

IV 研究成果に関する一欄表

研究成果の刊行に関する一覧表

書籍

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V 研究成果の刊行物・別冊

Menstrual Blood-derived Cells Confer Human Dystrophin Expression in the Murine Model of Duchenne Muscular Dystrophy via Cell Fusion and Myogenic Transdifferentiation [□]

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Duchenne muscular dystrophy (DMD), the most common lethal genetic disorder in children, is an X-linked recessive muscle disease characterized by the absence of dystrophin at the sarcolemma of muscle fibers. We examined a putative endometrial progenitor obtained from endometrial tissue samples to determine whether these cells repair muscular degeneration in a murine mdx model of DMD. Implanted cells conferred human dystrophin in degenerated muscle of immunodeficient mdx mice. We then examined menstrual blood-derived cells to determine whether primarily cultured nontransformed cells also repair dystrophied muscle. In vivo transfer of menstrual blood-derived cells into dystrophic muscles of immunodeficient mdx mice restored sarcolemmal expression of dystrophin. Labeling of implanted cells with EGFP and differential staining of human and murine nuclei suggest that human dystrophin expression is due to cell fusion between host myocytes and implanted cells. In vitro analysis revealed that endometrial progenitor cells and menstrual blood-derived cells can efficiently transdifferentiate into myoblasts/myocytes, fuse to C2C12 murine myoblasts by in vitro coculturing, and start to express dystrophin after fusion. These results demonstrate that the endometrial progenitor cells and menstrual blood-derived cells can transfer dystrophin into dystrophied myocytes at a high frequency through cell fusion and transdifferentiation in vitro and in vivo.

INTRODUCTION

Skeletal muscle consists predominantly of syncytial fibers with peripheral, postmitotic myonuclei, and its intrinsic repair potential in adulthood relies on the persistence of a resident reserve population of undifferentiated mononuclear cells, termed "satellite cells." In mature skeletal muscle, most satellite cells are quiescent and are activated in response to environmental cues, such as injury, to mediate postnatal muscle regeneration. After division, satellite cell progeny, termed myoblasts, undergo terminal differentiation and become incorporated into muscle fibers (Bischoff, 1994). Myogenesis is regulated by a family of myogenic transcription factors including MyoD, Myf5, myogenin, and MRF4 (Sabourin and Rudnicki, 2000). During embryonic development, MyoD and Myf5 are involved in the establishment of the skeletal muscle lineage (Rudnicki *et al.*, 1993), whereas myogenin is required for terminal differentiation (Hasty *et al.*, 1993; Nabeshima *et al.*, 1993). During muscle

repair, satellite cells recapitulate the expression program of the myogenic genes manifested during embryonic development.

Dystrophin is associated with a large oligomeric complex of glycoproteins that provide linkage to the extracellular membrane (Ervasti and Campbell, 1991). In Duchenne muscular dystrophy (DMD), the absence of dystrophin results in destabilization of the extracellular membrane-sarcolemma-cytoskeleton architecture, making muscle fibers susceptible to contraction-associated mechanical stress and degeneration. In the first phase of the disease, new muscle fibers are formed by satellite cells. After depletion of the satellite cell pool in childhood, skeletal muscles degenerate progressively and irreversibly and are replaced by fibrotic tissue (Cossu and Mavilio, 2000). Like DMD patients, the mdx mouse lacks dystrophin in skeletal muscle fibers (Hoffman *et al.*, 1987; Sicinski *et al.*, 1989). However, the mdx mouse develops only a mild dystrophic phenotype, probably because muscle regeneration by satellite cells is efficient for most of the animal's life span (Cossu and Mavilio, 2000).

Myoblasts represent the natural first choice in cellular therapeutics for skeletal muscle because of their intrinsic myogenic commitment (Grounds *et al.*, 2002). However, myoblasts recovered from muscular biopsies are poorly expandable in vitro and rapidly undergo senescence (Cossu and Mavilio, 2000). An alternative source of muscle progenitor cells is therefore desirable. Cells with a myogenic potential are present in many tissues, and these cells readily

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form skeletal muscle in culture (Gerhart et al., 2001). We report here that human dystrophin expression in the mdx model of DMD is attributed to cell fusion of mdx myocytes with human menstrual blood-derived stromal cells.

MATERIALS AND METHODS

Isolation of Human Endometrial Cells from Menstrual Blood

Menstrual blood samples ($n = 21$) were collected in DMEM with antibiotics (final concentrations: 100 U/ml penicillin/streptomycin) and 2% fetal bovine serum (FBS), and processed within 24 h. Ethical approval for tissue collection was granted by the Institutional Review Board of the National Research Institute for Child Health and Development, Japan. The centrifuged pellets containing endometrium-derived cells were resuspended in high-glucose DMEM medium (10% FBS, penicillin/streptomycin), maintained at 37°C in a humidified atmosphere containing 5% CO₂, and allowed to attach for 48 h. Nonadherent cells were removed by changing the medium. When the culture reached subconfluence, the cells were harvested with 0.25% trypsin and 1 mM EDTA and plated to new dishes. After 2–3 passages, the attached endometrial stromal cells were devoid of blood cells. Human EM-E6/E7/hTERT-2 cells, endometrium-derived progenitors, were obtained from surgical endometrial tissue samples and were immortalized by E6, E7, and hTERT-2 (Kyo et al., 2003). C2C12 myoblast cells were supplied by RIKEN Cell Bank (The Institute of Physical and Chemical Research, Japan).

Flow Cytometric Analysis

Flow cytometric analysis was performed as previously described (Terai et al., 2005). Cells were incubated with primary antibodies or isotype-matched control antibodies, followed by additional treatment with the immunofluorescent secondary antibodies. Cells were analyzed on an EPICS ALTRA analyzer (Beckman Coulter, Fullerton, CA). Antibodies against human CD13, CD14, CD29, CD31, CD34, CD44, CD45, CD50, CD54, CD55, CD59, CD73, CD90, CD105, CD117 (c-kit), CD133, HLA-ABC, and HLA-DR were purchased from Beckman Coulter, Immunotech (Marseille, France), Cytotech (Hellebæk, Denmark), and BD Biosciences Pharmingen (San Diego, CA).

In Vitro Lentivirus-mediated Gene (EGFP) Transfer into EM-E6/E7/hTERT-2 Cells

AQ:2 Infection of EM-E6/E7/hTERT-2 cells with lentivirus having a CMV promoter and EGFP reporter resulted in high levels of EGFP expression in all cells. Cells were analyzed for EGFP expression by flow cytometry (Miyoshi et al., 1997, 1998).

In Vitro Myogenesis

AQ:3 Menstrual blood-derived cells or EM-E6/E7/hTERT-2 cells were seeded onto collagen I-coated cell culture dishes (Biocoat, BD Biosciences, Bedford, MA) at a density of 1×10^5 /ml in growth medium (DMEM, supplemented with 20% FBS). Forty-eight hours after seeding onto collagen I-coated dishes, cells were treated with 5-azacytidine for 24 h. Cell cultures were then washed twice with PBS and maintained in differentiation medium (DMEM, supplemented with either 2% horse serum (HS) or 1% insulin-transferrin-selenium supplement [ITS]). The differentiation medium was changed twice a week until the experiment was terminated.

RT-PCR Analysis of EM-E6/E7/hTERT-2 Cells and Menstrual Blood-derived Cells

Total RNA was prepared using Isogen (Nippon Gene, Tokyo, Japan). Human skeletal muscle RNA was purchased from TOYOBO (Osaka, Japan). RT-PCR of Myf5, MyoD, desmin, myogenin, myosin heavy chain-Ix/d (MyHC Ix/d), and dystrophin was performed with 2 µg of total RNA. RNA for RT-PCR was converted to cDNA with a first-stand cDNA synthesis kit (Amersham Pharmacia Biotechnology, Piscataway, NJ) according to the manufacturer's recommendations. The sequences of PCR primers that amplify human but not mouse genes are listed in Supplementary Table 1. PCR was performed with TaKaRa recombinant Taq (Takara Shuzo, Kyoto, Japan) for 30 cycles, with each cycle consisting of 94°C for 30 s, 62°C or 65°C for 30 s, and 72°C for 20 s, with an additional 10-min incubation at 72°C after completion of the last cycle.

Immunohistochemical and Immunocytochemical Analysis

Immunohistochemical analysis was performed as previously described (Mori et al., 2005). Briefly, the sections were incubated for 1 h at room temperature with mouse mAb against vimentin (Cone V9, DakoCytomation, Fort Collins, CO). After washing in PBS, sections were incubated with horseradish peroxidase-conjugated rabbit anti-mouse immunoglobulin, diluted, and washed in cold PBS. Staining was developed by using a solution containing diaminobenzidine and 0.01% H₂O₂ in 0.05 M Tris-HCl buffer, pH 6.7. Slides were

counterstained with hematoxylin. In the cases of fluorescence, frozen sections fixed with 4% PFA were used. The antibodies against human dystrophin (NCL-DYS3; Novocastra, Newcastle upon Tyne, United Kingdom) or anti-human nuclei mouse mAb (clone 235-1, Chemicon, Temecula, CA) was used as a first antibody, and goat anti-mouse IgG conjugated with Alexa Fluor 488 or goat anti-mouse IgG antibody conjugated with Alexa Fluor 546 (Molecular Probes, Eugene, OR) was used as a second antibody.

Immunocytochemical analysis was performed as previously described (Mori et al., 2005), with antibodies to skeletal myosin (Sigma, St. Louis, MO; product no. M 4276), MF20 (which reacts with all sarcomere myosin in striated muscles, Developmental Studies Hybridoma Bank, University of Iowa, IA), α -sarcomeric actin (Sigma, product no. A 7811), and desmin (BioScience Products, Bern, Switzerland; no. 010031, clone D9) in PBS containing 1% bovine serum albumin. As a methodological control, the primary antibody was omitted. In the cases of fluorescence, slides were incubated with Alexa Fluor 546-conjugated goat anti-mouse IgG antibody.

