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Differentiation of the definitive endoderm from Wharton's Jelly Mesenchymal Stem Cells (WJMSC)

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Wharton's jelly mesenchymal stem cells (WJMSCs) are multipotent cells derived from human umbilical cord that are viewed as promising candidate source of mesenchymal stem cells for regenerative medicine. Apart from their accessibility, other advantages of WJMSCs include painless and non-invasive procedures to donor, immunomodulatory properties and faster self-renewal. In this study, we focused on differentiating WJMSCs into the definitive endoderm lineage as the first step in differentiation into endoderm derivatives, such as insulin-producing cells. The effect of various treatments on definitive endoderm differentiation was evaluated using real-time PCR to detect expression of four marker genes (i.e. *Sox17*, *FoxA2*, *Cxcr4* and *Cer*). Here, we show that a low concentration of activin A in combination with Wnt3a promote differentiation of WJMSCs toward definitive endoderm. These findings may be of value in designing new WJMSCs-based differentiation protocols for regenerative cell therapies.

Key words: activin A, definitive endoderm, differentiation, Wharton's jelly mesenchymal stem cells.

INTRODUCTION

The umbilical cord is a rich source of stem cells with high proliferation and differentiation capabilities (Weiss & Troyer, 2006). Wharton's jelly is the connective tissue of the umbilical cord lying between the amniotic epithelium and the umbilical vessels (Taghizadeh *et al*., 2011). Several protocols have been reported for isolation, culture and characterization of Wharton's jelly stem cells (WJMSCs) (Lu *et al*., 2006; Seshareddy *et al*., 2008; Koliakos *et al*., 2011). WJMSCs possess characteristics of both embryonic stem cells and adult stem cells as they possess pluripotency properties, as well as multipotent tissue maintenance (Carlin *et al*., 2006). WJMSCs appear to offer potential clinical advantages because of their unique beneficial properties (Bongso & Fong, 2012).

Although human Wharton's jelly mesenchymal stem cells (hWJMSCs) and human bone marrow mesenchymal stem cells (hBMMSCs) have common features such as fibroblast-like phenotype, nonhematopoietic surface markers (Can & Karahuseyinoglu, 2007), hypoimmunogenecity (Weiss *et al*., 2008; Tipnis *et al*., 2010) and multipotent plasticity (Fan *et al*., 2011), it seems that hWJMSCs have several different characteristics and advantages over hBMMSCs in cli-

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nical application (Bongso & Fong, 2012). hWJMSCs maintain the same multipotent differentiation potential with relatively higher colony-forming unit (CFU) formation and proliferation rates, higher telomerase activity, shorter population doubling time, and longer time to senescence, without loss of stem cell potency for longer periods of time after serial passaging (Troyer & Weiss, 2008; Fong *et al*., 2011; Taghizadeh *et al*., 2011).

Other features of hWJMSCs, as compared to hBMMSCs, include up-regulation of few early lineage markers specifically related to endoderm both in early and late passages (Hsieh *et al*., 2010; Nekanti *et al*., 2010), higher expression of early ectoderm markers (Prasanna & Jahnavi, 2011) and maintenance of stem cell properties after many passages (Bongso *et al*., 2008).

hWJMSCs have anticancer properties which include the expression a number of tumor suppressor genes (compared to other MSCs) and the ability to seek out and kill cancer cells (Ayuzawa *et al*., 2009; Ganta *et al*., 2009; Fong *et al*., 2011). In addition, WJMSCs, unlike embryonic stem cells (ESCs), do not form tumors when transplanted even though they exhibit properties related to ESCs. Specifically, WJMSCs express pluripotency markers (Oct-4, Sox-2, Nanog) (Carlin *et al*., 2006; Can & Karahuseyinoglu, 2007; La Rocca *et al*., 2009), stage specific embryonic antigen-1 (Tra-1-60, Tra-1-81, SSEA-1) (Fong *et al*., 2007), SSEA-4 and alkaline phosphatase (Fong *et al*., 2007, 2011).

Several studies have demonstrated the plasticity of hWJMSCs which was similar to that of other MSCs that are multipotent and capable of diverse lineage commitment (Xu *et al*., 2012). WJMSCs can be differentiated into a wide variety of tissues such as bone, cartilage, and adipose cells (Wang *et al*., 2004, 2011b; Sarugaser *et al*., 2005; Karahuseyinoglu *et al*., 2007). WJMSCs can also be differentiated into glial and neurons (Mitchell *et al*., 2003; Fong *et al*., 2007), cardiomyocytes (Corrao *et al*., 2012), endothelial cells (Wu *et al*., 2007), skeletal muscle cells (Connconi *et al*., 2006), hepatocytes (Scheers *et al*., 2011) and pancreatic islet-like clusters (Chao *et al*., 2008; Anzalone *et al*., 2011).

