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Differentiation of Human Umbilical Cord Wharton's Jelly-Derived Mesenchymal Stem Cells Into Germ-Like Cells In Vitro

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ABSTRACT

Recent studies have demonstrated that mesenchymal stem cells could differentiate into germ cells under appropriate conditions. We sought to determine whether human umbilical cord Wharton's jelly-derived mesenchymal stem cells (HUMSCs) could form germ cells in vitro. HUMSCs were induced to differentiate into germ cells in all-trans retinoic acid, testosterone and testicular-cell-conditioned medium prepared from newborn male mouse testes. HUMSCs formed ''tadpole-like'' cells after induction with different reagents and showed both mRNA and protein expression of germ-cell-specific markers Oct4 (POUF5), Ckit, $CD49_f$ (α6), Stella (DDPA3), and Vasa (DDX4). Our results may provide a new route for reproductive therapy involving HUMSCs and a novel in vitro model to investigate the molecular mechanisms that regulate the development of the mammalian germ lineage. J. Cell. Biochem. 109: 747–754, 2010.

KEY WORDS: HUMAN UMBILICAL CORD; MESENCHYMAL STEM CELL; GERM LIKE CELLS; IN VITRO DIFFERENTIATION

Human umbilical cord Wharton's jelly-derived mesenchymal stem cells (HUMSCs) possess pluripotent characteristics for indefinite proliferation and can be induced to differentiate into advanced derivatives of all three germ layers, such as bone, cartilage, fat, muscle, heart and brain cells [Mitchell et al., 2003; Ma et al., 2005; Fu et al., 2006; Wu et al., 2007; Karahuseyinoglu et al., 2008; Pereira et al., 2008]. Unlike the isolation of stem cells derived from other sources, such as embryos and bone marrow, that of HUMSCs is noninvasive and does not have surrounding moral or ethical issues [Denker, 1999; Romano, 2004]. Furthermore, HUMSCs have a relatively high proliferation rate and self-renewal capacity as compared with other adult stem cells [Izadpanah et al., 2006; Baksh et al., 2007]. Recent studies have shown that HUMSCs are immune suppressive in mixed lymphocyte assays and inhibit T-cell proliferation [Cho et al., 2008; Weiss et al., 2008]. Allogenic transplantation with HUMSCs is well tolerated in several models and can avoid immunological rejection [Cho et al., 2008]. Therefore, clinical application of HUMSCs has been considered promising for regenerative medicine.

Two unanswered questions in mammalian developmental biology are when and where the fate of the germ cell is specified. Germ cells are highly differentiated cells that participate in the complex processes of fertilization. They also retain pluripotent properties, which allows them to support the development of new individuals. Given these distinctive traits, scientists have long attempted to reproduce germ cell differentiation, or gametogenesis, in vitro [Conti and Giudice, 2008]. Under appropriate conditions, embryonic stem cells (ESCs), bone marrow stem cells(BMCs), and even stem cells derived from fetal porcine skin and rat exocrine pancreas, could be induced to differentiate toward the germ cell lineage. Moreover, the induced cells resemble germ cells morphologically and in some cases functionally; these germ cells can be produced in vitro [Hübner et al., 2003; Toyooka et al., 2003; Clark et al., 2004; Geijsen et al., 2004; Dyce et al., 2006; Lacham-Kaplan et al., 2006; Nayernia et al., 2006a,b; Danner et al., 2007]. Because the multipotent properties of HUMSCs are between that of ESCs and adult stem cells, the induction of germ cells from HUMSCs might be possible.

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Transplantation of stem cells for infertility has been a top research in resent years, especially for those of prereproductive or reproductive age who suffer to cancer illnesses, because one of the most devastating adverse effects of cancer treatments is damage to the reproductive system. It is demonstrated that xenotransplantation of germ cells is possible [Hill and Dobrinski, 2006; Kanatsu-Shinohara et al., 2008], and transplantation of ESCs and bone marrow or peripheral blood can form germ cells in vivo [Toyooka et al., 2003; Johnson et al., 2005; Nayernia et al., 2006a,b]. However, application of ESCs remains controversial for ethical reasons, and there still a debate on the survival of BMCs after transplantation to recipient mice [Eggan et al., 2006; Lassalle et al., 2008]. Considering to such an obstacle to ESCs and BMCs, finding an ideal source of stem cell which is neither ethical considering nor immunological rejection for infertility treatment becomes a new objective for researcher. HUMSCs might be the right candidate in this case.