Western Blotting

Western blot analysis was performed as previously described (Mori et al., 2005). Blots were incubated with primary antibodies (desmin, myogenin [Clone F5D, Santa Cruz Biotechnology], and dystrophin [NCL-DYSA, Novocastra]) for 1–2 h at room temperature. After washing three times in the blocking buffer, blots were incubated for 30 min with a horseradish peroxidase-conjugated secondary antibody (0.04 µg/ml) directed against the primary antibody. The blots were developed with enhanced chemiluminescence substrate according to the manufacturer's instructions.

Fusion Assay

EM-E6/E7/hTERT-2 cells (2500/cm²) or EGFP-labeled EM-E6/E7/hTERT-2 cells (2500/cm²) were cocultured with C2C12 myoblasts (2500/cm²) for 2 d in DMEM supplemented with 10% FBS and then cultured for 7 additional days in DMEM with 2% HS to promote myotube formation. The cultures were fixed in 4% paraformaldehyde and stained with a mouse anti-human nuclei IgG1 mAb and the mouse anti-human dystrophin IgG2a mAb (or anti-myosin heavy chain IgG2b mAb MF-20). The cells were visualized with appropriate Alexa-fluor-conjugated goat anti-mouse IgG1 and IgG2a (or IgG2b) secondary antibodies (Molecular Probes). Total cell nuclei were stained with DAPI (4',6-diamidino-2-phenylindole).

In Vivo Cell Implantation

Six- to 8-wk-old NOD/Shi-scid/IL-2 receptor $-/-$ (NOC, CREA, Shizuoka, Japan) mice and 6- to 8-wk-old mdx-scid mice were implanted with EM-E6/E7/hTERT-2 cells and menstrual blood-derived cells in seven independent experiments. The cells (2×10^7) were suspended in PBS in a total volume of 100 µl and were directly injected into the right thigh muscle of NOC mice or mdx-scid mice. The mice were examined 3 wk after cell implantation, and the right thigh muscle was analyzed for human vimentin and dystrophin by immunohistochemistry. The antibodies to vimentin and dystrophin (NCL-DYS3) react with human vimentin and dystrophin-equivalent protein, but not murine protein.

RESULTS

Surface Marker Expression of Endometrium-derived Cells

We investigated myogenic differentiation of primary cells without gene introduction from menstrual blood, because menstrual blood on the first day of the period is considered to include endometrial tissue. We successfully cultured a large number of primary cells from menstrual blood. Menstrual blood-derived cells showed at least two morphologically different cell groups: small spindle-like cells and large stick-like cells, regarded as being passage day (PD) 1 or 2 (Figure 1, A and B, respectively). Surface markers of the menstrual blood-derived cells were evaluated by flow cytometric analysis. Surface markers of EM-E6/E7/hTERT-2 cells (Figure 1C) and menstrual blood-derived cells (Figure 1D) were evaluated by flow cytometric analysis (Figure 1E). In these experiments, the cells were cultured in the absence of any inductive stimuli. EM-E6/E7/hTERT-2 cells were positive for CD13, CD29 (integrin β 1), CD44 (Pgp-1/ly24), CD54, CD55, CD59, CD73, and CD90 (Thy-1), implying that EM-E6/E7/hTERT-2 cells expressed mesenchymal cell-related antigens in our experimental setting. Menstrual blood-derived cells were positive for CD13, CD29, CD44, CD54, CD55, CD59, CD73, CD90, and CD105, implying that proliferating

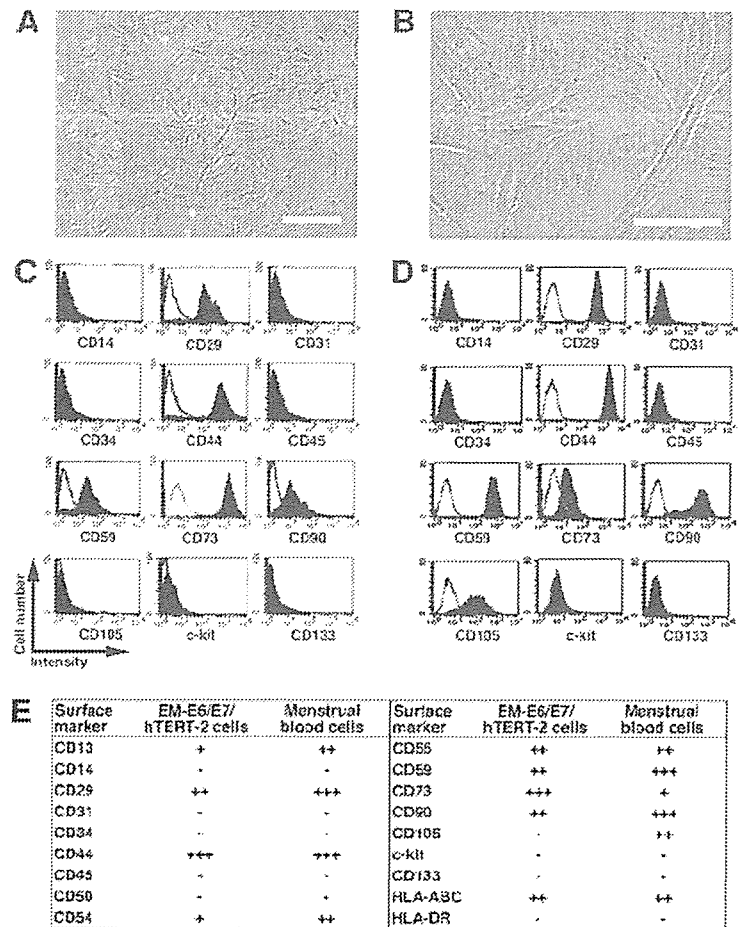


Figure 1. Surface marker expression of endometrium-derived cells. (A and B) Morphology of menstrual blood-derived cells, regarded as being PD 1 or 2. Scale bars, 200 μ m (A), 100 μ m (B). (C and D) Flow cytometric analysis of cell surface markers of EM-E6/E7/hTERT-2 cells (C) and menstrual blood-derived cells (D). (E) Further phenotypic analysis in EM-E6/E7/hTERT-2 cells and menstrual blood-derived cells are summarized. Peak intensity was estimated in comparison with isotype controls. +++, strongly positive (>100 times the isotype control); ++, moderately positive (<100 times but more than 10 times the isotype control), weakly positive (<10 times but more than twice the isotype control), -, negative (less than twice the isotype control).

erated and propagated cells express mesenchymal cell-related cell surface markers. Unlike EM-E6/E7/hTERT-2 cells, the menstrual blood-derived adherent cells were positive for CD105. EM-E6/E7/hTERT-2 cells and menstrual blood-derived cells expressed neither hematopoietic lineage markers, such as CD34, nor monocyte-macrophage antigens such as CD14 (a marker for macrophage and dendritic cells), or CD45 (leukocyte common antigen). The lack of expression of CD14, CD34, or CD45 suggests that EM-E6/E7/hTERT-2 cells and the menstrual blood-derived cell culture in the present study is depleted of hematopoietic cells. The cells were also negative for expression of CD31 (PECAM-1), CD50, c-kit, and CD133. The cell population was positive for HLA-ABC, but not for HLA-DR. These results demonstrate that almost all cells derived from endometrium are of mesenchymal origin or stromal origin.

Implanted Endometrium-derived Cells Induce De Novo Myogenesis in Immunodeficient NOG Mice

EM-E6/E7/hTERT-2 cells originate from the endometrial gland and are considered as endometrial progenitor cells or bipotential cells capable of differentiating into both glandular epithelial cells and endometrial stromal cells (Kyo *et al.*, 2003). To determine whether EM-E6/E7/hTERT-2 cells and menstrual blood-derived cells generate complete endometrial structure *in vivo*, like endometriosis, the cells without any treatment or induction were injected into the right thigh

muscle of immunodeficient NOG mice. PBS without cells was injected into the left thigh muscles as a control. We failed to detect any endometrial structure in the cell-injected site. Immunohistochemical analysis using an antibody specific to human vimentin, an intermediate filament associated with a mesenchymal cell, revealed that the injected EM-E6/E7/hTERT-2 cells (Figure 2, A-F) or menstrual blood-derived cells (Figure 2, G-L) extensively migrated or infiltrated between muscular fibers (Figure 2, arrowheads). To investigate if the donor cells between muscular fibers occur as a result of cell migration, we performed a time-course analysis of implanted cells, as probed by human-specific antibody to vimentin (Supplementary Figure 1). Donor cells at 3 h after implantation are observed at the injection site, which is considered to be due to just injection of cells. Cells at 1-3 wk after implantation are detected between myocytes in the muscle bundle or muscular fascicle as well as in the interstitial tissue, implying that the donor cells between myotubes result from cell migration. Interestingly, some of the vimentin-positive implanted cells exhibited round-shaped structure (Figure 2, D, F, and J, arrows), suggesting that endometrium-derived cells are capable of differentiating into myoblasts/myotubes, and can contribute to skeletal muscle repair in patients suffering from genetic disorders such as DMD, similar to previous reports for marrow stromal cells (Dezawa *et al.*, 2005) and synovial membrane cells (De Bari *et al.*, 2003).