Several reports demonstrated that WJMSCs do express the MSC markers such as CD90, CD105, CD73, CD29, and CD44 but do not express hematopoietic and endothelial markers (CD45, CD56, CD31 and CD34). In addition, WJMSCs may express CD117 (receptor for the stem cell factor), CD68 (macrophage-specific antigen) and CD14 (monocyte-specific marker) (La Rocca *et al*., 2012).

Mesenchymal stem cells have been shown to be hypoimmunogenic owing to the lack of HLA-DR and lack of co-stimulatory ligands implicated in activation of both T and B cell responses. MHC-Class I molecules are expressed at low levels and this could be a mechanism to protect them from natural killer cell (NK)-mediated lysis (Prasanna & Jahnavi, 2011). WJMSCs are a good peri-natal source of MSCs and their immune privilege properties are comparable to BMMSCs. WJMSCs, like BMMSCs, do not require tissue matching, and are less immunogenic than BMMSCs, and therefore they are compatible for transplantation therapy in allogeneic settings without immunorejection (Taghizadeh *et al*., 2011). Not only could WJMSCs be promising to generate mature cell types for organ repopulation, they have also been shown to stimulate reparative processes in the organs using multiple mechanisms. WJMSCs have been reported to secrete cytokines and other modulatory molecules which can reduce inflammation. This could benefit the higher survival of local cells and progenitors, as well as self-repair of tissues. Furthermore, secretion of matrix metalloproteinases may potentiate these cells to overcome fibrosis, due to the breakdown of excessive extracellular matrix released during the fibrotic process, and release of matrix-bound factors. Metalloproteinases may also play a role in mediating immunomodulatory processes, which trigger paracrine signaling cascades followed by peripheral immune tolerance without the need of direct cell-cell contact (La Rocca *et al*., 2012). WJMSCs may be an important source for cell therapy because of their high proliferative capacity, minimal chances of viral or bacterial contamination, easy donor accessibility, immunomodulatory activity and anticancer properties and lack of ethical issues (Anzalone *et al*., 2011; Fan *et al*., 2011; Bongso & Fong, 2012).

The definitive endoderm (DE), from which the pancreas arises, is one of the three germ layers –ectoderm, mesoderm and definitive endoderm– that form during gastrulation. Studies on mouse embryo indicate that the DE and mesoderm have a common biopotent progenitor, called mesoendoderm (Lowe *et al*., 2001; Shiraki *et al*., 2010). Mesoendoderm originates from anterior primitive streak that after migration and interaction with environment and surrounding cells generates definitive endoderm and mesoderm (D'Amour *et al*., 2005; Kroon *et al*., 2008).

Genetic studies have shown that transforming growth factor β (TGFβ) and Wnt/β-Catenin signaling pathways play crucial roles during mesoderm and endoderm formation in vertebrates (Semb, 2008). High levels of Nodal, a member of the TGFβ superfamily, lead to the expression of DE genes such as *Sox17*, *FoxA2* and *Cxcr4* and its low levels lead to the expression of mesodermal genes such as the Brachyury gene (Takenaga *et al*., 2007; Teo *et al*., 2011). Activin, another member of TGFβ superfamily, also binds to the same receptors as Nodal and triggers similar intracellular signaling events. Activin A treatment mediates cell transition through mesoendodermal pathway which is associated with the transient expression of Brachyury, N-Cadherin, Wnt3a and FGF4 (Liew, 2010). Wnt3a, also, directly induces Brachyury expression and promotes rapid and highly efficient cellular progression through primitive streak to DE (Mfopou *et al*., 2010).

A large number of studies have addressed differentiation of ESCs into DE (D'Amour *et al*., 2005; Takenaga *et al*., 2007; Sulzbacher *et al*., 2009; Brolén *et al*., 2010; Teo *et al*., 2011). However, a very limited number of studies have focused on differentiation of WJMSCs into DE and its derivatives (Anzalone *et al*., 2010, 2011). The aim of this study was differentiating WJMSCs into DE, which is the first step in *in vivo* differentiation towards insulin-producing cells and hepatocyte-like cells.

