the cell viability on scaffolds did not increase compared with 2D culture group.

3.3. Gene expression pattern of pancreatic endocrine genes

To demonstrate whether the hiPSCs had differentiated into IPCs, Real-Time PCR analysis was performed to investigate endocrine genes expression profiles for pancreatic cell markers in the mRNA level. Since genes expression analysis indicated no significant differences between the two controls, both of them were considered as one group. As indicated in Fig. 4, the expression of Glut2, Ngn3, Insulin, Glucagon, and Pdx1 genes in undifferentiated hiPSCs, as a control group, was low. After the hiPSCs differentiated into beta-like cells, Real-Time PCR analysis showed that the expression pattern of endocrine genes was significantly increased in the 2D culture of hiPSCs-derived IPCs in comparison with control group (undifferentiated hiPSCs). High expression of endocrine genes was detected in the cells cultured and differentiated on the PCL/PVA scaffold. HiPSCs-derived IPCs indicated a significant increase in Pdx1, Glucagon, Ngn3, and Insulin gene expression when they cultured in nanofibrous scaffolds compared to the 2D culture. Overall, these results indicated that the presence of a

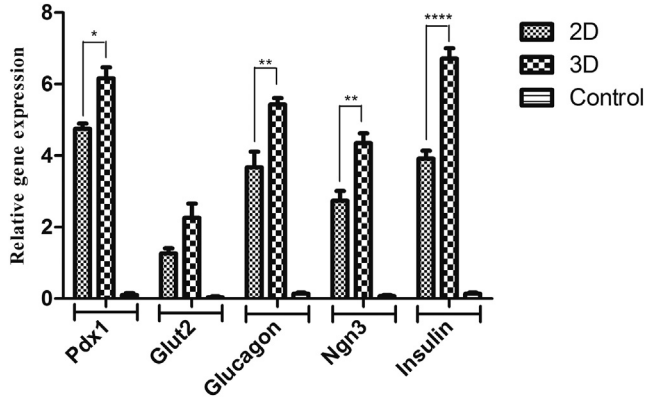

Fig. 4. Relative gene expression in end stage derived IPCs. The expression levels of pancreatic transcription factors such as Pdx1, Ngn3, Insulin, Glucagon, and Glut2 were analyzed at each group of differentiation into IPCs. Gene transcripts of 3D group are compared 2D group. Relative levels of gene expression were normalized to the human β2M. The value is shown in each graph as mean ± SEM. **P-value* < 0.05, ***P-value* < 0.01 and *****P-value* < 0.0001.

scaffold along with the induction media could conduct to effective differentiation of hiPSCs to beta-like cells and PCL/PVA nanofibrous scaffolds as a synthetic polymer can enhance and improve IPCs differentiation.

3.4. Immunofluorescence staining

To confirm pancreatic differentiation of hiPSCs into beta-like cells in the protein level, expression pancreatic-specific markers (Pdx1 and Insulin) was investigated by the immunostaining assay (Fig. 5a, b). Immunocytochemistry did not see any Insulin and Pdx1 genes expression in 3D and 2D control groups (data not shown). In the 2D experimental culture group, Weak or moderate expression of Insulin and Pdx1 was detected in the cytoplasm and nuclei, respectively, in differentiated IPCs on day 21. In a 3D experimental group, strong immune-reactivity of both markers was seen in the cytoplasm and nuclei of differentiated IPCs on day 21. Qunter-staining of the nucleus (blue) was done by DAPI. These results are displayed in Fig. 5a, b. The qualitative results of the immunostaining assay were quantitatively confirmed by ImageJ software (Fig. 5c).

