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The expression of cytochrome P4502J2 gene and 14, 15 epoxyeicosatrienoic acid level influence the amount of insulin secreted from human mesenchymal stem cell-derived insulin-producing cells

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Abstract

Background: Insulin-producing cells differentiated from human mesenchymal stem cells demonstrate limited glucose-stimulated insulin secretion. Cytochrome P450 2J2 and its product epoxyeicosatrienoic acids regulate β-cell function in the human pancreas. The aim of this study is to explore the expression pattern of cytochrome P450 2J2 gene and 14, 15 epoxyeicosatrienoic level along the differentiation of human bone marrow-derived mesenchymal stem cells into insulin-producing cells.

Results: The differentiated insulin-producing cells express high levels of pancreatic duodenal homeobox-1 and insulin gene mRNA. It secretes increasing amounts of C-peptide in response to increasing glucose concentrations than undifferentiated cells. The differentiated insulin-producing cells were found to express reduced amounts of cytochrome P450 2J2 gene mRNA and significant low level of 14, 15 epoxyeicosatrienoic acid than the undifferentiated cells. A strong positive correlation between 14, 15 epoxyeicosatrienoic concentrations and C-peptide released from the differentiated insulin-producing cells was noticed.

Conclusions: Cytochrome P4502J2 and its product 14, 15 epoxyeicosatrienoic might affect insulin secretion from differentiated insulin-producing cells.

Keywords: Insulin-producing cells, Cytochrome P4502J2, 14, 15 Epoxyeicosatrienoic acid, Pancreatic duodenal homeobox-1 mRNA, Insulin mRNA, Human mesenchymal stem cells

Background

Mesenchymal stem cells (MSCs), self-renewable multipotent stromal cells, are usually isolated with relative ease from several organs, including the liver, umbilical cord, and bone marrow (Vanella et al. 2010). Under certain culture conditions, bone marrow-derived MSCs (BM-MSCs) can be differentiated into insulin-producing cells (IPCs). It is characterized by expressing both the pancreatic β-cell developmental genes as pancreatic duodenal homeobox-1 (PDX-1) gene, and functional genes as insulin and glucagon genes (Zanini et al. 2011; Xie et al. 2009; Moriscot et al. 2005). MSC-derived IPCs are not only shown to secrete insulin in response to glucose, but also reverses hyperglycemia in drug-induced diabetic mice (Sun et al. 2007; Chen et al. 2004). Therefore, induction of IPCs offers an approach to supply donor β-cell sources for diabetes cell therapy and screen new anti-diabetic drugs (Xie et al. 2009; Sun et al. 2007; Volarevic et al. 2010).

However, the generation of IPCs from MSCs occurs at a low rate, and most of these induced cells show limited glucose-stimulated insulin secretion (GSIS), which limits basic and clinical applications (Xie et al. 2013). Consequently, understanding metabolic pathways related to

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IPC induction and insulin secretion has become an essential and vital issues.

Cytochrome P4502J2 (CYP2J2), an arachidonic acid epoxygenase, is widely expressed in various human tissues, such as the heart, lung, blood vessels, liver, kidney, ileuma, and jejunum, and highly expressed in pancreatic Langerhans cells (Xu et al. 2013; Zeldin et al. 1997).

CYP2J2-derived epoxyeicosatrienoic acids (EETs), a set of lipid intermediaries, have diverse biological and cytoprotective properties (Sodhi et al. 2009). EETs promote endothelial cell growth and angiogenesis and inhibit apoptosis (Wang et al. 2005). As well, EETs posse an anti-inflammatory effect via the suppression of NF-κB and IκB kinase activity (Node et al. 1999).

Moreover, a significant amount of EETs is produced in the human and rat pancreas, providing biochemical evidence that CYP epoxygenase product may regulate β-cell function (Zeldin et al. 1997). Upon production, 90% of EETs esterifies at the *sn-2* position of glycerophospholipids where they are stored and released according to the cell's needs (Karara et al. 1991). Free EETs are unstable, either transformed by soluble epoxide hydrolase (sEH) to the less active dihydroxyepoxytrienoic acids or undergo β oxidation (Abraham et al. 2014).

Earlier reports stated that human MSCs not only exhibit a considerable amount of CYP2J2 and synthesized significant levels of EETs (Kim et al. 2010), but their levels also modulate adipogenesis and cell differentiation in MSC-derived adipocytes (Vanella et al. 2011).

