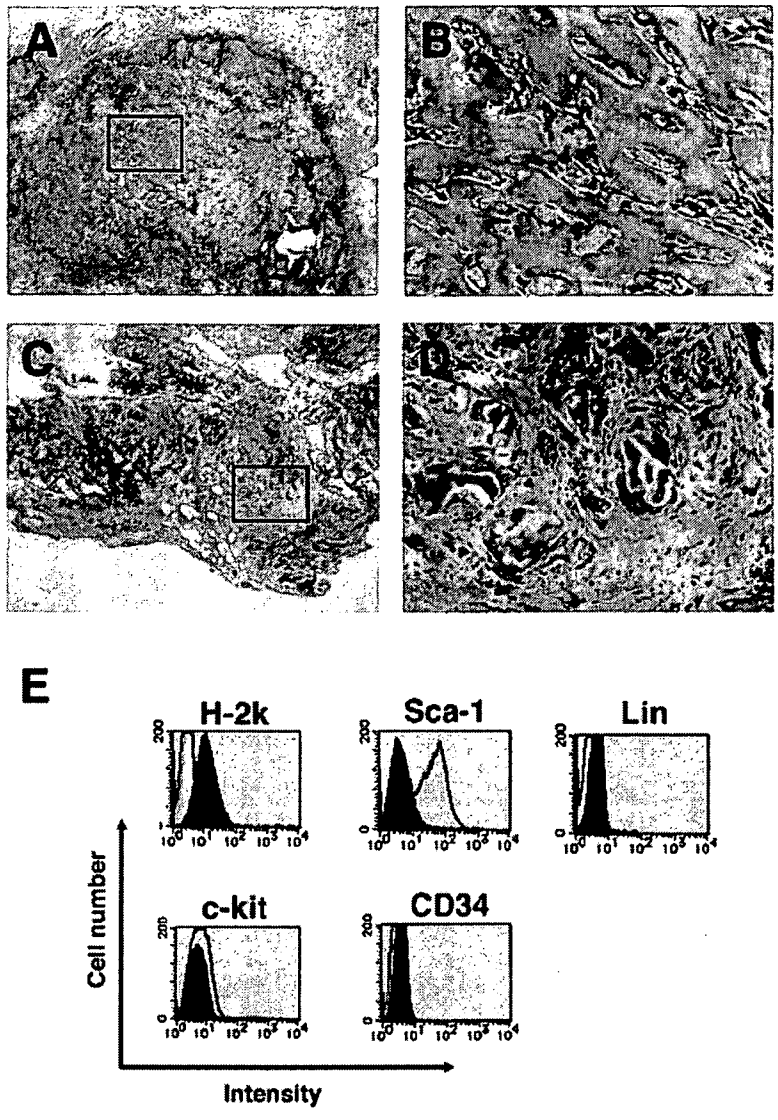


Fig. 2. Rejection of the implanted KUSA-A1 cells in an allogeneic combination. Microscopic view of the generated bone 18 days after the implantation of $5 \times 10^6$ KUSA-A1 cells, which were derived from C3H/He mice, into the subcutaneous tissue of syngeneic C3H/He mice (**A, B**) or allogeneic BALB/c mice (**C, D**). Hematoxylin and eosin stain. Parts B and D are higher magnifications of A and C, respectively. **E**: Alterations in cell surface antigens after implantation. Flow cytometric analysis was performed on KUSA-A1 cells (open peaks) and cultured mesenchymal cells obtained from KUSA-A1 ectopic bone (closed peaks) in BALB/c nu/nu mice. The mesenchymal cells were obtained from the KUSA-A1 ectopic bone, and analyzed by flow cytometry. One of major histocompatibility antigens, H-2k, was upregulated, and Sca-1 antigen was downregulated.

accounted for $7.0 \pm 1.7/10^6$ cells (Fig. 4E, middle). In contrast, no CFU-S was detected in the peripheral blood from the mice without KUSA-A1 cell implantation.

Since the upregulation of HSCs in the femurs from the KUSA-A1 cell-implanted mice was rather surprising to us, the time-course of the KSL cell numbers in the femurs from the mice with KUSA-A1 ectopic bone was investigated (Fig. 4F). The number of KSL cells started to increase at 3 weeks, continued to increase to 0.47% by 5 weeks, returned to the basal level, that is, 0.08% at 6 weeks, and then fell down to 0.07% at 7 weeks, implying that the HSC number is strongly correlated with the process of dynamic membranous osteogenesis at the implanted site.

## DISCUSSION

Bone remodeling occurs continuously throughout life, and HSCs may mobilize during this remodeling process. The finding in this study support such hypothesis that a very specific niche may be functionally enhanced by bone remodeling (Watt and Hogan, 2000), while a stable or static microenvironment does not support hematopoietic mobilization. For example, accelerated bone remodeling by physical exercise and Vitamin D intake trigger increasing mobilization of HSCs. On the other hand, lack of dynamic bone remodeling in bedridden elderly, astronauts, dieters, postmenopausal women, and patients immobilized for long periods results in downregulation of HSCs in bone marrow.

### Upregulation of HSCs by "dynamic" membranous ossification of implanted KUSA-A1 osteoblasts

The cell implantation-based strategy employed in this study revealed that increased niche size following subcutaneous implantation of an osteoblast cell line in syngeneic or immunodeficient mice resulted in

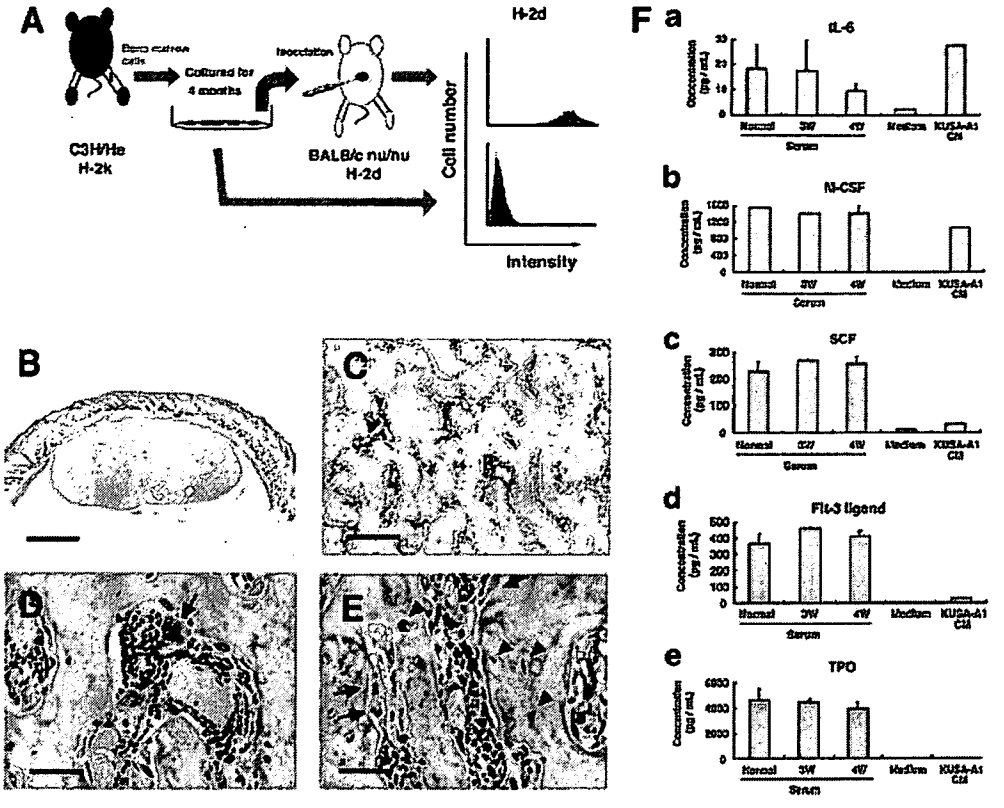


Fig. 3. Hematopoietic cells of host origin in the ectopic bone and serum levels of cytokines after subcutaneous implantation of KUSA-A1 cells. **A**: Flow cytometric analysis of the H-2d antigen in the hematopoietic cells of ectopic bone and KUSA-A1 cells in vitro. The hematopoietic cells in KUSA-A1 bone were examined for expression of the H-2d antigen of the host mice. **B–E**: Histopathological appearance of the hematopoietic cells used for flow cytometric analysis. Trilineage cells, that is, megakaryocytes (D, arrows), erythroblasts (D, E), and granulocytes (E), were observed. Osteoblasts and mature osteocytes are indicated by arrows and arrowheads, respectively (E). Scale bars: 2 mm (B), 400 μm (C), 100 μm (D, E). **F**: Serum levels of interleukin-6 (IL-6) (**a**), macrophage-colony stimulating factor (M-CSF) (**b**), stem cell factor (SCF) (**c**), fms-like tyrosine kinase-3 (Flt-3) ligand (**d**), and thrombopoietin (TPO) (**e**), measured by the ELISA method. The blood samples were obtained at 5 weeks after implantation.

increases in the HSC population. In the HSC population, both the CFU-S and KSL cells increased. The niche that regulates the generation and differentiation of the HSCs was formed following KUSA-A1 cell implantation, and subsequent membranous ossification in vivo. The enlarged area of niche, that is, the inner surface of bone during the dynamic process of membranous osteogenesis may account for the dramatic upregulation of HSCs in the host bone marrow. Once the osteogenic process is terminated, the number of osteoclasts decreases and no bone is remodeled. Furthermore, the number of the KSL cells returns to the basal level in host bone marrow. These facts suggest a correlation between the osteogenic process (Fig. 1C) and increasing number of KSL cells (Fig. 4F).

The source of the CFU-S in the peripheral blood of the mice implanted with KUSA-A1 osteoblasts may be the bone marrow of (a) the ectopic bone; (b) the host femur; (c) both the ectopic bone and the host femur (Fig. 4C). Mobilization of CFU-S from ectopic bone into the peripheral blood is the most likely cause since the induction of HSCs was accompanied by dynamic osteogenesis. The increased HSC number in the host bone marrow can be explained by HSC mobilization from ectopic bone into the peripheral blood. In the normal mice, such migration or mobilization of hematopoietic cells occurs during development. Hematopoietic events in the mouse begin in the yolk sac and aorta-gonad-mesonephros region at day 7 of gestation, and they shift the site to the fetal liver at mid-gestation followed by the bone marrow shortly before birth. The prevailing notion has been that this sequence reflects the migration of HSCs from the yolk sac to the definitive hematopoietic sites. Observation in this study, that is, the generation of ectopic bone in the subcutaneous tissues and the resultant migration of HSCs via the peripheral blood, seems to mimic above process during developments (Dzierzak et al., 1998).