MATERIALS AND METHODS

Isolation and culture of WJMSCs

Human umbilical cords were obtained from full-term caesarian patients with their consent. The entire process was done in sterile conditions. Proximal part of the umbilical cords close to the placenta (~15 cm) was cut and squeezed to drain the extra blood and then immersed into the handling medium (0.9% normal saline containing 200 U ml^{-1} penicillin and 200 μg ml^{-1} streptomycin). Samples were transferred to the laboratory on ice and washed twice in PBS and cut into 3-5 cm pieces. Amniotic membrane was carefully separated from Wharton's jelly (WJ) tissue. A longitudinal excision was made along the WJ tissue in order to expose blood vessels and using forceps vessels were separated. The WJ tissue was cut into explant pieces approximately 3-5 mm in size that weighed 10-20 mg. The edges of all explants were carefully trimmed and the explants were washed twice in PBS. For each umbilical cord, about 6 explant pieces were transferred to a 25 cm^2 tissue culture flask and incubated for 5 min in order to achieve a good attachment between the explant pieces and the flask. After incubation, 3 ml of pre-warmed DMEM/HG supplemented with 15% fetal bovine serum (FBS) was added to each flask. Flasks were incubated at 37˚C, 5% CO_2 for 72 hrs. After 72 hrs medium was changed carefully and 5-7 days later, explant buds were observed around pieces and cells were fed with fresh medium for 5 to 7 days following cell budding. The explant pieces were removed and cells were allowed to grow for 3-5 days. At this step cells could be harvested and expanded.

Osteogenic differentiation

Cells at third passage were cultured in a 6-well tissue culture plate containing DMEM-HG medium at a density of 3000 cells cm^{-2}. When cells reached 90% confluency, the medium was replaced with the osteogenic differentiation medium for 21 days. Osteogenic induction medium contains DMEM-HG supplemented with 10% FBS, 10 nM Dexamethazone (Sigma-Aldrich, St. Louis, MO), 10 nM β-glycerophosphate (Sigma-Aldrich, St. Louis, MO) and 0.05 nM L-ascorbic acid-2-phosphates (Sigma-Aldrich, St. Louis, MO). An equal number of cells were cultured in DMEM-HG for 21 days as control. The media were replaced every 4 days in both groups. After 21 days, cells were stained by Alzarin Red S (Sigma-Aldrich, St. Louis, MO) for assessment of calcium deposition and observed under a phase contrast inverted microscope.

Adipogenic differentiation

WJMSCs were cultured in 6-well plates at a density of 3000 cell cm^{-2} in DMEM-HG medium. At 90% confluency, the medium was replaced with adipogenic induction medium for 21 days. Adipogenic medium was composed of DMEM-LG supplemented with 10% FBS, 100 nM Dexamethazone (Sigma-Aldrich, St. Louis, MO), 0.05 nM L-Ascorbic acid 2-phosphate (Sigma-Aldrich, St. Louis, MO) and 50 μg ml^{-1} Indomethacine (Sigma-Aldrich, St. Louis, MO). The adipogenic induction was changed every 3 days. After 3 weeks, the formation of lipid-vacuoles was checked using Oil Red O staining. Briefly, the medium was removed and cells were washed with 2 ml PBS. Cells were fixed using 10% formalin and incubated for 25 min at room temperature. After fixation, formalin was removed and 2 ml of Oil Red O working solution was added to each well for 5 min, then cells were washed and observed using an inverted phase contrast microscope.

Flow cytometry

Mesenchymal stem cells were harvested at third passage and 1×10^6 cells were re-suspended in 2 ml PBS containing 5% FBS. Cells were incubated for 30 min at 4˚C with 5 µl anti-human antibodies against CD105, CD90, CD34 and CD45 in the dark. All antibodies were purchased from eBioscience (San Diego, CA). Cells were centrifuged and resuspended in 1 ml PBS and assessed using a Dako Galaxy flow cytometer and data were analyzed using FlowJo version 8.8.7 software (Treestar, OR).

Differentiation of definitive endoderm

When WJMSCs reached 80% confluency at their third passage, they were induced to differentiate into DE. Six groups were designed and in all groups, cell were cultured for 3 days in RPMI supplemented with 0 (day 1) and 0.2% (days 2-3) FBS. Cells were treated with two concentrations of activin A (20 and 100 ng ml^{-1}) and 25 ng ml^{-1} Wnt3a in four groups (Table 1).

RNA extraction and Reverse Transcription

After differentiation, cells were harvested and total RNA was extracted using Rneasy Mini kit (Qiagen, Valencia, CA). RNA concentration was measured using NanoDrop (Thermo Scientefic, Nanodrop, Wilmington, DE). In order to produce cDNA, oligo(dT) primers were used to prime the reverse transcription reactions and the synthesis was performed using cDNA synthesis kit (Fermentas). Initially, 200 ng RNA, 1 µl oligo (dT) primer (0.5 µg $µl^{-1}$) and nuclease-free water in 12 µl total volume were incubated at 65˚C for 5 min. Secondly, 4 µl ×5 Reaction Buffer, 1 µl Ribolock RNase Inhibitor (20 U $µl^{-1}$), 2 µl 10 mM dNTP Mix and 1 µl Reverse Transcriptase (200 U $µl^{-1}$) were added to a final reaction volume of 20 µl. Samples were incubated for 60 min at 42˚C. The reactions were terminated by incubation at 70˚C for 5 min.

