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Full Length Research Paper

Multipotent mesenchymal stem cells (MSCs) from human umbilical cord: Potential differentiation of germ cells

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Previous controversy exists as to whether umbilical cord (UC) can serve as a source of multipotent mesenchymal stem cells (MSCs) with their characteristics. Different methods and reagents have been used to induce the differentiation of UC-MSCs into functional cells. We investigated the isolation of UC-MSCs and their potential differentiation into neurons, cardiomyocytes and germ cells *in vitro*. The phenotypes, proliferation potential and markers of UC-MSCs were analysed by growth curves, RT-PCR and immunofluorescence, respectively. Then the cells were induced into neurons, cardiomyocytes and germ cells besides osteoblasts and adipocytes. Here, we report to obtain single cell-derived, clonally expanded MSCs that are of multilineage differentiation potential. The immunophenotype of these cells is consistent with those reported in bone marrow MSCs and embryonic stem cells (ESCs). Surprisingly, these cells can differentiate into cardiomyocytes, neural cells, even germ cells besides osteoblasts and adipocytes under appropriate induction conditions. Thus, these cells may be multipotent MSCs as evidenced by their ability to differentiate into cell types of all three germ layers. These cells may serve as an alternative source of MSCs to bone marrow and this will provide us a model to study the mechanism of cardiomyocyte, neural cell, even germ cell differentiation and new strategies for the therapy of infertility and sterility.

Key words: Multipotent, mesenchymal stem cells (MSCs), umbilical cord (UC), cardiomyocyte, neural cells; germ cells.

INTRODUCTION

Mesenchymal stem cells (MSCs) derived from bone marrow are well-characterized population of adult stem cells, which can form a variety of cell types, including fat cells, cartilage cells, bone cells, tendon cells and ligaments cells, muscles cells, skin cells and even nerve cells (Choong et al., 2007; Pittenger et al., 1999; Jiang et al., 2002). Bone marrow transplant has been used for a long time to treat leukemia and many types of cancer, as well as various blood disorders. Bone marrow contains a promising source of multipotent stem cells-mesenchymal stem cells (MSCs) and hematopoietic stem cells (HSCs) (Pittenger et al., 1999). Recently, it has been reported that murine MSCs derived bone marrow may trans-differentiate into gametes (sperms or follicles) *in vivo* and *in vitro*. These cells share genes typical of germ cells and proposed that bone marrow stem cells can migrate and colonize the ovaries to maintain a plentiful stock for reproduction and may differentiate into sperms in mice (Johnson et al., 2005; Nayernia et al., 2006). However, aspirating bone marrow from the donor is invasive, and especially, the differentiating potential and the number of MSCs derived from bone marrow decreases gradually with age (Lee et al., 2004). Therefore, many scientists have been looking for alternative sources of MSCs and

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found that umbilical cord blood (UCB) and UC maybe an excellent alternative source of bone marrow stem cells because these cells are younger than other adult stem cells (Barachini et al., 2009; Lee et al., 2004; Secco et al., 2008). Importantly, UC stem cell transplants are less prone to rejection issue than either bone marrow or peripheral blood stem cells and these 'waste' stem cells could be cryopreserved and stored in a stem cell bank for the donor, his families and others; What's more, these young stem cells have not yet developed the features that can be recognized and attacked by the recipient's immune system. Also, UC lacks well-developed immune cellswith less chances that the transplanted cells will attack the recipient's body, a problem called graft versus host disease (Liu et al., 2010). Both the versatility and availability of UC stem cells makes them a potent resource for transplant therapies (Kumar et al., 2006 ; Cutler et al., 2010).

UCB transplantation has been used in clinical practice for many years (Harris. 2008). Recently, scientists have shown that multipotent MSCs were obtained from UC and these MSC like cells have the ability to differentiate into multilineages including bone, adipocytes, osteoblasts and hepatocyte-like cells, and endothelial cells (Flynn et al., 2007; Lee et al., 2004; Secco et al., 2008; Zhang et al., 2009; Xu et al., 2010; Yoo et al., 2010). However, controversy exists as to whether UC contains real multipotent MSCs, which are capable of differentiating into cells of three different connective tissue lineages such as bone, cartilage, and adipose tissues, being the best candidates for tissue engineering of musculoskeletal tissues, and even differentiating into cardiomyocytes, neuron-like cells and hepatocytes (Lee et al., 2004; Kang et al., 2006; Orlandi et al., 2008; Secco et al., 2009). However, so far, no progress and evidences have been reported in the isolation and characterization of MSCs from UC to differentiate into functional germ cells. Therefore, the aim of this study is to investigate the possibility of obtaining clonally expanded MSCs that have the potential for nearly pluripotent differentiation including potential differentiation into germ cells.

MATERIALS AND METHODS

Culture of MSCs derived from UC

The MSCs derived from male term (post-natal) babies UC were provided by North Branch Bio-Technology Co., Ltd of Jiangsu (http://www.stemcellsbank.com.cn, Taizhou, Jiangsu Province, China). The cells were plated in coated tissue culture T75 flasks (Becton Dickinson) in StemPro® MSC SFM (Invitrogen) medium. The cell density was 1×10^5/ml. The cells were allowed to adhere for 2 days and non-adherent cells were washed out with medium changes. The media were used to initiate growth of the adherent UC-MSCs: DMEM/F12 (Invitrogen) with 10% fetal bovine serum (FBS, Hyclone), supplemented with 0.1 mM 2-mercaptoethanol (Invitrogen), penicillin (100 U/ml; Sigma), streptomycin (0.1 mg/ml; Sigma), and 2 mM glutamine (Invitrogen). Expansion of the cells was performed in the same media. Cells were incubated at 37°C, 5% CO_2 in a humidified atmosphere. When cells reached 80% confluency, they were detached with 0.125% trypsin (Invitrogen), centrifugated at 1500 rpm for 5 min, and replated at the ratio of 1:3 under the same culture conditions. The medium was first changed at 24 h after plating, and then changed at every other day.

To obtain single cell-derived, clonally expanded MSCs, the isolated plate-adhering fifth passage cells, were serially diluted and plated on to 96-well plates at a final density of 10 cells per well. Colonies that grew were cultured and tested for their differentiation potential. Separated fibroblast like colonies termed CFU-Fs were identified at a mean interval of 1 to 3 weeks after initial plating. To study mesenchymal cells obtained from an individual post-natal fetus, all colonies growing in a 60 mm plate were trypsinized and obtained. The adherent stromal bone-marrow fibroblasts like (CFU-F like colonies) were fixed with methanol and stained with Wright-Giemsa staining. Also the growth curve of seventh and ninth passage UC-MSCs were evaluated respectively.