Here, we demonstrate that HUMSCs can express markers characteristic of germ cells. This observation heralds a new thinking about HUMSCs as a source for reproductive medicine.

MATERIALS AND METHODS

ISOLATION AND EXPANSION OF HUMSCs

Protocols for sampling human umbilical cord were approved by the Institutional Review Board of Shantou University Medical College. The collection and expansion of HUMSCs were as previously described [Ma et al., 2005]. Briefly, umbilical cords were obtained from consenting patients delivering full-term male infants by cesarean section at the Second Affiliated Hospital of Shantou University Medical College. After the arteries and veins were removed, the remaining tissue – Wharton's jelly – was transferred to a sterile container in high glucose Dulbecco's modified essential media (H-DMEM; Gibco, USA) and diced into small fragments. The explants were transferred to 24-well plates in fresh growth medium (containing the H-DMEM along with 10% fetal bovine serum, 100 μg/ml penicillin, 100 mg/ml streptomycin, 1 μg/ml amphotericin B, 5 ng/ml epidermal growth factor (R&D, USA) and 5 ng/ml basic fibroblast growth factor (bFGF; [Sigma, USA]). The cells were left undisturbed for 5–7 days in a 37°C humidified incubator with 5% CO_2 to allow migration of cells from the explants, when the media was replaced every 2 days. When cells reached 80–90% confluence, they were harvested by use of a 0.05% trypsin/0.53 mM EDTA solution and replated into larger culture flasks at a 1:3 ratio.

PREPARATION OF TESTICULAR-CELL-CONDITIONED MEDIUM

Five- to 7-day-old Kunming male mice were killed, and the testes were removed from the body. The isolated testes were washed twice with 0.1 M phosphate-buffered saline (PBS) and centrifuged at 1,000 rpm for 5 min. After removal of the supernatant fractions, growth medium was added to the precipitate and triturated for dispersal into a single cell. The cells were suspended and divided in six-well culture dishes containing growth medium and incubated at 37°C in 5% CO_2 in air. Conditioning medium was collected on day 10 of primary culture and every 3 days afterward. The conditioning medium was centrifuged, filtered, and stored at −20°C or used immediately.

INDUCTION OF GERM CELLS FROM HUMSCs

HUMSCs at passages 3–6 were cultured in differentiation medium for induction of germ cells. Differentiation medium consisted of H-DMEM supplemented with 5% FBS, 50% filtered testicular-cell-conditioned medium, 2 μM all-trans retinoic acid (RA, Sigma) and 1 μM testosterone. Cells were maintained in this culture by replacing half the medium every 3–4 days.

FLOW CYTOMETRY

HUMSCs at passage 3 were analyzed by flow cytometry to determine the pluripotent cell characteristics. After trypsinization, approximately 10^6 cells were pelleted and resuspended in PBS and fixed with 4% buffered paraformaldehyde for 20 min at room temperature. Collected cells were then incubated with antibodies against phycoerythrin (PE)-conjugated CD29, CD59, and fluorescein isothiocyanate (FITC)-conjugated CD44 (all BD, USA). Cells were incubated in the dark for 30 min at 4°C. To detect the presence of Oct4, cells were permeabilized in PBS with 1% Triton X-100 for 10 min at room temperature after fixing and incubated with primary antibody against Oct4 (Santa Cruz Biotechnology, USA) overnight at 4°C. The cells were then stained in the dark with a fluorescent secondary antibody for 30 min at 4°C. Control samples were incubated with FITC and PE-conjugated mouse IgG1 isotype antibodies (Santa Cruz Biotechnology). After incubation, cells were washed with PBS and centrifuged to remove unbound antibody. Cells were resuspended in 1 ml PBS and analyzed by flow cytometry with use of EPICS XL (COULTER, USA).

