

MEF/KSF-conditioned culture medium: An effective method for *in vitro* culture of mouse dermal papilla cells with osteogenic differentiation potential

LIANG XU^{1*}, WENLAN GAO^{2*}, SHANSHAN BAI^{1*}, HUICHUAN DUAN¹, XIAOGANG PAN^{3**} and WEI WU^{1**}

¹Department of Plastic and Reconstructive Surgery, Shanghai Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine; ²Department of Stomatology, Shanghai Children's Medical Center, Shanghai Jiao Tong University School of Medicine; ³Department of Orthodontics, Shanghai Ninth Peoples' Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai 200011, P.R. China

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Abstract. Hair follicle stem cells are pluripotent and have a self-renewal capacity and multi-differentiation potential *in vitro*. As hair follicle stem cells can be easily sampled from the skin and hair of clinical patients at a considerable quantity, these cells have potential applications in wound repair and skin tissue engineering. Effective approaches for the *in vitro* culture and amplification of mouse hair follicle stem cells, as well as the *in vitro* osteogenic differentiation potential and cell source when obtaining mouse-separated cells were examined. Serial subculture was performed in different culture systems. Cell source was detected based on the relevant surface markers derived from mouse hair follicles at the gene and protein levels, and the differential potential was determined. The proliferative ability of hair follicle-derived stem cells obtained from mouse embryonic fibroblast (MEF)/keratinocyte serum-free medium (KSF)-conditioned medium was the

highest among all culture systems. The induced group had a stronger osteogenic differentiation potential compared with the non-induced group, indicating that the cells obtained from MEF/KSF-conditioned medium were cells derived from the hair follicle dermal papilla. Therefore, the strong osteogenic differentiation potential of the hair follicle-derived mesenchymal stem cells was screened with MEF/KSF-conditioned culture medium following amplification, and biological characteristics similar to those of hair follicle dermal papilla cells were observed.

Introduction

Hair follicle stem cells may serve as seed cells, as these are easy to obtain and abundantly available (1). Numerous studies have demonstrated the potential of hair follicle stem cells to differentiate into skin appendages such as epidermis, hair follicles, nerves and sebaceous and sweat glands and their ability to regenerate and repair skin and skin appendages (2-4). The beneficial effects of hair follicle stem cells on wound healing has been observed in a variety of reported clinical cases (5,6). Clinical results indicate the rapid healing of the dermal graft is due to the differential potential of hair follicle stem cells (7). Martínez *et al* (8) demonstrated that autologous transplantation of terminal hair follicles induces wound healing in chronic venous leg ulcers. Moreover, the outer root sheath cells and dermal papilla (DP) cells of hair follicles may be induced to differentiate into osteocytes (9), bone marrow cells (10), adipocytes (11), myocytes (12) and neurons (13) *in vitro*. However, it is currently not possible to specifically isolate and obtain hair follicle stem cells, due to their hybrid and dynamic cell components during the growth cycle (14). Currently, micro-isolation, culture methods and cell surface-specific markers are widely used to isolate hair follicle stem cells (15). Alkaline phosphatase (ALP), α -smooth muscle actin (α -SMA), SOX2 and neural cell adhesion molecule (NCAM) are highly expressed in DP cells, which are used in the identification of DP cells (16). Ohyama (17) labelled and purified hair follicle stem cells using laser-capture microdissection but failed to obtain

Correspondence to: Dr Wei Wu, Department of Plastic and Reconstructive Surgery, Shanghai Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine, 639 Zhi Zao Ju Road, Shanghai 200011, P.R. China
E-mail: dr.wuwei@qq.com

Dr Xiaogang Pan, Department of Orthodontics, Shanghai Ninth Peoples' Hospital, Shanghai Jiao Tong University School of Medicine, 639 Zhi Zao Ju Road, Shanghai 200011, P.R. China
E-mail: xgpan70@126.com

*,** Contributed equally

Abbreviations: MEF/KSF, mouse embryonic fibroblast/keratinocyte serum-free medium; DP cells, dermal papilla cells; HFSC, hair follicle stem cells; TGF, transforming growth factor; α -SMA, α -smooth muscle actin; NCAM, neural cell adhesion molecule; AKP, alkaline phosphatase; pan-CK, pan-cytokeratin

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monoclonal cells. Furthermore, in a previous study, these monoclonal cells were partially purified through screening with surface markers, but their viability and number decreased (18). Although microscopic operation is suitable for the purification of cells, this process is unpersuasive and time-consuming (19). In the present study, hair follicle cells were cultured via the mouse embryonic fibroblast (MEF)/keratinocyte serum-free medium (KSF) (MEF/KSF) culture system *in vitro* to detect their three-directional induction potentials. In addition, biological characteristic tests were performed to demonstrate that the cells were derived from DP cells, as well as to identify effective and convenient methods that could be used to culture hair follicle DP cells *in vitro*.