Real-Time PCR

Real-time polymerase chain reaction (RT-PCR) was performed using Applied Biosystems Step One Plus™ Real-Time PCR Systems (Foster City, CA). Specific primers and Power SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA) were used for RT-PCR. The PCR reaction consisted of 7.5 µl of SYBR Green PCR master mix, 0.4 µl of 10 mM forward and reverse primers (listed in Table 2), 3.7 µl of nuclease-free water, and 3 µl of template cDNA (200 ng) in a total volume of 15 µl. Initial enzyme activation was performed at 95˚C for 15 min, followed by 50 cycles of denaturation at 95˚C for 15 s, primer annealing at 60˚C for 30 s and extension at 72˚C for 30 s. Melting curve analysis was performed at 95˚C for 1 min, 60˚C for 30 s and 95˚C for 30 s. Relative gene expression was analyzed using the comparative CT method. Expression level of genes was normalized to the house-keeping gene, GAPDH. ANalysis Of VAriance (ANOVA) was used to examine the statistical significance of the differences observed among the treatments. Statistical analysis was performed using SPSS version 14 software (Chicago, IL).

RESULTS

Isolation, Culture and Characterization of hUCS-MSCs

The first step in our study was the isolation and characterization of MSCs from WJ. Explant technique was used to prevent the damage caused by mechanical and enzymatic methods. After 5-7 days, cell buds were observed around explants. In the following days, the cell morphology was changed from round to monolayer adherent spindle and fibroblast-like cells with a whirlpool-like arrangement. The cells treated with adipogenic induction medium, showed a lot of lipid vacuoles that were stained by Oil Red O (Fig. 1A). When cells cultured in osteogenic induction medium,

TABLE 1. Experimental design for Activin A and Wnt3a treatments

	Day 1: RPMI + 0% FBS	Days 2 and 3: RPMI + 0.2% FBS
Treatment 1	Activin A (20 ng ml^{-1})	Activin A (20 ng ml^{-1})
Treatment 2	Activin A (100 ng ml^{-1})	Activin A (100 ng ml^{-1})
Treatment 3	Activin A (20 ng ml^{-1}) + Wnt3a (25 ng ml^{-1})	Activin A (20 ng ml^{-1})
Treatment 4	Activin A (100 ng ml^{-1}) + Wnt3a (25 ng ml^{-1})	Activin A (100 ng ml^{-1})
Treatment 5	Activin A (100 ng ml^{-1})	Activin A (100 ng ml^{-1}) + Wnt3a (25 ng ml^{-1})
Treatment 6	Activin A (100 ng ml^{-1}) + Wnt3a (25 ng ml^{-1})	Activin A (100 ng ml^{-1}) + Wnt3a (25 ng ml^{-1})
Treatment 7 control	–	–