The expression of *CYP2J2* gene in differentiated IPCs is not yet investigated. Therefore, we aim in this study to investigate the expression pattern of *CYP2J2* gene and 14, 15 EET level along the differentiation of human bone marrow-derived MSCs into IPCs.

Methods

Isolation and culture of human bone marrow-derived MSC (HBM-MSCs)

Bone marrow aspirates (BMA) were obtained from the iliac crest of three non-diabetic adult volunteers (age 35–65 years), during hip replacement surgery under approved protocol by the faculty of medicine ethical committee, Suez Canal University. Written informed consent was obtained from the volunteers. The HBM-MSCs were isolated and cultured as previously described (Gabr et al. 2013).

In brief, mononuclear cells were isolated from BMAs diluted with low glucose DMEM (Dulbecco's modified Eagle's medium) by density gradient centrifugation in Ficoll-Paque 1.077 g/mL (Lonza BioWhittaker, Belgium). The cells, (5×10^6)/BMA, were cultured in a complete growth medium (low glucose (1 g/L) DMEM,10% heat-inactivated fetal bovine serum (FBS), 1% penicillin, 1% streptomycin [Lonza BioWhittaker, Belgium], and 1% L-glutamine (Gibco BRL, Life Technologies, UK). After 72 h, the non-adherent cells were discarded. When the adherent cells reached 80% confluence, it is trypsinized and expanded by re-plated for two passages (P1-P2), 8 days each.

Differentiation of HBM-MSCs into IPC

At P3, the homogenous, spindle-shaped, fibroblast-like MSCs were induced to differentiate into IPCs by a three-stage protocol described (Gabr et al. 2013). In short, MSCs were cultured in serum-free, glucose-rich (25 mmol/L) DMEM containing, firstly, 0.5 mmol/L β-mercaptoethanol (Sigma) for 2 days; secondly, 2 mmol/L L-glutamine, 2% B27 supplement (Gibco BRL, Life Technologies, UK), 1% non-essential amino acids, 20 ng/mL epidermal growth factor, and 20 ng/mL basic fibroblast growth factor (Sigma) for 8 days; and finally, 10 ng/mL activin-A, 10 ng/mL betacellulin, 10 mmol/L nicotinamide (Sigma), and 2% B27 supplement for another 8 days. The medium was replaced at the third day in every stage. At the end of the differentiation protocol (18 days), the cells become spherical and tend to form islet-like clusters.

Flow cytometry characterization of MSC cell markers

At P3, the cells were incubated with the following antibodies against CD105 (FITC), a positive marker of MSC and CD45 (PerCP), CD34 (PE) a negative markers of MSC (BioLegend, USA) as previously described (Gabr et al. 2013).

Evaluation of insulin, PDX-1, and CYP2J2 gene expression by real-time PCR

Total RNA was extracted from undifferentiated MSCs (control) at P3 and differentiating cells at days 18 by QIAamp RNA Blood Mini Kit (Valencia, CA, USA ca. no. 52304). Total RNA was converted to cDNA using QuantiTect Reverse Transcription Kit (Catalog no. 205311).

Quantitative real-time PCR amplification was done using QuantiTect SYBR Green PCR Kit (Catalog no. 204141, QIAGEN Inc., Valencia, CA, USA), and a step one Real-Time PCR System (ABI PRISM, Applied Biosystem, CA, USA). Relative gene expression was assessed using comparative cycle threshold (CT) to determine the fold change ($2^{-\Delta\Delta Ct}$) of gene expression between samples and normalized to an endogenous reference (*GAPDH*[1] for *insulin* and *PDX-1*, *GAPDH*[2] for *CYP2J2*) (Livak and Schmittgen 2001).

PCR reaction mix

One to 2 μl of the cDNA was amplified in 20 μL total volume including 10 μl of 2x Quantitec SYBR green PCR master Mix (QIAGEN, Valencia, CA), nuclease-free water, and 25 pmol of each of the following primer pair (Table 1).