Materials and methods

Configuration of MEF-conditioned medium. The current study was approved by the animal experimental ethical commission of Shanghai Ninth People's Hospital Affiliated to Shanghai Jiaotong University, School of Medicine [approval no. (2014)61]. Female mice (age, 12.5 weeks; weight, 30.0-35.0 g) were housed under a 12 h light/dark cycle, at temperatures of 23°C and a relative humidity of 40-60% humidity, with *ad libitum* access to food and water. Mice with confirmed pregnancy (Kunming mice; Institute of Laboratory Animal Science, Chinese Academy of Medical Science of PLA, China) were used. A total of 30 female mice and 25 fetal mice was used in the present study. The food and water intake and of animals were monitored once a day for their well-being. Animals will be euthanized using an intraperitoneal injection of 200 mg/kg sodium pentobarbital when they exhibited >20% loss of body weight or obvious stress, such as grooming their head and forepaws more than usual, exaggerated escape behaviors, or hiding more than usual. A Buprenorphine HCl, S.C. injection (0.05 mg/kg) was used 30 mins prior to surgery to reduce pain for the animals and one pregnant mouse was housed per cage to prevent fighting between mice. Anesthesia was performed using 80-100 mg/kg Ketamine and 10 mg/kg Xylazine. Mouse epidermis was harvested from fetal mice (embryo 13.5-14.5 day) collected from the abdominal cavity of female mice, and the pregnant mice were terminated. The samples were sheared into pieces, digested with 0.2% collagenase at 37°C for 4 h and resuspended. After filtration with a 200-mesh filter, the samples were centrifuged at 500 x g for 5 min at room temperature. The supernatant was discarded and the isolated MEF cells were resuspended in DMEM containing 10% FBS (Gibco; Thermo Fisher Scientific, Inc.). The cells were plated in a 10-cm Petri dish to obtain MEF cells. MEF cells (prior to P4 generation) that had reached 50% fusion were cultured for an additional 48 h after changing the DMEM containing 4.5 g/l D-glucose (Gibco; Thermo Fisher Scientific, Inc.) culture medium. The cell culture medium was subsequently centrifuged at 200 x g for 5 min and the obtained supernatant was filtered through a 0.22-mm needle filter. MEF/KSF medium (Gibco; Thermo Fisher Scientific, Inc.; 500 ml basal medium, 5 ml keratinocyte growth supplement and 5 ml penicillin/streptomycin solution)-conditioned culture medium was obtained after mixing MEF-conditioned medium with KSF culture medium without serum at a ratio of 1:1.

Isolation and culture of mouse hair follicle stem cells. Dorsal skin samples were obtained from sacrificed C57 mice (20 female mice; 6 weeks old; 18-20 g; Kunming, China) housed under a 12 h light/dark cycle, at a temperature of 23°C and a humidity 40-60%, with free access to water and food. Under aseptic conditions, samples were cut into pieces. Samples were subsequently soaked in chloramphenicol for 5 min and cultured overnight with 0.2% dispase II at 4°C. Following incubation, the pieces were dehaired with tweezers and soaked in 0.05% trypsin for digestion in a water bath on an oscillator at 40°C for 10 min. Trypsin was rapidly inactivated using DMEM containing FBS serum, and a cell suspension was obtained via filtration. The suspension was centrifuged at 500 x g for 5 min at room temperature and the supernatant was discarded. Following resuspension and re-centrifugation at 500 x g for 5 min at room temperature, the cells were counted using a hemocytometer and randomly distributed into the following four groups: KSF culture medium group (Group A), KSF + 10% FBS culture medium group (Group B), DMEM + 10% FBS culture medium group (Group C) and MEF/KSF-conditioned culture medium group (Group D). In each group, cells were seeded at a density of 1×10^4 cells/cm².

Identification of three-directional induction conditions for mouse hair follicle stem cells in vitro

Identification of conditions for cell induction and differentiation toward osteoblasts. Hair follicle stem cells were cultured at 37°C in an osteogenic induction medium (100 nmol/l dexamethasone, 50 μ mol/l levorotatory vitamin C and 10 nmol/l β -glycerophosphate; (Gibco; Thermo Fisher Scientific, Inc.). P1-MEF/KSF-HFSC (hair follicle stem cells) were subjected to Von Kossa. Cells were fixed using 4% paraformaldehyde for 30 min at room temperature, washed twice with PBS and dissolved using 100 mg of Silver nitrate in 2 ml water (5%). Aliquots (500 μ l) were added to each 6-well plate for 20-30 min under UV light and washed twice with water. Samples were then dissolved in 100 mg sodium thiosulfate in 2 ml water (5%) and added to each well for 5 min (500 μ l). Samples were washed twice. Nuclear Fast Red staining solution (500 μ l) was added, and cells were incubated for 5 min at room temperature. Samples were washed in triplicate and then subjected to and alizarin red staining. For this procedure, cells were fixed with 4% paraformaldehyde for 30 min at room temperature and incubated at room temperature for 15 min, rinsed cells three times with PBS and added to Alizarin red staining solution (1X Alizarin Red Stain Solution; cat. no. 2003999; EMD Millipore). After incubation at room temperature for at least 20 min, excess dye was removed, and samples were washed three times with PBS. Finally, 1-5 ml PBS was added to each well to prevent cells from drying. Procedures were performed on day 21 of cell culture, then observed under a light microscope (Leica Microsystems, Inc.; magnification, x40).

Identification of conditions for cell induction and differentiation toward adipocytes. Cells were cultured at 37°C in an adipogenic induction medium (Gibco; Thermo Fisher Scientific, Inc.) 1 mmol/l dexamethasone, 10 mg/l of recombinant human insulin, 0.5 mmol/l IBMX (3-Isobutyl-1-Methylxanthine) and 100 mol/l indomethacin).