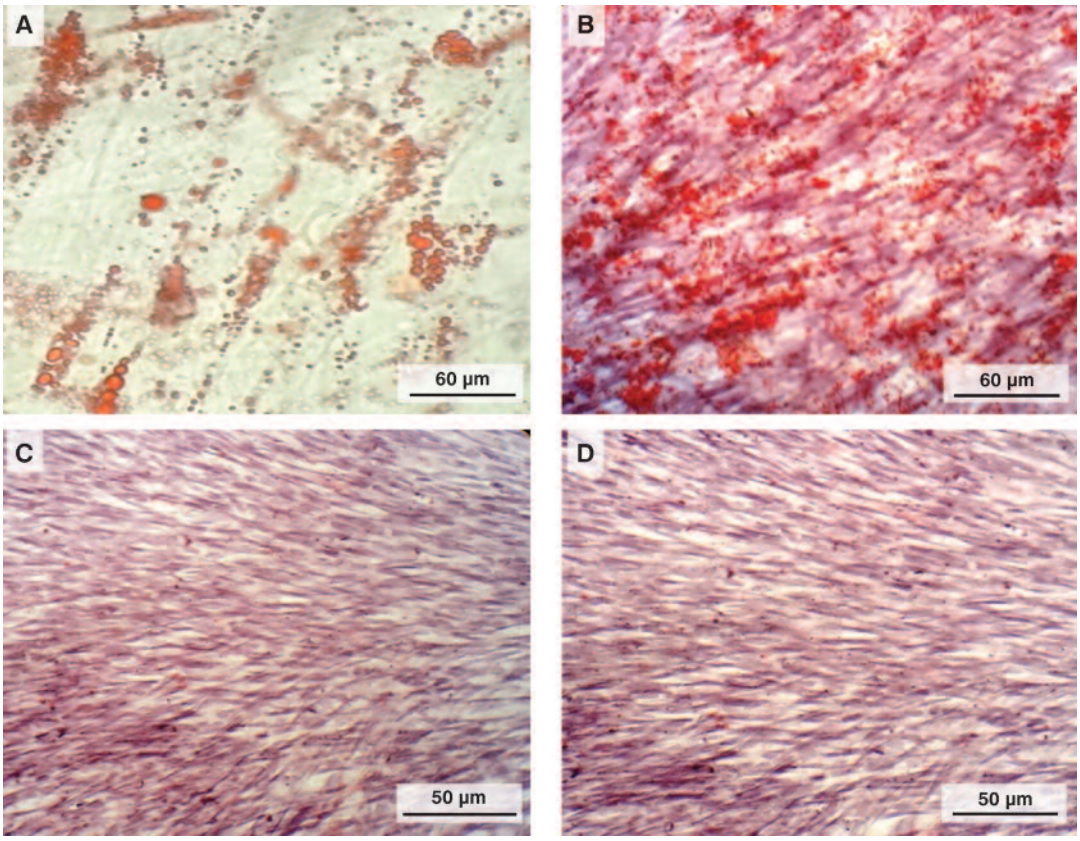

FIG. 1. Differentiation of WJMSCs into adipocytes and osteoblasts. After 21 days lipid droplets were formed in adipogenic medium and stained with Oil Red O staining (A) and calcium deposition was stained with Alizarin Red S staining (B) in osteogenic medium. Non-treated cells were stained as control (C & D).

deposition of calcium in the cells that were stained red by Alizarin Red staining was observed (Fig. 1B). Moreover, flow cytometric analysis of WJMSCs indicated high level expression of specific markers for MSCs, such as CD105 and CD90, but not expression of hematopoietic markers (CD34 and CD45) (Fig. 2).

Differentiation of WJMSCs to definitive endoderm

The first step in differentiation of pluripotent cells into pancreatic cells, similarly to *in vivo* condition, is development of definitive endodermal cells (D'Amour *et al*., 2006; Kroon *et al*., 2008; Sulzbacher *et al*., 2009). Nodal and Wnt signals are the major inducing factors in human and mouse cells that induce this differentiation. Several studies have used activin A to mimic the role of nodal. In addition, initial treatment with Wnt3a efficiently enhances DE induction of endoderm into transient mesoderm progenitor population.

In this study, we evaluated the effect of activin A in DE induction of WJMSCs by analyzing vertebrate DE markers. We applied low and high concentrations of activin A (20 ng ml^{-1} and 100 ng ml^{-1}) with or without Wnt3a. Additionally, the effect of simultaneous use of Wnt3a and activin A and the order of Wnt3a treatment was studied. The duration of activin A treatment was kept the same (3 days) in all treatments (Table 1). On the second day of activin A treatment, cell aggregates were observed (Fig. 3). At this stage, cells were shorter and their proliferation rate was lower compared with untreated control (Fig. 3).

The highest expression of *Sox17* was seen in treatment 3 (3.25 fold increase) which was significantly higher than that of all the other treatments, but treatment 5. *Sox17* expression was also significantly higher ($p < 0.05$) in treatments 1, 4, 5 and 6 than the untreated control (7) (Fig. 4A).

Treatments 3, 4 and 6 showed the highest expression of *FoxA2* (1.75, 1.68, and 1.62 fold increase, respectively) which was significantly higher than that of the untreated control (7) and treatment 1 ($p < 0.05$). Among them, treatment 3 expressed *FoxA2* significantly higher than that of treatments 2 and 5 (Fig. 4B).