RNA ISOLATION AND RT-PCR

Total RNA was isolated from HUMSCs and human sperm by use of Trizol reagent (Invitrogen, USA), according to the manufacturer's instructions. RT-PCR involved use of an RNA PCR Kit (AMV) v3.0 (Takara, Japan) following the manufacturer's instructions. The PCR conditions were 2 min at 94°C, then 35 cycles of 94°C for 30 s, 50–65°C for 30 s, 72°C for 30 s, and a final extension for 10 min at 72°C. The amplified PCR products were visualized by 1.2% (w/v) agarose gel electrophoresis and ethidium-bromide staining. The primers used for RT-PCR analyses were Vasa (NM_024415, 225 bp), (f) 5′-GAAGTGGCAGTGGAAGAA-3′, (r) 5′-CAAGAATAGTGTCGTATTTGTCG-3′; Oct4 (NM_002701,416 bp), (f) 5′-GAGAGCAACTCCGATGGG-3′, (r) 5′-CTCACTCGGTTCTCGACTG-3′; Stella (NM_199286,218 bp), (f) 5′-GCAGTCCTCAGGGAAATCG-3′, (r) 5′-CATCCATTAGACACGCAGAAAC-3′; Ckit (NM_000222.2, 345 bp), (f) 5′-AAGGACTTGAGGTTTATTCCT-3′, (r) 5′-CTGACGTTCATAATTGAAGTC-3′; α6 (259 bp), (f) 5′-TTTGGCGTGGCTGACTTAC-3′, (r) 5′-ATGGCAACATCACCTTCTATT-3′; and β-actin (NM_ 001101.2, 396 bp), (f) 5′-CACACTGTGCCCATCTACGA-3′, (r) 5′-TACAGGTCTTTGCGGATGTC-3′.

IMMUNOCYTOCHEMISTRY

For immunofluorescent localization of germ-cell markers under various culture conditions, HUMSC cultures were established on glass coverslips and treated with differentiation medium or control medium for 14 days; half the condition medium was changed to

fresh medium every 3–4 days. After 14 days' induction, cells were washed twice with PBS and fixed in 4% paraformaldehyde in PBS for 20 min. Cells were then washed three times with PBS and incubated for 10 min in PBS with 1% Triton X-100. Next, cells were blocked for 20 min in 5% BSA, then incubated with anti-Oct4 (Santa Cruz Biotechnology), anti-Stella (Santa Cruz Biotechnology), anti-α6 (Abcam, UK), anti-Vasa (R&D), or anti-Ckit (Zhongshan Co, China) for 2 h at 37°C or overnight at 4°C. Cells were then washed in PBS and incubated for 1 h at room temperature with rabbit anti-goat IgG-FITC (Boster, China) for Vasa, sheep anti-mouse IgG-Cy3 (Sigma) for α6 and Oct4, goat anti-rabbit IgG-FITC for Stella (Boster), or sheep anti-mouse IgG-FITC (Boster) for Ckit. Cells were then blocked, washed and incubated with DAPI (Sigma) for 5 min, then washed three times with PBS. The cells were then viewed under a confocal microscope.

Results

CHARACTERISTICS OF HUMSCs

At day 5–7 after the beginning of HUMSC primary culture, adherent fragments of Wharton's jelly were observed and spindle-shape cells started to migrate out from Wharton's jelly fragments (Fig. 1A). Half the medium was changed every 3–4 days until cells covered the culture area completely. The duration of primary culture was 10–14 days. HUMSCs were shown as fibroblast-like adhered cells, and most appeared to be flat, wide, and polygonal after passaging; this appearance did not change substantially up to passage 9 (Fig. 1B–D).

To determine whether HUMSCs could have multipotent potential, we assessed the expression of a number of markers associated with ESCs and adult stem cells by flow cytometry of cultured HUMSCs at passage 3. HUMSCs expressed the embryonic marker Oct4 (48.6%)