### Unexpected upregulation of MHC antigen after implantation of donor cells

Although most HSCs have been reported to express MHCs, that is, HLA in humans, and H-2 antigens in mice, no mesenchymal stem cells have been reported to express MHC antigens at least in vitro (Jiang et al., 2002). Since lack of these antigens on the cell surface may contribute to the induction of tolerance in these cells when transplanted in allogeneic combination, the complete rejection of the transplanted mesenchymal cells and de novo expression of the H-2 antigen after in vivo implantation was contrary to our expectation. We do not know the molecular mechanisms responsible for upregulated expression of H-2 and downregulated expression of Sca-1 after cell implantation, but care should be exercised when mesenchymal cells are implanted for therapeutic purposes, because membrane-bound molecules, including functionally essential molecules, might be modulated after implantation.

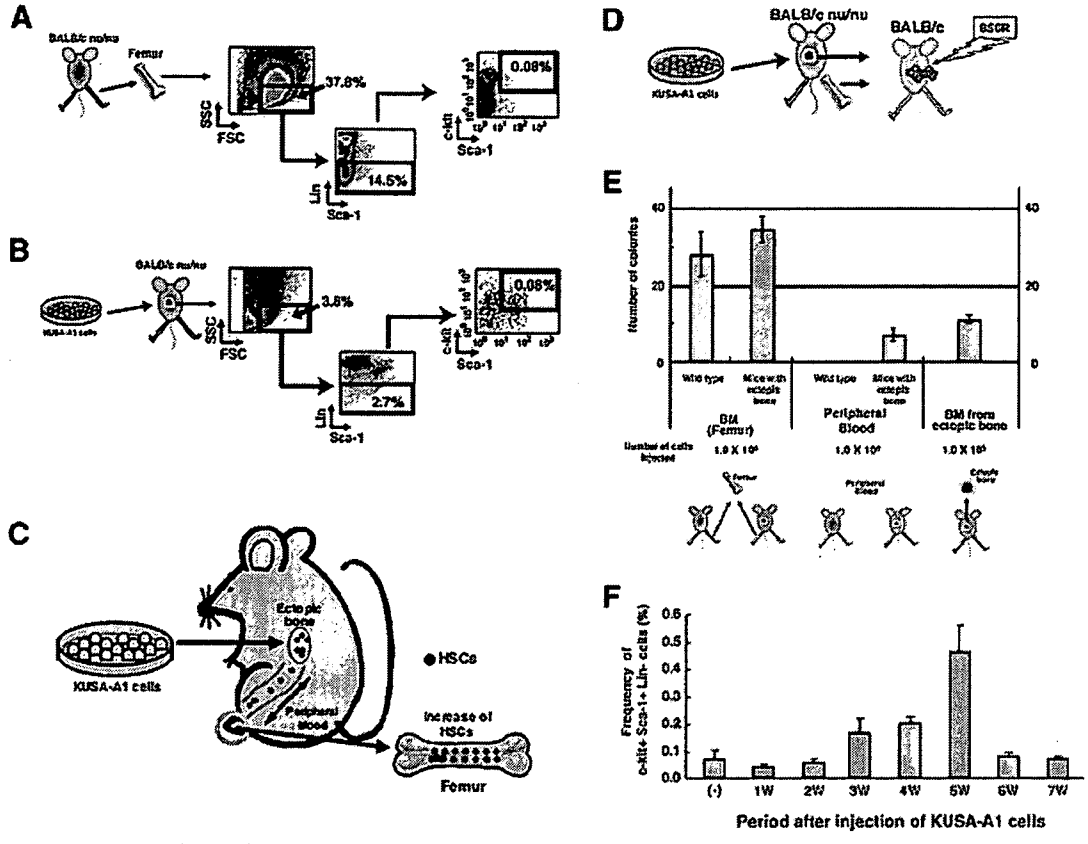


Fig. 4. Mobilization of c-kit$^+$ Sca-1$^+$ Lin$^-$ (KSL) cells in the ectopic bones generated by KUSA-A1 cells. **A** and **B**: Flow cytometric analysis of hematopoietic stem cell markers was performed on hematopoietic cells in the femur of BALB/c nu/nu mice (A) and the ectopic bone generated in BALB/c nu/nu mice (B). KSL cells accounted for 0.08% of the hematopoietic cells in KUSA-A1 bone. **C**: Proposed mechanism of HSC mobilization in the peripheral blood of mice with the ectopic bone, and the increased HSCs in the femur of mice implanted with osteoblasts. Osteoblastic cells whose number has been increased by local injection into the tissues support an increase in number of HSCs in both bone marrow and peripheral blood, as a result of an increase in size of the microenvironment or niche in vivo. The niche size defined by dynamic osteogenic process affects the number of stem cells. **D**: Experimental design to investigate mobilization of CFU-S in the peripheral blood of mice with the ectopic bone, and the proportion of KSL cells in the femur of mice implanted with KUSA-A1 cells. Hematopoiesis was induced in the ectopic bone by KUSA-A1 cell implantation (See Fig. 3). The hematopoietic cells in the ectopic bone and the host femur were analyzed for further CFU-S analysis in mice exposed to 850 cGy irradiation. **E**: CFU-S assay in the marrow cells of the femur of mice not implanted with any cells; the femoral marrow cells of mice with ectopic bone; peripheral blood cells of mice not implanted with cells; peripheral blood cells of mice with ectopic bone; marrow cells in ectopic bone. The blood samples were obtained at 5 weeks after implantation. The number of HSC or CFU-s increased to $11.2 \pm 0.8/1.0 \times 10^6$ cells in the KUSA-A1-induced ectopic bone. CFU-s in the peripheral blood increased to $7.0 \pm 1.7/10^6$ cells at day 12 after implantation of the KUSA-A1 cells while no CFU-s were detected in the peripheral blood from mice without cell implantation. **F**: Time course of the proportion of KLS cells in the femur of mice implanted with KUSA-A1 cells.

### Crucial role of marrow stromal subsets in HSC regulation

HSCs are a subset of bone marrow cells that are capable of self-renewal and of forming all types of blood cells. The increases in bone size generated by the spindle-shaped KUSA-A1 osteoblasts correlated with the increase in the number of HSCs. The osteoblasts and a subpopulation of the HSCs expressed N-cadherin, a cell-surface molecule that helps cells adhere to one another, and N-cadherin and -catenin may form important components of the interaction between HSCs and their niche (Zhang et al., 2003). The Notch signaling pathway is also known to regulate cell-fate decisions in many organisms (Calvi et al., 2003). Involvement of cytokine signaling in HSC regulation has been reported to be crucial to the development of blood-forming tissue in embryos. The doubling of bone size mirrored the increase in the HSC population in the mice implanted with KUSA-A1 cells.

The strategy to increase the size of the HSC population by implanting osteoblasts into the subcutaneous tissue to increase the osteoblast cell population may be proven to be of certain clinical value in the future. The concept that a microenvironment or niche controls HSCs may be useful for HSC expansion in vivo, and has potential implications for HSC harvesting and recovery after transplantation (Fig. 4C). Direct implantation of KUSA-A1 cells into syngeneic or immunodeficient mice, in order to better understand the interactions between HSCs and bone marrow, may therefore lead to the development of practical methods of manipulating stem cells and define a model for investigating the impact of the microenvironment on cell physiology (Li et al., 2000). Cellular and molecular identification using the strategy of niche-constituent cells or signaling pathways will provide pharmacological targets with therapeutic potential for stem-cell-based therapies.

### ACKNOWLEDGMENTS

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# Differentiation of Adult Stem Cells Derived from Bone Marrow Stroma into Leydig or Adrenocortical Cells

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**Adult stem cells from bone marrow, referred to as mesenchymal stem cells or marrow stromal cells (MSCs), are defined as pluripotent cells and have the ability to differentiate into multiple mesodermal cells. In this study, we investigated whether MSCs from rat, mouse, and human are able to differentiate into steroidogenic cells. When transplanted into immature rat testes, adherent marrow-derived cells (including MSCs) were found to be engrafted and differentiate into steroidogenic cells that were indistinguishable from Leydig cells. Isolated murine MSCs transfected with green fluorescence protein driven by the promoter of P450 side-chain cleaving enzyme gene (CYP11A), a steroidogenic cell-specific gene, were used to detect steroidogenic cell production *in vitro*. During *in vitro* differentiation, green fluorescence protein-positive cells, which had characteristics similar to those of Leydig cells, were found. Stable transfection of murine MSCs with a transcription factor, steroidogenic factor-1, followed by treatment with cAMP almost recapitulated the properties of Leydig cells, including the production of testosterone. Transfection of human MSCs with steroidogenic factor-1 also led to their conversion to steroidogenic cells, but they appeared to be glucocorticoid- rather than testosterone-producing cells. These results indicate that MSCs represent a useful source of stem cells for producing steroidogenic cells that may provide basis for their use in cell and gene therapy. (*Endocrinology* 147: 4104–4111, 2006)**

STEM CELLS ARE self-renewing elements with the capacity to generate multiple distinct cell lineages. They exist in various tissues, even in adults, and have been isolated from a variety of differentiated tissues, including bone marrow, umbilical blood, brain, and fat (1–6). Among these, bone marrow-derived mesenchymal stem cells (MSCs), also known as marrow stromal cells, are defined as pluripotent cells and have been shown to differentiate into adipocytes, chondrocytes, osteoblasts, and hematopoietic-supporting stroma both *in vivo* and *ex vivo* (1–3). Furthermore, they are able to generate cells of all three germ layers (7, 8). In addition to their multipotency for differentiation, MSCs have attracted considerable interest for use in cell and gene therapy because these cells can easily be obtained from adult marrow tissue (8–10).

The gonad and adrenal gland are the primary steroidogenic organs in mammals. In the gonad, male Leydig cells or female granulosa and theca cells are responsible for the production of androgens and estrogens. The adrenal cortex produces glucocorticoids and mineralocorticoids, although some androgens are also produced in many species, except rodents. These steroidogenic organs develop from the common adrenogenital primodium, which originates from the intermediate mesoderm (11). Fetal-type steroidogenic cells appear when the adrenogenital primodium differentiates into the adrenal cortex and the gonads of the two sexes. These are replaced by adult-type steroidogenic cells during the period between birth and puberty (12, 13), but these processes are poorly understood.

