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EMBRYONIC-LIKE STEM CELL DERIVED FROM ADULT BONE MARROW: IMMATURE MORPHOLOGY, CELL SURFACE MARKERS, ULTRAMICROSTRUCTURE AND DIFFERENTIATION INTO MULTINUCLEATED FIBERS IN VITRO

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Abstract – Embryonic-like stem cell (ELSC), expressing part of surface markers of human embryonic stem cells, may be a better candidate for cell therapy of degenerative muscular disease than mesenchymal stem cell (MSC). We isolated ELSC and MSC from bone marrow, respectively, and compared their differences in the characteristics and the capacity of myogenic differentiation. Results showed that ELSC could be isolated successfully from 3 adult bone marrow samples by using serum-free medium with 10ng/ml basic fibroblast growth factor (bFGF). At the same cell density, MSC could also be isolated from the same samples by using DMEM/F12 medium containing 10% new cattle serum. However, ELSC appeared as small, morphologically slenderer, upregulated expression of SSEA-4 and ultramicroscopically more immature than MSC derived from the same samples. Immunofluorescent staining and RT-PCR analysis showed ELSC weakly expressed Oct-4, Nanog-3 and Sox-2. Moreover, ELSC and MSC could be induced into long, multinucleated fibers expressing myogenin and myosin heavy chain (MHC) in myogenic differentiation medium, but by day 10, proportion of multinucleated fibers positive for MHC was respectively 25.0%±6.9% and 13.8%±7.6% in ELSC and MSC culture. These data suggest that bone marrow derived ELSC represent an ideal candidate for cell therapy of degenerative muscular disease.

Key words: Multipotential stem cell, microultrastructure, myogenic differentiation, muscle fibres.

INTRODUCTION

A stem cell is an immature cell capable of self-renewing and differentiating. Bone marrow is likely the major source of adult stem cells (7). Mesenchymal stem cell (MSC) were first isolated and described as fibroblast-like cells that reside in the bone marrow of vertebrate animals including humans more than 20 years ago by Friedenstein (4). In the past decade, although more and more progress has been done in understanding the plasticity of MSC and in their therapeutic potential in regenerative medicine, MSC derivation has not much changed from the original plastic-adherent culture. Because of a lack of good markers for isolation and purification of MSC, resulting so-called MSC were heterogenous (14), which has hampered the clinical application of MSC.

Recent researches have demonstrated that there were primitive stem cells expressed embryonic stem cell markers in adult bone marrow. Similar stem cells were isolated by different culture techniques and the cells were termed different names such as multipotential

Abbrevations: bFGF:basic fibroblast growth factor; **ELSC:**Embryonic-like stem cell; **MAPC:**multipotential adult progenitor cell; **MIAMI cell:**marrow-isolated adult multilineage inducile cell; **MHC:**myosin heavy chain; **MSC:**Mesenchymal stem cell; **NCS:**new cattle serum; **NEAA:**non-essential amino acids; **PBS:**phosphate-buffered saline; **PCR:**Polymerase chain reactions; **RER:**rough endoplasmic reticulum; **rhEGF:**recombinant human epidermal growth factor; **RT:**room temperature; **RT-PCR:**reverse transcriptase polymerase chain reaction; **SR:**serum replacement; **VSEL:**very small embryonic-like stem cell

adult progenitor cell (MAPC) (12), marrow-isolated adult multilineage inducile (MIAMI) cell (3), very small embryonic-like stem cell (VSEL) (8) and MSC-expressed FZD-9, Oct-4, Nanog-3 (1) so on. In order to describe these cells expressing embryonic stem cell markers conveniently, they were named embryonic-like stem cell (ELSC) in this paper. Specially, Battula V.L etal (1) succeeded in isolating MSC-expressed FZD-9, Oct-4, Nanog-3 (namely ELSC) by using a novel protocol that was originally designed for the expansion of human embryonic stem cell (hESC), and succeeded in demonstrating an ELSC cell line capable of differentiating into cells expressing mesodermal, endodermal and ectodermal markers in vitro, which is likely to open a new and encouraging road for isolating multipotent stem cells. Based on the more immature genetype, we hypothesized that ELSC was a better candidate for cell therapy of degenerative muscular disease than MSC because of its stronger myogenic differentiation potential.

In the present study, we compared the differences in the characteristics and the myogenic differentiation capacity in ELSC cultured in the knockout DMEM with MSC cultured in the traditional DMEM/F12 medium containing serum. We demonstrated that ELSC was more immature cell than MSC, and had higher myogenic differentiation capacity than MSC under the identical myogenic differentiation medium. Thus ELSC may represent an ideal candidate for cell therapy of degenerative muscular disease.

MATERIALS AND METHODS

Bone marrow

Adult human bone marrows were collected from the iliac crest of 3 patients (8-year-old, 13-year-old, 15-year-old) with their informed consent after approval of the ethics committee of Kunming general hospital of People's Liberation Army. The patients suffering from hematopoietic system disease were excluded. Approximately 30ml bone marrow were aspirated from each patient and mixed with 10ml anticoagulant solution containing 0.47% citric acid, 1.33% sodium citrate and 3% glucose.