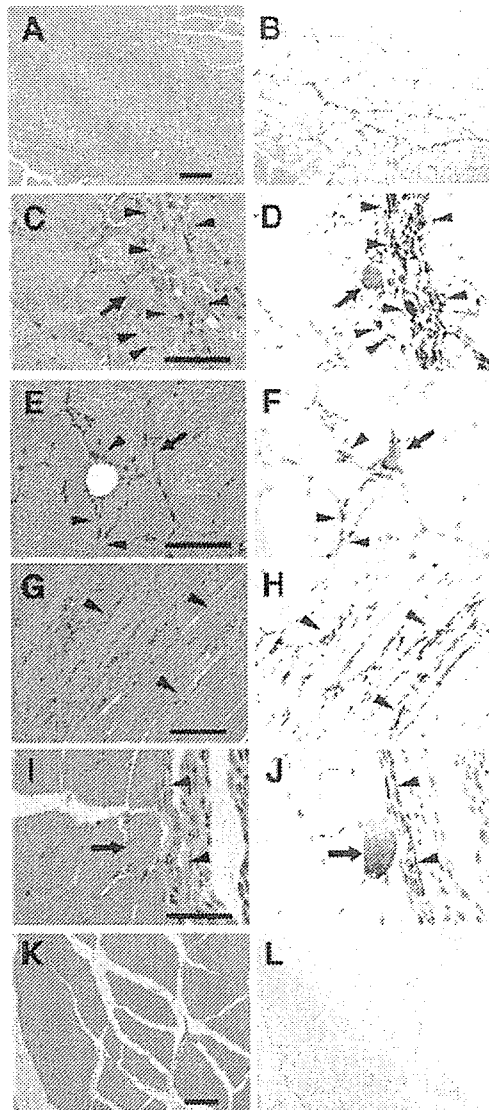


Figure 2. Implantation of endometrium-derived cells into the muscle of NOG mice. EM-E6/E7/hTERT-2 cells (A–F) or menstrual blood–derived cells (G–J) cultured in absence of any stimuli were directly injected into the right thigh muscle of NOG mice. Immunohistochemical analysis was performed using antibody that reacts to human vimentin but not to murine vimentin. (A, C, E, G, I, and K) hematoxylin and eosin stain (H&E; B, D, F, H, J, and L) immunohistochemistry. Note that vimentin-positive EM-E6/E7/hTERT-2 cells and menstrual blood–derived cells with a spindle morphology (C–J, arrowheads) extensively migrated into muscular bundles at 3 wk after injection, and some of the injected cells exhibited round structure (D, F, and J, arrows). Isotype mouse IgG1 served as a negative control (L). Scale bars, 100 μ m (A, B, K, and L), 50 μ m (C–F, I, and J), 90 μ m (G and H).

Induction of Myogenic Differentiation in Endometrial Progenitor Cells In Vitro

EM-E6/E7/hTERT-2 cells at 2 wk (cultured in the DMEM supplemented with 20% FBS) after exposure to different concentrations (5, 10, and 100 μ M) of 5-azacytidine were analyzed by immunostaining using anti-desmin antibody (Figure 3, A–F). The number of desmin-positive cells was

significantly higher in experimental groups with 5 or 10 μ M 5-azacytidine than in untreated control groups ($p < 0.05$). To investigate whether EM-E6/E7/hTERT-2 cells are capable of differentiating into skeletal muscle cells in vitro, the cells were exposed to 5 μ M 5-azacytidine for 24 h and then subsequently cultured in the DMEM supplemented with 2% HS (Figure 3, G–J) or serum-free ITS for up to 21 d (Figure 3K). Skeletal myoblastic differentiation of the cells was analyzed by evaluating expression of MyoD, Myf5, desmin, myogenin, MyHC-IIx/d, and dystrophin by RT-PCR. The MyoD, desmin, myogenin, and dystrophin genes were constitutively expressed, but MyHC-IIx/d and Myf5 genes were not. The decline of MyoD was observed in both the 2% HS (Figure 3, G and H) and the serum-free ITS (Figure 3K). The expression of MyHC, as determined by RT-PCR and immunocytochemistry, significantly increased with 2% HS (Figure 3, G–J) and serum-free ITS (Figure 3K). Immunocytochemical analysis indicated that α -sarcomeric actin (Figure 3I) and MyHC (Figure 3J) were detected in the cells incubated with 2% HS for 21 d.

In Vitro Myogenic Differentiation of Menstrual Blood-derived Cells

Menstrual blood–derived cells at 3 wk (cultured in DMEM supplemented with 20% FBS) after exposure to different concentrations (5, 10, and 100 μ M) of 5-azacytidine were analyzed by immunostaining using anti-desmin antibody (data not shown). The number of desmin-positive cells was significantly higher in experimental groups with 5 or 10 μ M 5-azacytidine than with 100 μ M 5-azacytidine; for further in vitro experiments, the menstrual blood–derived cells were exposed to 5 μ M 5-azacytidine for 24 h and then subsequently cultured in DMEM supplemented with low serum (2% HS) or serum-free ITS for up to 21 d (Figure 4). Myogenic potential of human menstrual blood–derived cells was analyzed by evaluating the expression of Myf5, MyoD, desmin, myogenin, MyHC-IIx/d, and dystrophin by RT-PCR. MyoD, desmin, and dystrophin genes were constitutively expressed in menstrual blood–derived cells, but MyHC-IIx/d and Myf5 were not (Figure 4A). For cells treated with 2% HS or serum-free ITS, the mRNA level of desmin and myogenin significantly increased after 3 d, and desmin steadily increased until day 21 (Figure 4, C and D). MyHC-IIx/d started to be expressed at a low level at day 21 of induction (Figure 4C). We then analyzed desmin expression by immunocytochemistry. Menstrual blood–derived cells were exposed to 5 μ M 5-azacytidine for 24 h and then subsequently cultured in DMEM supplemented with 20% FBS for up to 2 wk. Desmin was readily detected in colonies of the menstrual blood–derived cells (Figure 4B). Western blot analysis indicated that desmin, myogenin, and dystrophin were highly expressed in the cells incubated for 3 wk (Figure 4, E–G). These results suggest that menstrual blood–derived cells are, like the EM-E6/E7/hTERT-2 cells, able to differentiate into skeletal muscle.

Regeneration of Dystrophin by Cell Implantation in the DMD Model mdx-scid Mouse

To investigate whether human EM-E6/E7/hTERT-2 cells and menstrual blood–derived cells can generate muscle tissue in vivo, cells without any treatment or induction were implanted directly into the right thigh muscles of mdx-scid mice (Supplementary Figure 2). The left thigh muscles were injected with PBS as an internal control. After 3 wk, myotubes in the muscle tissues injected with human EM-E6/E7/hTERT-2 cells and menstrual blood–derived cells expressed human dystrophin as a cluster (Figure 5, A, C, and D, EM-

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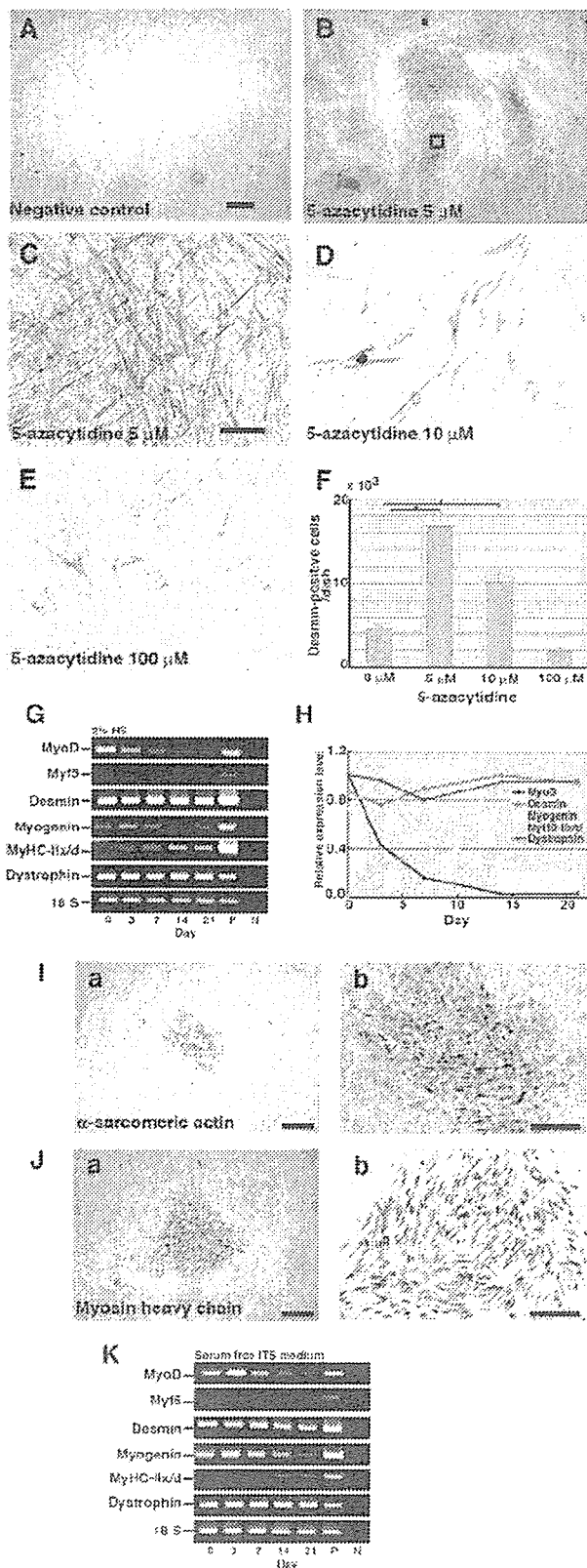


Figure 3. Expression of myogenic-specific genes during myogenic differentiation of EM-E6/E7/hTERT-2 cells. (A–F) Immunocytochemical

E6/E7/hTERT-2 cells, and 5B, menstrual blood-derived cells). Quantification analysis revealed that the percentage of dystrophin-positive myofibers after implantation of menstrual blood-derived cells was high, compared with that after implantation of EM-E6/E7/hTERT-2 cells (Figure 5E). Donor cells with EGFP fluorescence participated in myogenesis 3 wk after implantation (Supplementary Figure 3). EGFP-labeled EM-E6/E7/hTERT-2 cells became positive for human dystrophin (Figure 5C). Dystrophin was not detected in the muscle of mdx-scid mice and NOG mice without cell implantation because the antibody to dystrophin used in this study is human-specific, implying that dystrophin is transcribed from dystrophin genes of human donor cells but not from reversion of dystrophied myocytes in mdx-scid mice.