P1-MEF/KSF-HFSC were subjected to oil red staining on day 21 of cell culture.

Identification of conditions for cell induction and differentiation toward chondroblasts. Cells were cultured at 37°C in a chondroblast induction medium (Gibco; Thermo Fisher Scientific, Inc.; 1 mmol/l sodium pyruvate, 0.1 mmol/l vitamin C, 0.1 nmol/l dexamethasone, 10 ng/ml transferrin growth factor (TGF)- β 3, 6.25 μ g/ml recombinant human insulin, 6.25 μ g/ml transferrin, 6.25 ng/ml selenious acid, 1.25 ng/ml BSA and 5.35 μ g/ml linoleic acid). P0-MEF-HFSC cells were washed twice with PBS (pH 7.4) for 1 min on day 21 and fixed with 10% neutral formaldehyde after 30 min. The cells were observed under a microscope and imaged after the immunohistochemical detection of type II collagen. Samples were fixed with 4% paraformaldehyde in PBS for 15 min at room temperature. Aspirated fixative was rinsed twice in PBS for 5 min each, after which samples were permeabilized with 0.1–0.5% triton x-100 in PBS for 10 min. Aspirated triton x-100 was rinsed twice in PBS for 5 min each and incubated with 10% normal goat serum (cat. no. ab7481; Abcam) in PBS for 30 min at room temperature. Aspirated goat serum was incubated with sections and collagen type II primary antibodies (1:200; cat. no. ab188570; Abcam) overnight at 4°C or 1 h at 37°C. Samples were rinsed three times in PBS for 5 min each and incubated with HRP-conjugated secondary antibodies (1:500; Goat Anti-Rabbit IgG; cat. no. ab6721; Abcam) for 1 h at 37°C in the dark. Samples were subsequently rinsed three times in PBS for 5 min each in the dark. Samples were incubated with 1 μ g/ml DAPI at room temperature for 5 min and mounted with a drop of mounting medium.

Identification of the biological characteristics of hair follicle-derived cells from mice

Immunofluorescence staining. The culture medium from P1-MEF-HFSC cells was discarded, the cells were washed with PBS and fixed with 4% polyformaldehyde for 15 min at room temperature. Cells were washed with PBS and treated with 0.3% Triton after 20 min. The samples were washed in PBS and blocked with 10% goat serum (cat. no. ab7481; Abcam) for 1 h at room temperature, followed by an overnight incubation with 1:200 diluted K15, α -SMA primary antibody (cat. no. ab5694; Abcam) and NCAM monoclonal antibody (cat. no. ab204446; Abcam) at 4°C. Cells were washed with PBS and treated with secondary antibodies [1:500; Goat Anti-Rabbit IgG H&L (Alexa Fluor® 488); cat. no. ab150077; 1:500; Goat Anti-Rabbit IgG H&L (TRITC); cat. no. ab7087; Abcam] at 37°C for 30 min. The nucleus was stained with DAPI for 10 sec at 37°C. Images were acquired using a Leica immunofluorescences microscope (Leica Microsystems, Inc.; magnification, x40).

Alkaline phosphatase (AKP) staining. The culture medium was removed and P1-MEF-HFSC cells were washed with PBS. Cells were embedded in 10% formaldehyde (4°C) for 10 min at room temperature. After washing with distilled water, cells were incubated with a drop of a solution containing 20 mg naphthol AS-BI phosphate, 0.5 ml DMSO, 50 ml 0.2 mol/l veronal acetate buffer (pH 9.2) and 0.5 ml 1 mol/l 6-azo-pararosaniline (pH 9–10 adjusted with NaOH) at room

temperature for 45 min. The glass slide was washed with PBS, dried and mounted with glycerin gelatin (GGI-10X15ML; Sigma-Aldrich; Merck KGaA). Images were obtained with light microscope (Leica Microsystems, Inc.; magnification, x40).

Flow cytometry. The cell suspension obtained from P1-MEF/KSF-HFC cells following digestion was filtered through a 40- μ m filter to remove impurities, followed by centrifugation at 500 g or 5 min. Flow cytometry staining was subsequently performed (on the ice). Cell pellets were washed, resuspended in ice-cold fluorescence-activated cell sorting (FACS) buffer (pH 7.4; 0.1 M PBS; 1 mM EDTA and 1% BSA) and stained for markers with the following antibodies: Pan-cytokeratin (CK; 1:1,000; anti-pan Cytokeratin antibody; cat. no. ab234297; Abcam), α -SMA (1:1,000; cat. no. ab5694; Abcam) and NCAM (1:1,000; cat. no. ab204446; Abcam) were added for 30 min at 4°C. PBS buffer was used for the control group. The cells were washed twice with PBS and centrifuged at 500 x g for 5 min at 4°C. Subsequently, goat anti-rabbit (1:1,000) antibodies including: (Alexa Fluor® 488; cat. no. ab150077; Abcam), Goat Anti-Rabbit IgG H&L (Cy5®; cat. no. ab6564; Abcam) and Goat Anti-Rabbit IgG (FITC; cat. no. ab6717) were added and incubated for 30 min in the dark at 4°C. Samples were rinsed twice with PBS, centrifuged at 500 x g or 5 min at 4°C, resuspended in PBS containing 2% FBS and prepared for further analysis. The cells were analyzed using flow cytometry analysis software Flow Jo7.6.5 (FlowJo LLC).