RNA isolation and RT-PCR

RNA was extracted from 30×10^5 UC-MSCs and induced differentiated cells using Trizol (Qiagen, Beijing) according to the manufacturer's instructions. The mRNA was reverse transcribed to cDNA using Advantage RT-for-PCR (Takara, Dalian) based on the manufacturer's instructions. cDNA was amplified using a ABI GeneAmp PCR System 2400 (Takara, Dalian) at 94°C for 40 s, 56°C for 50 s, and 72°C for 60 s for 35 cycles, after initial denaturation at 94°C for 5 min. Semi-quantitative RT-PCR was carried out with 0.5 µl cDNA, 30 pmol each of forward and reverse primers and 2 units Platinum Taq polymerase (Takara,Dalian) in a final volume of 15 µl. The solution was incubated at 94°C for 2 min and then subjected to 35 cycles of amplification, each consisting of 95°C for 30 s, 52 to 58°C for 30 to 45 s (annealing) and 72°C for 60 s (primer extension). At the end of the temperature cycles the solution was incubated at 72°C for 10 min. The PCR products were subjected to electrophoresis on 1.0% (w/v) agarose gels containing 1 mg/ml ethidium bromide and the products were viewed and photographed under UV light. β-actin was used as an internal control. The primers used for RT-PCR analyses are shown in Table 1. Primers were designed to span exons to distinguish cDNA from genomic DNA products.

Flow cytometry analysis

For cell surface antigen phenotyping, fifth- to seventh-passage cells were detached and stained with fluorescein- or phycoerythrin-coupled antibodies and analyzed with FACS Calibur (Becton Dickinson). Cells were treated with TrypLE (Millipore, USA), harvested, and washed twice with culture medium. Before staining, cells were allowed to recover for 20 min in suspension. Cell staining was performed using mouse monoclonal antibodies followed by fuorescein isothiocyanate (FITC)-conjugated affinity-purifed mouse fuorochrome-conjugated isotype control antibodies, or FITC or phyco-erythrin (PE)-coupled antibodies against the common leukocyte antigen CD45 (Becton Dickinson, USA), the surface-expressed 5′-ectonucleotidase CD71 (Becton Dickinson), the β1-integrin CD29 (Becton Dickinson), CD73 (Becton Dickinson); CD105 (Becton Dickinson); CD11a (Becton Dickinson), CD90 (Becton Dickinson), CD166 (Becton Dickinson), CD117 (Abcam, UK), CD34 (Becton Dickinson) and CD44 (Becton Dickinson). All antibodies were used following the manufacturers' instructions. Binding of antibodies against the markers as primary antibodies was detected by anti-mouse immunoglobulin G (IgG) conjugate (Becton Dickinson), or isotype-specifc FITC- or PE-conjugated goat

Table 1. The primer sequences.

Gene	Forward primer	Reverse primer		Product size (bp)
Sox2	GCCCAGGAGAACCCCAAGAT	GGGTGCCCTGCTGCGAGTA	58	520
Oct4	GAAGCTGGACAAGGAGAAGCT	CATGCTCTCCAGGTTGCCTC	58	379
hTERT	GTGTGCTGCAGCTCCCATTTC	GCTGCGT CTGGGCTGTCC	58	264
Nanog	GCGAATCTTCACCAATG	TTTCTGCCACCTCTTAC	54	407
B-ACTIN	GCGGCATCCACGAAACTAC	TGATCTCCTTCTGCATCCTGTC	58	138
GATA4	TCCCTCTTCCCTCCTCAAATTC	TCAGCGTGTAAAGGCATCTG	54	193
Nkx2.5	AGCACTTCTCCGCTCACTTC	CCGTGCACAGAGTGGTACTG	60	232
Dazl	ATGAAAGATAAAACCACCAACC	TGTTGACAGCCTGGTCCACTGA	58	391
Stella	TCCCTCTTCCCTCCTCAAATTC	TCAGCGTGTAAAGGCATCTG	60	238
SCP3	CTAGAATTGTTCAGAGCCAGAG	GTTCAAGTTCTTTCTTCAAAG	60	247

anti-mouse IgG F(ab')2 fragments (Becton Dickinson). Results were analysed based on the mean percentage of positive cells and standard deviation from multiple experiments.

Spontaneous differentiation i*n vitro*

For the formation of EBs, 7th- to 15th-passage UC-MSCs were collected from the culture dish, dissociated by Tryple into single cells and resuspended at 3×10^5 cells/ml, in culture medium DMEM consisting of 10% FCS (Hyclone), 0.1 mM 2-mercaptoethanol (Sigma), 2 mM glutamine (Gibco) for 3 days. Then the cells were transferred into normal sterile culture dishes for 7 to 10 days. Cultures were observed each day and the three germ layer markers: NESTIN (ectoderm), BRACHUARY (mesoderm) and AFP (endoderm) were analysed by RT-PCR at 7 day EBs.

Generation of osteoblasts

11th- to 13th-passage cells were treated with osteogenic medium for 3 weeks with medium changes twice weekly. Osteogenesis was assessed at weekly intervals. Osteogenic medium consists of DMEM supplemented with 10^{-8} M dexamethasone (Sigma-Aldrich, St Louis, MO), 10 mM β-glycerol phosphate (Sigma-Aldrich), and 50 µg/ml ascorbic acid (Sigma-Aldrich). The potentiality of osteoblast differentiation was evaluated by Alizarin-red S staining. Briefly, cells were fixed with 4% paraformaldehyde (PFA) and stained with 1% Alizarin-red S (Sigma-Aldrich) solution in water for 10 min.

Generation of adipocytes

11th-to 13th-passage cells were treated with adipogenic medium for 1 to 3 weeks. Medium changes were carried out twice weekly and adipogenesis was assessed at weekly intervals. Adipogenic medium consists of DMEM supplemented with 0.25 mM 3-isobutyl-1-methylxanthine (IBMX; Sigma-Aldrich), 10^{-8} M dexamethasone (Sigma-Aldrich, St Louis, MO), 5 µg/ml insulin (Sigma-Aldrich), and 10% FBS (Hyclone). Adipocyte differentiation was tested by oil-red O staining. Cells were fixed with 4% PFA and stained with oil-red O (Sigma-Aldrich) for 10 min.

Generation of neural cells

5th- to 7th-passage cells, seeded at a density of 3000 cells/cm^2, were treated with neural stem cell medium including 20 ng/ml EGF (Chemicon), 10 ng/ml bFGF (Millipore), B27 (Invitrogen) and 1% Insulin-transferrin-Selenium (ITS, Invitrogen) in DMEM/F12 (Invitrogen) for 2 weeks. The neural cells were identified by morphology and immunohistochemistry with neuron specific enolase (NSE, 1:200, Millipore) and β-tubulin Ⅲ (1:1000, DSHB) antibodies respectively. Some induced samples were evaluated by aniline blue staining. Cells were fixed in 4% PFA for 30 min at room temperature, then stained with 5% w/v aniline blue in PBS (pH 3.5). Each slide was then washed with PBS, counted and examined under the inverted microscope at 200 magnification.