3.5. Insulin and C-peptide release and insulin content in response to glucose stimulation

In order to evaluate the ability of differentiated cells, insulin and C-peptide secretion of hiPSC-IPCs in response to various concentrations of glucose were done by utilizing an ELISA kit. The secretion of cultured hiPSCs in control groups detected very low levels of C-peptide and insulin in the exposure low or high of glucose concentrations. Both derived IPCs mediated through the procedure with 3D and 2D culture did not have a significant response to concentrations of glucose challenge from 5.5 to 15 mM compared to each other (*P-value* > 0.05). However, in differentiated cells with 3D and 2D culture, amounts of insulin secretions were increased by a concentration of 25 mM glucose, released 8.83 ± 0.65 and 6.1 ± 0.45 ng/10^6 cells (n = 6) per 60 min and the amounts of C-peptide were 41.3 ± 2.45 pmol/l and 32.6 ± 2.51 pmol/l (n = 6) respectively. Thus, our data in Fig. 6a, b indicated that differentiated cells with 3D culture in the concentration of 25 mM glucose challenge could significantly produce more insulin and C-peptide compared to differentiate cells with 2D culture (*P-value* < 0.05, *P-value* < 0.01). Moreover, the insulin content in iPSCs-derived IPCs was measured. The values for insulin in 5.5, 15 and 25 mM glucose concentrations in 2D group were 7.3 ± 3.5 ng/mg, 9.16 ± 2.32 ng/mg, 24.18 ± 1.8 ng/mg and values in 5.5, 15 and 25 Mm glucose for 3D group were 11.2 ± 1.2 ng/mg, 17.3 ± 4.4 ng/mg, 34.3 ± 1.2 ng/mg respectively. Thus, the obtained results confirmed that maturated iPSCs-derived IPCs in 3D group could produce significant amount of insulin as compared to 2D group (*P-value* < 0.5). These results are shown in Fig. 6c.

4. Discussion

The connection between extracellular matrix and islets is vital for islet function and survival. In a 2D culture (TCPS) of beta-like cells, low cell–matrix connection between cells and less spherical morphology of cells were reported. The beta-like cells require a suitable microenvironment for maintaining its spherical architecture, function and viability, which could be provided by a favorable nanofibrous scaffold. In the present study, we used PCL/PVA scaffold as a substrate for differentiation of iPSCs into beta-like cells. To evaluate the effect of this scaffold on iPSCs during pancreatic differentiation, we seeded the iPSCs in 3D culture (nanofibrous scaffolds) and TCPS with induction medium and further assessed cell morphology and function.

In order to induce IPCs, in this study, we used reagent growth factors such as bFGF, EGF, Activin-A and betacellulin. EGF and bFGF play an important role in cellular proliferation, survival and differentiation. EGF and bFGF, have been shown to be useful in IPCs differentiation (Lumelsky et al., 2001). Hardikar et al. reported that, several differentiation factors and growth factors tested on pancreatic progenitor cells, bFGF secreted by endocrine cells was found to be the chemo-attractant in the clustering of precursor cells (Hardikar et al., 2003). Activin proteins belong to the transforming growth factor-beta (TGF-β) superfamily. Activin-A can enhance and regulate a wide range of biological activities including glucose-stimulated insulin secretion in beta-cells from humans and rats in culture (Florio et al., 2000).

The invert and electron microscope images displayed, in both groups, during differentiation progression, the morphology of cells regularly changed and ultimately hiPSC-IPCs converted spherical-shaped cell that was the usual shape of islets of Langerhans. The milestone of our data along with our other study was 3D morphology of iPSCs-IPCs in nanofiberous scaffold at the end of stages that was completely similar to spherically shaped islets of Langerhans (Enderami et al., 2017b). This morphology has not seen in same studies during same periods (Khorsandi et al., 2015a). Our immunostaining analysis indicated the expression of insulin and Pdx1 in iPSCs-IPCs cultured on nanofiberous scaffold qualitatively and quantitatively were higher than 2D culture. These results reveal that PCL/PVA scaffold could improve homogenous and efficient differentiation iPSCs into beta-like cells. The induction of these expressions of endocrine markers during differentiating iPSCs on this scaffold, were also detected by our quantitative PCR analysis. When we focused on the expression of endocrine-specific transcription factors, we noticed the difference between 3D and 2D groups was statistically significant in endocrine specific markers in mRNA and protein level. The expression of Glucagon, Insulin, Pdx1, Ngn3 genes in 3D culture were significantly higher than 2D cultures, but about Glut-2 gene as a beta cell maturity marker, although this glucose transporter gene was expressed in both groups, there was no statistically significant difference between them. This result aligned with Enderami et al.'s study in 2017 (Enderami et al., 2017a). For this reason, the results (Fig. 6a, b) revealed that the differentiated cells secretions in response to the low level of glucose (5.5 mM) concentration between both experimental groups were not significant. We documented insulin secretion from differentiated iPSCs into beta-like cells after the cells were exposed to 5.5_25 mM glucose. However, the previous study indicated that glucose concentration (at 20–30 mM) is a potent stimulator for IPCs by secreting insulin *in vitro* (Aloysious and Nair, 2014). In agreement with the previous study that gelatin/dextran nanofibrous scaffold was used for differentiation of adipose stem cells to beta-like cells, our results showed that PCL/PVA promotes