To determine if dystrophin expression in the donor cells is due to transdifferentiation or fusion, immunohistochemistry with an antibody against human nuclei (Ab-HuNucl) and DAPI stain was performed. If dystrophin expression is explained by fusion, dystrophin-positive myocytes must be demonstrated to have both human and murine nuclei. We examined almost all the 7-μm-thick serial histological sections parallel to the muscular bundle (longitudinal section) of the muscular tissues by confocal microscopy and found that dystrophin-positive myocytes have nuclei derived from both human and murine cells in the longitudinal section of the myocytes (Figure 5D), implying that dystrophin expression is attributed to fusion between murine host myocytes and human donor cells, rather than myogenic differentiation of EM-E6/E7/hTERT-2 cells and menstrual blood-derived cells per se.

Detection of Human Endometrial Cell Contribution to Myotubes in an In Vitro Myogenesis Model

To simulate in vivo phenomena, human endometrial cells were cocultured in vitro with murine C2C12 myoblasts for 2 d under proliferative conditions and then switched to differentiation conditions for an additional 7 d. Figure 6A

analysis of EM-E6/E7/hTERT-2 cells using an antibody to desmin. (A) Omission of only the primary antibody to desmin serves as a negative control. (C) Higher magnification of inset in B. (F) Myogenic differentiation of EM-E6/E7/hTERT-2 cells with exposure to different concentrations (B, 5 μM; C, 5 μM; D, 10 μM; E, 100 μM) of 5-azacytidine. To estimate myogenic differentiation, the number of all the desmin-positive cells was counted for each dish (n = 3). Data were analyzed for statistical significance using ANOVA. EM-E6/E7/hTERT-2 cells were cultured in the DMEM supplemented with 2% HS (G–J; horse serum), and serum-free ITS (K). (G and K) RT-PCR analysis with PCR primers allows amplification of the human MyoD, Myf5, desmin, myogenin, myosin heavy chain type IIx/d (MyHC-IIx/d), and dystrophin cDNA (from top to bottom). RNAs were isolated from EM-E6/E7/hTERT-2 cells at the indicated day after treatment with 5-azacytidine. RNAs from human muscle and H₂O served as positive (P) and negative (N) controls. Only the 18S PCR primer used as a positive control reacted with the human and murine cDNA. (H) Time course of MyoD, desmin, myogenin, MyHC-IIx/d, and dystrophin expression in the cells incubated with 2% HS for up to 21 d after 5-azacytidine treatment. Relative mRNA levels were determined using Multi Gauge Ver 2.0 (Fuji Film). The signal intensities of MyoD, desmin, and dystrophin mRNA at day 0, myogenin mRNA at day 3, and MyHC-II/d mRNA at day 21 were regarded as equal to 100%. (I and J) The cells were exposed to 5 μM 5-azacytidine for 24 h and then subsequently cultured in DMEM supplemented with 2% HS for 21 d. α-Sarcomeric actin (I) and skeletal myosin heavy chain (J) was detected by immunocytochemical analysis. Scale bars, 2 mm (A and B), 300 μm (C–E), 900 μm (Ia and Ja), 425 μm (Ib and Jb).

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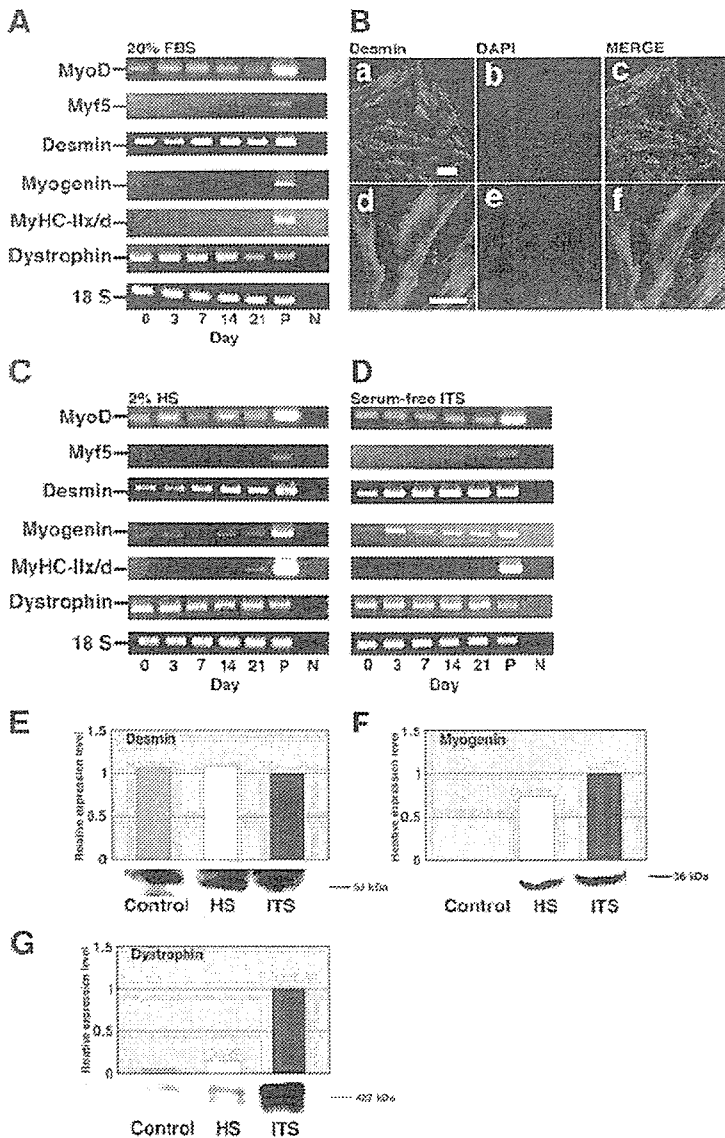


Figure 4. Expression of myogenic-specific genes in differentiated menstrual blood-derived cells. Menstrual blood-derived cells were cultured in DMEM supplemented with 20% FBS, 2% HS, or serum-free ITS medium. (A) RT-PCR analysis with PCR primers that allows amplification of the human MyoD, Myf5, desmin, myogenin, MyHC-IIx/d, and dystrophin cDNA (from top to bottom). RNAs were isolated from menstrual blood-derived cells at the indicated day after treatment with 5 μ M 5-azacytidine for 24 h. RNAs from human muscle and H₂O served as positive (P) and negative (N) controls. Only the 18S PCR primer used as a positive control reacted with the human and murine cDNA. (B) Immunocytochemical analysis using an antibody to desmin (a–f) was performed on the menstrual blood-derived cells at 2 wk after exposure to 5 μ M of 5-azacytidine for 24 h. The desmin-positive cells are shown at higher magnification (d–f). Merge of a and b is shown in c, and merge of d and e is shown in f. The images were obtained with a laser scanning confocal microscope. Scale bars, 200 μ m (a–c) and 75 μ m (d–f). (C and D) RT-PCR analysis of menstrual blood-derived cells on DMEM supplemented with 2% HS (C) or serum-free ITS medium (D) after exposure to 5 μ M 5-azacytidine for 24 h. (E–G) Western blot analysis was performed on the cells cultured in myogenic medium indicated for 21 d. The blot was stained with desmin (E), myogenin (F), and dystrophin (G) antibodies followed by an HRP-conjugated secondary antibody.

provides an example of how human and mouse nuclei in the EGFP-positive myotubes were detected. Multinucleated myotubes were revealed by the presence of specific human dystrophin (Figure 6, B and C) and myosin heavy chain (Figure 6D). Dystrophin was detected in cytoplasm in culture condition (Figure 6, B and C) despite evidence of cell surface localization in vivo. Human dystrophin and human nuclei were unequivocally identified by staining with antibodies to human dystrophin and human nuclei, whereas the numerous mouse nuclei present in this field, as shown by DAPI staining, are negative (Figure 6, B and C).