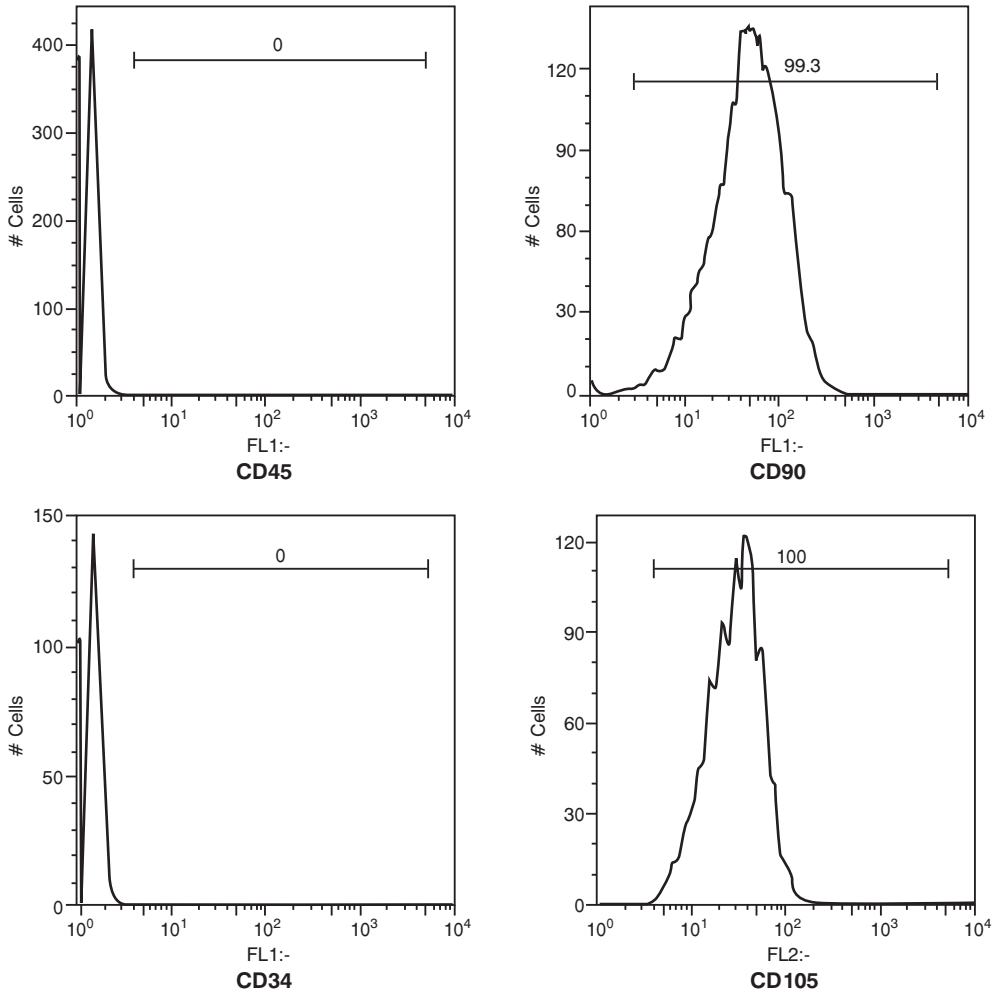

FIG. 2. Flow cytometric analysis of WJ-MSC surface antigens. Cells were cultured in DMEM-HG supplemented with 10% FBS and were analyzed by flow cytometry after three passages. Cells express CD90 and CD105 but do not express CD34 and CD45 (hematopoietic markers).

The highest expression of *Cxcr4* was observed in treatment 3 (3.1 fold increase compared with the untreated control) which was statistically significant compared to treatments 1, 2 and 7 (Fig. 4C). Expression of *Cxcr4* in treatment 4, 5 and 6 was also elevated but no significant difference was observed among them ($p > 0.05$).

Treatment 3 showed the highest expression of *Cer* (2.95 fold increases compared with the untreated control) which was significantly higher ($p < 0.05$) than that of treatments 2, 5 and 7 (Fig. 4D).

DISCUSSION

The aim of this study was differentiating WJMSCs into DE as a critical step in generating DE derivatives such as insulin-producing cells and hepatocyte-like cells. Several studies have demonstrated the feasibility of generating DE from ESCs (Kubo *et al.*, 2004; D'Amour *et al*., 2005; Takenaga et al., 2007; Chayosumrit *et al*., 2010). D'Amour *et al*. (2005) showed that using high concentrations of activin A with Wnt3a, and low concentrations of serum, a highly enriched definitive endoderm can be achieved. They developed a five-step protocol and differentiated ESCs into definitive endoderm, primitive gut tube, posterior foregut, pancreatic endoderm and endocrine hormone-expressing cells.

In spite of promising potential of WJMSCs, only a limited number of studies have been performed on the differentiation of these cells into insulin-producing cells (Chao *et al.*, 2008; Wu *et al*., 2009; Wang *et al*., 2011a, b; Tsai *et al*., 2012). None of these protocols has been designed based on a series of endodermal intermediates similar to what occurs during pan-