Generation of cardiomyocytes

After proliferating to nearly a layer, these cells were induced by 10^{-7} M RA (Sigma) and 0.75% DMSO (Sigma) in DMEM with 10% FBS, or treated with 10 µM 5-azacytidine (5-aza, Sigma, St. Louis) for 48 h, and then were induced by 10% FBS in DMEM. The induced cardiomyocytes were identified by morphology, immune-histochemistry with human cardiac a-actin (1:500, Sigma), CT3 (1:1000, DSHB), Islet1 (1:1000, DSHB) antibodies respectively. Also the induction efficiencies were evaluated by cardiac specific markers (cardiac α-actin, GATA4 and Nkx2.5) based on RT-PCR. The primers are shown in Table 1.

Potential differentiation of germ cells

The UC-MSCs were used for germ cell differentiation by using with 2×10^{-6} M RA and 10 ng/ml BMP4 in DMEM for 7-14 d (Danner et al., 2007). The induced cells were formed and identified by morphology and immunohistochemistry with VASA (1:1000, Abcam) and SCP3 (1:300, Santa Cruz) antibodies respectively, which are markers of germ cells, sperms or oocytes (Clark et al., 2004; Hua et al., 2009; Lacham-kaplan et al, 2006; Toyooka et al., 2003).The differentiation efficiencies were evaluated by expression of specific markers of germ cells (Dazl,Stella and SCP3) based on RT-PCR. The primers are shown in Table 1. Induced samples were evaluated by alkaline phosphatase (AP) described (Piedrahita et al., 1998). Briefly, culture plates were rinsed three times with PBS and fixed in 4% PFA for 10 min at room temperature. Fixed cells were washed three times with PBS and stained in naphtol AS-MX phosphate (200 µg/ml, Sigma) and Fast Red TR salt (1 mg/ml, Sigma) in 100 mM tris-buffer (pH 8.2) for 10 to 30 min at room temperature. Staining was terminated by washing cultures in PBS to evaluate the characteristics and count the number of AP positive cells or colonies.

Immunofluorescence

For staining of intracellular proteins, cells were fixed overnight with 4% PFA for 15 min at 4℃ and permeabilized with 0.1% Triton X-100 (Sigma-Aldrich) for 10 min. Slides and dishes were incubated with the mouse or rabbit primary antibodies against human Oct4 (1:500, Chemicon), Sox2 (1:200, Chemicon), Klf4 (1:200, Chemicon), C-myc (1:200, Chemicon), SSEA4 (1:200, Chemicon) and hTERT (1:100, Santa Cruz) for 1 hour at room temperature or overnight at 4℃, followed by fluorescein- or phycoerythrin-coupled goat anti-mouse or rabbit IgG secondary antibodies (1:500, Invitrogen) for 1 h. Between incubations, slides and dishes were washed with PBS. The nuclei of cells were counterstained with Hoechst33342, and then observed and analysed with Leica microscope.

RESULTS

UC-MSCs were generated from 98% of UC and the mean number of MSCs was 2.11×10^5/g in North Branch Bio-Technology Co., Ltd of Jiangsu Province (Taizhou, Jiangsu Province, China). The primary cells reached 60 to 80% confluence at 4 to 9 day. The individual UC-MSCs appeared as spindle-shaped-like fibroblasts and the cells were unique in their phenotypes and assumed a mono-layer configuration on reaching confluency during culture (Figure 1A). Giemsa staining of UC-MSCs at passage 5 to 8 indicated that most of the cells were of mononuclear fibroblast morphology and CFU-F colonies (Figures 1B to C). Overgrown confluent mononuclear cells in culture formed colonies (Figures 1D and E). Normally, these UC-MSCs were negative for AP staining (Figure 1F). The cell growth curve assay showed that these cells have the characteristics of rapid proliferation *in vitro*, and multiplied nearly 20 folds in 8 days (Figure 1G), with the mean population doubling (PD) time being 76.8±8.4 h (P0), 72.8±5.4 h (P2), 38.8 h (P7). These cells proli-ferated up to passage 20 and maintained normal MSC phenotypes. The mean clonal capacity of these cells was 15±5%. RT-PCR and immunofluoresce staining demon-strated that these cells expressed Oct4, Sox2, Klf4, Nanog, C-myc, SSEA4 and hTERT (Figures 1H and 2).

Immunophenotypic characterization of UC-derived fibroblast-like MSCs were extensively expanded, and characterization by flow cytometry revealed that the cells isolated by the described method were negative for CD71, CD34, CD45 (leukocyte common antigen), and CD117 (C-KIT), indicating these cells are not of hemato-poietic origin (Figure 3). UC-derived cells were found to be showed strong positive homogeneous staining for markers of mesenchymal progenitors at different passages (P1, P3, P4 and P6, Table 2).

These markers included being positive for integrins CD29 (β1-integrin), matrix receptors CD44 (hyaluronate receptor), CD90 (Thy-1) and CD105 (endoglin), CD73, CD166, and were negative for hematopoietic origin markers: CD34, CD14, CD71, CD45 and HLA-DR at different passages, which are consistent with the findings for bone marrow and cord blood MSCs (Yu et al., 2004; Rebelatto et al., 2008).

In vitro differentiation of osteoblasts and adipocytes from UC-derived MSCs

To investigate the osteogenic potential of the UC-derived cells, 11th-to 13th-passage cells were plated at a density of 2×10^3 cells/cm^2 and cultured under conditions appro-priate for inducing differentiation for each lineage. When induced to differentiate under osteogenic conditions, the spindle shape of UC-derived cells become flattened, broadened, and aggregated with increasing time of induction and formed mineralized matrix as evidenced by Alizarin red staining (Figure 4A and B). The mean percentage of Alizarin red staining was significantly higher in induced cultures than that untreated cells at 14 days (65±8% vs 10±5%, P<0.01%).

To assess the adipogenic potential, 11th to 13th-passage cells were plated at a density of 2×10^3cells/cm^2 and cultured in adipogenic medium. Morphologic changes in cells as well as the formation of neutral lipid vacuoles were noticeable as early as 1 week after induction and visualized by staining with oil-red O. The mean percentage of oil-red O staining was significantly higher in induced cultures than that untreated cells at 14 days (60±10% vs 10±5%, P<0.01%) (Figures 4C and D).

In vitro differentiation of neural and cardiomyocyte-like cells from UC-derived MSCs

Using the neural cell induction method, 5th- to 7 th-passage UC-derived cells were seeded at a density of 3×10^3 cells/cm^2 and tested for their neural differentiation potential. After 10 days of differentiation, 50% of cells in the plate acquired the morphology of neuroglial cells exhibiting a refractile cell body with extended neurite-like structures, 30% of cells had partially acquired the mor-phology of neural-like cells, and 10% of cells exhibited morphology resembling those of restricted precursors of the neuroectodermal lineage. 20% of cells stained positive for immunofluorescence assay against β-tubulin Ⅲ and NSE (Figures 5A, B, C) at 6 days after differentiation and increased to approximately 50% by day 10. Also, 80% induced cells were positive for aniline blue staining (Figure 5D).