One approach to resolving the complexities of organogenesis is to use stem cells as a model system for differentiation. In this study, the differentiation of MSCs into steroidogenic cells was examined *in vivo* and *in vitro* by several methods. A number of studies have reported that the injection of MSCs into some tissues leads to the differentiation of the injected cells into tissue-specific cells, probably due to the microenvironment near the injection sites. To determine whether MSCs are able to differentiate into steroidogenic cells, we injected a purified population of rat MSCs into the prepubertal rat testis and examined the fate of these cells by immunohistochemistry. In addition, the spontaneous differentiation of MSCs to specific cells can be monitored by the expression of specific genes in the differentiated cells. One such experimental approach, known as a promoter-sorting method, is to use fluorescence-activated cell sorting (FACS) to select green fluorescence protein (GFP)-positive MSCs in which the expression of GFP is under the control of the promoter of a gene that is expressed in a cell type-specific fashion. In this study, to demonstrate the emergence of steroidogenic cells from isolated MSCs *in vitro*, a GFP expression vector driven by the CYP11A promoter (CYP11A is a

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Abbreviations: ES, Embryonic stem; FACS, fluorescence activated cell sorting; GFP, green fluorescence protein; hMSC, human MSC; 3β-HSD I, 3β-hydroxysteroid dehydrogenase I; 17β-HSD III, 17β-hydroxysteroid dehydrogenase III; mMSC, murine MSC; MSC, mesenchymal stem cell; P450arom, cytochrome P450 aromatase; P450c17, cytochrome P450 17α-hydroxylase; P450c21, cytochrome P450 steroid 21-hydroxylase; P450scc, P450 side-chain cleaving enzyme; SF, steroidogenic factor; StAR, steroidogenic acute regulatory protein.

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gene encoding the cholesterol side-chain cleavage enzyme, an essential enzyme for steroidogenesis) was integrated into the MSCs, and GFP-positive MSCs were then separated by fluorocytometry. Finally, to achieve the efficient differentiation of the isolated MSCs *in vitro*, the orphan nuclear receptor, steroidogenic factor (SF)-1 was ectopically expressed in MSCs. MSCs successfully differentiated into steroidogenic cells using any of these procedures. These results indicate that MSCs represent a useful source of stem cells for producing steroidogenic cells that may provide basis for their use in cell and gene therapy.

## Materials and Methods

### *Animals*

GFP transgenic rats [SD TgN(act-EGFP)OsbCZ-004] were kindly provided by Dr. M. Okabe (Osaka University, Osaka, Japan). Sprague Dawley rats were purchased from Sankyo (Shizuoka, Japan). At all times, the animals were treated according to National Institutes of Health guidelines. The donor animals used in this study were generally 4–5 wk old, and the recipient animals were 3 wk old.

### *Histology and immunofluorescence analysis*

Immunohisto- and cytochemical staining with antirat P450 side-chain cleaving enzyme (P450scc) (C-16; Santa Cruz Biotechnology, Santa Cruz, CA), antimouse 3β-hydroxysteroid dehydrogenase I (3β-HSD I) (kindly provided by Dr. A. Payne, Stanford University Medical Center, Stanford, CA), antipig cytochrome P450 17 α-hydroxylase (P450c17) (kindly provide by Dr. D. Hales, University of Illinois at Chicago, Chicago, IL) or anti-GFP (Medical & Biological Laboratories Co., Ltd.) were performed on 10-μm frozen sections or cultured cells on glass slides using standard protocols. Appropriate Cy3- or fluorescein isothiocyanate-conjugated secondary antibodies (Sigma, St. Louis, MO) were used for detection.

### *Cell culture, stable transfection, and hormone assay*

MSCs from GFP transgenic rats were collected and cultured as described by Pochampally *et al.* (14). Mouse (KUM9) (15) or human (hMSC-hTERT-E6/E7) (16) bone marrow-derived MSCs were maintained in Iscova's MEM or DMEM with 10% fetal calf serum. Plasmid DNA was transfected using the LipofectAmine PLUS reagent (Invitrogen, Carlsbad, CA) or calcium phosphate coprecipitation. Cells were used for the experiments after 10–12 passages, and steroid hormone production was sustained for at least 4 months. The levels of each steroid hormone in the media were measured by RIA.

### *Transplantation*

Bone marrow cells from TgN(ActbEGFP) transgenic rats ($1 \times 10^6$) were injected into the testes of 3-wk-old SD rats. Two to three weeks after transplantation, testes were removed to examine histochemically survival and differentiation of transplanted cells.

### *Plasmid construction*

A 2.3-kb fragment of the human CYP11A (P450scc gene) promoter that functions specifically in steroidogenic organs (17) was obtained by PCR using pSCC2300-LacZ (kindly provided by Dr. B. C. Chung, Institute of Molecular Biology, Taipei, Taiwan) as a template and integrated into a promoter-less pEGFP-1 vector (CLONTECH, Palo Alto, CA). The *Eco*RI-*Stu*I restriction fragment, containing the CYP11A promoter-GFP, was then excised and inserted into *Eco*RI and *Swa*I site of pPUR (CLONTECH). The expression vector for rat SF-1 cDNA containing the entire coding region was generated by RT-PCR and subcloned into pIRES-puro2 vector (CLONTECH).

### *FACS analysis and cell purification*

Cells were harvested by treatment with 0.25% trypsin/EDTA, after which they were neutralized with DMEM with 10% fetal calf serum, washed twice with PBS, and filtered through a 35-mm pore size nylon screen. FACS analysis was performed on a flow cytometer with a 488-nm argon laser and GFP-positive cells were isolated.

### *RT-PCR and real-time PCR*

Total RNA from the cultured cells was extracted using the Trizol reagent (Invitrogen). RT-PCR was performed as described previously (18). The reaction mixture was subjected to electrophoresis in a 1.5% agarose gel, and the resulting bands were visualized by staining with ethidium bromide. Real-time PCR was performed as described by Rutledge and Cote (19). Reagents for real-time PCR were purchased from Applied Biosystems (Warrington, UK), except for SYBER green PCR master mix (QIAGEN, Valencia, CA). Reactions were carried out and fluorescence was detected on a GeneAmp 7700 system (Applied Biosystems). The primers used are shown in Table 1.

### *Western blot analysis*

The extraction of protein from the cultured cells and subsequent quantification was performed as described previously (20). Equal amounts of protein (50 μg) were resolved by 12.5% SDS-PAGE and transferred to polyvinylidene difluoride membranes. Western blot analyses of SF-1, steroidogenic acute regulatory protein (StAR), P450scc, 3β-HSD I, P450c17, and β-tubulin were carried out with antisera directed against SF-1 (Ad4BP, kindly provided by Dr. K. Morohashi, National Institute of Basic Biology, Okazaki, Japan), StAR (kindly provided by Dr. W. Miller, University of California, San Francisco, CA) (21), P450scc (kindly provided by Dr. B. C. Chung) (22), 3β-HSD I (kindly provided by Dr. A. Payne), P450c17 (kindly provided by Dr. D. Hales) (23), and β-tubulin (D-10, Santa Cruz). ECL Western blot reagents (Amersham Pharmacia Biotech, Piscataway, NJ) were used for detection.

## Results

### *Transplantation of rat bone marrow mesenchymal stem cells*

In the prepubertal testis, fetal-type Leydig cells are replaced by adult-type Leydig cells, which originate from mesenchymal precursor cells that are present in the testicular interstitium (12). To determine whether MSCs can be engrafted into the testis and converted into steroidogenic cells we took $1 \times 10^6$ bone marrow cells from TgN(ActbEGFP) transgenic rats that had been maintained in culture (Fig. 1A) and injected them into the testes of 3-wk-old SD rats. As shown in Fig. 1C, donor engraftment was confirmed (100%) at various periods after transplantation (1–4 wk). A histochemical examination revealed that the GFP-positive cells present in the testes were located in the interstitium and were not observed within the seminiferous tubules (Fig. 1D). An immunohistochemical study showed that most of the GFP-positive cells in the interstitium were also positive for three Leydig cell markers, P450scc (Fig. 1E), 3β-HSD I, and P450c17 (data not shown). These results indicate that donor derived-plastic adhered marrow cells had in fact differentiated into steroidogenic Leydig-like cells *in vivo*.

### *Gene promoter sorting*

Although these data suggest that the injected stem cells differentiated into Leydig cells, the apparent stem cell plasticity may also be explained by possible cell-nuclear fusion between donor and recipient cells, as has been recently suggested (24). Therefore, we next performed *in vitro* experiments to determine whether purified murine MSCs (mMSCs), KUM9 (15), have the capacity to differentiate into steroidogenic cells. To detect a cell population committed to

**TABLE 1.** Primers for RT-PCR and real-time PCR

| Gene | Sequence | Gene | Sequence |
|---|---|---|---|
| RT-PCR | | RT-PCR | |
| SF-1 | F-CGCACAGTCCAGAACAACAAGCA<br>R-CGGTTAGAGAAGGCAGGATAGAG | hHSD3b2 | F-CAGTGTGCCAGTCTTCATCT<br>R-AGCAGGAAGCCAATCCAGTA |
| mStAR | F-GAAGGAAAGCCAGCAGGAGAACG<br>R-CTCTGATGACACCACTCTGCTCC | hP450c17 | F-CATGCTGGACACACTGATGC<br>R-GGTTGTATCTCTAAAATCTGT |
| mP450scc | F-TTCCGCTTTTCCTTTGAGTCCAT<br>R-GTGTCTCCTTGATGCTGGCTTTC | hHSD17b3 | F-GCAGATTTTACAAAAGATGACAT<br>R-TCATGGGCAAGGCAGCCACAGGT |
| mHSD3b1 | F-ACTGCAGGAGGTCAGAGCT<br>R-GCCAGTAACACAGAATACC | hP450c21 | F-TGCCTGCCTATTACAAATGT<br>R-GGTGAAGCAAAAAAACCACG |
| mHSD3b6 | F-TCTGGAGGAGATCAGGGTC<br>R-GCCCGTACAACCGAGAATATT | hP45011 b1 | F-ACATTGGTGCGCGTGTTCCTC<br>R-GAGACGTGATTAGTTGATGGC |
| mP450c17 | F-AAATAATAACACTGGGGAAGGC<br>R-TGGGTGTGGGTGTAATGAGATGG | hP450 11b2 | F-TACAGGTTTTCCTCTACTCG<br>R-AGATGCAAGACTAGTTAATC |
| mP450c21 | F-AGAGGATCCGCTTGGGGCTGC<br>R-GGAGGAATTCCTTATGGATGGC | hP450aro | F-CTGGAAGAATGTATGGACTT<br>R-GATCATTTCCAGCATGTTTT |
| mP450 11b1 | F-TCACCAAATGTATCAAGAATGTGT<br>R-CCATCTGCACATCCTCTTTCTCTT | β-Actin | F-GGGAAATCGTGCGTGACATTAAG<br>R-TGTGTTGGCGTACAGGTCTTTG |
| mP450 11b2 | F-CCAACAGATGTATCTGGAAGGTGC<br>R-CCATCTGCACATCCTCTTGCCTCA | hIGF-2 | F-AGTCGATGCTGGTGCTTCTACCTCTT<br>R-TGCGGCAGTTTTGCTCACTTCCGATT |
| mLHR | F-CTCCACCTATCTCCCTGTC<br>R-TCTTTCTTCGGCAAATTCCTG | Real-time PCR | |
| mACTHR | F-GCTCCAAGGATCATTTACTTGC<br>R-CGCCAGGAGGCTTAACATAAC | mP450scc | F-CCAGTGTCCCCATGCTCAAC<br>R-TGCATGGTCCTTCCAGGTCT |
| GAPDH | F-ACCACAGTCCATGCCATCAC<br>R-TCCACCACCCTGTTGCTGA | mHSD3b1 | F-TAACAATTTAACAGCCCCTCCTAAGG<br>R-ATCCAGCCATGGTCAACACA |
| GFP | F-TGACCACCCTGACCTACGGCGT<br>R-GGTAGTGGTTGTCGGGCAGCA | mHSD3b6 | F-AAACCATCCTCCACTGTTCTAGCT<br>R-TGGAGATGGTCAGCCACAAG |
| mHSD17b3 | F-ATTTTACCAGACAAGACATCT<br>R-GGGGTCAGCACCTGAATAATG | mP450c17 | F-AGTTTGCCATCCCGAAGGA<br>R-CTGGCTGGTCCATTCATTT |
| mP450aro | F-TCAATACCAGGTCCTGGCTA<br>R-GTATGCACTGATTCACGTTC | mHSD17b3 | F-TGGGACAATGGGCAGTGAT<br>R-GCCAACTCAAATGAATAGGCTTTC |
| hStAR | F-GAGAGTCAGCAGGACAATGG<br>R-CTGGTTGATGATGCTCTTGG | β-Actin | F-CAACCGTGAAAAGATGACCCAGATC<br>R-AGTCCATCACAATGCCTGTGGTAC |
| hP450scc | F-TAGTGTCTCCTTGATGCTGG<br>R-GAAAGGAAGTGTTCACCACG | | |