Table 1 Primers used in this study

Gene	Primers
Insulin	F: 5′CTACCTAGTGTGCGGGGAAC3′, R: 5′ CACAATGCCACGCTTCTG3′
PDX-1	F: 5′GAGCTGGCTGTCATGTTGAA3′, R: 5′AGTGGTTGAAGCCCCTCAG3′
GAPDH[1]	F: 5′TGCTGGCGCTGAGTACGTCG3′, R: 5′TGACCTTGGCCAGGGGTGCT3′
CYP2J2	F: 5′CTCCTACTGGGCACTGTCGC3′, R: 5′TGGGCCTCCTCCTGAAT3′
GAPDH[2]	F: 5′CCAGGTGGTCTCCTCTGACTTC3′, R: 5′TCATACCAGGAAATGAGCTTGACA3′

PCR cycling conditions

Insulin: Initial heat activation 95 °C, 15 s; 30 cycles of denaturation 94 °C, 30 s; annealing 61 °C, 30 s; extension 72 °C, 30 s.

PDX-1: Initial heat activation 95 °C, 15 s; 30 cycles of denaturation 94 °C,30 s; annealing 57 °C,30 s; extension 72 °C, 30 s.

CYP2J2: Initial heat activation 95 °C, 15 s; 45 cycles of denaturation 95 °C, 15 s; annealing 56 °C, 30 s; extension 72 °C, 40 s.

Reactions were performed on a step one Real-Time PCR System (ABI PRISM, Applied Biosystem, CA, USA).

Determination of insulin secretion by differentiated MSCs

Insulin secretion was determined indirectly by evaluating C-peptide release in culture media in response to increasing glucose concentrations. Cells at the end of differentiation were initially incubated for 3 h in glucose-free Krebs-Ringer bicarbonate buffer (KRB) followed by incubation in 3.0 mL of KRB containing 5.5, 12, or 25 mM/L glucose for 1 h. Supernatant C-peptide concentrations were assayed using Human C-Peptide ELISA Kit (Biovendor, cat no. RIS005R) according to the manufacturer's instructions.

Determination of 14, 15-DHET level

To investigate the function of CYP2J2, the concentration of 14, 15-dihydroxyeicosatrienoic acid (14, 15-DHET), the stable EET metabolite, was measured. Cell culture media of differentiated and undifferentiated MSCs were centrifuged at 1500 rpm for 10 min at 4 °C. Cell supernatant was collected, and 14, 15-DHET concentration was determined by 14, 15 EET/DHET ELISA Kit (AB175812, ABCAM, USA), according to the manufacturer's instructions.

Determination of 14, 15-DHET level in insulin-producing cells

To evaluate the relation between 14, 15-DHET level and C-peptide release, both 14, 15-DHET and C-peptide levels were measured in the cell supernatant of differentiated MSCs obtained from the three donors and incubated in 3.0 mL of KRB containing 25 mM/L glucose for 1 h by 14, 15 EET/DHET ELISA Kit (AB175812, ABCAM, USA), and Human C-Peptide ELISA Kit (Biovendor, cat no. RIS005R) according to the manufacturer's instructions.

Data analysis

Statistical analysis was performed using SPSS V22.0 and Microsoft Excel 2007. Non-parametric data were evaluated by Kruskal-Wallis test. A P value of < 0.05 was considered significant.

Results

Morphological characterization of cultured cells

Adherent cells were detected 24 h after culture. The appearance of spindle-shaped MSC-like adherent cells was noticed 3–7 days (3.7 ± 1.6) after onset of culture. With time, the colonies of spindle-shaped adherent MSC-like cells survive and predominate (Fig. 1).

Phonotypical characterization of cultured cells at the end of expansion stag

The surface antigens on the P3 HBM spindle-shaped MSC-like cells were analyzed by flow cytometry. It showed them expressing statistically significant high levels of CD105 compared to CD34 and CD45 ($P < 0.05$) (Fig. 1).

Validation of IPCs derived from HBM-MSCs

P3 HBM-MSCs were induced to differentiate into IPCs by a three-step, 18-day protocol. At day 6 of differentiation, the cells become shorter and began to collect, forming cellular aggregates. With contentious culture, spheroid epithelial-like cells tend to aggregate into islet-like cluster (Fig. 2).

mRNA expression of pancreatic development transcription factor, *PDX1*, and endocrine-related marker gene, *insulin*, were analyzed by quantitative real-time PCR. The gene transcripts of the differentiated cells are compared with that of undifferentiated hMSCs (control). The expression of *PDX-1* and *insulin* genes in the differentiated cells was significantly higher than undifferentiated cells ($P < 0.05$) (Table 2).