To determine whether UC-derived cells can differen-tiate into cardiomyocyte-like cells *in vitro*, cells were allowed to grow to 60% confluence prior to induction. The cuboidal or multinucleus morphology of cardiomyocyte-like cells were observed as early as 10 days after culturing under cardiac conditions in the presence of 5-AZA. The expression of cardiac cells associated genes: cardiac β-actin, Islet1 and CT3 were detected at the indicated time points analysed by immunofluorescent

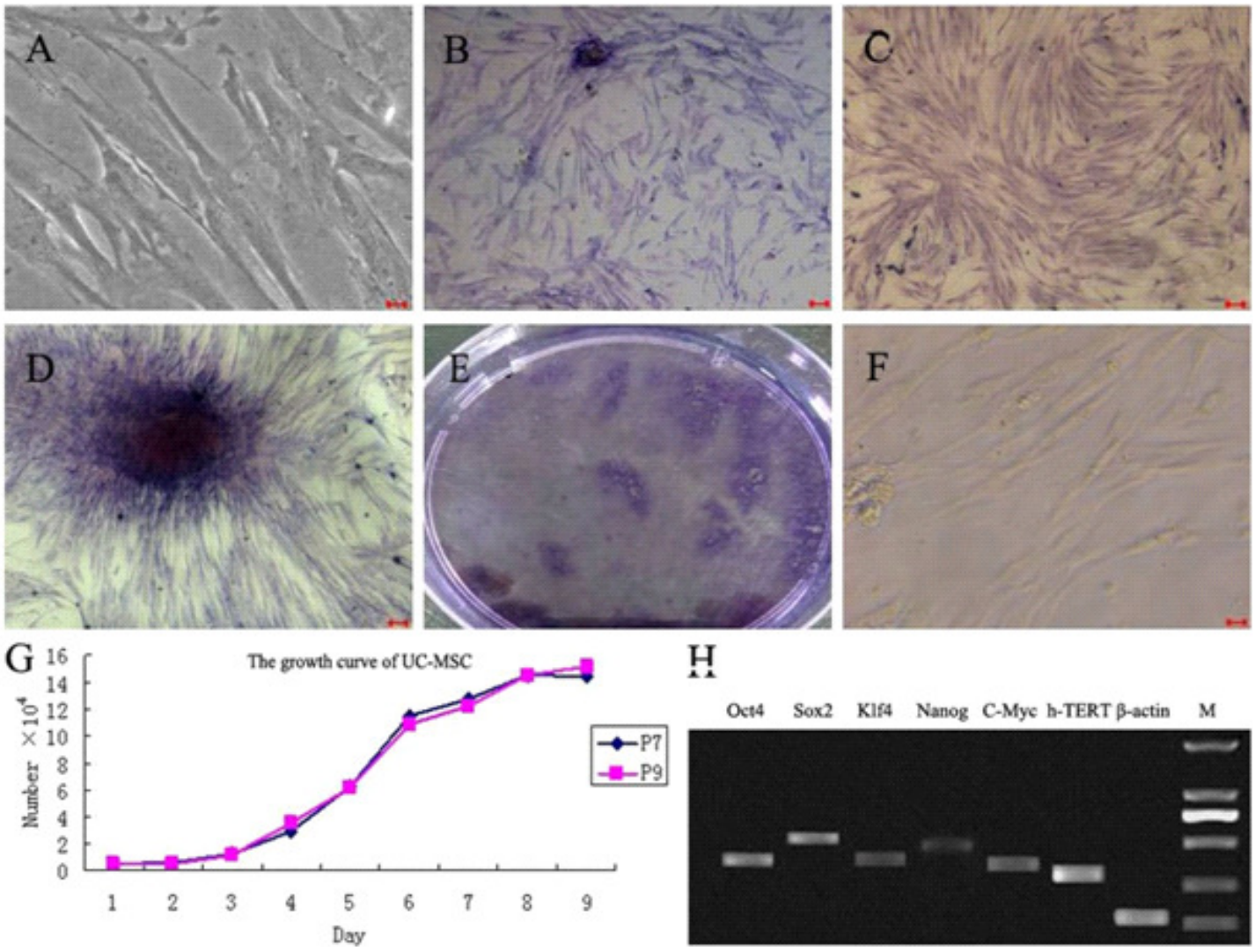

Figure 1. Characterization of UC-MSCs. The spindle fibroblast like UC-MSCs (A), Bar=50 μm; Giemsa staining showed that UC-MSCs were fibroblast and mononuclear and CFU-Fs were formed (B, C), Bar=100 μm; UC-MSCs formed compact colonies identified with Giemsa staining (D,E), Bar=100 μm. UC-MSCs were negative for AP staining (F), Bar=100 μm. G, The growth curve of UC-MSCs at Passage 7 and 9. H, Specific pluripotent markers of MSCs were analysed by RT-PCR (Oct4, Sox2, Klf4, Nanog, C-myc and hTERT,β-actin was used as internal control).

staining (Figure 6). Expressions of cardiac specific markers-Nkx2-5 and cardiac β-actin were up-regulated induced by 5-aza or RA in combination with DMSO compared with the untreated group based on semi-quantitative RT-PCR. However, GATA4 was only increased in 5-AZA induced cells. Under cardiac conditions expression of β -actin, an early developmental marker gene of cardiomyocytes, was detectable by day 7 and remained detectable up to day 15.The expression of CT3, a late marker gene of cardiomyocytes, was detected by day 14 and increased with time of differentiation. Undifferentiated cells did not express cardiac a-actin or CT3. These results demonstrated that human UC-MSCs may differentiate into cardiomyocytes in our induced cultures (Martin-Rendon et al., 2008).

In vitro differentiation of germ cells from UC-derived MSCs

To investigate the potential of UB-MSC for differentiation into germ cells, the cells were treated with 2×10^{-6} M RA. It was found that there were some round cells and a small number of germ cells formed in the treated cultures. The expression of the meiotic and germ cell markers SCP3 and VASA increased in RA-treated cultures compared with the untreated group based on semi-quantitative RT-PCR and immunofluorescent staining. Some round and spindle-shaped cells derived after the treatment of UC-MSCs with RA showed expressions of specific markers such as SCP3 and VASA by immunohistochemical analysis (Figure 7). After RA