Isolation of ELSC and MSC

To prepare ELSC and control, erythrocytes of bone marrow were lysed in ammonium chloride solution (0.8% NH_4Cl and 0.1mM EDTA) (Sigma chemical company, STLOUS, USA) for 2min at room temperature (RT). Mononuclear cells were collected by centrifugation at 1000g for 5min and rinsed twice with phosphate-buffered saline (PBS) and divided equally into two groups. Cells were expanded according to the modification of Battula's protocol (1). Briefly, one group of mononuclear cells (1×10^7) were suspended in serum-free medium and transferred to gelatin-coated flask (Corning company, NY, USA) for the isolation of ELSC. The serum-free medium is knockout DMEM (Invitrogen company, Karlsruhe, Germany) supplemented with 20% serum replacement (SR) (Invitrogen), 2mM L-glutamine (Invitrogen), 1% non-essential amino acids (NEAA) (Invitrogen), 0.1mM 2-mercaptoethanol (Sigma) and 5 or 10ng/ml basic fibroblast growth factor (bFGF) (Cell Systems, Remagen, Germany). Coating of flasks was performed by adding 5ml of 0.1% gelatin (Sigma)/H_2O solution and incubated for 30min at RT. After incubation, the remaining solution was removed and air dried overnight at RT. As a control, at the same cell density, the other group of mononuclear cells (1×10^7) were suspended in DMEM/F12 medium (HyClone Laboratories Inc., Logan, UT) containing 10% new cattle serum (NCS) (Invitrogen) and transferred to uncoated flasks (Corning) for the isolation of MSC. The two groups of cells grown at 37°C and 5% CO_2 and were photographed every day under inverted Olympus microscope. Culture mediums were replaced with the respective fresh mediums every 4 days, and nonadherent cells were removed. Upon reaching approximately 80% confluence, adherent cells were passaged by treatment with trypsin/EDTA (Invitrogen). Passage 2 resulting adherent cells were analyzed for expression of stem cell markers including Oct-4, Nanog-3, Sox-2, SSEA-4 and CD34 by Immunostaining or reverse transcriptase polymerase chain reaction (RT-PCR) or flow cytometry analysis.

Ultramicrostructural examination

For ultramicrostructural analysis, pellets of passage 4 ELSC and passage 4 MSC were fixed with 2.5% glutaraldehyde in 0.1M phosphate buffer, pH7.4, for 24h at 4°C and then postfixed with 1% OsO_4 in the same buffer for 1h at RT. Samples were dehydrated and embedded in epoxy resin. Ultrathin sections were counterstained with lead citrate and uranyl acetate and ultramicrostructural observations were carried out using an energy filtered transmission electron microscope Philips CM120.

In vitro myogenic differentiation

To induce myogenic differentiation of ELSC, they were cultured in turn in the serum-free medium and the myogenic differentiation medium (SKGM-2 Kit, lot no.CC-3245, Lonza company, Basel, Switzerland) which consists of 100ml skeletal muscle growth medium, 10ml fetal bovine serum, 2ml glutamine, 0.1ml recombinant human epidermal growth factor (rhEGF), 0.1ml dexamethasone, 0.1ml gentamicin sulfate amphotericin-B according to manufacturer's specifications. In brief, passage 2 ELSC were collected and resuspended in the serum-free medium and plated into gelatin-coated six-well cell culture dishes (Corning) at a density of 2×10^4 cell/well. After 12h of culture, the medium was replaced with the myogenic differentiation medium. The differentiation medium was changed every 3 days with fresh medium. By using the same protocol, at the same density, MSC, cultured in 10% NCS DMEM/F12 medium for 12h, were used for myogenic differentiation induction. After 5 or 10 days of induction culture, the cells were used for immunostaining analysis of myogenic markers including MyoD, myogenin, myosin heavy chain (MHC). Likewise, passage 2 ELSC and MSC were cultured in $25cm^2$ cell culture flask (Corning) by above mentioned method, respectively. After 10 days of induction culture, the cells were collected for RT-PCR analysis. Uninduced ELSC and MSC were used as negative control respectively.

Comparison of myogenic differentiation rate

In order to investigate myogenic differentiation rate, according to the method reported (myogenic differentiation rate=number of fibers positive for MHC/total number of counted cells) (2), myogenic differentiation rate of 3 bone marrow samples were assessed by counting the number of fibers positive for MHC in six randomly encountered low-powered fields (n=6×3=18). Comparison of myogenic differentiation rate was carried out between ELSC and MSC by t-test.

Immunostaining analysis

For intracellular expression of Oct-4, Nanog-3, Sox-2, MHC, MyoD and Myogenin, cells were fixed with 4% paraformaldehyde at 4°C for 10min and permeabilized with 0.5% Triton X-100 for 10min. For cell surface expression of CD34, cells were only fixed with 4% paraformaldechyde at 4°C for 10min. Fixed or permeabilized cells were rinsed three times in PBS and incubated at RT for 10min in goat/rabbit serum or blocking solution consisting of PBS, 3%H_2O_2 and subsequently incubated at 4°C for 10h with primary antibody (all from Santa Cruz biotechnology, California, USA) including rabbit-anti-human CD34, Oct-4, Nanog-3, MyoD, goat-anti-human Sox-2 and mouse-anti-human myogenin, MHC. Then cells were rinsed once in PBS to remove the primary antibody. For the analysis of CD34, Oct-4, Nanog-3 and Sox-2, the cells were incubated with FITC-goat-anti-rabbit IgG or FITC-rabbit-anti-goat IgG (Invitrogen) for 30min at RT. For myogenin, MyoD and MHC, the cells were incubated with a secondary HRP-conjugated rabbit/mouse antibody (Gene company, USA). Unbound secondary antibody was removed with PBS washing. Negative control was stained as described above except that the primary antibody was excluded. Fluorescence was analyzed using a Nikon 80i microscope with Applied Imaging Cytovision software. While the other stained cells were photographed with an Olympus microscope.