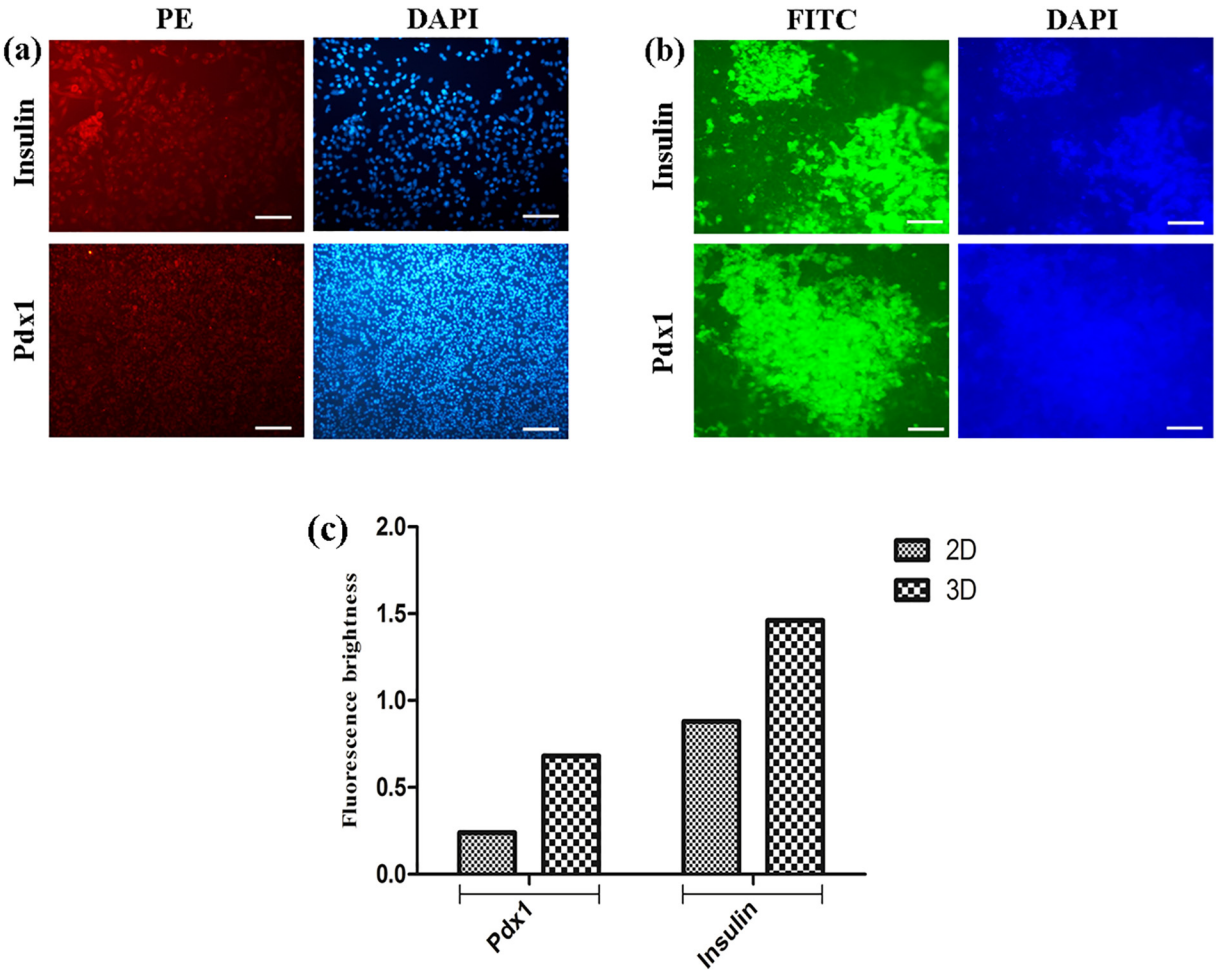

Fig. 5. Immunocytochemical analysis in end stage derived IPCs. Immunofluorescence analysis detected nuclei localization of Pdx1, and cytoplasmic localization of Insulin in differentiated IPCs at day 14 in the 2D group (a) and 3D group (b). Counter-staining of nucleus (blue) was performed by DAPI. Bar graph quantification of the immunocytochemistry assay. Each bar represents the relative value of insulin and Pdx1 markers, differences were observed between 3D and 2D groups. Scale bars are 100 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