F, Forward; R, reverse.

the steroidogenic lineage, we first introduced a human CYP11A1 promoter/GFP gene construct into the mMSCs. This was accomplished by using a 2.3-kb fragment of the promoter region of the human CYP11A1 (a gene that encodes cytochrome P450scc, cholesterol side-chain cleavage enzyme), which has been shown to selectively drive transgene expression to adrenal and gonadal steroidogenic cells (17). In some of the transformed cell lines, GFP fluorescence was detected, as shown in Fig. 2, B and C, but the number of GFP-expressing cells was very low. Thus, GFP-positive cells were enriched by sorting with flow cytometry (Fig. 2E, 1–5% of total cells). As shown in Fig. 2, F and G, enriched GFP-positive cells were also positive for P450scc, indicating that a very small but distinct portion of the mMSCs had spontaneously differentiated into cells that produce the steroid hormone-synthesizing enzyme. Further analysis of the differentiated cells revealed the expression of several genes that are specific to testicular Leydig cells, as shown in Fig. 2H. These include a nuclear orphan receptor SF-1, 3β-HSD types I and VI, and LH receptor (Fig. 2H, lane SCC+). LH receptor and 3β-HSD VI are known to be typical markers for androgen producing cells, such as Leydig cells (25). These observations further support the *in vivo* findings that rodent MSCs have the capacity to differentiate into Leydig-like cells in the testis.

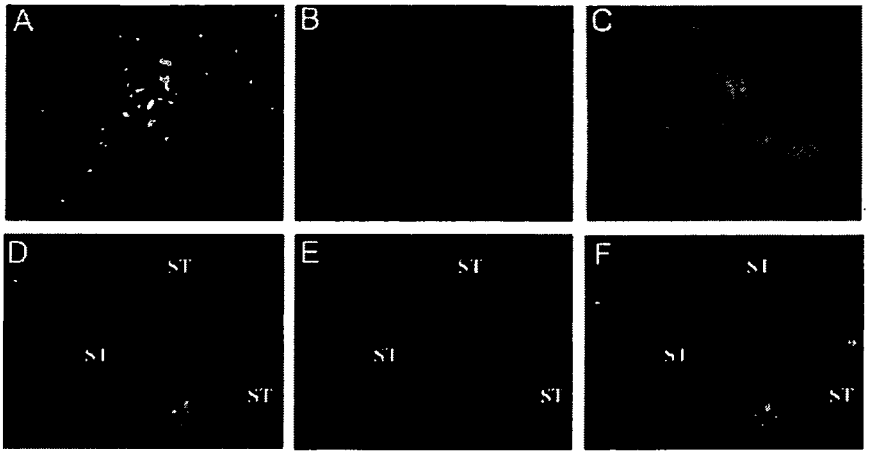


FIG. 1. Transplantation of GFP-positive MSCs into the testis. A, Fluorescence view of MSCs from a green rat 3 d after the first passage. Fluorescence microscopic view of testis before (B) or 3 wk after (C) MSC transplantation. Double staining of frozen sections from the testis 5 wk after MSC transplantation with anti-GFP (D) and anti-P450scc (E) antibodies. F, Merged fluorescent image of D and E. ST, Seminiferous tubule.

*Stable transfection of SF-1 into mouse MSCs*

It is noteworthy that SF-1 expression was induced in the GFP-positive cells (Fig. 2H). SF-1, also known as Ad4BP, regulates the cell-specific expression of a variety of proteins that are involved in steroidogenesis, in addition to its roles in reproduction and gonadal differentiation (26). Therefore, we next examined the effects of the stable transfection of SF-1 to mMSCs. Various cell lines that stably express SF-1 were isolated. As shown in Fig. 3C, SF-1-induced morphological changes in the cells, such as the accumulation of numerous lipid droplets. However, the transformed cells did not express steroidogenic enzyme genes or produce any steroid

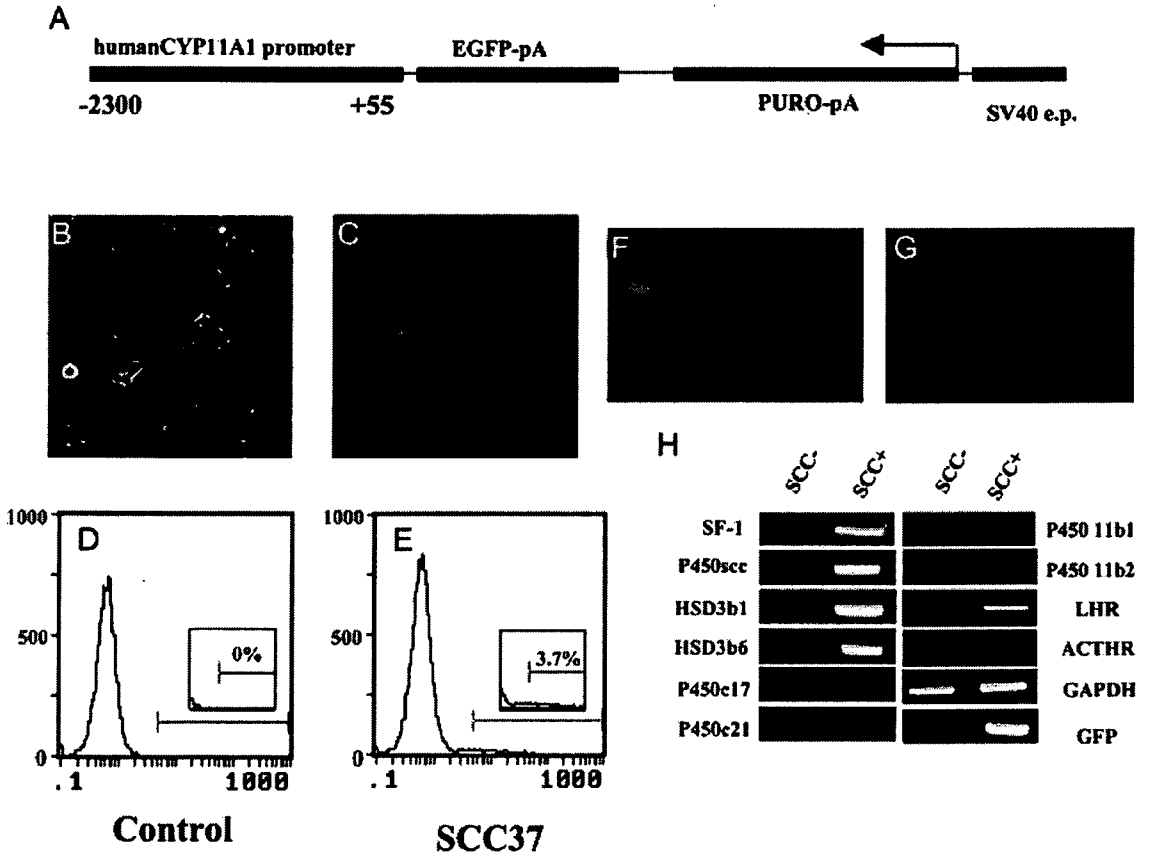


FIG. 2. Spontaneous differentiation of KUM9 into steroidogenic cells. A, Schematic representation of the SCC-reporter gene (SCC-GFP). The SCC-GFP reporter plasmid contains the 2300-bp upstream sequence of the human CYP11A1 gene and the puromycin-*N*-acetyltransferase gene (PURO-pA) driven by the Simian virus 40 early promoter (SV40 e.p.). Phase-contrast (B) and fluorescent (C) images of mMSCs transfected with SCC-GFP and selected by puromycin are shown. Flow cytometric analysis of enhanced GFP (EGFP) expression in KUM9 transfected with control-GFP (D) or SCC-GFP (E) are shown. KUM9-derived cells expressing GFP (F) under the control of the human CYP11A1 promoter were immunocytochemically stained with anti-P450scc antibody (G). H, SCC-GFP-positive (SCC+) and negative (SCC−) populations were sorted and analyzed for various marker genes by RT-PCR.