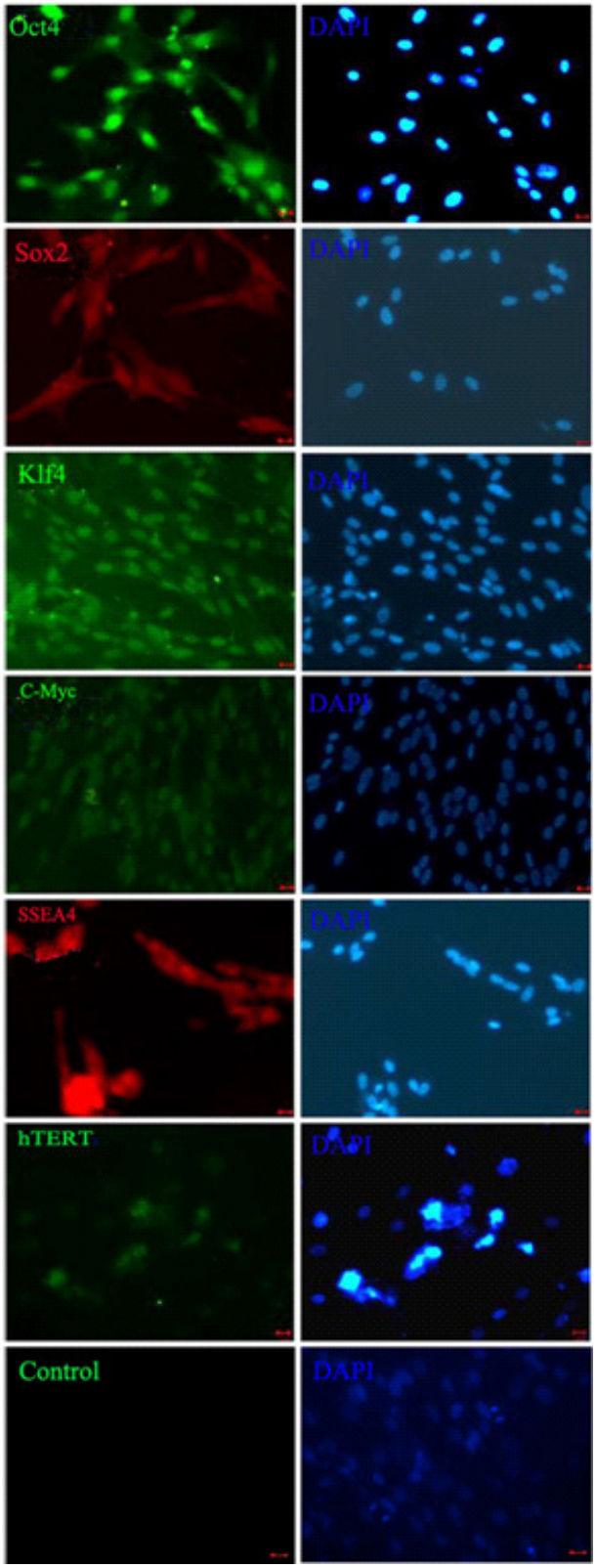

Figure 2. Immunofluorescence analysis of UC-MSCs. UC-MSCs were positive for pluripotent ESC markers:Oct4, Sox2, Klf4; C-myc; SSEA4 and hTERT; the right column were the Hoechst33342 nuclear staining for the left column, Control (Negative control). Bar=20 μm.

treatment, an increase in the number of cells which expressed VASA was detected compared with untreated cells (20±5% vs 8±5%, P<0.01%). However, no typical spermatid-like cells were observed in RA treated and it has not yet been determined whether these male germ cells can enter meiosis and form functional gametes. These results indicated that small subpopulations of UC-MSCs are able to differentiate into germ cells.

DISCUSSION

The morphology of these cells from UC resembles that of MSCs isolated from the bone marrow and other tissues (Lee et al., 2004; Pittenger et al., 1999; Gonzalez et al., 2009). MSCs have been studied extensively and many independent research groups worldwide have successfully isolated MSCs from a variety of sources, most commonly, from the bone marrow (Deet et al., 2001; Pittenger et al., 1999; Sottile et al., 2002). However, controversy exists over whether such stem cells are present in UC, and to date, little evidence in the literature substantiates the existence of such cells in UC. Erices et al. (2000) and Goodwin et al. (2001) independently reported the successful isolation of progenitor cells of mesenchymal origin from UC, whereas Mareschi et al. (2001) concluded against such findings.

MSCs are fibroblast-like in morphology, self-renewable, and capable of differentiating into, at least, three connective tissue lineages of the mesoderm including bone, cartilage, and adipocytes (Lee et al., 2004; Pittenger et al., 1999). We have shown in the present study that a plate adherent population of fibroblast-like cells isolated from UC, indeed, can be extensively clonally expanded *in vitro* while retaining the potential to differentiate, under *in vitro* conditions, into multiple lineages of the mesoderm (Rebelatto et al., 2008). Goodwin et al. (2001) showed that these cells also expressed neural markers, a heterogeneous plate-adhering population from total mononuclear cells and the presence of neural progenitors in UC has been demonstrated (Sanchez-Ramos et al., 2001). In this study, we demonstrated that our relatively more homogeneous UC-derived MSCs exhibited typical MSC phenotype analysed by morphology, RT-PCR analysis, and histochemical, cytochemical, and immune-cytochemical evaluations (Lee et al., 2004; Pittenger et al., 1999). These cells expressed Oct4, Sox2, Klf4, C-myc and hTERT, which are markers of pluripotent ESCs and induced pluripotent stem cells, and also these cells may differentiate into osteoblats, adipocytes, neuroglial-like, cardiomyocyte-like cells and germ cells. This finding demonstrated that UC-MSCs share some features with MSCs (Baharvand et al., 2010; Panepucci et al., 2004). However, these UC-MSCs did not proliferate as pluripotent stem cells, iPSCs and cannot form teratomas in nude mice in our study and previous reports