differentiation of glucose-responsive beta-like cells from iPSCs. In this study, a significant increase in the secretion of insulin upon glucose stimulation in PCL/PVA nanofibrous scaffold iPSCs derived IPCs in comparison to TCPS was recognized. This finding describes that mature IPCs can be generated from iPSCs in 3D cultures. Khorsandi et al.'s reported that, compared to adipose-derived mesenchymal stem cell-derived IPCs in the 2D culture, the insulin release from mesenchymal stem cell-derived IPCs in the 3D culture showed a nearly 3.8-fold increase when exposed to a high glucose concentration (Khorsandi et al., 2015b). In our study, iPSCs-derived IPCs in the 3D culture indicated 1.5-fold increases in the insulin secretion compared to the 2D culture of iPSCs-derived IPCs. In the present study, the showed results of endocrine lineage markers expression in mRNA and protein level evaluation were aligned with the other studies in the field of stem cell differentiation to beta-like cells (Enderami et al., 2017b; Lahmy et al., 2014; Khorsandi et al., 2015b).

The exact cellular and molecular events that PCL/PVA scaffold exerts on iPSCs during differentiation to beta-like cells were not evaluated in this study. However, previous studies have revealed that 3D culture conditions increase global gene expression, cell differentiation, cell viability and proliferation. It has been reported that in 3D culture, this cellular behavior may be related to physiological conditions and interactions of seeded cells with an extracellular matrix and neighboring cells (Meng et al., 2014; Page et al., 2013). Besides the increase in the expression of endocrine genes, Baharvand et al., have revealed cellular organelles involved in protein synthesis and secretion also increase in the 3D culture (Baharvand et al., 2004). Several studies indicated the high potential application of electrospun nanofibers fabricated from various synthetic polymers in combination with a variety of stem cells in pancreatic tissue engineering (Enderami et al., 2017b; Fazili et al., 2016; Hoveizi et al., 2014).

The result suggests that the nanofibrous scaffold efficiently mimics the role of ECM by preparing a positive environment for IPCs. This condition obtainable by the nanofibrous scaffold is due to the presence of biocompatible and biodegradable polymer, the good porosity and lack of toxic agents. The PCL/PVA nanofibrous scaffold is more favorable for maintaining the structure and morphology, thereby enhancing the function and survival of IPCs.

In summary, the results of this study introduced a new approach for the differentiation of hiPSCs to beta-like cells with PCL/PVA scaffold for the first time. Differentiation in this scaffold system is much more efficient than monolayer culture system in the mRNA and protein levels, insulin and C-peptide secretion, and response to glucose challenge test. In addition, this nanofibrous scaffold could be a feasible alternative to ECM and be very useful for pancreatic tissue engineering. However, more research is required to investigate the exact cellular and molecular mechanisms of this scaffold function *in vivo*.