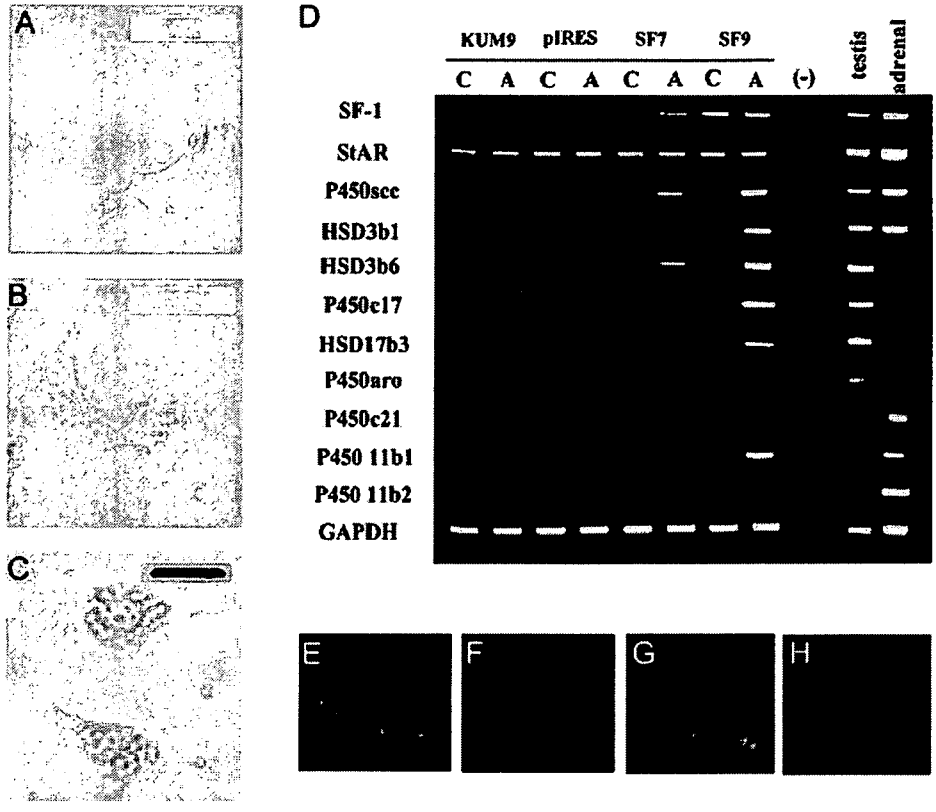


FIG. 3. Differentiation of KUM9 into steroidogenic cells. Phase-contrast images of mMSCs untransfected (A) or stably transfected with the control (B) or the SF-1-expression (C) vectors are shown. An immunoblot analysis was performed with an antibody against SF-1 (*insets*). D, RT-PCR analysis of each clones cultured with or without 8-bromoadenosine-cAMP (8br-cAMP) for 7 d (C: without 8br-cAMP; A: with 8br-cAMP). Data were compared with those from mouse adrenal and testicular tissues. Fluorescent images of 4′,6′-diamino-2-phenylindole staining (E and G) and a P450scc immunostaining (F and H) of KUM9 transfected with the SF-1-expressing vector and then cultured with (G and H) or without 8br-cAMP (E and F).

hormones (Fig. 3D and Table 2). Therefore, we next added cAMP to the cultures because cAMP is known to induce steroidogenesis in a number of steroidogenic cell lines. Treatment of confluent cultures with cAMP was found to induce both P450scc mRNA (Fig. 3D) and protein (Fig. 3H) in the transformed cell lines, SF7 and SF9, whereas no induction was observed in untransfected (KUM9) or vector-transfected (pIRES) mMSCs (Fig. 3D). Treatment of the cells for a period of 7 d further induced the expression of other steroidogenic enzyme genes, as shown in Fig. 3D. Several cell lines showed similar expression patterns (two of which are shown in Fig. 3D).

3β-HSD types I and VI were induced 3 d after cAMP treatment (Fig. 4). In the testis, the formation of testosterone is dependent on 3β-HSD activity, and isoform types I and VI have been shown to be expressed in the adult mouse testis (27). P450c17 and 17β-hydroxysteroid dehydrogenase III (17β-HSD III) were induced 5 d after the treatment (Fig. 4). It is interesting to note that the order of induction of the enzymes is similar to the sequential order for the steroid hormone synthetic pathway. 3β-HSD enzymes are essential for the production of progesterone, and P450c17 and 17β-HSD III are both required for the production of testosterone in testicular Leydig cells. Consistent with the expression pattern of the steroidogenic enzymes, testosterone was the major sex steroid hormone produced in the transformed cell line, SF9, when treated with cAMP for 7 d (Table 2). Two adrenal-specific steroid hormones, glucocorticoids and mineralocorticoids, were not detected in these cells. These results clearly demonstrate that the stable expression of SF-1 and the addition of cAMP induced the differentiation of mMSCs into steroidogenic cells and that these cells have properties that are similar to those of testicular Leydig cells.

**TABLE 2.** Production of steroid hormones by MSCs stably expressing SF-1 (SF9-KUM9 or SF4-hMSC) in the presence (+) or absence (−) of 8br-cAMP (ng/ml)

| Cell (cAMP) | Progesterone | Testosterone | Estradiol | Glucocorticoid | Aldosterone |
|---|---|---|---|---|---|
| pIRES-KUM9 (−) | N.D. | N.D. | N.D. | N.D. | N.D. |
| pIRES-KUM9 (+) | N.D. | N.D. | N.D. | N.D. | N.D. |
| SF9-KUM9 (−) | N.D. | N.D. | N.D. | N.D. | N.D. |
| SF9-KUM9 (+) | 24.3 ± 4.25 | 1.6 ± 0.29 | N.D. | N.D. | N.D. |
| pIRES-hMSC (−) | N.D. | N.D. | N.D. | N.D. | N.D. |
| pIRES-hMSC (+) | N.D. | N.D. | N.D. | N.D. | N.D. |
| SF4-hMSC (−) | N.D. | N.D. | N.D. | N.D. | N.D. |
| SF4-hMSC (+) | 270 ± 82.5 | 17.5 ± 0.20 | 0.21 ± 0.11 | 520 ± 200 | 1.56 ± 0.42 |

Data are means and SEM values of at least duplicate assays. N.D., No detectable values.

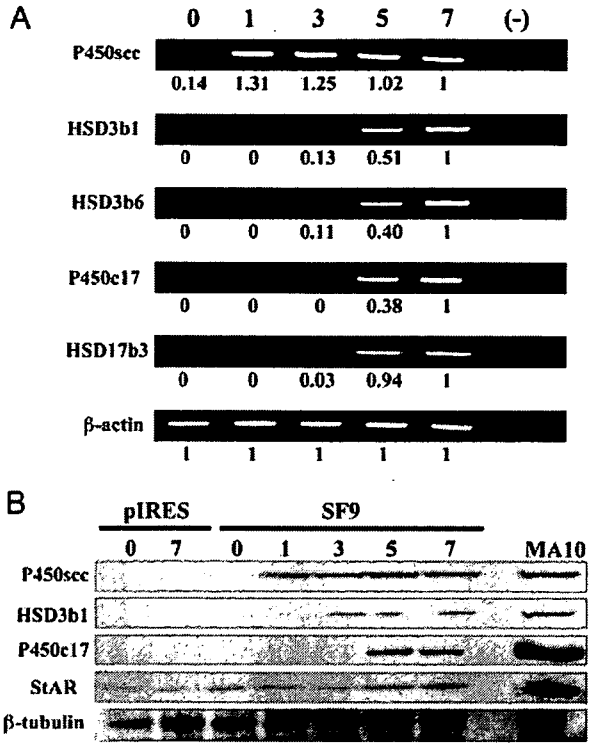


FIG. 4. Time-dependent induction of steroidogenic enzymes by cAMP. KUM9 cells stably transfected with SF-1-expression (SF9) or control (pIRES) vector were cultured and treated with 8-bromoadenosine-cAMP for the indicated times. A, P450scc, 3β-HSD I, 3β-HSD VI, P450c17, and 17β-HSD III mRNA levels were analyzed by RT-PCR and real-time PCR. Real-time PCR data are the mean values of at least triplicate assays. The 7-d value was arbitrarily taken as 1.0. B, Immunoblot analyses were performed with antibodies against StAR, P450scc, 3β-HSD I, P450c17, and β-tubulin using the same lysates. The data were compared with that from MA-10 cells treated with cAMP (4 h).

*Stable transfection of SF-1 into human MSCs*

We next examined the issue of whether the same approach could also be used to induce the differentiation of human MSCs (hMSCs) into steroidogenic cells. Similar to the results obtained with mMSCs, hMSCs (hMSC-TERT-E6/E7) expressed no steroidogenic enzymes or StAR before transfection with SF-1 even after cAMP treatment (Fig. 5). After SF-1 transfection, all the transformed cell lines became positive for StAR gene expression, and the expression levels were further increased by cAMP treatment. Most of the steroidogenic enzymes, P450scc, 3β-HSD II, P450c17, cytochrome P450 steroid 21-hydroxylase (P450c21), cytochrome P450 aromatase (P450arom), and cytochrome P450 steroid 11 β-hydroxylase, were also substantially induced by cAMP stimulation. A significant difference between mMSCs and hMSCs was the strong expression of the P450c21 gene in the case of hMSCs. This caused a difference in the kinds of steroids produced by mMSCs and hMSCs. As listed in Table 2, glucocorticoids were the major steroids produced by the transformed hMSCs, hSF4, whereas testosterone was the major product from the transformed mMSCs, mSF9. The hSF4 cells mainly produced cortisol, the major glucocorticoid produced by the human adrenal gland. These results clearly demonstrate that the stable expression of SF-1 and subsequent cAMP treatment induced the differentiation of hMSCs into steroidogenic cells. In addition, the cortisol-producing cells also expressed ACTH receptors and can respond to ACTH for the quick production of cortisol at nanomolar levels (data not shown).

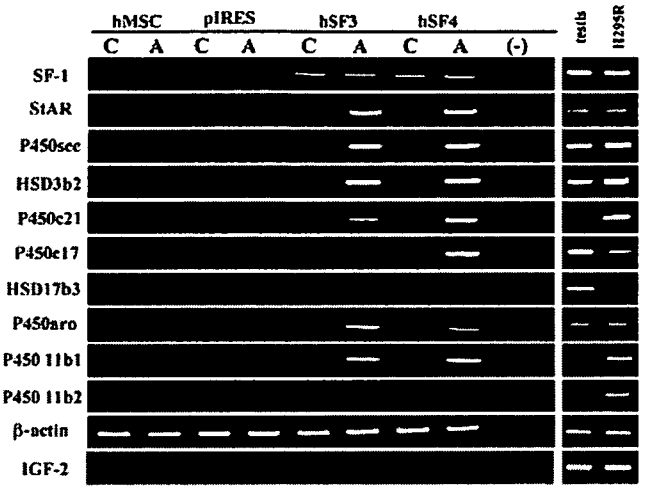


FIG. 5. Induction of steroidogenic enzymes in hMSCs. hMSCs were stably transfected with the control (pIRES) or SF-1-expression (SF3, -4) vector. RT-PCR analysis of each clone was cultured with or without 8-bromoadenosine-cAMP (8br-cAMP) for 7 d (C: without 8br-cAMP; A: with 8br-cAMP). The data were compared with that from human testis and NCI-H295R, a human adrenocortical tumor cell line, treated with cAMP (24 h).