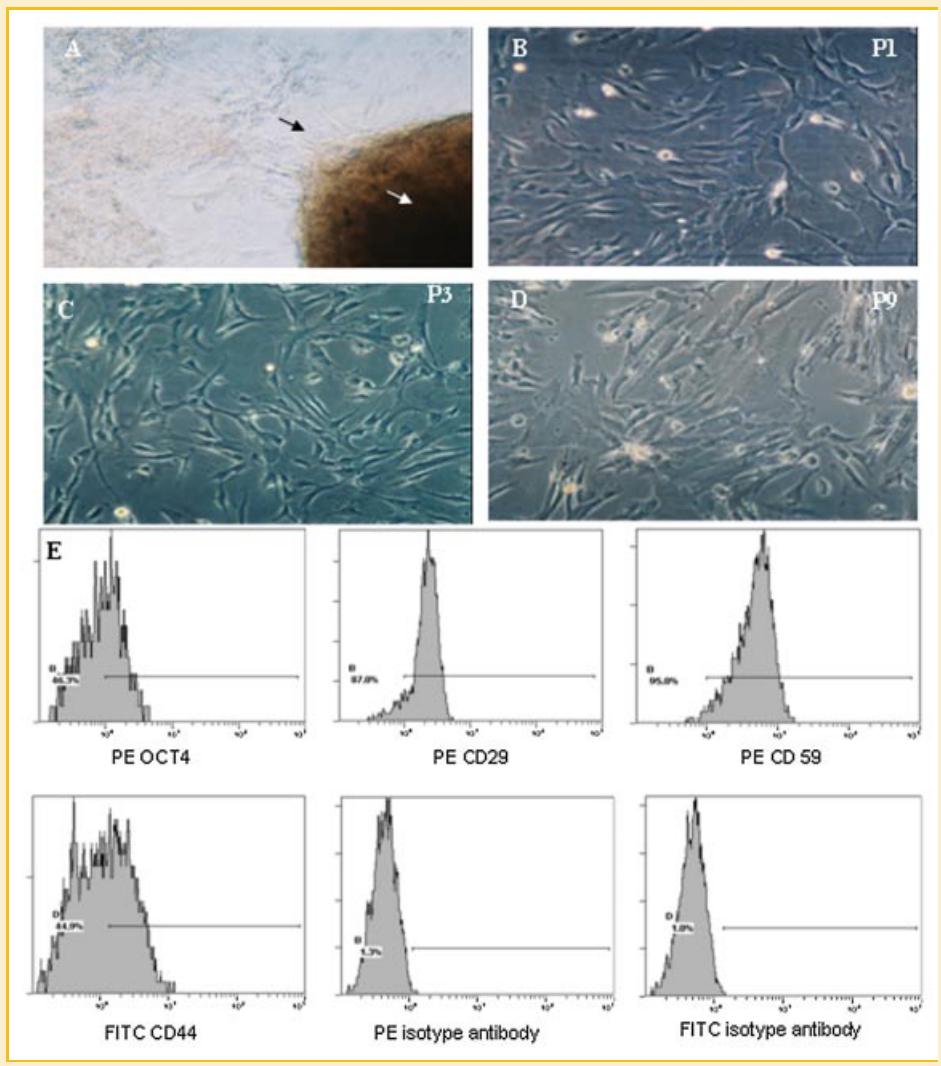

Fig. 1. A–D: Morphology of human umbilical cord mesenchymal stem cells (HUMSCs). Primary culture of HUMSCs at day 7; the tissue was adherent (white arrowheads) and cells migrated out from adherent tissue of Wharton's jelly (black arrowheads) (A). Fibroblast-like HUMSCs at passage 1 (B), 3 (C), and 9 (D). Magnification (100×); (E) Phenotypes of HUMSCs at passage 3. Cells were labeled with PE- or FITC-conjugated antibodies and examined by flow cytometry. Cells were positive for Oct4, CD29, CD44, CD59. Data are representative of three independent experiments.