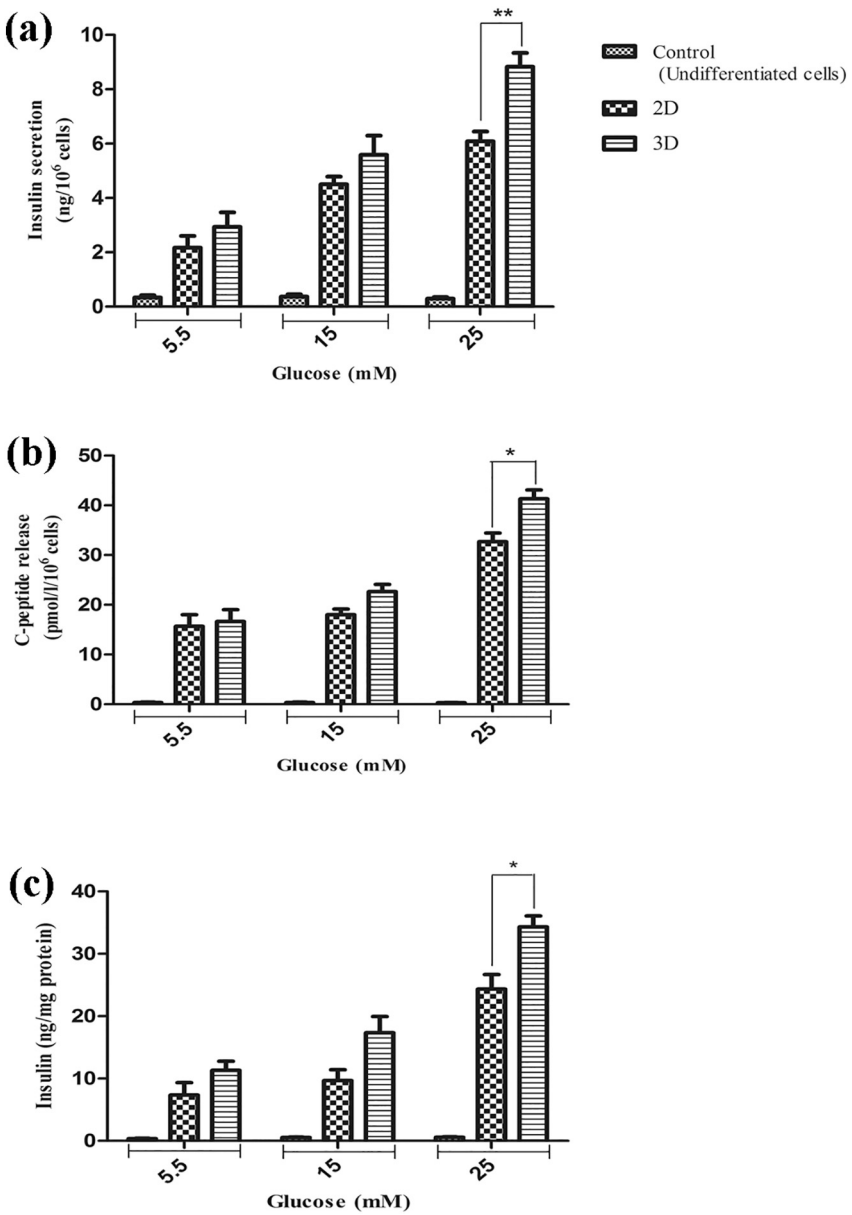

Fig. 6. *In vitro* insulin and C-peptide response assay in end stage derived IPCs. (a and b) insulin secretion and C-peptide release changes in two groups of IPCs and control group in response to various concentrations of glucose from 5.5 to 25 mM. (c) Intracellular insulin content in each concentration of glucose that normalized with total cellular protein. Values are expressed as mean ± SEM. (n = 6). **P-value* < 0.05 and ***P-value* < 0.01.

Conflict of interest

There is no conflict of interest in this study.