DISCUSSION

Skeletal muscle has a remarkable regenerative capacity in response to an extensive injury. Resident within adult skeletal muscle is a small population of myogenic precursor cells (or satellite cells) that are capable of multiple rounds of proliferation (estimated at 80–100 doublings), which are

able to reestablish a quiescent pool of myogenic progenitor cells after each discrete regenerative episode (Mauro, 1961; Schultz and McCormick, 1994; Seale and Rudnicki, 2000; Hawke and Garry, 2001). Although muscle regeneration is a highly efficient and reproducible process, it ultimately is exhausted, as observed in senescent skeletal muscle or in patients with muscular dystrophy (Cussoni *et al.*, 1997; Cossu and Mavilio, 2000). In the present study, we investigated the myogenic potential of human endometrial tissue-derived immortalized EM-E6/E7/TERT-2 cells and primary cells derived from human menstrual blood. Human menstrual blood-derived cells proliferated over at least 25 PDs (9 passages) for more than 60 d and stopped dividing before 30 PDs. This cessation of cell division is probably due to replicative senescence or shortening of telomere length. Cell life span of menstrual blood cells is relatively short when compared with human fetal cells (Imai *et al.*, 1994; Terai *et al.*, 2005), and this shorter cell life span may be attributed to shorter telomere length of adult cells (i.e., endometrial stro-

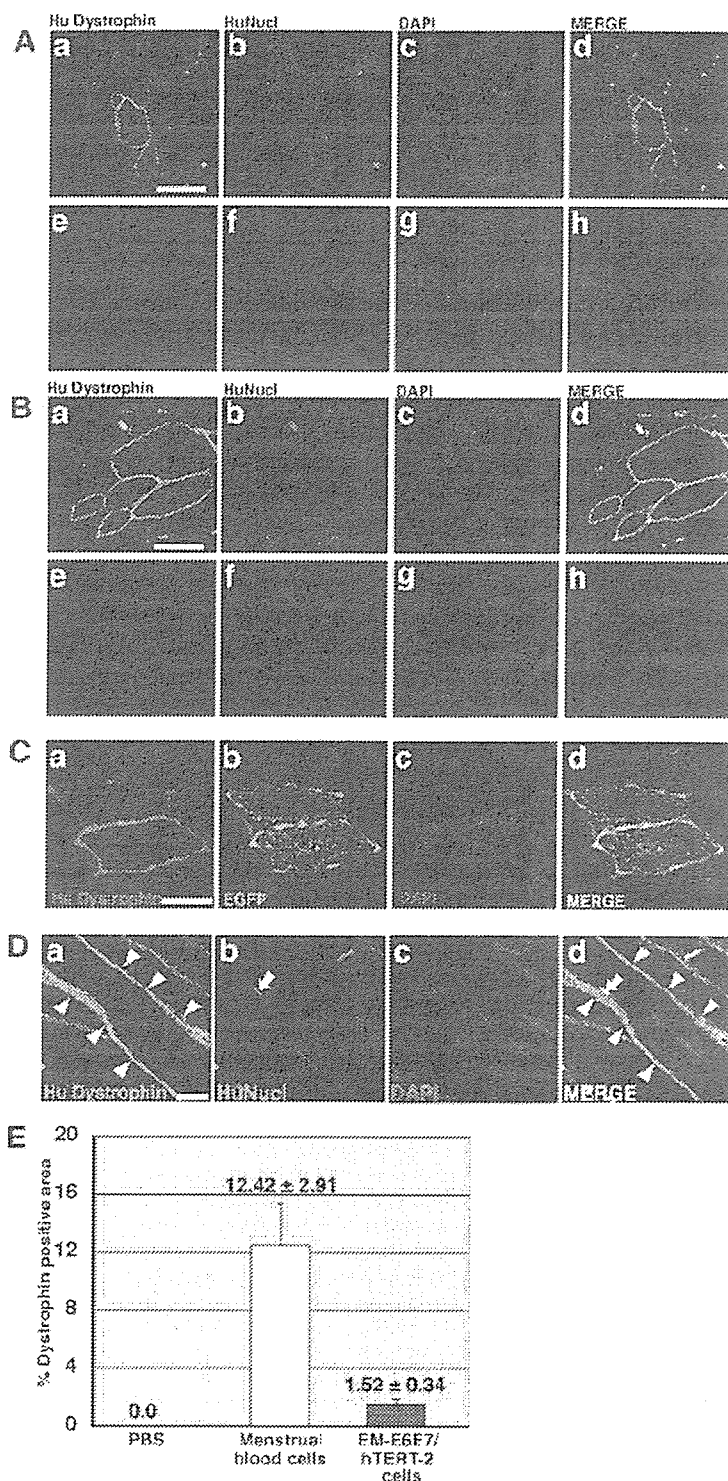


Figure 5. Conferral of dystrophin to mdx myocytes by human endometrial cells. (A and B) Immunohistochemistry analysis using an antibody against human dystrophin molecule (green), human nuclei (HuNucl, red), and DAPI staining (blue) on thigh muscle sections of mdx-scid mice after direct injection of EM-E6/E7/hTERT-2 cells (A) or menstrual blood-derived cells (B) without any treatment or induction. (C) ECFP-labeled EM-E6/E7/hTERT-2 cells without any treatment or induction were directly injected into the thigh muscle of mdx-scid mice. Immunohistochemistry revealed the incorporation of implanted cells into newly formed ECFP-positive myofibers, which expressed human dystrophin 3 wk after implantation. (A and B) As a methodological control, the primary antibody to dystrophin was omitted (e and f). (D) Immunohistochemistry analysis using an antibody against human dystrophin molecule (green, arrowheads), human nuclei (HuNucl, red, arrow), and DAPI staining (blue) on thigh muscle sections of mdx-scid mice after direct injection of human EM-E6/E7/hTERT-2 cells without any treatment or induction. (A and B) Merge of a–c is shown in d, and merge of e–g is shown in h. (C and D) Merge of a–c is shown in d. Scale bars, 50 μ m (A and B), 20 μ m (C and D). (E) Quantitative analysis of human dystrophin-positive myotubes. Menstrual blood-derived cells or EM-E6/E7/hTERT-2 cells without any treatment or induction were directly injected into thigh muscle of mdx-scid mice. The percentage of human dystrophin-positive-myofiber areas was calculated 3 wk after implantation of the EM-E6/E7/hTERT-2 cells or menstrual blood-derived cells. Injection of PBS without cells into mdx-scid myofibers was used as a control.

mal cells) at the start of cell cultivation, as is the case with hematopoietic stem cells (Suda *et al.*, 1984).

Menstrual blood-derived cells had a high replicative ability similar to progenitors or stem cells that display a long-term self-renewal capacity and had a much higher growth rate in our experimental conditions than marrow-derived

stromal cells (Mori *et al.*, 2005). In addition, the myogenic potential of menstrual blood-derived cells, i.e., a high frequency of desmin-positive cells after induction, is much greater than expected. The higher myogenic differentiation ratio can be explained just by alteration of cell characteristics from epithelial and mesenchymal bipotential cells or heter-

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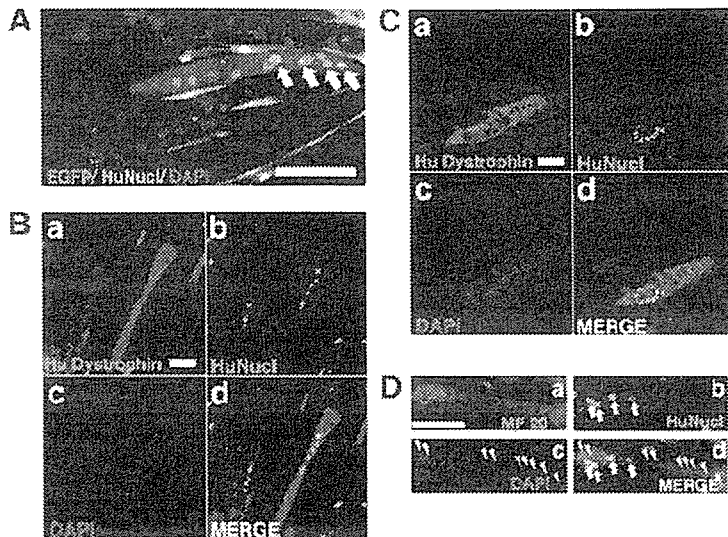


Figure 6. Detection of human endometrial cell contribution to myotubes in an in vitro and in vivo myogenesis model. EGFP-labeled EM-E6/E7/hTERT-2 cells (A) or EM-E6/E7/hTERT-2 cells (B) or menstrual blood-derived cells (C and D) were cocultured with C2C12 myoblasts for 2 d under conditions that favored proliferation. The cultures were then changed to differentiation media for 7 d to induce myogenic fusion. (A) Myotubes were revealed by EGFP (green); human nuclei were detected by antibody specific to human nuclei (HuNucl, red, arrows). (B–D) Myotubes were revealed by specific human dystrophin mAb NCL-DYS3 (B and C, red) or anti-myosin heavy chain mAb ME-20 (D, red). (D) Human nuclei were detected by antibody specific to human nuclei (HuNucl, green, arrows). Total cell nuclei in the culture were stained with DAPI (blue, arrowheads). (B–D) Merge of a–c are shown in d. The cultures were then changed to differentiation media for 7 d to induce myogenic fusion. Scale bars, 100 μ m (A–D).

ogeneous populations of cells to cells with the mesenchymal phenotype in our cultivation condition, as determined by cell surface markers (Figure 1, C–E). MyoD-positive cells are present in many fetal chick organs such as brain, lung, intestine, kidney, spleen, heart, and liver (Gerhart *et al.*, 2001), and these cells can differentiate into skeletal muscle in culture. Constitutive expression of MyoD, desmin, and myogenin, all markers for skeletal myogenic differentiation in both immortalized EM-E6/E7/hTERT-2 cells and menstrual blood-derived cells, implies either that most of these cells are myogenic progenitors or that these cells have myogenic potential. Expression of MyoD, one of the basic helix-loop-helix transcription factors that directly regulate myocyte cell specification and differentiation (Edmondson and Olson, 1993), occurs at the early stage of myogenic differentiation, whereas myogenin is expressed later, related to cell fusion and differentiation (Aurade *et al.*, 1994).