Human MSCs also expressed P450arom as in the case of the human adrenocortical carcinoma NCI-H295R cell line (Fig. 5), whereas normal adrenal cells do not express it (28). However, hSF3 or -4 did not express IGF-II, an adrenocortical tumor marker. It has recently been shown that P450arom is expressed in human bone marrow stroma cells under certain conditions (29). Thus, it is probable that the expression of P450arom in hMSCs was not the result of a malignant phenotype or the differentiation of the cells by SF-1 and cAMP treatment.

*Stable transfection of SF-1 into cells other than MSCs*

We next examined the effects of transfection of SF-1 into several cell lines other than MSCs, *i.e.* a human cell line HEK293, murine embryonic stem cells, and murine cell lines F9 and NIH3T3. None of the transfected cell lines autonomously produced steroid hormones, although some were induced to express the P450scc and 3β-HSD genes (Fig. 6).

## Discussion

The findings presented herein demonstrate that rodent MSCs have the potential to differentiate into steroidogenic cells with characteristics that are very similar to testicular Leydig cells. It has been postulated that mesenchymal progenitors of Leydig cells are present in the testicular interstitium (12). Immature Leydig cells are gradually replaced by mature Leydig cells that are thought to differentiate from these mesenchymal progenitors during the prepubertal period. In fact, the injection of MSCs into the testis during this critical period caused the differentiation of MSCs into steroidogenic cells that were indistinguishable from Leydig cells. Concerning the *in vivo* experiments, the possibility of

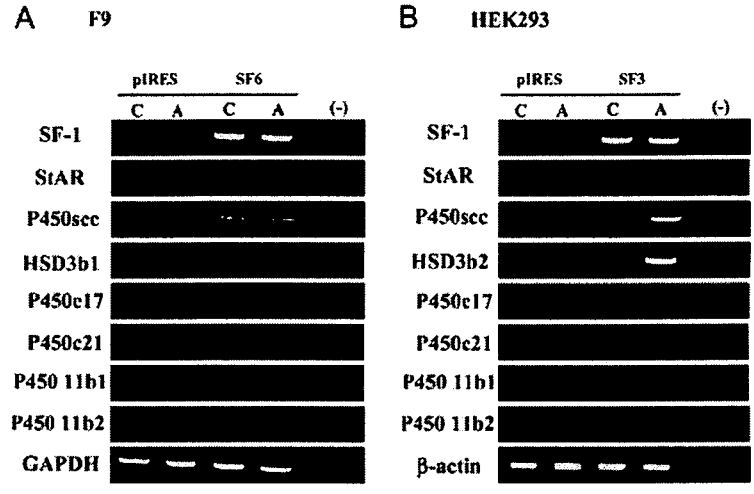


FIG. 6. Stable transfection of SF-1 and cAMP treatment for F9 (A) and HEK293 cells (B). RT-PCR analysis of steroidogenesis-related genes in each stable cell line transfected with SF-1 or pIRES (control) cultured with or without 8-bromoadenosine-cAMP (8br-cAMP) for 7 d (C: without 8br-cAMP; A: with 8br-cAMP).

cell fusion between donor MSCs and recipient testicular Leydig cells or their progenitor cells cannot be excluded. However, it should be emphasized that very small but distinct portions of mMSCs underwent spontaneous differentiation into Leydig-like cells *in vitro*. Lo *et al.* (30) demonstrated, by means of a cell transplantation assay, the presence of stem cells or progenitors for Leydig cells. Therefore, our data strongly suggest that bone marrow-derived MSCs share common properties with testicular MSCs or Leydig cell progenitors. Conversely, testicular MSCs or Leydig cell progenitors might also have pluripotent characteristics, similar to bone marrow-derived MSCs, as has been reported for some other MSCs (4, 31).

In addition, transfection of cultured mMSCs with SF-1 followed by cAMP stimulation resulted in their differentiation into Leydig cells. The same procedure also led to the successful induction of hMSCs into steroidogenic cells. In this case, however, most of the cell lines expressing SF-1 largely produced glucocorticoids rather than testosterone. This was mainly due to the strong induction of P450c21 gene expression in the hMSCs. To investigate the issue of whether hMSCs are able to differentiate into Leydig cells, we also injected hMSCs to the testis of nude mice or rats (data not shown). Unfortunately, the human cells did not survive for more than several weeks in the rodent testis.

Because the established cell lines need much longer times than general steroidogenic cells to produce steroid hormones by cAMP stimulation in this study, we speculate that cAMP treatment of this study is necessary for the induction of the cellular differentiation rather than direct stimulation of gene transcription of steroidogenic enzymes.

In hMSCs, the stable expression of SF-1 and cAMP treatment induced the expression of the StAR gene, which is essential for the transfer of cholesterol from the outer to the inner membrane of mitochondria in which the conversion of cholesterol to steroid hormones begins (21). The same treatment failed to induce StAR gene expression in several cell lines (other than MSCs) including embryonic stem (ES) cells and therefore failed to induce any steroid hormones. The expression of the P450scc or 3β-HSD gene was induced at low levels in some of them, however (Fig. 6). It has been reported that the stable transfection of SF-1 into ES cells results in morphological changes and the induction of P450scc enzyme expression, (32). No autonomous production of steroid hormones was observed, however, probably because of the deficiency of cholesterol storage and mobilization and the lack of StAR protein expression (32). Therefore, our present observations suggest that MSCs, but not ES cells, are excellent precursors of steroidogenic cells. In contrast to human cells, StAR was constitutively expressed in KUM9 as well as the freshly isolated rat MSCs (our unpublished data). Therefore, we speculate that StAR gene expression is not always under the control of SF-1, and the pattern of expression may be different between species, even in the same tissues. In addition to the steroidogenesis, the movement of cholesterol to the inner mitochondrial membrane is also important for its metabolism, because one of the rate-determining steps, the 27-hydroxylation of cholesterol, is catalyzed by sterol 27-hydroxylase, which is located in the inner mitochondrial membrane (33, 34). Cholesterol metabolites, such as oxysterols have been proposed to be potential regulators of genes in cholesterol homeostasis (33). We found that sterol 27-hydroxylase mRNA was detectable in rat and mouse MSCs (data not shown), suggesting that it is involved in cholesterol metabolism. Therefore, it is assumed that the StAR protein in KUM9 is present to promote the cholesterol metabolism, despite the fact that steroidogenesis does not take place. In support of this hypothesis, ectopic expression of the StAR protein increases the metabolism of cholesterol in rat primary hepatocytes (34).

Gondo *et al.* (35) recently reported that the adenovirus-mediated forced expression of SF-1 transforms primary long-term cultured murine bone marrow cells into ACTH-responsive steroidogenic cells. In contrast to our observation obtained from murine MSCs, their steroidogenic cells produce both gonadal and adrenal steroids. There are two possible explanations for their results: 1) their cells were a mixed adrenal/gonadal phenotype or 2) were a mixture of adrenal or gonadal phenotypic cells. The latter seems to be more likely because our study clearly demonstrated the differentiation of adult stem cells derived from both murine and human into gonadal or adrenal steroidogenic cells. Therefore, with respect to the difference between mouse and human cells, we assume that the mouse MSCs used in our study were already committed to the gonadal lineage, whereas the hMSCs were already committed to

the adrenal lineage. In support of this hypothesis, it has frequently been reported that MSCs are heterogeneous populations that have a different differentiation potential (1, 2, 10). In a future study, the same treatment of various mouse or human MSCs need to be carried out, followed by observations of whether both adrenal and gonadal phenotypes are obtained. This might also provide a tool for revealing the pathway leading to the differentiation of the cells into adrenal or gonadal steroidogenic cells.

In summary, we demonstrate here that MSCs have the capacity to differentiate into steroidogenic cells, both *in vivo* and *in vitro*. MSCs represent not only a powerful tool for studies of the differentiation of the steroidogenic lineage but may also offer a possible clinical stem cell resource for diseases of steroidogenic organs.

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# Immortalization of human myogenic progenitor cell clone retaining multipotentiality

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**Abstract**

Human myogenic cells have limited ability to proliferate in culture. Although forced expression of telomerase can immortalize some cell types, telomerase alone delays senescence of human primary cultured myogenic cells, but fails to immortalize them. In contrast, constitutive expression of both telomerase and the E7 gene from human papillomavirus type 16 immortalizes primary human myogenic cells. We have established an immortalized primary human myogenic cell line preserving multipotentiality by ectopic expression of telomerase and E7. The immortalized human myogenic cells exhibit the phenotypic characteristics of their primary parent, including an ability to undergo myogenic, osteogenic, and adipogenic terminal differentiation under appropriate culture conditions. The immortalized cells will be useful for both basic and applied studies aimed at human muscle disorders. Furthermore, immortalization by transduction of telomerase and E7 represents a useful method by which to expand human myogenic cells in vitro without compromising their ability to differentiate.


*Keywords:* Muscle satellite cell; Stem cell; Immortalization; Myogenesis; Multipotentiality; Regeneration; Cell transfer therapy; Telomerase; E7; Papillomavirus

Telomere length is stably maintained through the action of telomerase in germ line and cancer cells, but not in most somatic tissues. The progressive shortening of telomeres in most human somatic cells culminates in replicative senescence in vitro [1–3]. Proliferative capacity has been correlated with telomere length in human muscle satellite cells [4], and the number of population doublings is limited when human myogenic cells are grown in vitro. Although the cells remain viable, the telomeres shorten during culture, and the cells cease to divide.

The fatal loss of muscle in patients with Duchenne muscular dystrophy (DMD) is caused by a decline in the capability of muscle to regenerate. Because DMD myogenic cells undergo an extra number of cell divisions, they undergo premature replicative senescence during childhood. DMD myogenic cells from muscle biopsies grow poorly in vitro, and rapidly undergo senescence [5,6]. Whether the premature senescence of DMD myogenic cells is attributable to telomeric attrition remains controversial [6,7], but telomere shortening could be involved in the premature senescence of human myogenic cells from DMD patients [8].

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Previous attempts have been made to extend the replicative capacity of human myogenic cells using viral oncoproteins such as simian virus 40 (SV40) large T antigen (Tag). However, while the transformation due to Tag was sufficient to delay senescence, it failed to induce cell immortality. In addition, extension of the life span often coincided with dedifferentiation and differentiation defects [9]. To circumvent these problems, a temperature-sensitive mutant Tag was expressed in human myogenic cells [10]. Because temperature-dependent inactivation of mutant Tag is achieved at 39 °C, mutant Tag does not prevent terminal differentiation of human myogenic cells at 39 °C. However, a leak of Tag activity might still be sufficient to alter gene expression and cause mutations in human myogenic cells. In addition, the effect of exposure to high temperatures on phenotypic stability in human myogenic cells remains to be determined.

Not all but some types of human cells appear to be immortalized by the expression of the reverse transcriptase component of human telomerase (hTERT) alone without transformation of cell properties [1,11]. Thus, it might be expected that human myogenic cells could be immortalized by introduction of hTERT, but previous attempts to reconstitute telomerase by ectopic expression of hTERT did not result in extension of the replicative life span in normal (the present study) and DMD human myogenic cells [12,13].