References

Abdulazeez, S.S., 2015. Diabetes treatment: a rapid review of the current and future scope of stem cell research. Saudi Pharm. J. 23 (4), 333–340.
Aghazadeh, Y., Nostro, M.C., 2017. Cell therapy for type 1 diabetes: current and future strategies. Curr. Diab. Rep. 17 (6), 37.
Aloysious, N., Nair, P.D., 2014. Enhanced survival and function of islet-like clusters differentiated from adipose stem cells on a three-dimensional natural polymeric scaffold: an in vitro study. Tissue Eng. A 20 (9–10), 1508–1522.
Amer, L.D., Mahoney, M.J., Bryant, S.J., 2014. Tissue engineering approaches to cell-based type 1 diabetes therapy. Tissue Eng. B Rev. 20 (5), 455–467.
Ardeshirylajimi, A., Dinarvand, P., Seyedjafari, E., Langroudi, L., Adegani, F.J., Soleimani, M., 2013. Enhanced reconstruction of rat calvarial defects achieved by plasma-treated electrospun scaffolds and induced pluripotent stem cells. Cell Tissue Res. 354 (3), 849–860.
Ardeshirylajimi, A., Soleimani, M., Hosseinkhani, S., Parivar, K., Yaghmaei, P., 2014. A comparative study of osteogenic differentiation human induced pluripotent stem cells and adipose tissue derived mesenchymal stem cells. Cell J. (Yakhteh) 16 (3), 235.
Association AD, 2016. 2. Classification and diagnosis of diabetes. Diabetes Care 39 (Supplement 1), S13–S22.
Baharvand, H., Hashemi, S.M., Ashtiani, S.K., Farrokhi, A., 2004. Differentiation of human embryonic stem cells into hepatocytes in 2D and 3D culture systems in vitro. Int. J. Dev. Biol. 50 (7), 645–652.
Chen, S., Borowiak, M., Fox, J.L., Maehr, R., Osafune, K., Davidow, L., et al., 2009. A small molecule that directs differentiation of human ESCs into the pancreatic lineage. Nat. Chem. Biol. 5 (4), 258–265.
Ellis, C., Ramzy, A., Kieffer, T.J., 2017. Regenerative medicine and cell-based approaches to restore pancreatic function. Nat. Rev. Gastroenterol. Hepatol. 14 (10), 612–628.
Enderami, S.E., Mortazavi, Y., Soleimani, M., Nadri, S., Biglari, A., Mansour, R.N., 2017a. Generation of insulin-producing cells from human-induced pluripotent stem cells using a stepwise differentiation protocol optimized with platelet-rich plasma. J. Cell. Physiol. 323 (10), 2878–2886.
Enderami, S.E., Soleimani, M., Mortazavi, Y., Nadri, S., Salimi, A., 2017b. Generation of insulin-producing cells from human adipose-derived mesenchymal stem cells on PVA scaffold by optimized differentiation protocol. J. Cell. Physiol. 233 (5), 4327–4337.
Enderami, S.E., Kehtari, M., Abazari, M.F., Ghoraeian, P., Nouri Aleagha, M., Soleimanifar, F., et al., 2018. Generation of insulin-producing cells from human induced pluripotent stem cells on PLLA/PVA nanofiber scaffold. Artif. Cells Nanomed. Biotechnol. 1–8.
Fazili, A., Gholami, S., Zangi, B.M., Seyedjafari, E., Gholami, M., 2016. In vivo