RT-PCR analysis

Total RNA was extracted from the cells by using TRIZOL reagent (Invitrogen) and quantified spectrophotometrically. Complementary DNA for PCR was reverse transcribed from 5 μg of total RNA by using oligo (dT)-15 primer and the reverse transcription system kit (Invitrogen). GAPDH was used as an endogenous control to normalize the mRNA level. PCR were carried out by using specific primers designed from published cDNA sequences with following parameters: 94°C for 4min//94°C for 30sec, 60°C for 30sec, 72°C for 1min (45 cycles) //72°C for 7min. Negative controls consisted of PCR reactions conducted with ultrapure water instead of cDNA or without reverse transcription. PCR products were electrophoresed in 1% agarose gel containing ethidium-bromide and photographed under UV light. These primers for RT-PCR were: Oct-4 (151bp) F:CAGGAGTCCCAGGACATGAA and R:GTGGTCTGGCTGAACACCTT; Nanog-3 (153bp) F:CTGTGATTTGTGGGCCTGAA and R:TGTTTGCCTTTGGGACTGGT; Sox-2 (340bp) F:TAGCACTTGTTGCCAGAACG and R:AAGCCGCTCTTCTCTTTTCC; Myogenin (565bp) F:GCCACAGATGCCACTACTTC and R:CAACTTCAGCACAGGAGACC; MHC (172bp) F:TCGAGGAACTACGAGCCACT and R:TCCATCTCTCCCTGGATTTG; MyoD (181bp) F:AAGCGCCATCTCTTGAGGTA and R:GCGAGAAACGTGAACCTAGC; GAPDH (313bp) F:GCCAAGGTCATCCATGACAACTTTGG and R:GCCTGCTTCACCACCTTCTTGATGTC.

Flow cytometry analysis

Passage 2 ELSC were trypsinized and 1×10^6 cells were aliquoted into FACS tubes (BD Bioscience, San Jose, CA). Cells were rinsed twice with a cold buffer solution (DPBS, 1% FBS, at pH7.4) and incubated with mouse-anti-human SSEA-4 or appropriate isotypematched controls (Santa Cruz) for 30 min at 4°C. Subsequently, cells were rinsed three times with a cold buffer solution and then incubated with FITC-goat anti-mouse IgG (Invitrogen). Cells were rinsed again twice with the cold buffer solution and fixed with 1% paraformaldehyde until analysis with FACScan. As a control, passage 2 MSC was analized by using the same protocol.

Statistics

Parametric variables were expressed as mean and standard deviations and compared by Student's test. A p value of <0.05 was considered significant.

RESULTS

Isolation and characterization of ELSC

According to the protocol reported by Battula et al (1), equal quantities of bone marrow-derived mononuclear cells plated into coated or uncoated culture flasks, 7-10 days later, adherent cells could be noticed in gelatin-coated culture flask by using serum-free medium, namely knockout DMEM supplemented with 20%SR, 5ng/ml bFGF, 2mM L-glutamine, 0.1mM mercaptoethanol and 1%NEAA, but ELSC was successfully isolated from only one out of three bone marrow samples. By increasing the concentration of bFGF from 5ng/ml to 10ng/ml, ELSC isolation from three samples was successful. At the same cell density, MSC could also be isolated from the same three bone marrow samples in uncoated flask by using the serum-containing medium, namely DMEM/F12 medium containing 10% NCS. However, there were distinct differences in cell morphology between ELSC and MSC. Compared with the morphology of MSC grown in DMEM/F12 medium with 10% NCS, primary ELSC, grown in serum-free medium, appeared as small, morphologically slenderer and homogeneous indicating that these cells are less differentiated (Fig. 1A). After passages, ELSC grew well-distributed in flask (Fig. 1B) and were more readily expanded in vitro and showed morphologically slenderer after passage 10 (Fig. 1C). And primary MSC showed different morphologies: a spindle-shaped morphology and another epithelioid, polygonal or round morphology (Fig. 1D). With passaging the