The present study tested different combinations of hTERT, Bmi1, and human papillomavirus type 16 genes E6 and E7 to immortalize primary human myogenic cells. Our results indicate that constitutive expression of hTERT and E7 immortalizes primary human myogenic cells. Furthermore, the immortalized human myogenic cells express myogenic lineage markers and conserve the multipotentiality that the parental cells possess.

## Materials and methods

*Cell culture.* The human myogenic cell clone Hu5 was isolated from normal subcutaneous muscle tissue of a 42-year-old woman [14] and maintained at 37 °C under 10% $CO_2$ in dishes coated with type I collagen (Sumilon, Tokyo, Japan) and containing primary cultured myocyte growth medium (pmGM) consisting of Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% fetal bovine serum (FBS), 2% Ultroser G (Biosepra, Cedex-Saint-Christophe, France), and glucose (4.5 mg/ml). Cells were plated at $2\times 10^5$/35-mm dish and cultured in pmGM. For induction of myogenic differentiation the medium was changed to primary cultured myocyte differentiation medium (pmDM) after 48 h. pmDM consists of the chemically defined medium TIS [15,16] supplemented with 2% FBS. For low density culture, cells were plated at $1\times 10^5$/35-mm dish in the indicated experiment.

For induction of terminal osteogenic differentiation, cells were cultured in DMEM supplemented with 10% FBS, glucose (4.5 mg/ml), and a combination of 10 mM β-glycerophosphate (βGP) (Sigma, St. Louis, MO) and recombinant human bone morphogenetic protein (BMP2) (500 ng/ml) (Strathman Biotech, Hamburg, Germany, or PeproTech EC, London, UK). The cells were stained with the calcium-staining dye alizarin red S (0.01%, Sigma) [14]. To induce adipogenic differentiation, we cultured myogenic cells in DMEM supplemented with 10% FBS, glucose (4.5 mg/ml), and 200 μM γ-linolenic acid (Sigma, St. Louis, MO) for up to 5 days. The cells were stained with oil red O (0.3%, Sigma) [14].

*Preparation and infection of recombinant retroviruses.* The full-length human *bmi1* cDNA was cloned from K562 cells by RT-PCR using the forward primer 5′-ACGCGTCGACCGCCATGCATCGAACAACGA GAAT-3′ and the reverse primer 5′-CGGATCCTCAACCAGAA GAAGTTGCTG-3′. The PCR product was cloned into pCLXSN to generate pCLXSN-bmi1. pCLXSN-16E6E7 was constructed by inserting an *Eco*RI–*Bam*HI segment containing *HPV-16 E6* and *E7* into pCLXSN. pCMSCVpuro comprises a CMV/LTR fusion promoter, a packaging signal Psi+, and the multicloning sequence from pCLXSN (Imgenex Co., San Diego, CA) followed by a PGK-puro cassette and a 3′ long terminal repeat of a murine embryonic stem cell virus from pMSCVpuro (Clontech, Palo Alto, CA). *HPV-16 E7* and *hTert* cDNA [17] segments were recombined in the retroviral vectors to generate pCMSCVpuro-hTERT and pCLXSN-16E7. Production of recombinant retroviruses has been described [18]. Briefly, a retroviral vector and packaging construct, pCL-10A1, was co-transfected into 293 T cells by using TransIT-293 (Mirus Co., Madison, WI) according to the manufacturer's instructions, and the culture fluid was harvested at 48–72 h post-transfection. The titer of the recombinant viruses was greater than $5\times 10^5$ drug-resistant colony forming units/ml on HeLa cells.

*Establishment of immortalized cells and clonal cultures.* Hu5 cells were transfected with retroviral vectors encoding hTERT, Bmi1, HPV-16 E7, or HPV-16 E6 and E7 as described [11]. Briefly, 1 ml of the culture fluid was added to Hu5 cells seeded on type I collagen-coated 6-well plates in the presence of polybrene (4 μg/ml). Following inoculation with viruses, the infected cells were selected in the presence of 0.5 μg/ml puromycin or 400 μg/ml G418. For single-cell cloning, 1000 transfected Hu5 cells were plated on a 100 mm culture dish coated with collagen and then cultured in medium comprising equal volumes of pmGM and conditioned medium from high-density cultures of precloned transfected Hu5 cells. Colonies were isolated and expanded for experimentation. The immortalized human myogenic cell clone E18 will be available from RIKEN BioResorce Center (http://www.brc.riken.go.jp).

*Karyotyping of immortalized cell clone.* After incubation in pmGM supplemented with 2 μM colcemid at 37 °C for 10 h, E18 cells were trypsinized and incubated in 0.5 ml of 1% sodium citrate for 15 min. This was followed by addition of 0.5 ml of Carnoy's fixative (3:1 by vol; methanol:acetic acid). The fixed cells were then spun down and resuspended in 0.5 ml of Carnoy's fixative. Metaphase chromosomes were stained with 10% Giemsa solution (Wako Pure Chem., Osaka, Japan) for 10 min.

*Immunofluorescence and immunochemical analyses of myogenic lineage marker proteins.* Cultured cells were grown on collagen-coated coverslips (Iwaki, Tokyo, Japan) for immunofluorescence or immunocytochemical analysis. The cells were fixed with 4% paraformaldehyde for 10 min at room temperature or placed on ice, respectively, and were then incubated for 18–66 h at 4 °C with primary antibodies. Primary antibodies included those to mouse MyoD (Novocastra, Newcastle, UK), sarcomeric MHC [19], nestin [20] (kindly provided by Y. Arimatsu), and desmin (Progen, Heidelberg, Germany). Secondary antibodies were biotinylated or Cy3-labeled antibodies to mouse or rabbit immunoglobulin G (Jackson ImmunoResearch Laboratory, Bar Harbor, ME). The biotinylated antibodies were detected with streptavidin-conjugated horseradish peroxidase. The peroxidase reaction was performed with 3,3-diaminobenzidine (Sigma). Cell nuclei were stained with 2, 4-diamidino-2-phenylindole dihydrochloride *n*-hydrate (DAPI) (0.5 μg/ml, Sigma) or hematoxylin (Wako Pure Chem., Osaka, Japan). Samples were visualized using an upright microscope (model BX50; Olympus) and a CCD camera (DP50; Olympus). Images were post-processed using Adobe Photoshop (Adobe Systems, San Jose, CA).

*Telomerase activity.* Telomerase activity was detected using a non-radioisotopic method [21] with a TRAPeze telomerase detection kit (Intergen, Purchace, NY), according to the manufacturer's instructions. One microgram of cell protein lysed in CHAPS buffer was used for the assay. PCR products separated in 12.5% polyacrylamide gels were stained with SYBR Green I (Cambrex Co., NY), and visualized with the LAS3000 CCD-Imaging System (Fujifilm Co. Ltd., Tokyo, Japan) on a UV transilluminator.

Table 1
Sequences of PCR primers and amplification conditions

| Target gene | Primer | Sequence (5′ → 3′) | Annealing temperature (°C) | Amplification cycles (bp) | Product size |
|---|---|---|---|---|---|
| Telomerase reverse transcriptase | hTert-F | GGAAGCAGAGGTCAGGCAGC | 58 | 28 | 719 |
| | hTert-R | AGAGCAGCGTGGAGAGGATG | | | |
| Human papilloma virus-16 E6 | HPV16 E6-F | GCAACAGTTACTGCGACGTG | 53 | 28 | 234 |
| | HPV16 E6-R | GGACACAGTGGCTTTTGACA | | | |
| Human papilloma virus-16·E7 | HPV16 E7-F | GATGGTCCAGCTGGACAAGC | 53 | 24 | 143 |
| | HPV16 E7-R | GTGCCCATTAACAGGTCTTC | | | |
| Glyceraldehyde-3-phosphate dehydrogenase | hGAPDH-F | GGGCTGCTTTTAACTCTGGT | 56 | 20 | 702 |
| | hGAPDH-R | TGGCAGGTTTTTCTAGACGG | | | |

*Reverse transcription and polymerase chain reaction (RT-PCR).* Total RNA was extracted from cultured cells with TRIzol-LS (Life Technologies, Rockville, MD), treated with RNase-free DNase (RQ-1; Promega, Madison, WI), and then reverse transcribed with the use of a Ready-To-Go You-Prime First-Strand Beads (Amersham Pharmacia Biotech, Buckinghamshire, UK) and random hexamers as primers. To determine ectopic expression of the transduced genes hTERT, E6, and E7, targeted genes were amplified by PCR with the primers listed in Table 1.

*Detection of ossification.* Paraformaldehyde-fixed cultured cells in 35-mm dishes were stained with the calcium-specific dye Alizarin Red S (0.01%, Sigma) for 30 min. Images of stained dishes were obtained with a digital scanner (Scanjet 5p; Hewlett-Packard, Palo Alto, CA) and then post-processed using Adobe Photoshop.

## Results and discussion

### *Immortalization of primary human myogenic progenitor cells*

We previously isolated a primary human myogenic progenitor cell clone, Hu5, from the healthy subcutaneous muscle of a nondystrophic woman [14]. In contrast to primary cultured mouse myogenic cells [22], Hu5 cells cease proliferation and undergo replicative senescence after 10–12 passages after cloning. They also exhibit compromised myogenic differentiation potential prior to replicative senescence. To circumvent alterations of their characteristics during in vitro culture, we set out to immortalize the cells in vitro.