differentiation of mesenchymal stem cells into insulin producing cells on electrospun poly-L-lactide acid scaffolds coated with *Matricaria chamomilla* L. oil. Cell J. (Yakhteh) 18 (3), 310.
Florio, P., Luisi, S., Marchetti, P., Lupi, R., Cobellis, L., Falaschi, C., et al., 2000. Activin A stimulates insulin secretion in cultured human pancreatic islets. J. Endocrinol. Investig. 23 (4), 231–234.
Greggio, C., De Franceschi, F., Figueiredo-Larsen, M., Gobaa, S., Ranga, A., Semb, H., et al., 2013. Artificial three-dimensional niches deconstruct pancreas development in vitro. Development 140 (21), 4452–4462.
Hardikar, A.A., Marcus-Samuels, B., Geras-Raaka, E., Raaka, B.M., Gershengorn, M.C., 2003. Human pancreatic precursor cells secrete FGF2 to stimulate clustering into hormone-expressing islet-like cell aggregates. Proc. Natl. Acad. Sci. 100 (12), 7117–7122.
Hoveizi, E., Nabiuni, M., Parivar, K., Ai, J., Massumi, M., 2014. Definitive endoderm differentiation of human-induced pluripotent stem cells using signaling molecules and IDE1 in three-dimensional polymer scaffold. J. Biomed. Mater. Res. A 102 (11), 4027–4036.
Khorsandi, L., Nejad-Dehbashi, F., Ahangarpour, A., Hashemitabar, M., 2015a. Three-dimensional differentiation of bone marrow-derived mesenchymal stem cells into insulin-producing cells. Tissue Cell 47 (1), 66–72.
Khorsandi, L., Khodadadi, A., Nejad-Dehbashi, F., Saremy, S., 2015b. Three-dimensional differentiation of adipose-derived mesenchymal stem cells into insulin-producing cells. Cell Tissue Res. 361 (3), 745–753.
Kondo, Y., Toyoda, T., Inagaki, N., Osafune, K., 2017. iPSC technology-based regenerative therapy for diabetes. J. Diabetes Investig. 9 (2), 234–243.
Lahmy, R., Soleimani, M., Sanati, M.H., Behmanesh, M., Kouhkan, F., Mobarra, N., 2014. miRNA-375 promotes beta pancreatic differentiation in human induced pluripotent stem (hiPS) cells. Mol. Biol. Rep. 41 (4), 2055–2066.
Lumelsky, N., Blondel, O., Laeng, P., Velasco, I., Ravin, R., McKay, R., 2001. Differentiation of embryonic stem cells to insulin-secreting structures similar to pancreatic islets. Science 292 (5520), 1389–1394.
Mahboudi, H., Kazemi, B., Soleimani, M., Hanaee-Ahvaz, H., Ghanbarian, H., Bandehpour, M., et al., 2018. Enhanced chondrogenesis of human bone marrow mesenchymal stem cell (BMSC) on nanofiber-based polyethersulfone (PES) scaffold. Gene 643, 98–106.
Maheshwari, S.U., Kumar, S.V., Nagiah, N., Uma, T.S., 2013. Electrospinning of polyvinylalcohol–polycaprolactone composite scaffolds for tissue engineering applications. Polym. Bull. 70 (11), 2995–3010.
Mansour, R.N., Barati, G., Soleimani, M., Ghoraeian, P., Nouri Aleagha, M., Kehtari, M., et al., 2018. Generation of high-yield insulin producing cells from human-induced pluripotent stem cells on polyethersulfone nanofibrous scaffold. Artif. Cells Nanomed. Biotechnol. 1–7.
Meng, X., Leslie, P., Zhang, Y., Dong, J., 2014. Stem cells in a three-dimensional scaffold environment. Springerplus 3 (1), 80.
Page, H., Flood, P., Reynaud, E.G., 2013. Three-dimensional tissue cultures: current trends and beyond. Cell Tissue Res. 352 (1), 123–131.
Piran, M., Enderami, S.E., Piran, M., Sedeh, H.S., Seyedjafari, E., Ardeshirylajimi, A., 2017. Insulin producing cells generation by overexpression of miR-375 in adipose-derived mesenchymal stem cells from diabetic patients. Biologicals 46, 23–28.
Raikwar, S.P., Zavazava, N., 2012. PDX1-engineered embryonic stem cell-derived insulin producing cells regulate hyperglycemia in diabetic mice. Transp. Res. 1 (1), 19.
Rathmann, W., Giani, G., 2004. Global prevalence of diabetes: estimates for the year 2000 and projections for 2030. Diabetes Care 27 (10), 2568–2569.
Ravi, M., Paramesh, V., Kaviya, S., Anuradha, E., Solomon, F., 2015. 3D cell culture systems: advantages and applications. J. Cell. Physiol. 230 (1), 16–26.
Shen, J., Cheng, Y., Han, Q., Mu, Y., Han, W., 2013. Generating insulin-producing cells for diabetic therapy: existing strategies and new development. Ageing Res. Rev. 12 (2), 469–478.
Takahashi, K., Tanabe, K., Ohnuki, M., Narita, M., Ichisaka, T., Tomoda, K., et al., 2007. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell 131 (5), 861–872.
Wei, R., Yang, J., Liu, G.-Q., Gao, M.-J., Hou, W.-F., Zhang, L., et al., 2013. Dynamic expression of microRNAs during the differentiation of human embryonic stem cells into insulin-producing cells. Gene 518 (2), 246–255.
Xue, J., He, M., Liu, H., Niu, Y., Crawford, A., Coates, P.D., et al., 2014. Drug loaded homogeneous electrospun PCL/gelatin hybrid nanofiber structures for anti-infective tissue regeneration membranes. Biomaterials 35 (34), 9395–9405.
Zarekhalili, Z., Bahrami, S.H., Ranjbar-Mohammadi, M., Milan, P.B., 2017. Fabrication and characterization of PVA/Gum tragacanth/PCL hybrid nanofibrous scaffolds for skin substitutes. Int. J. Biol. Macromol. 94, 679–690.
Zhang, D., Jiang, W., Liu, M., Sui, X., Yin, X., Chen, S., et al., 2009. Highly efficient differentiation of human ES cells and iPS cells into mature pancreatic insulin-producing cells. Cell Res. 19 (4), 429–438.