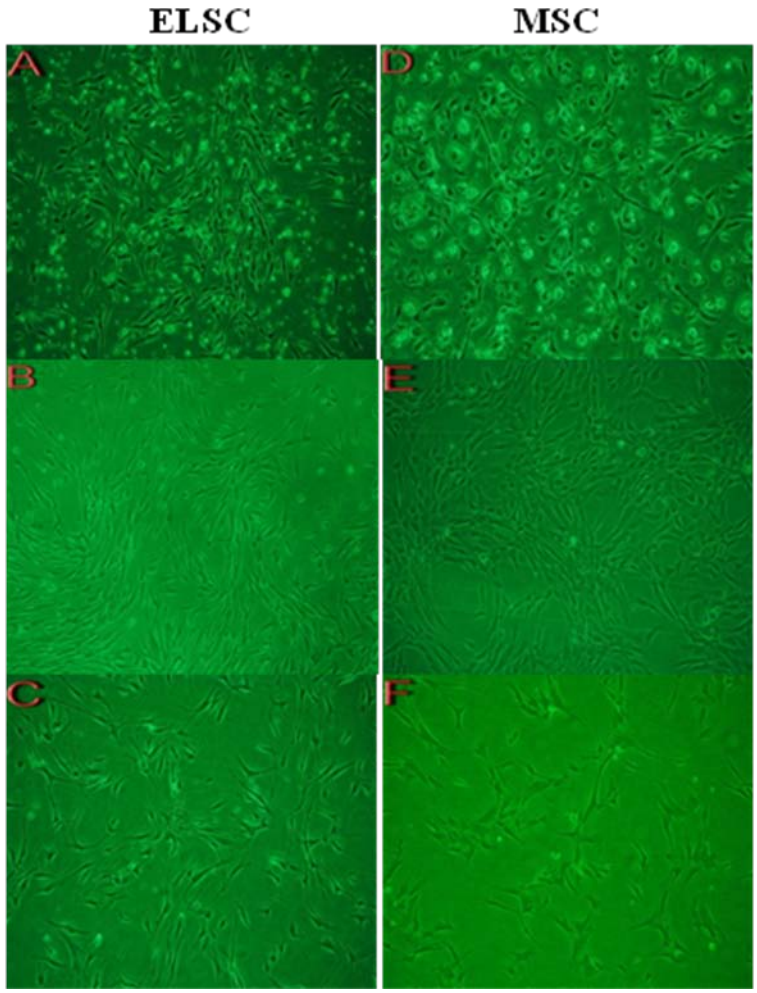

Figure 1. Morphological characterization of ELSC and MSC.
For isolation of ELSC and MSC, 1×10^7 mononuclear cells from the same bone marrow were cultured in the serum-free medium containing 10ng/ml bFGF and in DMEM/F12 containing 10%NCS, respectively. (A) The resulting cells (namely primary ELSC) appeared as small, morphologically slenderer and homogeneous shape cultured for 8 days in the serum-free medium. (B) Passage 3 ELSC grew well-distributed in the serum-free medium. (C) Passage 10 ELSC appeared as slenderer. (D) The resulting cells (namely primary MSC) showed different morphologies including spindle-shaped, epithelioid, polygonal or round shape cultured for 6 days in the serum-containing medium. (E) Passage 3 MSC with homogeneous shape grew in contagious distribution. (F) Passage 10 MSC appeared as obvious morphological alterations of aged cells. (A,B,C,D,E,F original magnification, ×40)