At the end of the differentiation protocol, the differentiated cells were found to secrete increasing amounts of C-peptide in response to increasing glucose concentrations ($P < 0.05$). C-peptide is released when pro-insulin is cleaved to insulin, and as such, it has been considered as a marker for insulin secretion (Table 3).

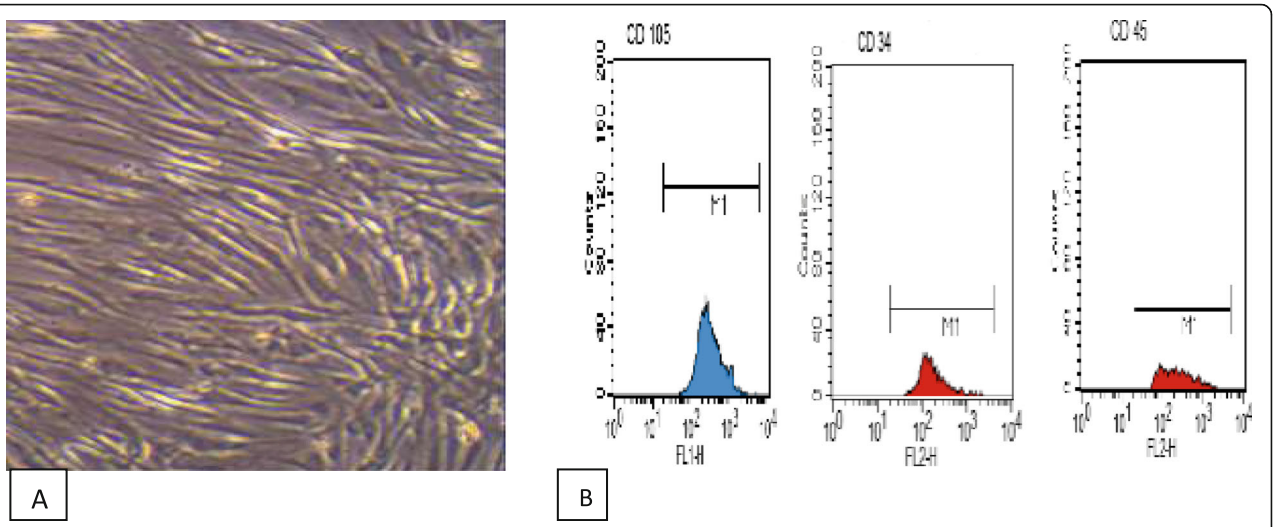

Fig. 1 **a** Undifferentiated spindle-shaped HBM-derived MSCs at the end of expansion phase (80% confluence, × 100). **b** Surface expression of the CD105, CD34, CD45, on HBM-MSC cells at P3. CD105 (endoglin and SH2) was expressed in 60.3 ± 3.6 of cells, while CD34 (hematopoietic stem cell marker) and CD45 (common lymphocytes antigen) were shown to be expressed in 25.1 ± 3.9 and 19.1 ± 1.6 of cells respectively ($P = 0.001$*)

CYP2J2 gene expression and 14, 15-DHET level before and after differentiation

The differentiated IPCs express non-significant reduced amounts of *CYP2J2* gene mRNA than the undifferentiated HBM-MSCs cells ($P = 0.31$) (Table 2).

In addition, the 14, 15-DHET, which measured to represent levels of EETs production, was reduced significantly in the differentiated IPCs than the undifferentiated MSCs cells ($P < 0.05$) (Table 2).

Relation between 14, 15 DHET concentration and C-peptide release

Increasing 14, 15-DHET concentration was associated with increase in C-peptide released from differentiated IPCs incubated in KRB containing 25 mM/L glucose (Table 4).

Non-parametric Spearman correlation between 14, 15-DHET concentrations and C-peptide released from IPCs shows statistically significant strong positive correlation between them (rho = 1.000, $P < 0.01$) (Fig. 3).

Discussion

Earlier studies reported an induction of limited functional insulin-producing cells, from bone marrow-derived MSCs of human origin, upon re-programming in vitro (Xin et al. 2016; Gabr et al. 2015; Jafarian et al. 2015).