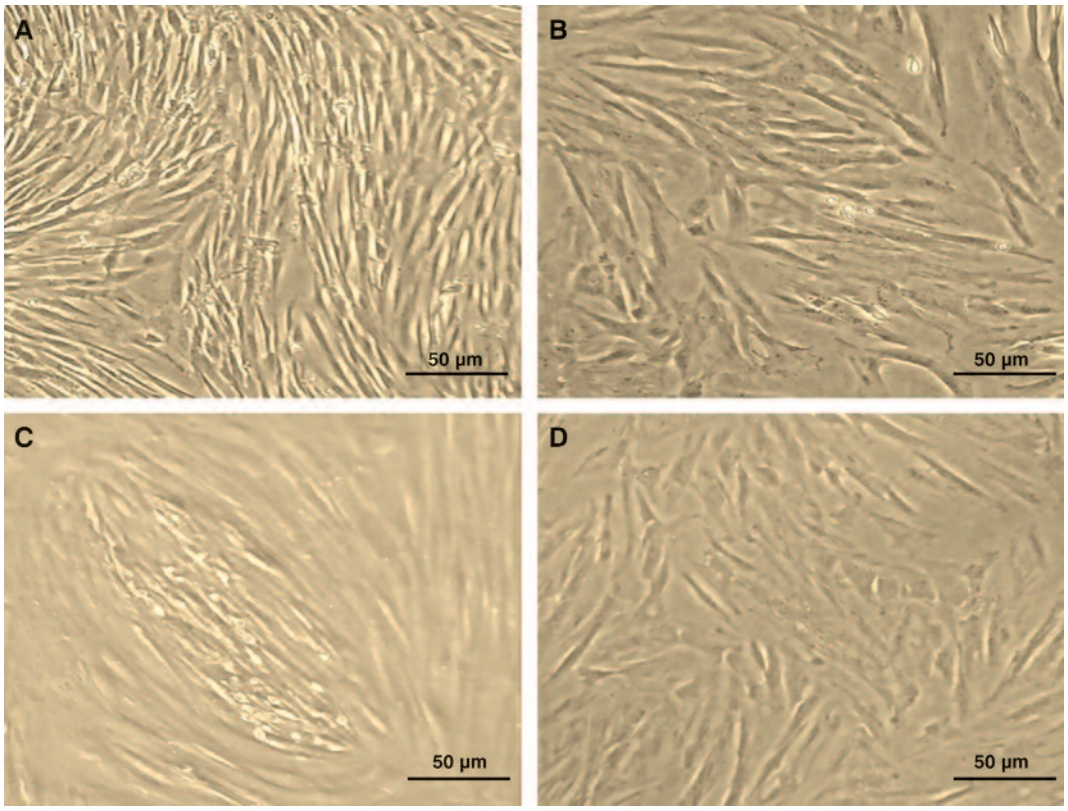

FIG. 3. Morphology of WJMSCs during differentiation to DE represented as phase-contrast images. (A) untreated control; (B) First day of treatment; (C) second day of treatment; (D) third day of treatment.

creatic development *in vivo*. However, designing a protocol based on *in vivo* condition may be the appropriate physiological pathway to differentiate WJMSCs into insulin-producing cells.

In this study, different treatments were tested to induce differentiation of WJMSCs into DE *in vitro*. We characterized the differentiation process at the RNA level using real-time PCR. Expression of *Sox17* and *Cxcr4* were assessed as DE markers and *FoxA2* and *Cer* were assessed as anterior DE markers. The phosphoinositol 3-kinase (PI-3-kinase) signaling pathway is activated in the presence of IGF and insulin.

TABLE 2. Primers used for real-time PCR

Gene	Primer	Sequence (5′-3′)	PCR lengh
GAPDH (NM_002046.4)	*GAPDH*-F TG *GAPDH*-R	GTATCGTGGAAGGACTCA CCTGCTTCACCACCTTCTTG	290 bp
Fox A2 (NM_153675.2)	*FoxA2*-F *FoxA2*-R	AATGGACCTCAAGGCCTACGAACA AGTTCATAATGGGCCGGGAGTACA	170 bp
Sox 17 (NM_022454.3)	*Sox17*-F *Sox17*-R	TGGACCGCACGGAATTTGAACA TTGCAGTAATATACCGCGGAGCTG	157 bp
Cer (NM_005454.2)	*Cer*-F *Cer*-R	TTGCTTTGGGAAATGCGGGTCT AGTTCAGTGGCAAGTGCATCGT	113 bp
Cxcr4 (NM_001008540.1)	*Cxcr4*-F *Cxcr4*-R	ACTGGCATTGTGGGCAATGGAT GAAACAGGGTTCCTTCATGG	105 bp