Reverse transcription PCR (RT-PCR). Cell culture media from P1-MEF/KSF-HFC cells and primary cultures of neuronal cells obtained from mice via digestion (control group) were discarded and cells were washed with PBS. Brain samples from mice were used as control group. The samples were treated with 1 ml TRIzol® (cat. no. 15596018; Thermo Fisher Scientific, Inc.) reagent on ice, followed by incubation for 5 min at 4°C with RNase for the inactivation of RNA enzymes (all the materials used for RNA treatment were performed as previously described (20). The samples were transferred into 1.5-ml Eppendorf tubes, followed by the addition of 400 μ l chloroform. The mixtures were shaken well for 15 sec and placed on ice for 15 min. The mixtures were centrifuged at 12,000 x g at 4°C for 15 min and 500 μ l supernatants were transferred into new Eppendorf tubes, followed by treatment with 500 μ l ice-cold isopropanol. The samples were mixed, placed on ice for 10 min, centrifuged at 12,000 x g and 4°C for 10 min. The supernatants were discarded and the pellets were treated with 70% alcohol. The mixtures were centrifuged at 7,500 x g and 4°C for 10 min. The supernatants were discarded and the residual liquid was absorbed with filter papers. The pellets were dried for 10 min and suspended in 20 μ l double-distilled water treated with Diethyl pyro carbonate (cat. no. 4387937; Thermo Fisher Scientific, Inc). The absorbance values at 260 nm were measured and the concentration of RNA was adjusted to 1 μ g/ μ l. These samples were stored at -80°C.

A total of 2 μ g total RNA was used for the reverse transcription reaction. The reaction conditions were: 2 μ l reverse transcription buffer (10X), 4 μ l 25 mM MgCl₂, 2 μ l 10 mM dNTP, 1 μ l Oligo dT-adaptor primer, 0.5 μ l RNA enzyme

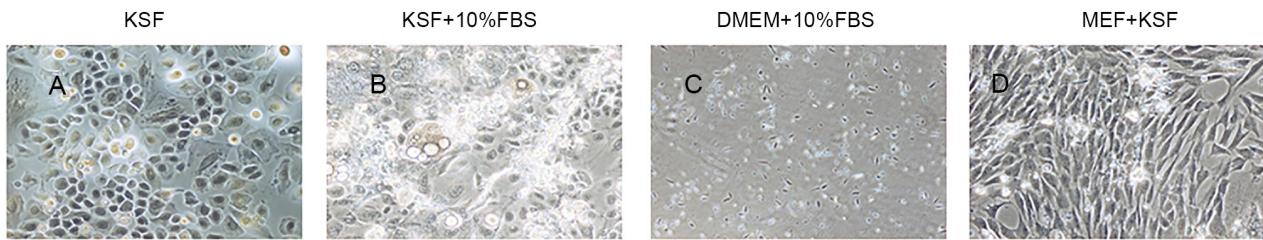


Figure 1. Morphology of hair follicle stem cells cultured in different culture systems. (A) KSF medium. (B) KSF medium + 10% FBS; numerous structures of lipid droplets were observed. (C) DMEM + 10% FBS. (D) MEF-conditioned medium. Reverse-phase microscope; magnification, x100. KSF, keratinocyte serum-free.

inhibitor (40 U/ μ l) and 0.5 μ l AMV reverse transcriptase (5 U/ μ l). The final volume was measured to 20 μ l with water. The RT reaction was performed under following conditions: 37°C for 10 min, 42°C for 1 h, 99°C for 5 min and 4°C for 5 min.

The product (2 μ g) was used for PCR amplification of the target gene fragment. PCR reaction conditions were as follow: 2 μ l PCR buffer (10X), 150 μ l MgCl₂ (25 mM), 0.5 μ l 10 mM dNTP, 0.25 μ l Taq enzyme (5 U/ μ l; cat. no. 9012-90-2; Sigma-Aldrich; Merck KGaA) and 0.3 μ l upstream and downstream primers (12.5 pmol/ μ l). The primers used were as follows: SOX-2 forward, 5'-GCGGAGTGGAACTTTTG TCC-3' and reverse, 3'-GGGAAGCGTGTACTTATCCTT CT5'; NCAM forward, 5'-AGAAATCAGCGTTGGAGA GTCC-3' and reverse, 3'-TCGTCATCATTCACACCACT-5'; α -SMA forward, 5'-CCCAGACATCAGGGAGTAATG G-3' and reverse, 3'-TCTATCGGATACTTCAGCGTCA-5'; β -actin forward, 5'-GTGACGTTGACATCCGTAAAGA-3' and reverse, 3'-GCCGGACTCATCGTACTCC-5'. The final volume was measured to 20 μ l with water. The PCR program was as follows: Initial denaturation at 94°C for 4 min, followed by 35 cycles of degeneration at 94°C for 30 sec, annealing temperature for 45 sec, 72°C for 45 sec; followed by final extension step at 72°C for 10 min. Electrophoresis identification was performed with 10 μ l PCR products on 1.5% agarose gel and the remaining PCR products were restored. DNA marker (5 μ l) was loaded in the same gel and used under a UV transilluminator to visualize the PCR product in the agarose gel. β -Actin was chosen as the reference gene for control. The results of PCR were analyzed using FIJI image processing software (version, -v2.0.0-rc-59/1.51n; National Institutes of Health)

Statistical analysis. All data are presented as the mean \pm SD (n=3) and the statistical analyses were analyzed using the statistical software Statistical Package for the Social Sciences (SPSS version 19.0; IBM Corp.). An unpaired Student's t-test was applied to analyze the difference between groups. P<0.05 was considered to indicate a statistically significant difference.