In the current study, in an attempt to elucidate the reasons under this observation, we studied the expression of *CYP2J2* gene and 14, 15 EET level, previously

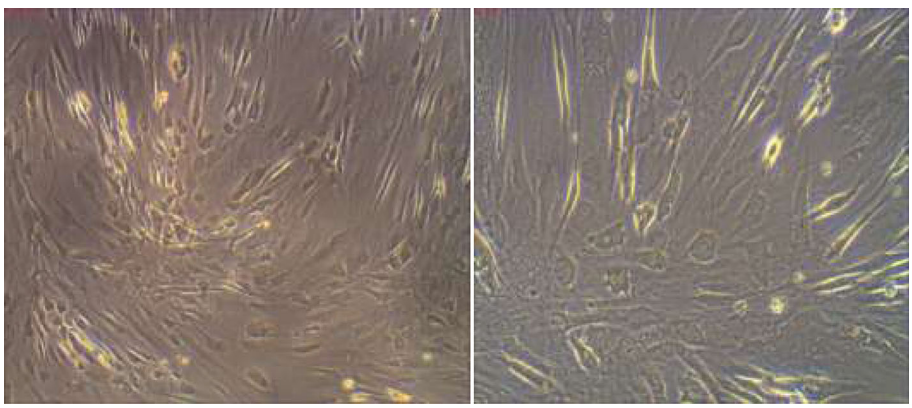

Fig. 2 Differentiation of hMSCs into insulin-producing cells (IPCs). HBM-MSCs at P3 were induced to differentiate into IPCs by an 18-day protocol. **a** After 14 days of induction, small round cell aggregates were formed. **b** At day 18 (the end of differentiation), more islet-like clusters and round epithelial-like cells appeared

Table 2 Relative expression of *PDX-1*, *insulin*, *CYP2J2* genes, and 14, 15-DHET conc. level before and after differentiation of HBM-MSCs to IPCs

	Undifferentiated HBM-MSCs	Differentiated IPCs	*P* value
PDX-1	1.0 ± 0.03	5.5 ± 0.9	0.046*
Insulin	1.0 ± 0.02	3.0 ± 0.2	0.04*
CYP2J2	1.0 ± 0.37	0.84 ± 0.01	0.31
14-15DHET (pg/mL)	4.85 ± 0.6	3.36 ± 0.4	0.005*

*Statistically significant difference (*P* value < 0.05)

reported to be involved in the regulation of pancreatic β-cell function (Luo and Wang 2011), before and after differentiation of HBM-derived MSCs into IPCs.

For this purpose, HBM-derived plastic adherent cells, isolated from adult healthy donors, were selected (Friedenstein et al. 1976). These cells were expanded for two passages, after the original seed of bone mononuclear cells, to get more uniform hMSCs (Sun et al. 2007; Xin et al. 2016). At the end of the expansion stage, the majority of cells attain spindle-shaped morphology as well as are being positive for MSC marker CD105 (Dominici et al. 2006).

Yet, small populations of the cells were positive for either CD34 or CD45, a result contradicting other researchers (Gabr et al. 2013; Xin et al. 2016; Karnieli et al. 2007). However, considering CD34 and CD45 negative markers for MSCs is debatable. Several studies provided persuasive evidence that uncultured BM-MSCs are CD34+, and they tend to gradually lose CD34 expression during prolonged cell culture (Simmons and Torok-Storb 1991; Suga et al. 2007; Mouiseddine et al. 2008; Stolzing et al. 2012; Lin et al. 2012).

On the other hand, MSCs derived from different adult sources were found to express detectable level of CD45, ranging from 8 to 26% (15%), in early passages, which decreases significantly with successive expansion (Gang et al. 2010; Maleki et al. 2014). Cell culture-associated loss of CD34 and CD45 expression may attribute to either down-regulation of CD34/45 proteins or production of modified forms in cultured cells that are nonreactive with CD34/45 antibodies (Watt et al. 1987; Fina et al. 1990).

Other factors could contribute to the different MSC cell surface marker profiles. It may depend on MSC tissue source, medium composition, or passage number (Hass et al. 2011).