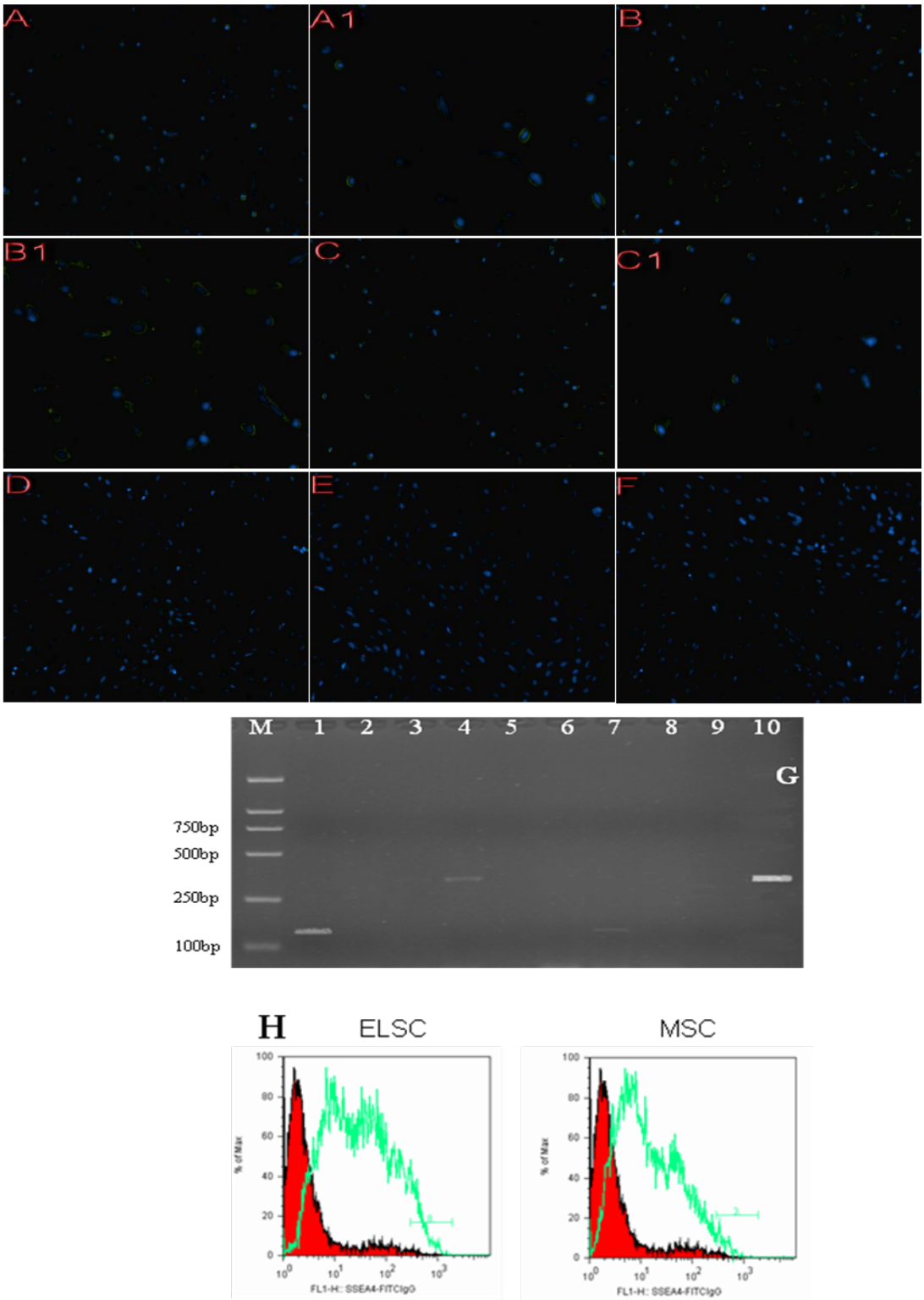

Figure 2. Expression of pluripotency markers in both ELSC and MSC.
Passage 2 ELSC, cultured in the serum-free medium supplemented with 10ng/ml bFGF, were used for Immunostaining or RT-PCR or flow cytometry analysis. As a control, passage 2 MSC, cultured in DMEM/F12 containing 10% NCS, were also analyzed. (A) green showed weak expression of Oct-4 in ELSC; (A1) magnification of a part of (A); (B) green showed weak expression of Nanog-3 in ELSC; (B1) magnification of a part of (B); (C) green showed weak expression of Sox-2 in ELSC; (C1) magnification of a part of (C); (D) no expression of Oct-4 in MSC; (E) no expression of Nanog-3 in MSC; (F) no expression of Sox-2 in MSC; (A,B,C,D,E,F original magnification, ×100). (G)Expression of pluripotent markers Oct-4(151bp), Nanog-3(153bp) and Sox-2(340bp) at the RNA level analyzed by RT-PCR. (lane M: 2000bp DNA marker; lane 1: Nanog-3 expression in ELSC; lane 2: No expression in MSC; lane 3: Negative control for Nanog-3; lane 4: Sox-2 expression in ELSC; lane 5: No expression in MSC; lane 6: Negative control for Sox-2; lane 7: Oct-4 expression in ELSC; lane 8: No expression in MSC; lane 9: Negative control for Oct-4; lane 10: GAPDH expression in MSC). (H) Flow cytometry analysis showed level of SSEA-4 expression was higher in ELSC than in MSC.

epithelioid population rapidly disappeared from culture and MSC grew in contagious distribution (Fig. 1E) and underwent obvious senescence after passage 10 (Fig. 1F).

The resulting adherent cells were transferred into six-well dishes for analyzing the expression of stem cell markers. Immunofluorescent staining results showed no expression of CD34 in both ELSC and MSC cultured in serum-free medium with 10ng/ml bFGF and in DMEM/F12 medium with 10% NCS, respectively (not shown), which indicated they were not haemopoietic stem cell. Importantly, ELSC, grown in serum-free medium supplemented with 10ng/ml bFGF, displayed induced expression of Oct-4, Nanog-3 and Sox-2 (Fig. 2A, A1, B, B1, C, C1). And as expected, expression of pluripotency genes Oct-4, Nanog-3 and Sox-2 were confirmed by RT-PCR analysis (Fig. 2G). Based on known expression of SSEA-4 in ELSC and in MSC (1, 5), we investigated expression level of SSEA-4 in both ELSC and MSC by flow cytometry analysis, and the results showed expression level of SSEA-4 was 7.91±0.49% and 3.15±0.68% in ELSC and MSC, respectively. Obviously, SSEA-4 level was higher in ELSC than in MSC. (Fig. 2H).

Ultramicrostructural examination of ELSC

ELSC expressed some of pluripotency markers. We decided to observe ultramicrostructure of ELSC grown in serum-free medium supplemented with 10ng/ml bFGF. Under transmission electron microscope, most of the passage 4 ELSC revealed prominent flower-like pseudopodium on the cell membrane, abundant rough endoplasmic reticulum (RER) profile in the cytoplasm. As a control, compared with that of ELSC, passage 4 MSC revealed a smooth cell membrane, distinctive vacuoles at the periphery of the cytoplasm and significant RER cisternae with moderately electron-dense secretory material (Fig. 3a, b, c, d, e).