Results

Growth conditions of adherent cells in different cultured systems. Hair follicle stem cells from the same batch were distributed and cultured in four different systems. Of these, small cell clones appeared after 3 days in Group B and

Group D, but after 7 days in Group A. Moreover, no small cell clone formation was observed after incubation of cells in Group C. Adherent cells in Group A appeared to be typical epidermal-like cells characterized by small size, close arrangement and a cobblestone-like shape. (Fig. 1A).

The number of clones for cells from the Group B was low; however, the growth condition of cells was excellent, as indicated by the consistent morphology, fusiform and polygonal appearance and longer cytoplasmic projections. (Fig. 1B).

Cell cloning in Group C was not notable and the cell growth was decreased. A few cells adhered to the dish in group C during planting. In addition, the cells died of aging without obvious cell clone formation (Fig. 1C).

It was indicated that cells in Group D exhibited the strongest proliferative ability. Adherent cells formed flat polygons and exhibited aggregative growth. Cell morphologies were similar to those of hair follicle DP cells and dermal sheath cells (21). The majority of cell clones formed from day 3 and these cells had proliferative abilities from day 5. (Fig. 1D).

Growth and passage condition of hair follicle-derived cells cultured in MEF-conditioned medium. Group D cells exhibited strong proliferative and formative abilities and the dish was confluent in 5-7 days. These cells were sub-cultured, and it was indicated that the cells retained their strong proliferative abilities and were able to reach their next generation after 3 days. Moreover, cell proliferation decreased from generation 6 and the cells stopped proliferating at generation 7 (Fig. 2).

Identification of induction ability of P1-MEF/KSF-HFSC cells in vitro

Identification of the differentiation potential of osteoblasts. The results of alizarin red staining suggested that both osteo-induced and non-induced groups exhibited diffused matrix mineralization, but this was less obvious in the non-induced group. Compared with the non-induced group, Alizarin red staining indicated a larger and increased level of black calcium nodules in the osteo-induced group (Figs. 3A and B). In addition, von Kossa staining granules of varying sizes were arranged in groups around the nucleus in the majority of cells from the lipid-induced group (Fig. 3C and D).

Identification of the differentiation potential of lipoblasts. The results also indicated that only a few cells from the non-induced group exhibited lipoblasts granules on day 21 following oil red O staining (Fig. 3E and F),

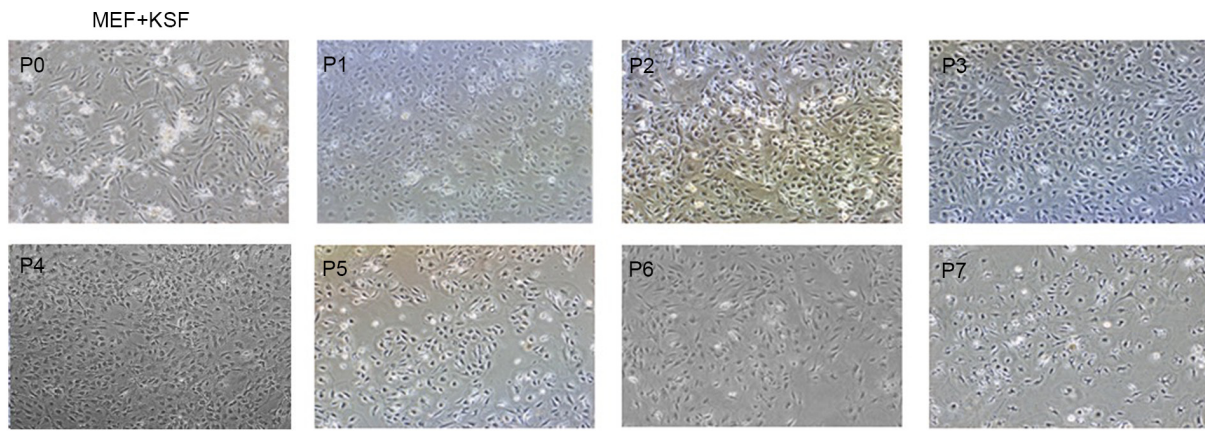


Figure 2. Morphology of hair follicle stem cells cultured in MEF-conditioned medium from original passage to passage 7. Reverse-phase microscope; magnification, x40. MEF, mouse embryonic fibroblast; KSF, keratinocyte serum-free.