Table 3 In vitro C-peptide release in response to increasing glucose concentration

	Glucose 5.5 mM	Concentration 12 mM	25 mM	*P* value
Human C-peptide release (ng/μg protein/hour) (Mean ± SD)	2.7 ± 0.8	3.8 ± 0.8	8.7 ± 1.5	0.04*

*Statistically significant difference (*P* value < 0.05)

Table 4 14, 15-DHET concentrations and C-peptide released from differentiated IPCs incubated in KRB containing 25 mM/L glucose

Donor	14, 15-DHET (pg/mL)	C-peptide (ng/μg protein/hour)	*P* value
1*	3.8	10	
2*	3.28	9.1	
3*	3.1	7.1	
Mean ± SD	3.36 ± 0.4	8.7 ± 1.5	*0.000

*Statistically significant difference (*P* value < 0.05)

Since, in the current study, the cells were expanded for two passages (16 days), which is less than that reported in other studies, five passages (25 days) in Xin et al. (Xin et al. 2016), 2 p (28 days) in Jafarian et al. (Jafarian et al. 2015), and 28 days in Karnieli et al. (2007), which could explain the difference in MSC phenotypic picture. Although the expansion period in the current study was the same as in Gabr et al. (Gabr et al. 2013), the use of L-glutamine in the culture media of this study may contribute to the discrepancy between the results.

In this study, HBM-MSCs were induced to differentiate into IPCs using soluble factors, three-stage protocol that mimicked in vivo pancreatic β-cell formation and development (Gabr et al. 2013). The differentiation is confirmed by the fact that differentiated cells expressing *insulin* and *PDX-1* genes, and secreting C-peptide in response to escalating glucose concentrations, results in concordance with other studies (Gabr et al. 2013; Xin et al. 2016).

In the current study, we assayed the C-peptide release from the differentiated cells to ensure that the IPC cells were synthesizing insulin. Previous studied had argued that the release of insulin from such cells does not indicate intrinsic insulin production. They suggest that insulin could be absorbed from the utilized culture

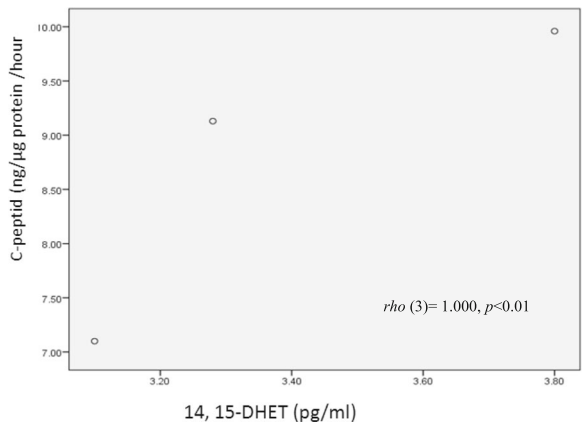

Fig. 3 Spearman correlation between 14, 15 EET concentrations and C-peptide amount released from differentiated IPCs incubated in 25 mM/L glucose

media (B27 containing additional insulin), sequestrated, and re-released upon glucose stimulation (Rajagopal et al. 2003; Hansson et al. 2004; Mc Kiernan et al. 2007).

However, the amount of C-peptide released from IPCs in the current study (8.7 ng/μg protein /hour/25 mM glucose) is modest, representing ≈ 28% of equivalent quantity of C-peptide in pancreatic islet cells at 25 mM glucose concentration (Gabr et al. 2013), a result comparable to that reported by Gabr et al. (Gabr et al. 2013).

Taking into consideration, the IPCs in the current study secrete C-peptide in a glucose-sensitive manner. It is proposed that the defect could be in the insulin release regulatory mechanisms, rather than the mechanism of cellular glucose sensing.

Glucose-stimulated insulin release is a complex process involving full oxidation of glucose by respiratory chain and production of ATP; rise in ATP/ADP ratio results in closer of the K_{ATP}-channels, results in depolarization of the plasma membrane followed by opening of the voltage-operated Ca $^{2+}$ channels, and leads to influx of and increase of the intracellular Ca^{2+}, which direct insulin granule translocation, and eventually insulin exocytosis (Klett et al. 2013a).