Acquisition or recovery of dystrophin expression in dystrophic muscle is attributed to two different mechanisms: 1) myogenic differentiation of implanted or transplanted cells and 2) cell fusion of implanted or transplanted cells with host muscle cells. Recovery of dystrophin-positive cells is explained by muscular differentiation of implanted marrow stromal cells and adipocytes (Dezawa *et al.*, 2005; Rodriguez *et al.*, 2005). In contrast, implantation of normal myoblasts into dystrophin-deficient muscle can create a reservoir of normal myoblasts that are capable of fusing with dystrophic muscle fibers and restoring dystrophin (Mendell *et al.*, 1995; Terada *et al.*, 2002; Wang *et al.*, 2003; Dezawa *et al.*, 2005; Rodriguez *et al.*, 2005). In this study using menstrual blood-derived cells, our findings—that the implantation of immortalized EM-E6/E7/hTERT-2 cells and menstrual blood-derived cells improved the efficiency of muscle regeneration and dystrophin delivery to dystrophic muscle in mice—is explained by both possibilities or the latter possibility alone, because cells expressing human dystrophin had both murine and human nuclei, located in the center and periphery of dystrophic muscular fiber, respectively (Figures 5D, in vivo, and 6, A–D, in vitro).

DMD is a devastating X-linked muscle disease characterized by progressive muscle weakness attributable to a lack of dystrophin expression at the sarcolemma of muscle fibers (Mendell *et al.*, 1995; Rodriguez *et al.*, 2005), and there are no

effective therapeutic approaches for muscular dystrophy at present. Human menstrual blood-derived cells are obtained by a simple, safe, and painless procedure and can be expanded efficiently in vitro. In contrast, isolation of mesenchymal stem cells/mesenchymal cells from other sources, such as bone marrow and adipose tissue, is accompanied by a painful and complicated operation. Efficient fusion systems of our immortalized human EM-E6/E7/hTERT-2 cells and menstrual blood-derived cells with host dystrophic myocytes may contribute substantially to a major advance toward eventual cell-based therapies for muscle injury or chronic muscular disease. Finally, we would like to reemphasize that human menstrual blood-derived cells possess high self-renewal capacity, whereas biopsied myoblasts capable of differentiating into muscular cells are poorly expandable in vitro and rapidly undergo senescence (Cossu and Mavilio, 2000).

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Hyaline Cartilage Formation and Enchondral Ossification Modeled With KUM5 and OP9 Chondroblasts

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Abstract What is it that defines a bone marrow-derived chondrocyte? We attempted to identify marrow-derived cells with chondrogenic nature and immortality without transformation, defining "immortality" simply as indefinite cell division. KUM5 mesenchymal cells, a marrow stromal cell line, generated hyaline cartilage *in vivo* and exhibited enchondral ossification at a later stage after implantation. Selection of KUM5 chondroblasts based on the activity of the chondrocyte-specific cis-regulatory element of the collagen $\alpha 2(\text{XI})$ gene resulted in enhancement of their chondrogenic nature. Gene chip analysis revealed that OP9 cells, another marrow stromal cell line, derived from macrophage colony-stimulating factor-deficient osteopetrotic mice and also known to be niche-constituting cells for hematopoietic stem cells expressed chondrocyte-specific or -associated genes such as type II collagen $\alpha 1$, Sox9, and cartilage oligomeric matrix protein at an extremely high level, as did KUM5 cells. After cultured OP9 micromasses exposed to TGF- $\beta 3$ and BMP2 were implanted in mice, they produced abundant metachromatic matrix with the toluidine blue stain and formed type II collagen-positive hyaline cartilage within 2 weeks *in vivo*. Hierarchical clustering and principal component analysis based on microarray data of the expression of cell surface markers and cell-type-specific genes resulted in grouping of KUM5 and OP9 cells into the same subcategory of "chondroblast," that is, a distinct cell type group. We here show that these two cell lines exhibit the unique characteristics of hyaline cartilage formation and enchondral ossification *in vitro* and *in vivo*. *J. Cell. Biochem.* 9999: 1–15, 2006. © 2006 Wiley-Liss, Inc.

Key words: Hyaline cartilage; chondroblasts; enchondral^{1,2} ossification; bioinformatics; gene chip

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The concept of regenerative medicine refers to the cell-mediated restoration of damaged or diseased tissue, and practically, regeneration of bone and cartilage may be one of the most accessible approaches. Candidate cell sources for regeneration of tissue include embryonic stem cells, fetal cells, or adult cells such as marrow stromal cells [Bianco and Robey, 2000], each of which has both benefits and drawbacks. Multipotent mesenchymal stem cells proliferate extensively, and to maintain the ability to differentiate into multiple cell types such as osteoblasts, chondrocytes, cardiomyocytes, adipocytes, and myoblasts in vitro [Umezawa et al., 1992; Pittenger et al., 1999; Bianco and Robey, 2000]. Marrow-derived stromal cells are also able to generate cardiomyocytes and endothelial cells [Makino et al., 1999], neuronal cells [Kohyama et al., 2001], and adipocytes [Umezawa et al., 1991]. Thus, marrow stromal cells are expected to be a good source of cell therapy in addition to embryonic stem cells and fetal cells [Pittenger et al., 1999].

In adults, chondrocytes maintain the extracellular matrix that gives cartilage its unique mechanical properties. Chondrocytes are long-lived and the development of new cells that are capable of producing cartilage *de novo* (i.e., chondroblasts) is not a normal part of adult cartilage physiology. A better understanding of the molecular mechanisms that regulate post-natal chondroblast differentiation would have a high impact on the design of strategies for cartilage repair. Cultures are commonly made from suspensions of cells dissociated from cartilage. Cartilage-derived cells in primary cultures can be removed from the culture dish and made to proliferate to form a large number of so-called secondary cultures: in this way, these cells may be repeatedly subcultured for weeks or months. Such cells often display many of the differentiated properties appropriate to their origin: the phenotype of the differentiated chondrocyte is characterized by the synthesis, deposition, and maintenance of cartilage-specific extracellular matrix molecules, including type II collagen and aggrecan [Archer et al., 1990; Hauselmann et al., 1994; Reginato et al., 1994]. The phenotype of differentiated chondrocytes is unstable in culture and is rapidly lost during serial monolayer subculturing [Benya and Shaffer, 1982; Lefebvre et al., 1990; Bonaventure et al., 1994]. This process is referred to as "dedifferentiation" and is a

major impediment to the use of mass cell populations for cell therapy or tissue engineering of damaged cartilage. However, when cultured three-dimensionally in a scaffold such as agarose, collagen, or alginate, redifferentiated chondrocytes start to reexpress the chondrocytic differentiation phenotype.

This study was undertaken to obtain bone marrow-derived chondroblastic cell lines that retain critical *in vivo* cell functions. Previous studies showed that it was possible to obtain lines of bone marrow-derived mesenchymal stem cells, mammary gland epithelial cells, skin keratinocytes, and pigmented epithelial cells that retained critical *in vivo* cell functions. By implanting cells into immunodeficient mice, we identified a newly isolated KUM5 chondroblastic cell line capable of *in vivo* hyaline-type chondrogenesis and serendipitously found that OP9 cells derived from osteopetrotic mice and also known as a niche-constituting cells for hematopoietic stem cells had chondrogenic potential.

MATERIALS AND METHODS

Cell Culture and Chondrogenic Differentiation

The cells were cultured in the growth medium (GM): Dulbecco's modified Eagle's medium (DMEM) with high glucose supplemented with 10% fetal bovine serum for KUM5 cells; α -MEM supplemented with 10% serum (BIOWEST, lot number: S03400S1820) for OP9 cells. For chondrogenic induction of pellet culture [Johnstone et al., 1998], both KUM5 and OP9 cells were cultured in the chondrogenic medium (CM): DMEM-high glucose containing 0.1 μ M dexamethasone, 1 mM sodium pyruvate, 0.17 mM ascorbic acid-2-phosphate, 0.35 mM proline, 6.25 μ g/ml bovine insulin, 6.25 μ g/ml transferrin, 6.25 μ g/ml selenous acid, 5.33 μ g/ml linoleic acid, and 1.25 mg/ml BSA (BioWhittaker). In the chondrogenic differentiation, the combination of one or several growth factors was added to the CM: TGF- β 3 10 ng/ml, BMP2 50 ng/ml, BMP4 50 ng/ml, BMP6 50 ng/ml, BMP7 50 ng/ml, PDGF 50 ng/ml, hyaluronic acid 250 ng/ml. The cells and the pellets were maintained at 37°C with 5% CO₂.

Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM)

The pelleted micromasses were examined by SEM and TEM. The micromasses were coated

with gold using a Sputter Coater (Sanyu Denshi Co., Tokyo, Japan) for SEM. The gas pressure was set at 50 mtorr, the current was 5 mA, and the coating time was 180 s. The samples were examined with a scanning electron microscope (JSM-6400F; JEOL, Ltd., Tokyo, Japan) operated at a voltage of 3 kV. For TEM, the micromasses and cell implants were initially fixed in PBS containing 2.5% glutaraldehyde for 24 h, and were embedded in epoxy resin. Ultrathin sections were double stained with uranyl acetate and lead citrate and were viewed under a JEM-1200EX transmission electron microscope (JEOL, Ltd.).

Flow Cytometric Analysis

Flow cytometric analysis was performed as previously described [Ochi et al., 2003; Mori et al., 2005; Terai et al., 2005].

Preparation and Transfection of Plasmid

The Venus gene (gift from Miyawaki) was obtained by BamHI and NotI digestion of Venus/pCS2 [Nagai et al., 2002]. The Venus gene was then cloned between the BamHI and NotI sites of pBluescriptII SK (-), excised by SalI and NotI digestion, and inserted between the XhoI and NotI sites of the p742-LacZ plasmid [Tsumaki et al., 1996], from which the LacZ gene was excised by XhoI and NotI digestion. This was named p742-Venus-Int plasmid. Transfection was performed using LipofectAmine 2000 (Invitrogen) according to the manufacturer's instructions.

Isolation of KUM5 Chondroblast

Cells were transfected with p742-Venus-Int plasmid and were cultured for 72 h. Venus-positive cells were sorted using the cell sorter (EPICS ALTRA, Beckman Coulter).