ELSC differentiation into muscle fibers in vitro

Having established ELSC with more immature phenotype and the recent discovery of ELSC capable of differentiating into cells expressing mesodermal, endodermal, and ectodermal markers in vitro, we decided to test whether ELSC can be differentiated into muscle fibers in vitro and to compare its capacity of myogenic differentiation with that of MSC. Myogenic differentiation *in vitro* was induced and myogenic differentiation rate was calculated. Long multinucleated muscle fibres positive for MHC in ELSC, cultured for 5 days in myogenic differentiation medium, could be detected by immunostaining analysis. After 10 days, more and more muscle fibers-positive for MHC and myogenin could be observed. Expression of muscle-specific genes MHC and myogenin were confirmed by RT-PCR analysis. As a control, MSC, cultured for 10 days in the myogenic differentiation medium, also differentiated into long, multinucleated muscle fibers positive for MHC and myogenin. Expression of MyoD could not be detected in both ELSC and MSC cultured in the myogenic differentiation medium for 5 days and 10 days by immunostaining and by RT-PCR analysis, respectively (Fig. 4A, B, C, D, E, F, G, H, I, J, K). As expected, No evidence of any myogenic differentiation was detected in non-induced ELSC and in non-induced MSC (not shown). However, By day 10, myogenic differentiation rate was respectively 25.0%±6.9% and 13.8%±7.6% in ELSC and in MSC cultured in the same myogenic differentiation medium. Obviously, the capacity of myogenic differentiation in ELSC is higher than parallel MSC cultured at the same densities in the same myogenic differentiation medium for 10 days, and the difference in myogenic differentiation rate was significant ($t<0.05$).

DISCUSSION

It is well known that hESC are expanded in serum-free medium, and the presence of bovine serum will induce the differentiation of hESC. It is a novel protocol to expand human bone marrow cells by using the method for hESC expansion. Our results showed that ELSC were more readily isolated from adult human bone marrow by serum-free medium supplemented with 10ng/ml bFGF than 5ng/ml bFGF. These ELSC were different from MSC isolated from the same bone marrow by using DMEM/F12 medium containing 10% NCS. ELSC had smaller and slenderer, homogeneous shape, and weakly expressed embryonic stem cell markers Oct-4, Nanog-3 and Sox-2. Consistent with the previous report (1, 5), MSC also expressed SSEA -4 that is commonly used as a marker for hESC, but its expression level of SSEA-4 was lower than that of ELSC. These results indicated that the knockout-DMEM medium supplemented with bFGF could induce or upregulate expression of pluripotency markers and that bFGF was an important factor affecting isolation of ELSC. In fact, as an important mitogenic factor, bFGF