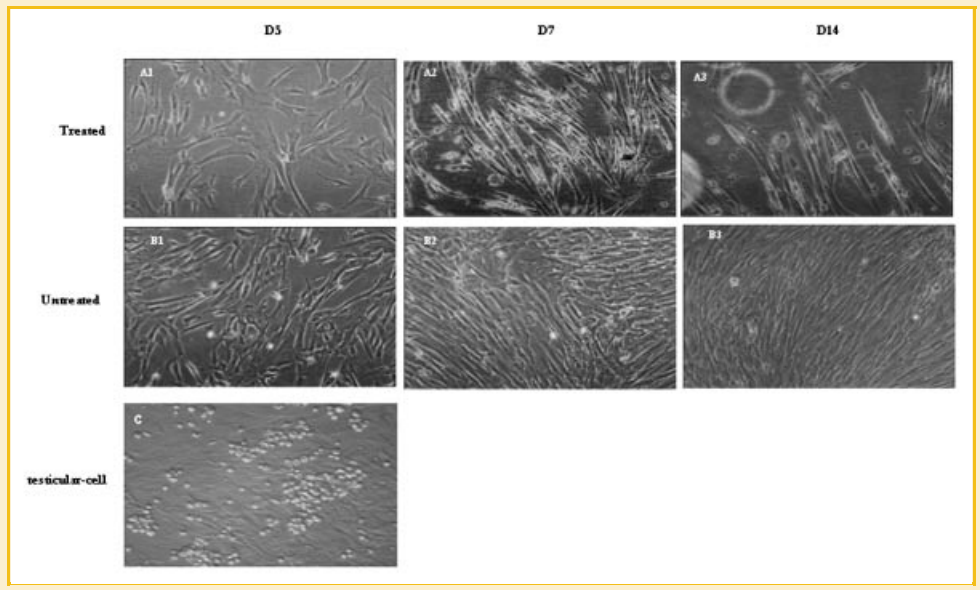

Fig. 2. A,B: Morphological changes of culture-differentiated HUMSCs (A1–3) compared with undifferentiated cells (B1–3) at days 3 (A1,B1), 7 (A2,B2), and 14 (A3,B3). Magnification (100×); (C) Primary culture of testicular cells from newborn mice at day 5. Germ cell shows a round morphology and coheres to adherent fibroblast-like somatic cells. Magnification (100×).

and antigens associated with pluripotent adult stem cells, including CD29 (87.7%), CD44 (52%), and CD59 (95.5%) (Fig. 1E).

TESTICULAR CELL CULTURE

After 24-h culture, testicular cells started to adhere and proliferate robustly within 5–7 days. As shown in Figure 2C, on day 5 after testicular cell culture, we observed two distinct cell phenotypes: round germ cells and fibroblast-like somatic cells. After the testicular-cell-conditioning medium was collected, the number of round germ cells was gradually reduced(data not shown); collection of the medium was discontinued after 30 days' culture [Lacham-Kaplan et al., 2006].

MORPHOLOGICAL CHANGES IN HUMSCs AFTER INDUCTION

To induce HUMSCs into germ cells, HUMSCs were treated with RA and testosterone in testicular-cell-conditioned medium, and morphological changes were observed every day under a phase-contrast microscope. After 7 days' incubation, the cells typically appeared as slender spindles and formed a ''tadpole-like'' shape (Fig. 2A2). However, the flat, wide or polygonal cells in the initial induction (Fig. 2A1) almost disappeared at 7 days' incubation, and their density was looser than that of untreated cells cultured in basic culture medium (Fig. 2B2). The morphology of these tadpole-like cells did not change significantly up to 14 days' treatment (Fig. 2A3). The control group of untreated HUMSCs were 100% confluent on day 14 (Fig. 2B3).

EXPRESSION OF GERM-CELL-SPECIFIC GENES IN TREATED HUMSCs

RNA was prepared from induced HUMSCs at days 0 (D0), 3 (D3), 7 (D7), and 14 (D14) after treatment. RT–PCR was performed to detect the expression of germ cell specific genes Oct4, Ckit, $CD49_f$, Stella, and Vasa. Oct4 was highly expressed in all detected samples (Fig. 3A), Ckit, $CD49_f$, and Stella were detected in sperm and treated