In Vivo Cell Implantation Assay

To determine the ability of cultured cells to differentiate in vivo, freshly scraped cells ($2-3 \times 10^7$ cells) were subcutaneously inoculated into Balb/c nu/nu mice (Sankyo Laboratory, Hamamatsu, Japan) as previously described [Umezawa et al., 1992]. Animals were sacrificed by cervical dislocation between 1 and 8 weeks after inoculation. The subcutaneous specimens were dissected at various times after implantation and fixed and decalcified for 1 week in 10% EDTA (pH 8.0) solution. After dehydration in ascending concentrations of ethanol and xylene,

the implants were embedded in paraffin. The paraffin sections were then deparaffinized, hydrated, and stained with hematoxylin and eosin, alcian blue, or toluidine blue. Paraffin sections were immunohistochemically stained with anti-type II collagen antibodies (Daiichi Fine Chemical Co., Ltd., Tokyo, Japan, Product No. F-57).

All animals received humane care in compliance with the "Principles of Laboratory Animal Care" formulated by the National Society for Medical Research and the "Guide for the Care and Use of Laboratory Animals" prepared by the Institute of Laboratory Animal Resources and published by the US National Institutes of Health (NIH Publication No. 86-23, revised 1985). The operation protocols were accepted by the Laboratory Animal Care and Use Committee of the Research Institute for Child and Health Development (2003-002).

Gene Chip Expression Analysis

Mouse-genome-wide gene expression was examined with the Mouse Genome MOE430A Probe array (GeneChip, Affymetrix), which contains the oligonucleotide probe set for approximately 23,000 full-length genes and expressed sequence tags (ESTs), according to the manufacturer's protocol (Expression Analysis Technical Manual and GeneChip small sample target labeling Assay Version 2 technical note. <http://www.affymetrix.com/support/technical/index.affx>). Total RNA was isolated with an RNeasy mini-kit (Qiagen, Chatsworth, CA). Double-stranded cDNA was synthesized, and the cDNA was subjected to in vitro transcription in the presence of biotinylated nucleoside triphosphates. The biotinylated cRNA was hybridized with a probe array for 16 h at 45°C, and the hybridized biotinylated cRNA was stained with streptavidin-PE and scanned with a Hewlett-Packard Gene Array Scanner. The fluorescence intensity of each probe was quantified by using the GeneChip Analysis Suite 5.0 computer program (Affymetrix). The expression level of a single mRNA was determined as the average fluorescence intensity among the intensities obtained with 11 paired (perfect matched and single nucleotide-mismatched) probes consisting of 25-mer oligonucleotides. If the intensities of mismatched probes was very high, gene expression was judged to be absent (A), even if high average fluorescence was obtained with the GeneChip Analysis Suite 5.0 program. The

level of gene expression was determined with the GeneChip software as the average difference (AD). Specific AD levels were then calculated as percentages of the mean AD level of six probe sets for housekeeping genes (β -actin and GAPDH). Further data analysis was performed with the Genespring software version 5 (Silicon Genetics, San Carlos, CA). To normalize the staining intensity variations among chips, the AD values for all genes on a given chip were divided by the median of all measurements on that chip. To eliminate changes within the range of background noise and to select the most differentially expressed genes, data were used only if the raw data values were less than 100 AD and gene expression was judged to be present by the Affymetrix data analysis.

Hierarchical Clustering and Principal Component Analysis

To analyze the gene expression data in an unsupervised manner by gene chip array, we used agglomerative hierarchical clustering and principal component analysis (PCA) (<http://lgsun.grc.nia.nih.gov/ANOVA/>). The hierarchical clustering techniques classify data by similarity and their results are represented by dendrogram. PCA is a multivariate analysis technique which finds major pattern in data variability. Hierarchical clustering and PCA were performed to group mesenchymal cells obtained from bone marrow into subcategories. Expression data of 244 cell surface marker genes (Supplementary Table I), 34 fat-associated genes (Supplementary Table II), 36 cartilage-associated genes (Supplementary Table III) dotted onto the gene chip were used for analysis.

RESULTS

Pelleted Micromass Culture of KUM5 Cells

KUM5 cells, one of the cloned lines of cells, were found to exhibit chondrogenesis *in vivo* within 4 weeks after direct injection. This possible chondrogenic cell line was subcloned by the limiting dilution method to obtain a cell line capable of forming elastic, fibrous or hyaline cartilage. When cultured in monolayer, KUM5 cells had a fibroblast-like morphology, and their doubling time was approximately 29.7 h. After reaching confluence, the cells had larger nucleus and cytoplasm, and generated so-called "chondrogenic nodules." We per-

formed the micromass culture of KUM5 cells in the GM or the CM, and continued the pelleted micromass culture for up to 10 weeks (Fig. 1A). The cells were equally embedded in the extracellular matrix, and the extracellular matrix of the KUM5 pellet culture did not show metachromasia with toluidine blue staining in the GM and the CM. Since transforming growth factor (TGF)- β and bone morphogenetic protein (BMP) are involved in chondrogenesis and osteogenesis [Fujii et al., 1999; Maeda et al., 2004], we used TGF- β 3 and BMPs on KUM5 culture. Exposure of the cells to TGF- β 3 augmented the metachromatic toluidine blue staining in the KUM5-micromass (Fig. 1A,B). BMP2 dramatically enhanced this TGF- β 3-induced differentiation, that is, caused stronger metachromatic staining and enlarged metachromatic area. To determine the effect of other cytokines on the TGF- β 3-induced chondrogenic differentiation, we added BMP4, BMP6, BMP7, PDGF, or hyaluronic acid to the CM supplemented with TGF- β 3. BMP4, BMP6, and BMP7 enhanced the TGF- β 3-induced chondrogenic differentiation in a manner similar to BMP2 (Fig. 1C,D). With exposure to BMP2, the number of the post-mitotic daughter cells in the cell nest increased, matrix became more abundant, and hypertrophic chondrocytes became larger at higher magnification (Fig. 1E). In contrast, PDGF inhibited the TGF- β 3 and BMP4-induced differentiation, as determined by toluidine blue staining (Fig. 1C_e,D_e). To confirm the chondrogenic differentiation histologically, we examined the ultrastructural analysis of the cartilaginous micromasses. Extracellular matrix was abundantly deposited over KUM5 cells, or the surface of the generated micromass. The cells covering the micromass showed a flattened shape (Fig. 3A,B). The KUM5 chondrocytes inside the micromass showed an oval or round structure, had cellular processes, and were embedded in the hypertrophic chondrocytes. Abundant rough endoplasmic reticulum and a small number of mitochondria were observed in the KUM5 chondrocytes (Fig. 3C).

Gene Chip Analysis of the KUM5 and OP9 Chondroblasts

To clarify the specific gene expression profile of marrow stromal cells, we compared the expression levels of approximately 23,000 genes in the KUM5, 9-15c, KUSA-O, KUSA-A1, H-1/A, and OP9 cells [Umezawa et al., 1992; Nakano

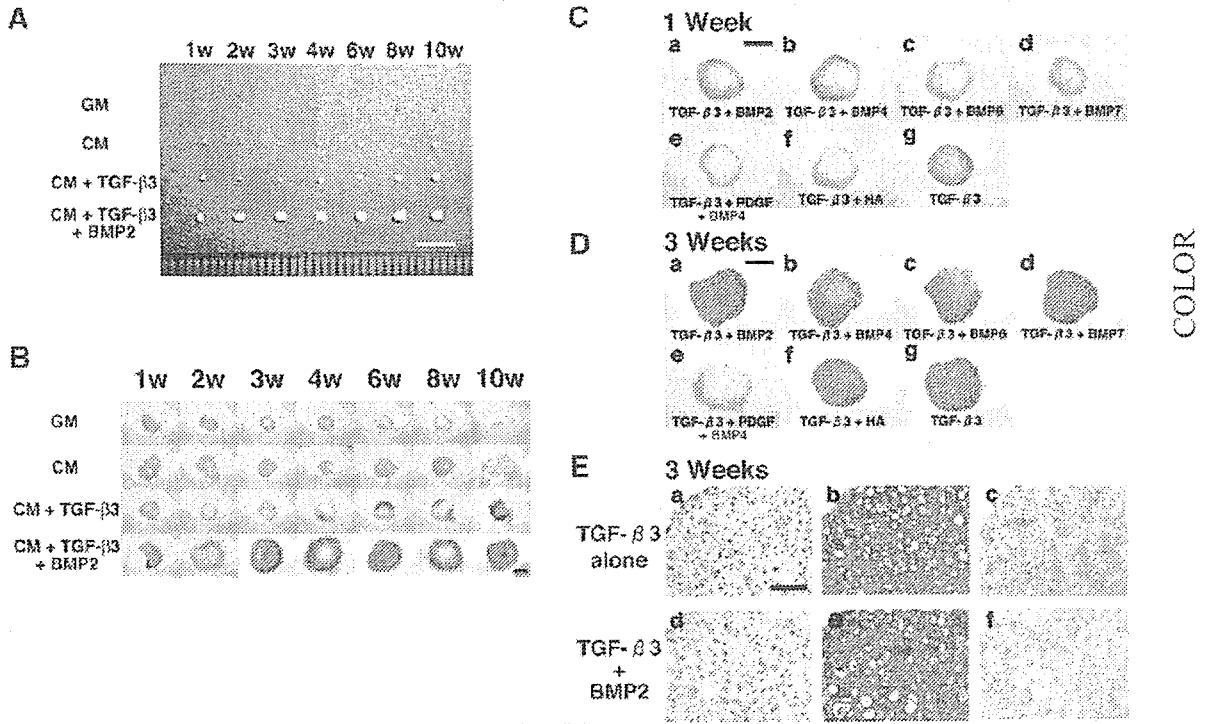


Fig. 1. In vitro chondrogenesis of KUM5 cells. **A,B:** Time-course analysis of growth factors-induced matrix production in KUM5 cells. Macroscopic view of KUM5 chondrogenic nodules which were generated after pellet culture for 1–10 weeks in the GM or the CM supplemented with or without growth factors as indicated (see “Cell culture” Section in Materials and Methods) (**A**) and Toluidine blue stained section (**B**). BMP2 drastically enhanced TGF- β 3-induced matrix production of KUM5 cells.