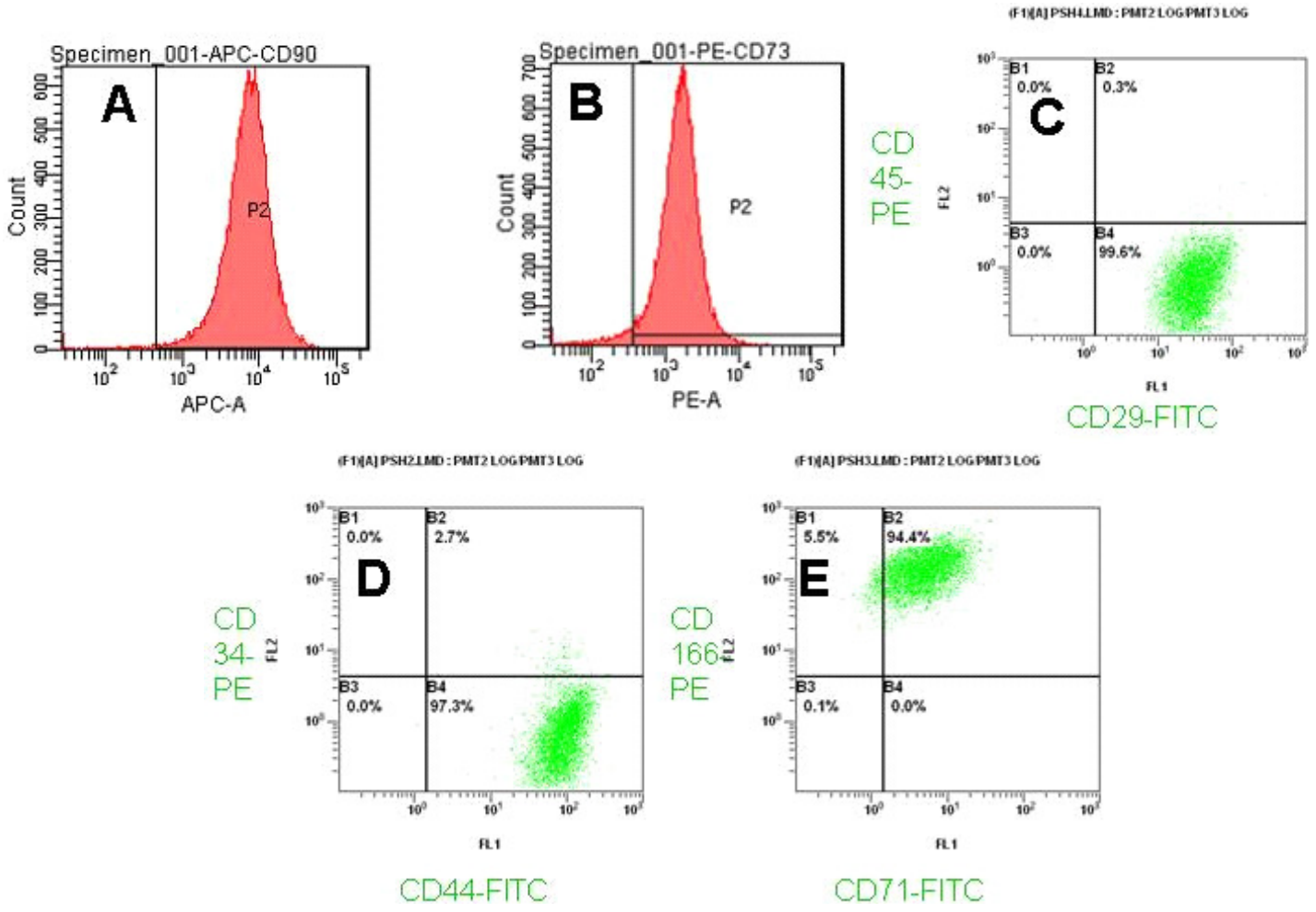

Figure 3. FACS analysis showed that UC-MSCs were positive for CD90, CD29, CD44, CD166, and negative for CD34, CD45, CD71(F-G).

Table 2. UC-MSCs were analysed with FACS at different passages.

Passage	CD73$^+$ (%)	CD90$^+$ (%)	CD105$^+$ (%)	CD14$^+$ (%)	CD34$^+$ (%)	CD45$^+$ (%)	HLA-DR+ (%)
P1	93.5	94.6	97.5	0.5	0.9	1.4	1.2
P3	96.6	99.8	98.6	0.4	0.9	0.3	1.2
P4	92.4	99.4	97.4	0.5	0.5	0.4	0.8
P6	92.7	99.6	98.6	0.2	0.2	0.1	0.2

(Baharvand et al., 2010; Goodwin et al., 2001; Xu et al., 2010). Therefore, we named them as multipotent UC-MSCs.

This report showed that UC does contain MSCs, even contain some pluripotent cells. With the approach reported in this study, it is possible to obtain single cell-derived, expanded MSCs from UC with remarkable potential to differentiate into multiple lineages of mesodermal and non-mesodermal origin. With this technique, the application of UC can be further extended and used as a new alternative source of MSCs to bone marrow (Lee et al., 2004). The self-renewal capacity of these cells is remarkable and is expected of multipotent stem cells. Flow cytometric analysis showed that these cells were negative for various hematopoietic lineage markers but positive for human MSC markers, as well as other various other integrins and matrix receptors. They were consistent with that reported in the literature for the bone marrow counterpart, indicating the MSC nature of these UC-derived cells (Lee et al., 2004). In addition to the potential of MSCs to differentiate into multiple lineages of the mesoderm, recent reports in the literature have indicated that bone marrow MSCs are capable of transdifferentiating into germ layer boundaries (Kopen et