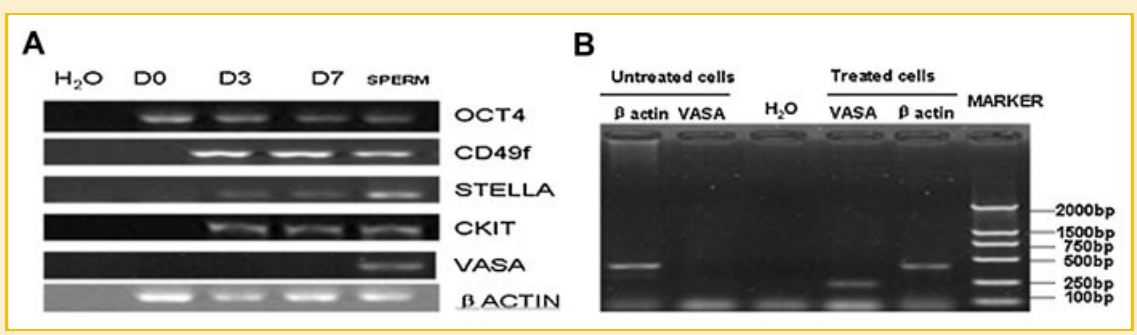

Fig. 3. RT-PCR analysis of HUMSCs expressing germ-cell-specific genes Oct4, Ckit, CD49f, and Stella before and during induced differentiation (A) and Vasa expression after 14 days' induction (B). D0, the day before differentiation; D3, D7, days 3 and 7, respectively, of differentiation. RNA isolated from human sperm served as a positive control. H_2O, no-template negative control.

cells at D3 and D7 but not at D0 (Fig. 3A). Vasa was expressed in differentiated cells at D14 (Fig. 3B).

At day 14 after incubation with differentiation media, the expression of germ-cell-related genes Oct4, Ckit, $CD49_f$, Stella and Vasa in differentiated and undifferentiated HUMSCs was detected by immunohistochemical analysis (Fig. 4). Oct4 was present in both treated (Fig. 4A2) and untreated cells (Fig. 4A1). However, Ckit (Fig. 4B2), $CD49_f$ (Fig. 4C2), Stella (Fig. 4D2), and Vasa (Fig. 4E2) were detected only in treated cells and rarely seen in untreated cells.