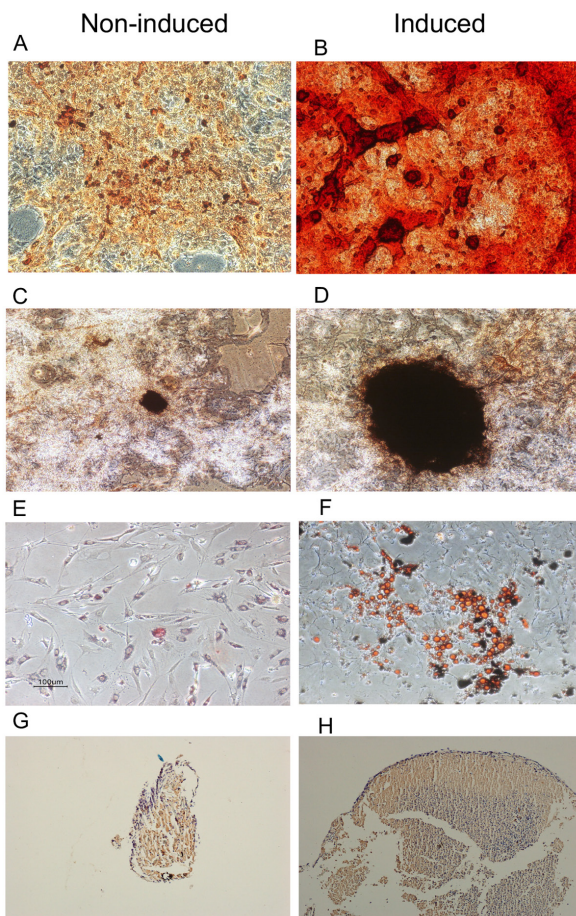


Figure 3. MEF-HFSCs showed osteogenic ability *in vitro*. Alizarin red staining of (A) non-induced MEF-HFSC and (B) osteo-induced MEF-HFSC. VonKossa staining of (C) non-induced MEF-HFSC and (D) lipid-induced MEF-HFSC. Oil red staining of (E) non-induced MEF-HFSC and (F) chondro induced MEF-HFSC. Immunohistochemistry staining of (G) non-induced MEF-HFSC and (H) chondro induced MEF-HFSC. Reverse-phase microscope; magnification, x40. MEF, mouse embryonic fibroblast; HFSC, hair follicle stem cells.

Identification of the differentiation potential of chondroblasts. The non-induced group did not exhibit a stable pallet structure, and a complicated structure was observed from the slice. However, a pallet structure was observed in cells from

the chondro-induced group. The pallet slice subjected to immunohistochemical collagen II staining was stained brown (Fig. 3G and H).

Identification of MEF-HSFC surface markers

Identification of cell surface markers of primary (P1 and P2 generation) cells. Results from the AKP staining were accurately observed and imaged. All cells from each generation adhered and exhibited the expression of AKP, SMA and NCAM (Fig. 4A) except for K15 expression as this is not observed in cells cultured by MEF medium.

Result of RT-PCR. The mRNA expression of SOX-2, NCAM and α -SMA were significantly higher in cells from the P1 generation compared with cells from the control group (Fig. 4B and C).

Result of flow cytometry. The flow cytometry results in the HFSC group suggested that Pan-CK, α -SMA, and NCAM accounted for 9.7, 91.3 and 95.8% of all cells, respectively (Fig. 4D). Quantification of flow cytometry results are presented in Fig. 4E. The expression of Pan-CK was only 9.7%, but α -SMA and NCAM levels were significantly increased.

Discussion

Bone defects are common and may be associated with trauma, infection, tumor resection and congenital diseases (22). Bone tissue engineering, conceptualized in the 1980s, is an emerging method that is used to repair bone defects (23) and involves the application of cells, synthetic materials and cytokines for *in vivo* tissue regeneration and *in vitro* construction (24). Seed cells have attracted increased attention and mesenchymal stem cells derived from bone marrow (25), amniotic fluid (26), umbilical cord blood (27), adipose tissue (28), muscles (29), dental pulp (30) and hair follicles are currently being investigated as seed cells for bone tissue engineering.

Hair follicles containing epithelial and mesenchymal stem cells, which are useful for cellular research (31,32). Through a series of cell labelling experiments, Cotsarelis (18) revealed that a hair follicle stem cell population may be located in the hair bulb and in the bulge region of the hair follicle outer root