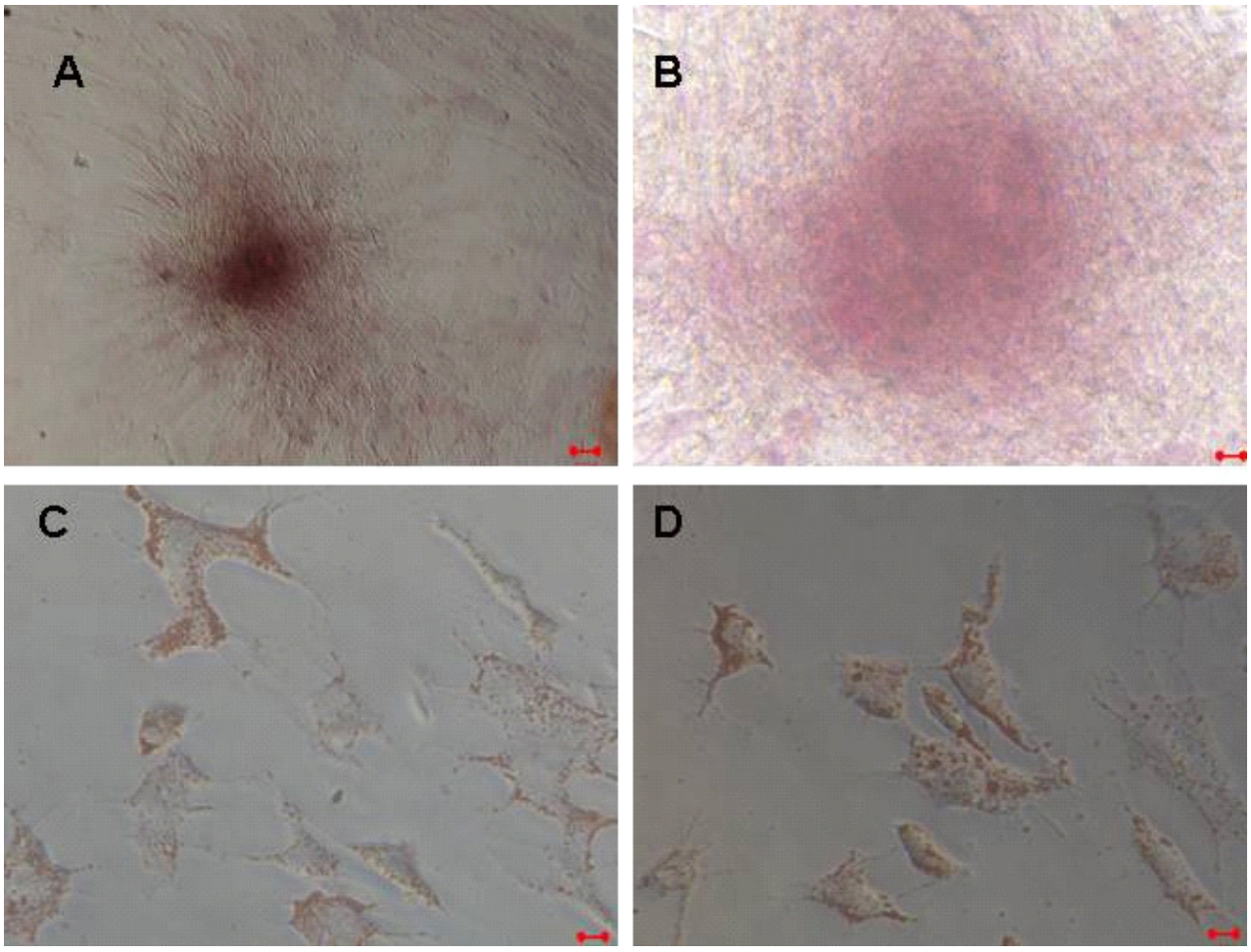

Figure 4. Osteoblast and adipocyte like cells were formed after induction. Aggregates were positive for Alizarin red staining (A, B); Lipid vacuoles were noticeable and visualized by staining with oil-red O in treated cells (C, D).

al., 1999; Rebelatto et al., 2008).

Most reports showed that the UC-derived cells may differentiate into mesoderm cell types including osteoblasts, chondrocytes and adipocytes under *in vitro* conditions (Lee et al., 2004; Pittenger et al., 1999). However, little progress and controvercial evidences have been reported in the isolation, characterization, and differentiation of MSCs from UC into functional neural, cardiomyocyte and germ cells (Lee et al., 2004; Rebelatto et al., 2008). Under neurogenic conditions, UC-derived cells exhibited the morphology of neural cells and expressed specific markers of neuroglial cells, which was confirmed by immunofluorescence assays for neural-specific proteins: β-tubulin II and NSE.

When UC-derived cells were cultured under cardiac conditions, most cells acquired a cuboidal morphology as opposed to the fibroblast-like morphology of undifferentiated cells. Cardiac β-actin, CT3 and Islet1 were detectable treated by 5-AZA or RA and DMSO. Previous studies showed that cardiac β-actin and CT3 were specific markers of cardiomyocytes (Rangappa et al., 2003; Orlandi et al., 2009). Nkx2-5 and/or Islet1 positive cardiac progenitors contribute to proepicardium during heart development (Zhou et al., 2008). These results demonstrated that human UC-MSCs may differentiate into cardiomyocytes in our induced cultures (Martin-Rendon et al., 2008).

Previous reports have shown that ESCs, fetal porcine skin stem cells and bone marrow MSCs can differentiate into germ cells *in vitro* (Clark et al., 2004; Dyce et al., 2006; Hua et al., 2009; Lacham-kaplan et al., 2006; Nayernia et al., 2006). Stem cells may provide a new potential source of male and female germ cells that could be used for infertility and sterility. However, the mechanisms are unclear and the efficiency of germ like cells derivation of stem cells is still low, with few could go through or complete the meiosis phase (Nayernia et al., 2006; Hua and Sidhu, 2008; Hua et al., 2009). In this study, small number of cells were positive for specific germ cell markers-SCP3 and VASA, and levels of expression of Stella, Dazl and SCP3 were increased inRA treated compared to control. These genes are

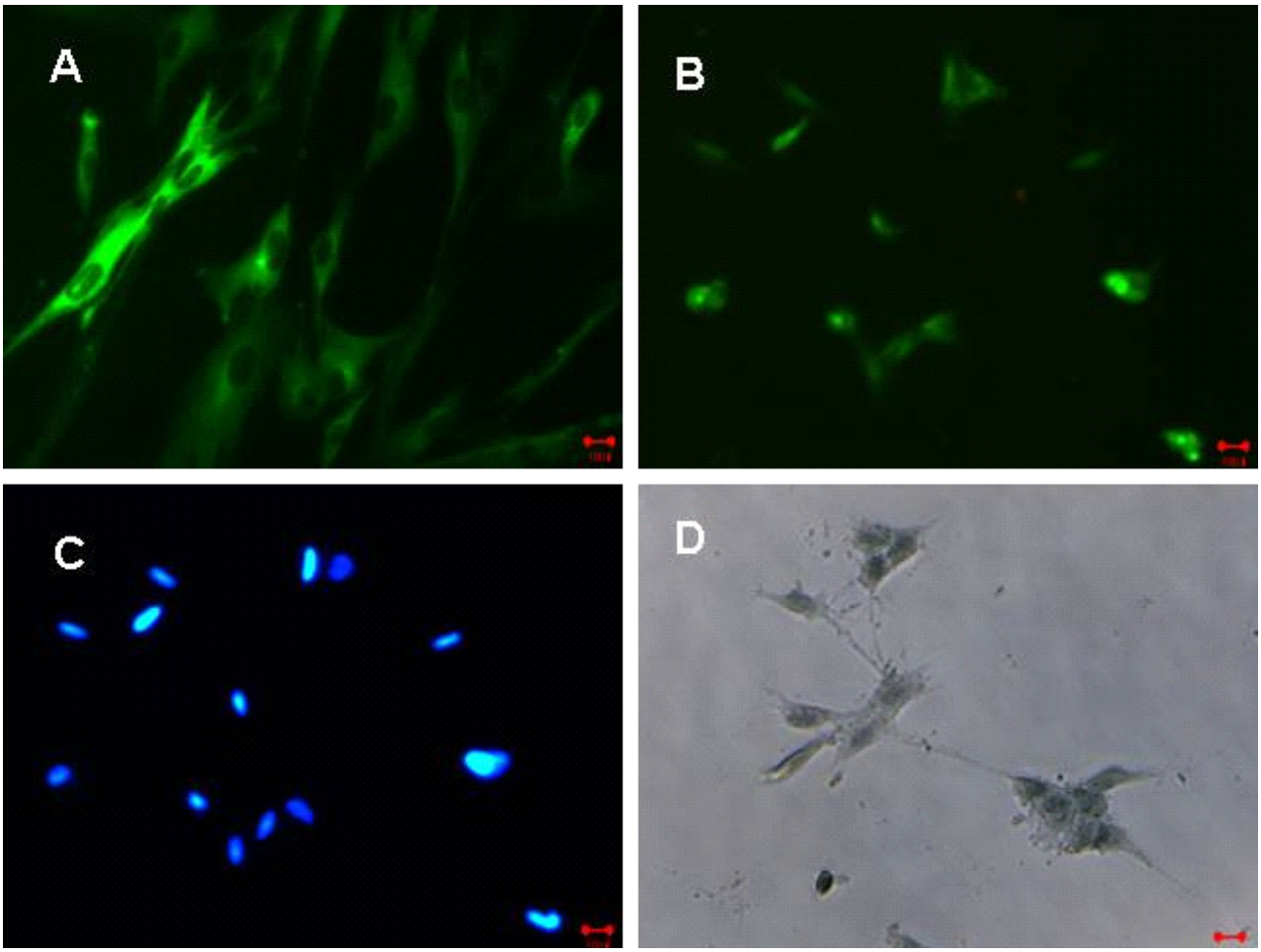

Figure 5. Neural like cells were formed after induction. A, β-tubulin Ⅲ positive cells; B, NSE positive cells; C, The nuclei of induced cells were stained with Hoechst33342; D, Neural like cells were positive for aniline blue staining.

expressed specifcally in PGCs, spermatogonial stem cells and male germ cells (Clark et al., 2004). The expression of germ cell markers in treated UC-MSCs was consistent with previous observations in ESCs and bone marrow MSCs (Nayernia et al., 2006). They indicated spontaneous differentiation of part or all of the population of MSCs and ESCs into germ cells *in vitro* induced by RA. These results demonstrated that UC-MSCs are pluripotent similar as VESL pluripotent cells derived from bone marrow and with the capacity to differentiate into germ cells (Lu et al., 2008).