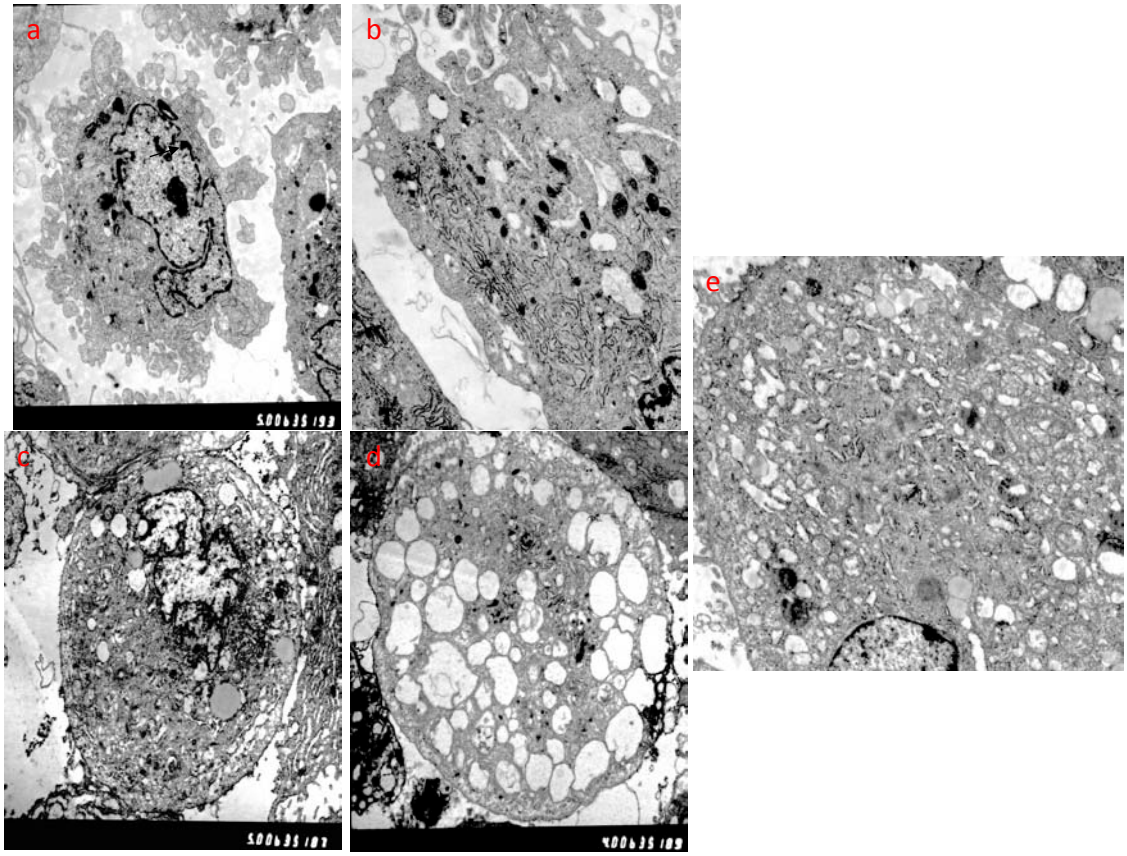

Figure 3. Transmission electron microscope analysis of ELSC and MSC.
Passage 4 ELSC and passage 4 MSC were used for transmission electron microscope analysis. Major ELSC features included (a) prominent pseudopodium on the cell membrane, and (b) rough endoplasmic reticulum (RER) profile in the cytoplasm. Major MSC features included (c)smooth cell membrane, (d) distinctive vacuoles, and (e) dilated RER cisternae with secretory material. (a, c, d, original magnification, ×5000. b, e original magnification, ×12000)

played wide-ranging roles in the regulating and controlling of cell development by not only promoting proliferation of stem cell but also inhibiting differentiation of stem cell and maintaining its stemness (15, 17). In this study, increasing the concentration of bFGF could help to isolating ELSC, which may be attributed to promoting proliferation role of bFGF. ELSC had immature phenotype and expressed some pluripotency markers, which may be attributed to inhibiting differentiation role of bFGF. In other words, it is likely that bFGF, as it does to hESC (17) and haemopoietic stem cell (18), also inhibits the differentiation of ELSC and preserves repopulating ability of ELSC in serum-free cultures. However, mechanism of bFGF is unknown.

In this paper, we observed for the first time significant differences in ultramicrostructure between ELSC and MSC. ELSC have characteristic and prominent pseudopodium on the cell membrane and RER profile in the cytoplasm. Major MSC features included smooth cell membrane, distinctive vacuoles and significant dilated RER cisternae with secretory material. Our results are similar to the recent related report (11). It is known that RER is the important place for synthesizing secretory proteins. Accordingly, cell feature of dilated RER cisternae with secretory material indicated MSC are actively synthesizing proteins for their growth or differentiation. The distinctive vacuoles could be evocative of an intense endocytotic activity. In contrast, the dominant pseudopodium on the ELSC membrane may be related to the expression of some proteins such as cell surface markers. The appearance of little dilated RER cisternae indicated that ELSC is a more primitive and metabolically quiescent state. Based on the significant differences in the ultramicrostructure, primitive stem cell derived from bone marrow could be identified by transmission electron microscope.