Previous studies considered the role of CYP2J2 and its derivative EETs in the control of insulin secretion. Inhibition of cytochrome P_{450} decreased glucose-stimulated insulin release and arginine-stimulated insulin from isolated rat pancreatic islets (Zeldin et al. 1997; Falck et al. 1983; Chen et al. 2013). EETs, and their metabolites through activation of peroxisome proliferator-activated receptors (PPAR) *γ*, increase calcium mobilization and insulin secretion, which is mediated by the free fatty acid receptor, G-protein-coupled transmembrane 40 (GPR 40), and GLUT2 expression in pancreatic β-cells (Wray and Bailey 2007; Kim et al. 2013).

In addition, the glycophospholipd-EETs were reported to increase glucose-stimulated-insulin secretion more than free EETs (Klett et al. 2013b; Gangadhariah et al. 2017). Furthermore, decrease EET deprivation, by inhibiting sEH or deleting its encoding gene (*Ephx2*), results in increased insulin sensitivity and insulin secretion and increases islet survival (Luo et al. 2010).

In the current study, the HBM-MSC-derived IPCs display decreased activity of CYP2J2, as addressed by a decrease in *CYP2J2* mRNA expression, and a significant decrease in 14, 15 EET level compared to the undifferentiated hMSCs. In addition, although of small sample size, a statistically significant strong positive correlation between 14, 15 EET concentration and C-peptide released from differentiated IPCs cells was found, as higher concentrations of 14, 15 EET are accompanied by increase in C-peptide released.

As a result, the low amount of insulin released from differentiated IPCs, in the current study, could be attributed to the relatively low 14, 15 EET level in the differentiated cells. 14,15 EET acts in an autocrine manner to affect the intracellular Ca^{2+} concentration via promoting Ca^{2+} influx through TRPV4, or TRPC3, and TRPC6 channels (transient receptor potential cation channel) (Campbell and Fleming 2010). In addition, evidence exists that 14, 15 EET markedly alter the activity of K_{ATP} channel (Lu et al. 2001). In pancreatic islets, K_{ATP} channels are primarily composed of four pore-forming potassium subunits (Kir6.2) and four sulfonylurea receptor regulatory subunits (SUR1). Accumulative data suggests that K_{ATP} channels have a role in exocytosis (Ashcroft and Rorsman 2013). SUR1 binds to the exocytosis-regulating protein EPAC2 (exchange protein directly activated by cAMP 2) and other exocytotic proteins, such as syntaxin-1A, and the Ca^{2+} sensor piccolo involved in exocytosis (Kang et al. 2011; Shibasaki et al. 2004).

Decreased expression of *CYP2J2gene* was also reported by Kim et al. (Kim et al. 2010) and Vanella et al. (Vanella et al. 2010). In such studies, the expression of *CYP2J2* and EET level in hMSC-derived adipocytes were significantly lower than the undifferentiated cells.

It is worth noting that our finding of decreased 14, 15 EET that is associated with low insulin secretion from differentiated IPC contradicts with the previous study (Falck et al. 1983) that associates 14, 15 EET to glucagon rather than insulin secretion. The difference in that study is probably due to the use of rat islets that contains glucagon-secreting cells, in contrast to differentiated IPCs in our study. A limitation of this study is the small sample size used in correlation expermints. Further studies with larger sample size are neded to validate the results.

Conclusions

Our findings suggest that CYP2J2-derived 14, 15 EET could affect insulin and C-peptide secretion from HBM-MSC-derived IPCs. Further studies are required to verify whether interventions to increase 14, 15 EET by EET agonists and sEH inhibitors could potentiate insulin secretion from MSC-derived IPCs.

Abbreviations

14, 15-DHET: 14, 15-Dihydroxyeicosatrienoic acid; BM-MSCs: Bone marrow-derived MSCs; CYP2J2: Cytochrome P4502J2; EETs: Epoxyeicosatrienoic acids; GSIS: Glucose-stimulated insulin secretion; hMSCs: Human mesenchymal stem cells; IPCs: Insulin-producing cells; PCR: Polymerase chain reaction; PDX-1: Pancreatic duodenal homeobox-1

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Availability of data and materials
All relevant data are included within the paper. Raw data are available from the corresponding author for researchers who meet the criteria for access to confidential data.

Authors' contributions
The authors contributed equally in this research. All authors read and approved the final manuscript.

Ethics approval and consent to participate
The study was approved by the faculty of medicine ethical committee, Suez Canal University.
A written informed consent was obtained from the participants.

Consent for publication
Not applicable

Competing interests
The authors declare that they have no competing interests.

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