Conclusion

This study demonstrated the isolation and characterization of a non-hematopoietic MSC population from the human UC and provides evidences that UC-MSCs have the ability to give rise to non-hematopoietic cells with characteristics of osteoblast, adiopcytes, neural cells, cardiomyocytes and even germ cells. Therefore, these cells may serve as a novel alternative source of multipotent autologous stem cells for cell replacement therapies in various diseases and provide a model to study the mechanism of germ cells differentiation and new strategies for the therapy of infertility and sterility.

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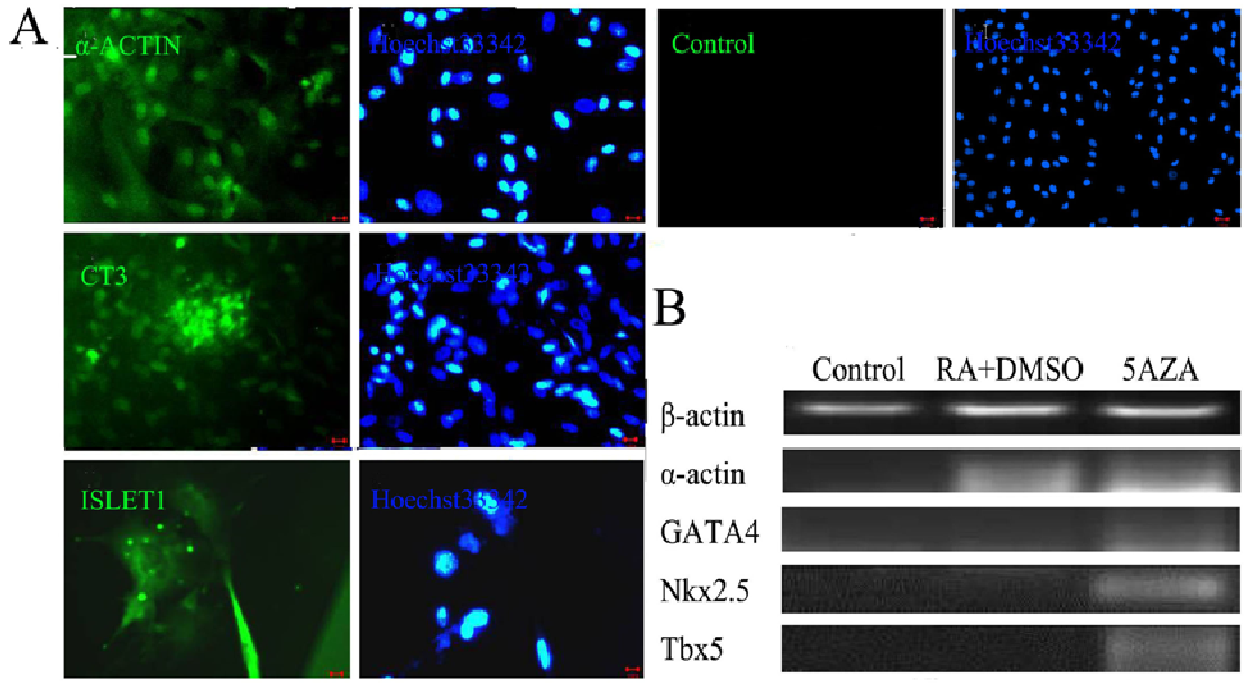

Figure 6. A, Cardiomyocyte-like cells were formed in induced cultures. Cardiac α-ACTIN, CT3 and ISLET1 positive cells were formed in the treated cultures (left lane); The nuclei of cells were stained with Hoechst33342 respectively in the right lane. Control (Negative control). B, Cardiac specific markers analysed by semi- RT-PCR showed that cardiac α-actin was up-regulated induced by RA in combination with DMSO or 5-AZA; GATA4, Nkx2 and Tbx5 were increased in 5-AZA induced cells (G).

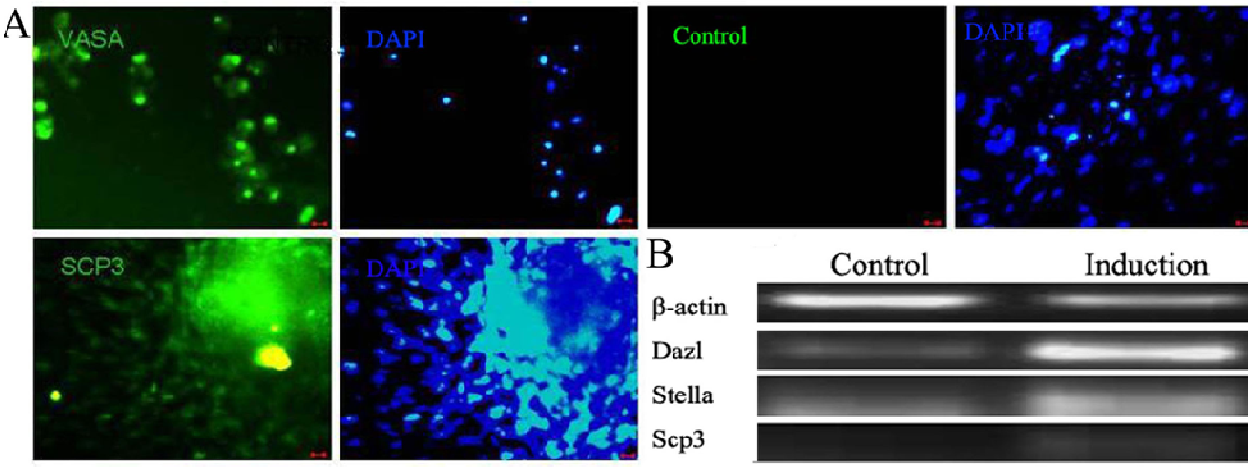

Figure 7. Immunofluorescence analysis of the germ cell differentiation after induction.The percentage of VASA and SCP3 positive cells were increased compared to control. The nuclei of cells were stained with Hoechst33342 in the right lane. Germ cell specific markers analysed by semi- RT-PCR showed that Dazl, Stella and Scp3 were up-regulated induced by RA(B).

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