C,D: Toluidine blue stained section of KUM5 chondrogenic nodules in the pellet culture exposed to growth factors as indicated for 1 week (**C**) or 3 weeks (**D**). **E:** Higher magnification of KUM5 chondrogenic pellet exposed to TGF- β 3 (**a–c**), or TGF- β 3 and BMP2 (**d–f**) for 3 weeks. **a, d:** Hematoxylin and Eosin stain; **b, e:** Toluidine blue stain; **c, f:** Alcian blue stain. Scale bars: 5 mm (**A**), 500 μ m (**B, C, D**), 100 μ m (**E**).

et al., 1994]. (<http://1954.jukuin.keio.ac.jp/umezawa/chip/sugiki>) by using the Affymetrix gene chip oligonucleotide arrays (Table I). RNAs were isolated from cell lines cultured in the GM without any induction of differentiation to perform the gene chip analysis. Of the 23,000 genes represented on the gene chip, chondrocyte-specific- or associated-genes such as type II collagen α 1, Sox9, and cartilage oligomeric matrix protein were more strongly expressed in KUM5 cells than in other marrow-derived mesenchymal cells. Surprisingly, OP9 cells [Nakano, 1996] also expressed these chondrocyte-specific or -associated genes at higher levels: the type II collagen α 1, and cartilage oligomeric matrix protein genes were expressed in OP9 cells at more than tenfold higher levels than in 9–15c mesenchymal stem cells, KUSA-O osteo-adipogenic progenitor cells, H-1/A pre-adipocytes, or even KUM5 chondroblasts. These results implied that KUM5 and OP9 cells have increased chondrogenic potential.

Pelleted Micromass Culture of OP9 Cells

We performed the pellet culture of OP9 cells in the GM and continued the culture for up to 10 weeks (Fig. 2A). The cells were equally embedded in the extracellular matrix and the extracellular matrix of the OP9 pellet culture did not show metachromasie with the toluidine blue stain in the GM (data not shown). With exposure to TGF- β 3, the cells in the peripheral zone generated cartilage and exhibited adipocyte-like morphology in the center (Fig. 2Bg,Cg). Next, we investigated the effect of BMP2 in the pellet culture of OP9 cells. The CM with TGF- β 3 and BMP2 dramatically induced the chondrogenic differentiation (Fig. 2A,Ba,Ca), that is, the pellet cells produced abundant extracellular matrix (Fig. 2D) and caused deeper metachromatic staining and an enlarged metachromatic area (Fig. 2Db). Additionally, we examined the effect of other cytokines on the differentiation of OP9 cells

TABLE 1. Cartilage-Associated Genes Expressed in KUM5 and OP9 Cells in Comparison With Other Marrow Stromal Cells

| Probe set | Genbank | Description | 8-15c | | | KUSA-O | | | KUSA-A1 | | | H1A | | | OP9 | | | KUM5 | | |
|--------------|-----------|---|-------|--------|--------|--------|--------|-----|---------|-----|--------|-------|--------|-----|--------|-----|--------|-------|--------|--------|
| | | | Flags | Raw | Raw | Flags | Raw | Raw | Flags | Raw | Raw | Flags | Raw | Raw | Flags | Raw | Raw | Flags | Raw | Raw |
| 1420567_a_at | NM_031163 | Procollagen, type II, alpha 1 | A | 28 | 197 | A | 98 | A | 46 | P | 1,739 | P | 85 | A | 110 | P | 1,123 | P | 1,123 | Bmp1a |
| 1428571_at | AK004383 | Procollagen, type IX, alpha 1 | P | 65 | 118 | P | 99 | P | 57 | P | 132 | P | 21,954 | P | 18,640 | P | 18,640 | P | 18,640 | Hsp1 |
| 1422953_at | NM_009925 | Procollagen, type X, alpha 1 | A | 13 | 39 | A | 16 | A | 104 | A | 218 | A | 2,851 | P | 382 | P | 382 | P | 382 | Frsip |
| 1418599_at | BB608814 | Procollagen, type XI, alpha 1 | A | 88 | 682 | P | 4,264 | P | 5,069 | P | 2,851 | P | 1,892 | P | 1,892 | P | 1,892 | P | 1,892 | Comp |
| 1419327_at | NM_016685 | Cartilage oligomeric matrix protein | A | 120 | 111 | A | 64 | A | 167 | A | 167 | A | 167 | A | 167 | A | 167 | A | 167 | Comp |
| 1442665_at | NM_007883 | Decorin | A | 176 | 36 | A | 273 | A | 238 | A | 238 | A | 238 | A | 238 | A | 238 | A | 238 | Den |
| 1416405_at | BC019302 | Biglycan | P | 12,600 | 11,817 | P | 11,011 | P | 12,922 | P | 12,922 | P | 21,954 | P | 18,640 | P | 18,640 | P | 18,640 | Hsp1 |
| 1443327_at | NM_007424 | Aggrecan 1 | A | 70 | 118 | A | 105 | A | 127 | A | 84 | A | 84 | A | 84 | A | 84 | A | 84 | Hsp1 |
| 1416321_s_at | BC019776 | Proline arginine-rich and leucine-rich repeat | P | 196 | 69 | P | 899 | P | 1,092 | P | 1,092 | P | 2,169 | P | 382 | P | 382 | P | 382 | Frsip |
| 1415839_at | NM_021365 | Fibronectin | P | 383 | 353 | M | 11,543 | P | 16,526 | P | 16,526 | P | 16,526 | P | 16,526 | P | 16,526 | P | 16,526 | Fmod |
| 1418745_at | NM_012070 | Osteonectin | P | 288 | 60 | P | 1,849 | P | 2,189 | P | 2,189 | P | 347 | P | 743 | P | 743 | P | 743 | Omd |
| 1415943_at | BC010560 | Syndecan 1 | P | 1,182 | 2,449 | P | 1,358 | P | 1,607 | P | 1,607 | P | 4,704 | P | 1,789 | P | 1,789 | P | 1,789 | Sdc1 |
| 1417912_at | A126682A | Syndecan 2 | P | 732 | 1,236 | P | 2,940 | P | 4,388 | P | 4,388 | P | 606 | P | 2,089 | P | 2,089 | P | 2,089 | Sdc2 |
| 1420653_at | NM_011530 | Syndecan 3 | P | 382 | 347 | P | 680 | P | 942 | P | 942 | P | 305 | P | 762 | P | 762 | P | 762 | Sdc3 |
| 1417654_at | NM_011521 | Syndecan 4 | P | 306 | 281 | P | 344 | P | 343 | P | 343 | P | 305 | P | 320 | P | 320 | P | 320 | Sdc4 |
| 1424860_at | BC077717 | SH-Box containing gene 9 | P | 120 | 5 | P | 59 | A | 27 | A | 27 | A | 1,844 | P | 183 | P | 183 | P | 183 | Sdc9 |
| 1420995_at | BM346342 | Transforming growth factor, beta receptor 1 | P | 769 | 703 | P | 657 | P | 963 | P | 963 | P | 1,395 | P | 602 | P | 602 | P | 602 | Tgfr1 |
| 1426444_s_at | S69114 | Transforming growth factor, beta receptor 2 | P | 562 | 746 | P | 1,068 | P | 1,189 | P | 1,189 | P | 688 | P | 1,132 | P | 1,132 | P | 1,132 | Tgfr2 |
| 1425820_at | AF038601 | Transforming growth factor, beta receptor 3 | P | 435 | 398 | P | 475 | P | 313 | P | 313 | P | 555 | P | 1,015 | P | 1,015 | P | 1,015 | Tgfr3 |
| 1422912_at | NM_007664 | Bone morphogenetic protein 4 | P | 1,048 | 846 | P | 6,470 | P | 7,266 | P | 7,266 | P | 1,739 | P | 2,890 | P | 2,890 | P | 2,890 | Bmp4 |
| 1425492_at | BM246246 | Bone morphogenetic protein receptor, type 1A | P | 1,486 | 815 | P | 1,089 | P | 1,164 | P | 1,164 | P | 1,164 | P | 1,164 | P | 1,164 | P | 1,164 | Bmpr1a |
| 1420847_s_at | NM_010287 | Fibroblast growth factor receptor 2 | P | 839 | 656 | P | 1,864 | P | 1,998 | P | 1,998 | P | 992 | P | 3,593 | P | 3,593 | P | 3,593 | Fgfr2 |
| 1417271_s_at | NM_007923 | Endoglin | A | 247 | 187 | A | 40 | A | 116 | A | 116 | A | 223 | P | 1,371 | P | 1,371 | P | 1,371 | Eng |
| 141314_s_at | L08431 | Vascular cell adhesion molecule 1 | P | 462 | 39 | A | 28 | A | 92 | A | 92 | A | 812 | P | 583 | P | 583 | P | 583 | Vcam1 |

The raw data from the gene chip analysis are available at our laboratory's web site (<http://1984-jukuin.keio.ac.jp/omezawar/hip/sugiki/>). Flag indicates the presence or absence of gene expression determined by presence/absence call (Affymetrix).
P (presence); gene is expressed. M (marginal); gene is marginally expressed. A (absence); gene is not expressed.