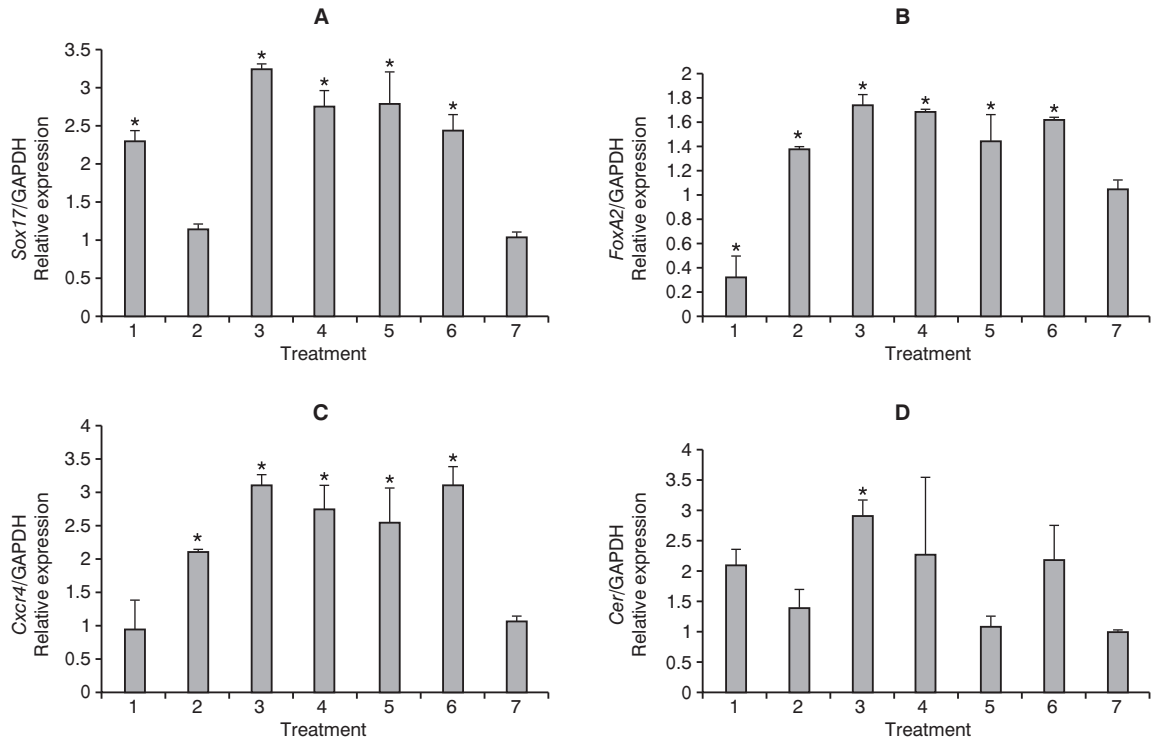

FIG. 4. RT-PCR analysis of the relative expression of 4 key genes during a 3 day treatment toward definitive endoderm differentiation. Treatments are the same as Table 1. (A) Relative expression of *Sox17*. All treatments, except treatment 2, were statistically significant compared to the control (7). (B) Relative expression of *FoxA2*. All treatments were significantly higher than control, except for treatment 1 which was significantly lower than the control. (C) Relative expression of *Cxcr4*. All treatment differed significantly when compared with the control (except treatment 1). (D) Relative expression of *Cer*. Only treatment 3 was statistically significant different when compared to the control.

Activation of this signaling pathway maintains cells in self-renewing state. To remove the self-renewing state, during the initial stage of WJMSC differentiation, PI-3-kinase signaling must be inhibited (Tsaniras & Jones, 2010). For this reason, we used serum-free medium. As shown in Figure 4, although treating cells at low concentration of activin A (treatment 1) induced the expression of *Sox17* and *Cer*, it had no significant effect on *FoxA2* and *Cxcr4*. At high concentration of activin A (treatment 2), *FoxA2* and *Cxcr4* were up-regulated but expression of *Sox17* and *Cer* did not increase compared to the untreated control. Comparison of Figure 4A-D suggests that activin A (20 ng ml^{-1}) application with Wnt3A (25 ng ml^{-1}), treatment 3, significantly up-regulates the expression of all the tested markers. Although comparison of treatments 4, 5 and 6 implicated that the tested markers were up-regulated to some extent, this up-regulation was not always observed for all markers and it was not always as significant as treatment 3.

We found that time and duration of Wnt3a application had no significant effect on WJMSCs differentiation. Regardless of time and duration, the presence of Wnt3a, compared with the treatments that only had activin A, induced upregulation of DE markers.

In conclusion, previous reports on human and mouse ES cells indicated that high concentrations of activin A (50-100 ng ml^{-1}) in the absence or at very low concentrations of serum promote effective DE induction (Kubo *et al*., 2004; Shim *et al*., 2007). In addition, treatment with low activin A concentrations (5-20 ng ml^{-1}) was sufficient to maintain human ES cells in pluripotent state (Vallier *et al*., 2005). Our results suggest that in WJMSCs, lower concentrations of activin A with Wnt3a up-regulate DE markers to a higher extent and more effective than high concentration. According to our results, the most efficient treatment in the induction of WJMSCs differentiation was the application of 20 ng ml^{-1} activin A and 25 ng ml^{-1} Wnt3a for 24 hrs and removal of Wnt3a and extending the activin A treatment for 48 hrs. This treatment could be used in future studies as the first step in WJMSCs differentiation into insulin-producing cells for regenerative cell therapies.

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