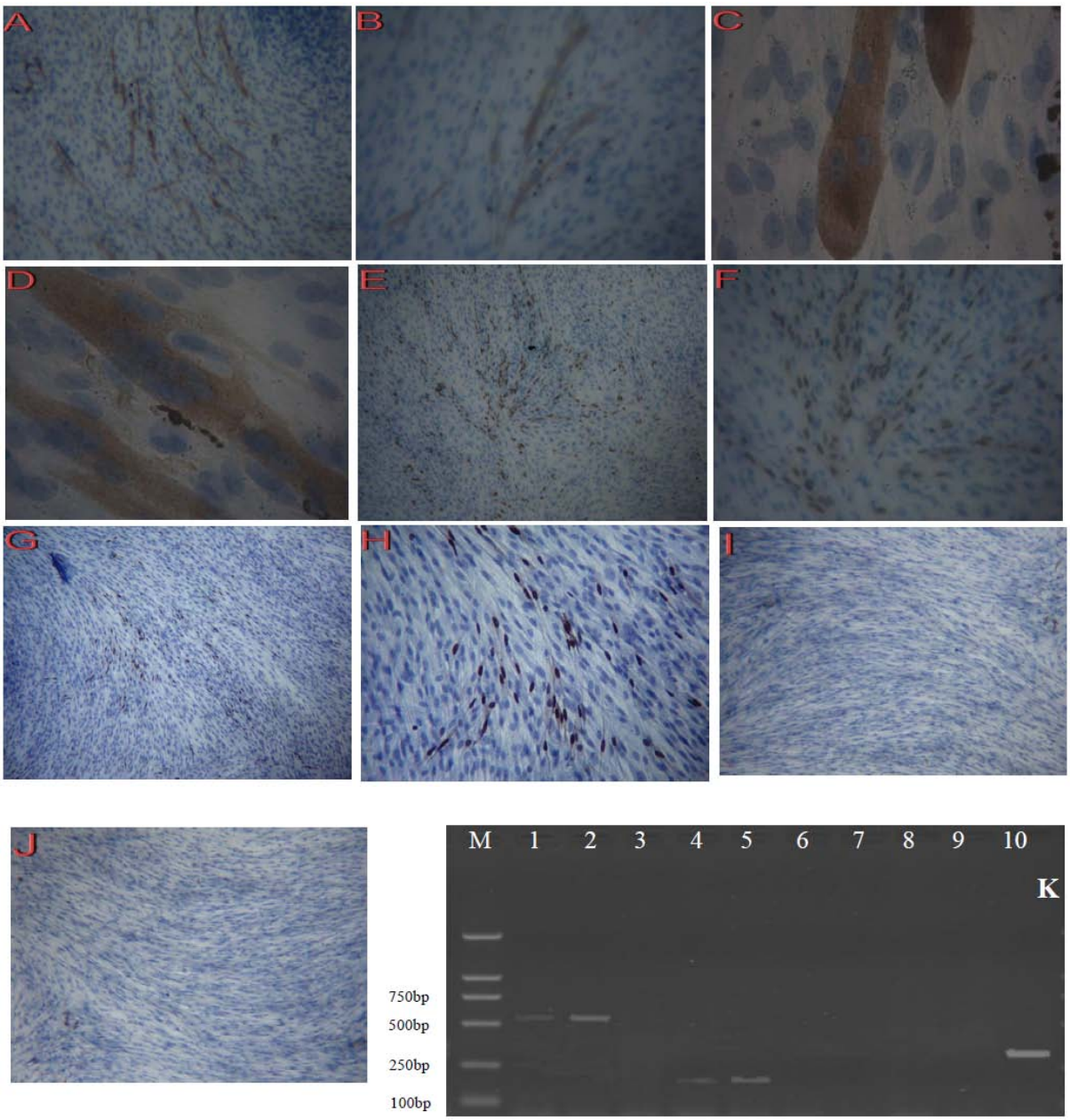

Figure 4. Expression of muscle-specific markers in ELSC and in MSC cultured for 10 days in the myogenic differentiation medium

At a density of 2×10^4 cell/well, passage 2 ELSC and MSC were cultured for 12h in the serum-free medium and serum-containing medium, respectively. The medium was replaced with the myogenic differentiation medium. After 10 days, expression of MHC, MyoD and myogenin were analyzed by immunostaining and RT-PCR. (A) the brown showed expression of MHC in ELSC; (B) the brown showed expression of MHC in MSC; (C)the brown showed long, three nucleated fibers; (D) the brown showed long, multinucleated fibers; (E) and (F)the brown showed expression of myogenin in ELSC; (G) and (H) the brown showed expression of myogenin in MSC; (I) no expression of MyoD in MSC; (J) no expression of MyoD in ELSC. (A, E, G, I, J original magnification, ×40. B, F, H original magnification, ×100. C, D, original magnification, ×400.). (K) Expression of myogenin gene (565bp), MyoD gene (181bp) and MHC gene (172bp) in ELSC and in MSC cultured for 10 days in the myogenic differentiation medium. (lane M: 2000bp DNA marker; lane 1: Myogenin expression in MSC; lane 2: Myogenin expression in ELSC; lane 3: negative control for myogenin; lane 4: MHC expression in MSC; lane 5: MHC expression in ELSC; lane 6: negative control for MHC; lane 7: No expression of MyoD in MSC; lane 8: No expression of MyoD in ELSC; lane 9: No expression of MHC in non-induced ELSC; lane 10: GAPDH expression in MSC.

5-azacytidine was generally used as inducer for myogenic differentiation of stem cells (16, 6), whose mechanism has been regarded as the activation of myogenic regulatory factors by causing hypomethylation of random DNA residues (13). Because hypomethylation of DNA could lead to tumorigenesis, it is obvious that medium containing 5-azacytidine is not suitable for induction of stem cell. In addition, it was also reported that 5-azacytidine could not induce MSC-derived from human bone marrow into myotube (2, 9). In this study, we succeeded in in vitro induction of ELSC and MSC into long, multinucleated fibers by using myogenic differentiation medium supplemented with rhEGF but not 5-azacytidine. These multinucleated fibers expressed MHC and myogenin but not MyoD. However, the myogenic differentiation rate was significantly higher in ELSC than that in MSC, which suggested ELSC was an ideal candidate for cell therapy of degenerative muscular diseases. Skeletal muscle growth medium without 5-azacytidine was a safer and more efficient inducer. Interestingly, it is reported that presence of rhEGF could negatively regulate myogenic differentiation of stem cell because it promotes proliferation of cells (10). Obviously, presence of rhEGF did not affect the myogenic differentiation of cells in this study, and the possible reasons include: (1) different stem cells used in the different experiments may hold different capacity of myogenic differentiation; (2) rhEGF may regulate the differentiation of stem cell by itself or cooperate with other growth factors. We don't know why no expression of MyoD is detected. One possible explanation is that the presence of rhEGF affects expression of MyoD.

In summary, consistent with the previously reported findings, our current experiment results demonstrated ELSC, expressing some of human embryonic stem cell markers, could indeed be isolated from adult bone marrow by using serum-free medium. These ELSC had more immature characteristics and stronger capacity of myogenic differentiation than parallel MSC under the same myogenic differentiation medium, which suggest that ELSC is an ideal candidate for the cell therapy of degenerative muscular disease. Since it is possible that ELSC arise in vitro through genetic or epigenetic changes, the relationship of ELSC to MSC or hESC or primitive stem cells such as MAPC, MIAMI cell and VSEL and so on is not clear. However, our results at least showed some standard for identifying adult bone marrow-derived multipotent stem cells carrying hESC markers and a method for myogenic differentiation in vitro. More importantly, ELSC has strong promise in reparative medicine for cell therapy of degenerative muscular disease and are free of the ethical concerns rose by the use of hESC as well as could be used for autologous implantation.

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