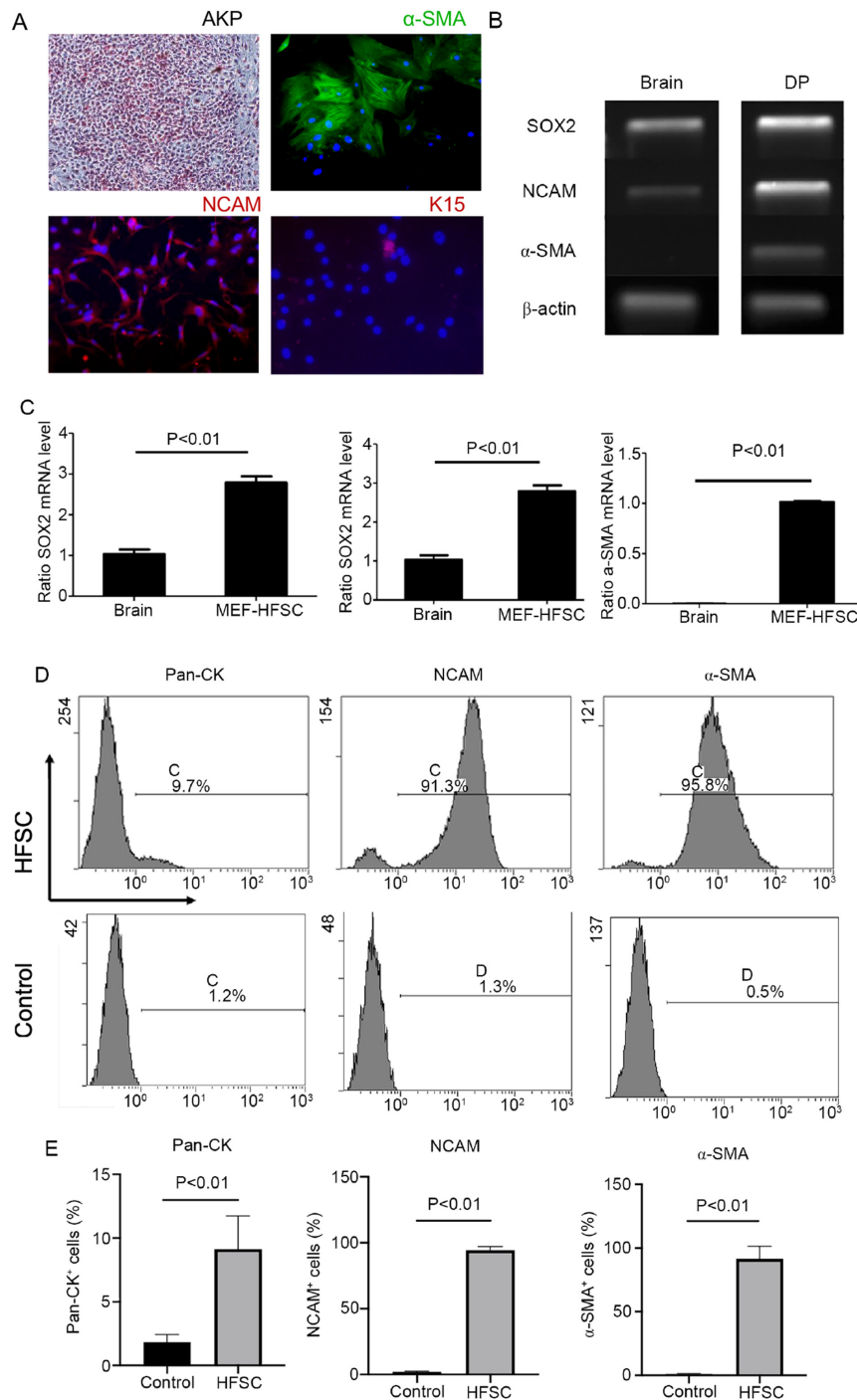


Figure 4. Identification of markers from MEF-HFSC. Staining for (A) AKP (upper left; magnification, x40), α -SMA (upper right; magnification, x100), NCAM (lower left; magnification, x100) and K15 (lower right; magnification, x100). Immunofluorescence staining. (B and C) reverse transcription-PCR of SOX2, NCAM and α -SMA from MEF-HFSC. (D and E) Flow cytometry for pan-CK, NCAM and α -SMA from MEF-HFSC. α -SMA, α -smooth muscle actin; NCAM, neural cell adhesion molecule; MEF/KSF, mouse embryonic fibroblast/keratinocyte serum-free medium; AKP, alkaline phosphatase; pan-CK, pan-cytokeratin.

sheath cells. Hair follicle stem cells were first isolated by extracting hair follicles from the human scalp and digesting them with pancreatic enzymes. Moreover, the outer root sheath cells were cultured through the collagen-coating method used by Kurata *et al* (33). However, the known methods of amplification and purification of DP cells are associated with a number of different limitations. To address this issue, the present study aimed to enrich hair follicle-derived mesenchymal stem cell populations and subject these cells to osteogenic differentiation for applications in tissue engineering.

KSF follicular epithelium stem cell culture medium and DMEM+100S mesenchymal stem cell culture medium was used to culture embryonic stem cells that were used for the growth of hair follicle-derived stem cells. Human embryonic stem cells have been successfully cultured via feeder layer isolation and cultivation of MEF by Thomson *et al* (34) in 1998. MEF cells are normally grown as feeder layer cells to preserve the multi-directional differentiation potential of primary generation cells in long-term cultures, due to their high availability, ease of preparation and remarkable

effect (35). Furthermore, Chin *et al* (36) used MEF cells and MEF-conditioned medium in 2007 and suggested that pigment epithelium-derived factor (PEDF), insulin-like growth factor binding protein (IGFBP)-2, IGFBP-7, monocyte chemoattractant protein-1 (MCP-1) and interleukin-6 (IL-6) served as growth factors in MEF-conditioned medium, as well as facilitated the low differentiation of embryonic stem cells. Based on these findings, MEF/KSF conditioned medium was used to culture and amplify hair follicle cells in the present study.

In the current study, cells lacking proliferative and adherent abilities revealed that no adipocytes and fibroblasts contained in hypodermis tissues were infiltrated in the cell suspension obtained via the sampling and enzyme digestion process in DMEM+10% FBS medium. In addition, the three types of phenotypes obtained under three different culture conditions facilitated the development of general conclusions based on the cellular morphologies. For example, cells cultured in KSF-conditioned medium may be hair follicle-derived epithelium stem cells and those cultured in KSF+100S medium may be hair follicle mesenchymal cells with stronger adipogenic potential. Furthermore, adherent cells cultured in MEF/KSF-conditioned culture medium are analogous to bone marrow-derived mesenchymal stem cells and hair papilla cells obtained using the combination of microsurgery and enzyme digestion. The adherent cell population was considered to be the hair follicle-derived mesenchymal stem cells and exhibited stronger proliferative and osteoblastic ability, as expected from the findings of cell induction and identification studies. Therefore, the results suggested that MSF/KSF-conditioned culture medium was useful for screening hair follicle-derived mesenchymal stem cells with osteoblastic capacity. However, the proliferation assay of cells cultured in MSF/KSF-conditioned culture medium requires further study, so follow-up experiments are required to verify these results.