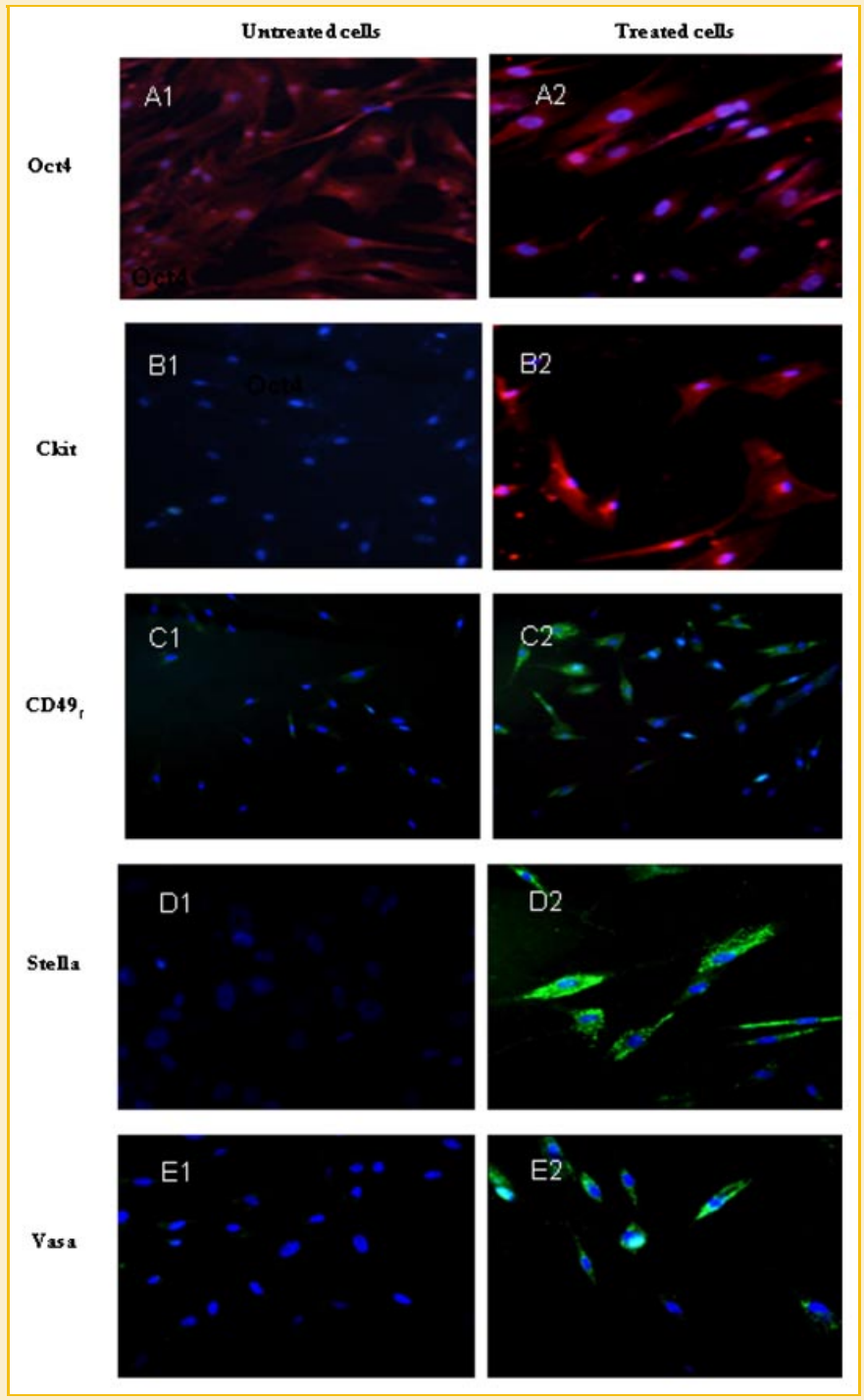

Fig. 4. Immunofluorescence location of germ-cell-specific markers Oct4 (red A1,A2), Ckit (red B1,B2), CD49f (green C1,C2), Stella (green D1,D2), Vasa (green E1,E2) in differentiated cells (A2,B2,C2,D2,E2) after 14 days' treatment, and undifferentiated cells (A1,B1,C1,D1,E1). Nuclei are shown in blue, DAPI (4′,6′-diamidino-2-phenylindole) staining. Original magnification: 400× (A1,A2,B1,B2,D1,D2,E1,E2), 200× (C1,C2).

DISCUSSION

Recent studies have shown that somatic stem cells have a more flexible differentiation potential than expected under suitable conditions. HUMSCs are a primitive stromal-cell population that can differentiate into all three germ layers. Germ cells of males or females could generate from ESCs and somatic stem cells such as bone-marrow stem cells, pancreatic stem cells and stem cells derived from fetal porcine skin. Here, we demonstrate the generation of human germ-like cells from HUMSCs.

We have previously reported on the isolation of stem cells from human umbilical cord Wharton's jelly. These stem cells possess properties of MSCs and can differentiate into nerve-like cells on culture with *Salvia miltiorrhiza* or β-mercaptoethanol [Ma et al., 2005]. As was expected from previous reports [Ma et al., 2005; Wu et al., 2007], we found that HUMSCs highly expressed Oct4, CD29, CD44, and CD59. Therefore, our starting population of cells contained pluripotent cells because Oct4 expression is restricted to pluripotent ESCs and germ cells [Schöler et al., 1990; Pesce et al., 1998], and CD29, CD44, CD59 antigens are considered associated with pluripotent adult stem cells.

Modeling germ-cell development in vitro has been studied for decades, although no standard culture system exists for the expansion of germline progenitor cells and their differentiation into mature gametes in vitro [Childs et al., 2008]. Recent studies have shown that germ cells can be generated successfully from ESCs and somatic stem cells under a suitable culture medium. Here, we differentiated HUMSCs into germ cells by incubating them in an artificial microenvironment simulating germ-cell development in vivo. This conditioning medium includes testicular-cell-conditioned medium, RA and testosterone. The testis is an abundant source of numerous growth factors such as bone morphogenetic protein 4, leukemia inhibitory factor, bFGF, stem cell factor, and growth differentiation factor-9 (GDF-9), which are needed for the development of germ cells [Takabayashi et al., 2001; Creemers et al., 2002; Pellegrini et al., 2003; Huleihel and Lunenfeld, 2004]. Recent studies have shown that testicular-cell-conditioned medium supports differentiation of ESCs into germ cells [Lacham-Kaplan et al., 2006]. RA, an active derivative of vitamin A, influences germ cell differentiation and is required for the transition into meiosis for both female and male germ cells [Koubova et al., 2006]. RA receptors (RARs) are expressed in both Sertoli cells and germ cells [Eskild et al., 1991], which can be stimulated by RA. Retinoids are involved in the regulation of testicular functions, which appear to be necessary for spermatogenesis [Livera et al., 2002] and the development of spermatocytes through early stages of meiosis [Akmal et al., 1997]. RA promotes differentiation of ESCs and bone-marrow cells into germ cells [Geijsen et al., 2004; Nayernia et al., 2006a,b; Drusenheimer et al., 2007; Kerkis et al., 2007], Furthermore, testosterone promotes differentiation of ESCs into cardiomyocytes and is essential for spermatogenesis in vivo [Griswold, 1998; Goldman-Johnson et al., 2008]. Cultured ESCs in conditioning medium containing testosterone and RA can generate male germ cells [Silva et al., 2009]. In the present study, we found HUMSCs changed morphologically after incubation in this culture system, although we could not observe the round cell type that had been reported by other groups [Hübner et al., 2003; Toyooka et al., 2003; Clark et al., 2004; Geijsen et al., 2004; Dyce et al., 2006; Lacham-Kaplan et al., 2006; Nayernia et al., 2006b; Danner et al., 2007]. Perhaps long-term culture is needed for the round cell shape. However, the expression of germ-cell-specific genes such as Oct4, Ckit, $CD49_f$, Stella, and Vasa in the differentiated HUMSCs confirmed that HUMSCs can differentiate into germ cells.