Hair follicle stem cells are derived from skin and hair and are widely used in tissue engineering, due to their abundant source and ease of isolation. Aside from their ability to differentiate into skin and skin appendages, hair follicle stem cells have the potential to differentiate into osteocytes, adipocytes, myocytes, chondrocytes and neurons (37). Prior to the study on hair follicle stem cells, *in vitro* osteo-induction of bone marrow-derived mesenchymal stem cells from mice was performed with the osteogenic induction system and the results of AKP staining (2 weeks later) and alizarin red staining (3 weeks later) were positive for the osteo-induced group, but negative for the non-induced group (Data not shown). Therefore, the induction and detection system for osteogenic differentiation containing vitamin C, sodium β -glycerophosphate and dexamethasone was beneficial.

In the present study, no positive results for osteo-induction were observed for cells from KSF and KSF+10% FBS groups, suggesting that these groups lacked the osteogenic potential. Therefore, these cells were considered to be epithelium-derived stem cells that exhibited only the ability of unidirectional differentiation in the hair follicle. Furthermore, MEF/KSF-conditioned culture medium was used for the induction of cells from primary (P1 and P2) generations, and strong and weak positive results were identified for cells from the induced and non-induced groups, respectively. Therefore,

the components of MEF/KSF-conditioned culture medium may exhibit effects on osteo-induction but failed to produce positive results when used to induce cells from subsequent generations; this finding may be associated with the loss of stem cell potential during cell passaging. It has been previously reported that DP and dermal sheath cells from hair papilla of Wistar rats may exhibit osteogenic and adipogenic abilities (3). Moreover, Bajpai *et al* (38) observed an increase in the intracellular concentrations of Runx2, ALP and osteonectin following osteo-induction of human hair follicle mesenchymal cells. The osteogenic potential peaked in the eighth generation of cultured cells and started to decline from generation 11. Collectively, these results are in line with those reported in the present study.

The current study detected the sources of cell markers in cultures of hair follicle stem cells *in vitro*. Hair follicle stem cells with osteogenic potential are located at the bulge region of the epithelium of the hair follicle tissue and hair papilla from the mesenchymal tissue (39). DP cells located in the basal region of hair follicles are considered to be an effective source of adult mesenchymal stem cells and used for the engineering of hair follicles and skin (40), and also bone (41), muscle (42) and nerve tissue (43). Therefore, it was hypothesized that DP cells are mesenchymal cell-derived cells obtained from the DP region of the hair follicle.

The markers of epithelium-derived cells from the hair follicle and dermis-derived cells of mice were evaluated and the results of the present study revealed that pan-CK and K15, which are surface markers of epithelium-derived cells, were absent. However, the cells exhibited positive results for AKP, α -SMA and NCAM (44,45), which are surface markers of hair follicle DP-derived cells. PEDF in MEF medium may enhance the expression of AKP in DP cells, and it has been previously demonstrated that pigment epithelium-derived factor enhances AKP expression in human mesenchymal stem cells (46). NCAM expression was most likely induced by insulin-like growth factor binding protein, which is highly expressed in MEF. Lyles *et al* (47) identified changes in insulin like growth factor II, which may regulate muscle NCAM expression during embryonic development. SOX2 is a member of the SOX HMG box family and is expressed in early stage in embryonic development to maintain pluripotency and self-renewal in embryonic stem (ES) cells (48). It has also been previously demonstrated that the of AKP (AKP is a membrane bound enzyme) activity and colony formation positively correlates with self-renewal potential of human ES cells (49). These results may indicate how the expression of this cell marker of DP cells was increased in MEF medium. To avoid any false-positive results caused by nonspecific antibody staining, typical epithelial and dermal cell markers were selected for analysis using flow cytometry. The results indicated that mesenchymal tissue-derived cells accounted for >90% of cells obtained from MEF/KSF-conditioned medium.

The aforementioned results demonstrated that MEF/KSF-HFSC may be sourced from DP of hair follicles. To identify the multi-lineage differentiation capacity of DP cells, adipocytes and chondroblasts originating from mesoderm differentiation were used for induction. It was indicated that MEF-HFSC may be spontaneously induced into osteoblasts, adipocytes, and chondroblasts in the absence of specific

conditions, as previously reported by Jahoda *et al* (9). However, the speculation that MEF/KSF-HFSC cells may be DP cells requires further examination.

In conclusion, the present study suggested that MEF/KSF-conditioned medium was an effective method for *in vitro* culturing of mouse dermal papilla cells with osteogenic differentiation potential.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

LX carried out the molecular genetic studies and drafted the manuscript. WG conducted the literature search and cell culture work. SB, XP and HD were involved in performing the histology experiments. LX and WW supervised the experiments and performed the analysis and interpretation of data. LX and WW are responsible for confirming the authenticity of raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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