To analyze germ cell characteristics of induced HUMSCs, we examined the expression of mRNA and protein markers diagnostic of germ cell development at different stages of HUMSC differentiation. Oct4 is a specific germline protein [Schöler et al., 1990] and is expressed in normal and dysgenetic human gonads [Rajpert-De Meyts et al., 2004]. RT-PCR and immunocytochemistry revealed that Oct4 was expressed persistently in both differentiated and undifferentiated HUMSCs. Stella is the most specific marker for primordial germ cells (PGCs) [Saitou et al., 2002; Sato et al., 2002]. Stella expression appears in specified PGCs around embryo day 7.5 (E7.5) and is maintained in males until E15.5 and in females until E13.5 [Sato et al., 2002; Payer et al., 2003]. Ckit is a germ-cell-enriched gene highly expressed in PGCs. It is expressed in early spermatogenic cells and in later stages of spermatogenesis, specifically in the acrosomal granules of the round spermatids and the acrosomal region of testicular spermatozoa [Sandlow et al., 1997]. Intergrin α6 is the surface marker of spermatogonial stem cells [Shinohara et al., 1999]. We found the mRNA expression of Stella, Ckit, and α6 in differentiated HUMSCs at D3 and D7 and the protein expression of Stella, Ckit, and α6 in differentiated HUMSCs at D14. Vasa mRNA and protein levels are abundant and specific in germ cells of both sexes throughout development. In humans, Vasa protein is present in migrating primordial germ cells. During normal spermatogenesis, Vasa expression is relatively weak to intermediate in spermatogonia, strong in spermatocytes/spermatids, and absent in spermatozoa [Castrillon et al., 2000]. Sorting Vasa-positive cells from differentiated ES cells and transplantation these cells into testes of infertile mice can produce mature sperm in vivo [Toyooka et al., 2003]. In this study, we observed Vasa expressed in differentiated HUMSCs at D14.

Taken together, our study shows that HUMSCs can differentiate into germ cells in vitro. Whether our HUMSC-derived germ-like cells can undergo meiosis and form functional sperm or oocytes needs further investigation. One of the important but unresolved question in our study is how to purify the germ-like-cells from differentiated HUMSCs, so that we can track easily to observe the changes of the differentiate cells, including the morphology. It has been shown that germ cells can isolate from differentiated ES cells or BM cells by cell trapping, that differentiated cells contain the GFP or LacZ transgene, and the reporter genes were under the control of a promoter of the germ cell specific gene, such as DDX4, Staller, stra8 [Toyooka et al., 2003; Nayernia et al., 2006a]. This may be available to HUMSCs derive germ cells in our further investigation. Alternatively these data in our study may support the results generated by other groups that germ cells can be induced from somatic stem cells. Moreover, recent studies demonstrated that transplanting bone marrow stem cells into infertile mice can generate germ-like cells in genital organs [Johnson et al., 2005; Nayernia et al., 2006a]. Here, we

predicted that HUMSCs could be a promising source of cell therapy in reproductive medicine.

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