Some types of human cells appear to be immortalized by introduction of hTERT alone, but others require inactivation of tumor suppressors in addition [1,11]. Although expression of hTERT in Hu5 cells by retrovirus-mediated gene transfer provided telomerase activity (Figs. 1A and B), it was insufficient to overcome replicative senescence (data not shown) [13,23]. We therefore transfected the cells with retroviruses encoding hTERT and either HPV-16 E7 alone or both E6 and E7 because E6 targets p53 for degradation and E7 inactivates the retinoblastoma protein Rb [11]. The resulting cells, designated Hu5/E and Hu5/EE, respectively, proliferated continuously and did not undergo replicative senescence. They were maintained as multiple clones, for which the chromosomal positions and copy numbers of the transfected genes varied. Ectopic expression of both hTERT and Bmi1, a member of the polycomb group family of proteins that suppresses transcription of the cyclin-dependent kinase inhibitor (*p16Ink4a*) gene, also

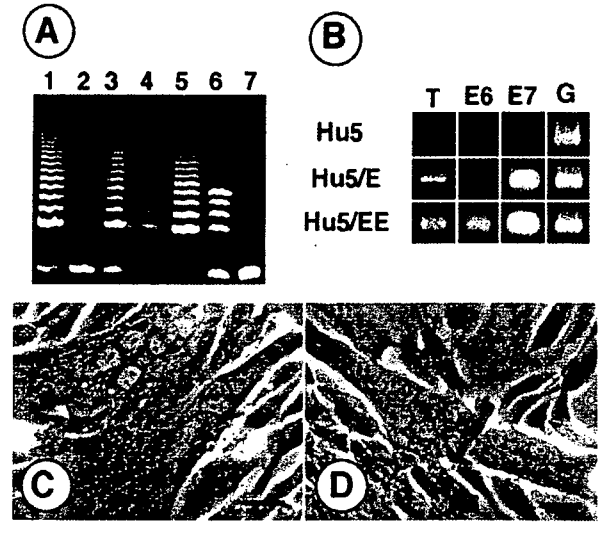


Fig. 1. Immortalization of human myogenic progenitor cells. (A) TRAP assay of parental and immortalized Hu5 cells for telomerase activity. Lanes: (1) HeLa cells (positive control), (2) Hu5 cells (passage 9), (3) Hu5 cells transduced with a retrovirus encoding hTERT alone, (4) Hu5 cells transduced with a retrovirus encoding hTERT and HPV-16 E7 (Hu5/E cells, passage 15), (5) Hu5 cells transduced with a retrovirus encoding hTERT and HPV-16 E 6/E7 (Hu5/EE cells, passage 12), (6) TSR-8 cells (positive control), (7) negative control (no cell lysate). (B) RT-PCR analysis of *hTert* (T) and *HPV-16 E6* and *E7* gene expression in parental and immortalized Hu5 cells. The human glyceraldehyde-3-phosphate dehydrogenase gene (G) served as an internal control. (C and D) Immortalized Hu5 cell derivatives Hu5/E (C) and Hu5/EE (D) were cultured in pmDM for up to 6 days and examined by phase-contrast microscopy. Scale bars, 20 μm.

resulted in a substantial extension of the life span of Hu5 cells, however, these Hu5/B cells retained little capacity for myogenic terminal differentiation in vitro and appeared to senesce within 20 passages. In a previous study, the constitutive expression of hTERT and Bmi1 in primary myogenic cells from a nondystrophic individual yielded immortalized clones, although these clones exhibited a differentiation block in vitro [12]. The life span of the primary human myogenic cells expressing hTERT and Bmi1 could be markedly extended, but immortalization is likely to occur at low frequency.

In contrast to the parental Hu5 cells, both Hu5/E and Hu5/EE cells exhibited telomerase activity (Fig. 1A).

RT-PCR analysis revealed the expression of both *hTert* and *HPV-16 E7* genes in Hu5/E cells and the expression of *hTert*, *E6*, and *E7* genes in Hu5/EE cells (Fig. 1B). To examine the myogenic differentiation potential of Hu5/E and Hu5/EE cells, we cultured them for up to 6 days in pmDM. Most Hu5/E cells differentiated into prominent myotubes (Fig. 1C), whereas only a small proportion of Hu5/EE cells underwent myogenic differentiation (Fig. 1D).

We next examined the expression of myogenic lineage marker proteins in the immortalized Hu5 cells by immunofluorescence analysis. First, the expression of a master gene for myogenesis, *MyoD*, was examined. MyoD was present in the nuclei of parental Hu5 cells (Figs. 2A and D) and a major population (approximately 80%) of Hu5/E cells (Figs. 2B and E); however, their expression was detected in only a minor population (10–20%) of Hu5/EE cells (Figs. 2C and F). Desmin and nestin, which are present primarily in proliferating parental Hu5 cells (Figs. 2G and J), were each expressed in Hu5/E cells (Figs. 2H and K); however, their expression was restricted to only a minor population (10–20%) of Hu5/EE cells (Figs. 2I and L). The immortalized line Hu5/E thus largely retains the original phenotype of the parental Hu5 cells. To date, Hu5/E and Hu5/EE cells have been cultured for >80 passages corresponding to more than 200 population doublings. We therefore conclude that they represent immortalized human myogenic progenitor cells that have retained their myogenic differentiation potential.

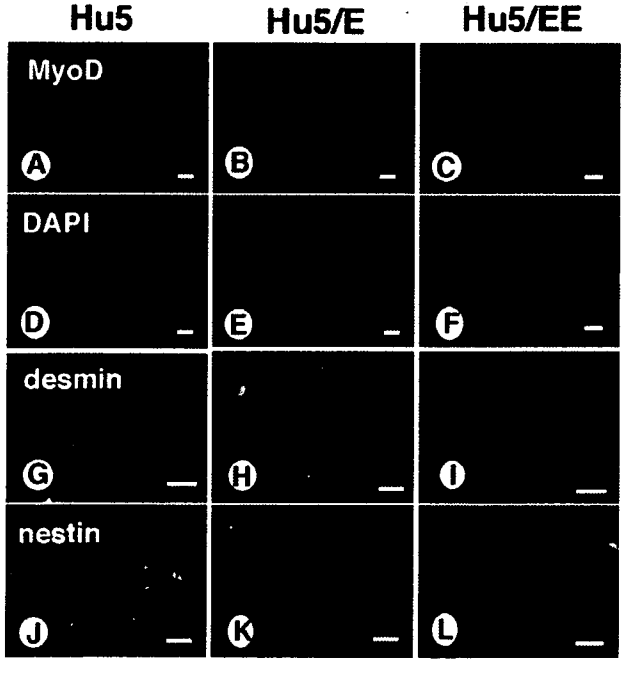


Fig. 2. Expression of myogenic proteins in primary and immortalized human myogenic cells. Undifferentiated Hu5 (A, D, G, and J), Hu5/E (B, E, H, and K), and Hu5/EE (C, F, I, and L) cells were subjected to immunofluorescence analysis with antibodies to MyoD (A through C), desmin (G through I), or nestin (J through L). Nuclei in the same fields as those in (A) through (C) were visualized by DAPI staining in (D) through (F), respectively. Nuclei in (G) through (L) were also revealed by DAPI staining (blue). Scale bars, 20 μm.

*Establishment of immortalized human myogenic cell clones*

The immortalized Hu5 cells, Hu5/E, and Hu5/EE cells contained multiple clones in which expression levels of the transduced genes varied. Furthermore, it is conceivable that differentiation-defective variant cells are included in the immortalized Hu5 cell population. To remove putative differentiation-defective cells from the culture, we isolated clones derived from individual Hu5/E cells, but not Hu5/EE cells, because a major fraction of Hu5/EE cells represented a nondifferentiating phenotype. First, the myogenic differentiation potential of each clone was determined by its ability to differentiate into myotubes in vitro on achieving confluence.

One of the Hu5/E-derived clones retaining myogenic differentiation potential, E18, was subjected to further analyses, although similar results were obtained with the eight other independent clones derived from individual Hu5/E cells. The doubling time of E18 cells was estimated at 34.7 ± 4.1 h based on their growth rate when cultured in pmGM. Karyotypic analyses of E18 cells were performed at 26 passages. All of the twenty E18 cells tested contained 46 chromosomes (Fig. 3). Therefore, E18 cells exhibited normal diploidy throughout in vitro culture and the immortalization process. The muscle lineage markers desmin and nestin were expressed in similar amounts in both parental Hu5 and immortalized E18 cells (data not shown).

Next, the expression of *MyoD* was examined in the immortalized Hu5 cell clone E18. MyoD was detected at a high level in less than 30–40% of E18 cells under the low cell density culture conditions (Figs. 4C and D) but at a lower level compared with that apparent in parental primary Hu5 cells (Figs. 4A and B). However, under the high cell density culture conditions, MyoD expression was up-regulated and detected at a high level in more than 80% of E18 cells (Figs. 4E and F). The immortalization process might result in the reduction of MyoD expression in E18 in a cell density-dependent manner. Taken together,

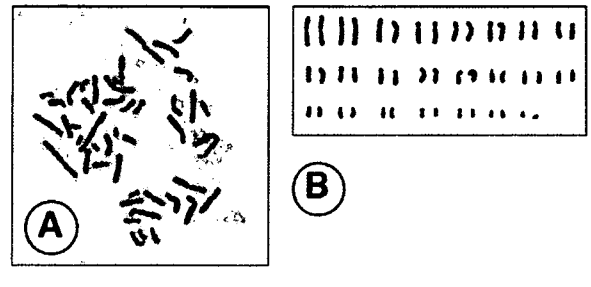


Fig. 3. Karyotypic analysis of immortalized human myogenic cell clone E18. (A and B) E18 cells were treated with colcemid (2 μM) for 10 h. Metaphase chromosomes were visualized by Giemsa staining (A). All of the twenty E18 cells tested exhibited 46 chromosomes (B).

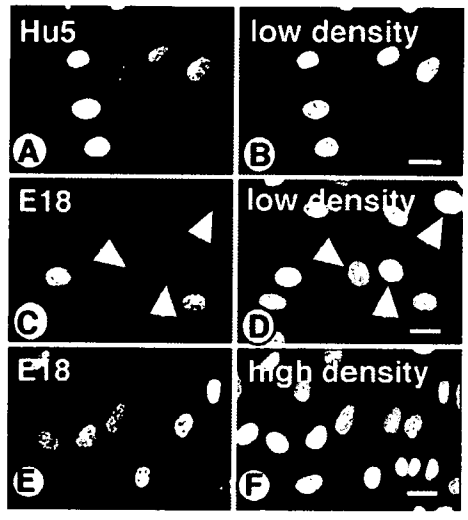


Fig. 4. Expression of MyoD in primary and immortalized human myogenic progenitor cell clones. Undifferentiated Hu5 cultured under a low-density condition (A and B) and E 18 cells cultured under low—(C and D) or high—(E and F) density conditions were subjected to immunofluorescence analysis with antibodies to MyoD (A, C, and E). Nuclei in the same fields as those in (A, C, and E) were visualized by DAPI staining in (B, D, and F), respectively. MyoD levels were at a reduced level in 60–90% of E18 cells under low-density conditions (arrowheads in C and D). Scale bars, 20 μm.

the results described here indicate that the immortalized line E18 largely retains the myogenic phenotype represented by parental Hu5 cells.

Mouse myogenic cells preserve the ability to undergo myogenic, osteogenic, and adipogenic differentiation [14]. E18 also underwent myogenic, osteogenic, and adipogenic terminal differentiation under the appropriate culture conditions that are somewhat different from those for mouse myogenic cells (Figs. 5A, B and C): induction of differentiation on day 2 of growing culture, medium containing 10% FBS, and higher concentration of γ-linolenic acid for adipogenesis. Hu5 and Hu5/E cells also underwent osteogenic terminal differentiation, although Hu5/EE cells lost the ability to undergo any terminal differentiation (Fig. 5A).

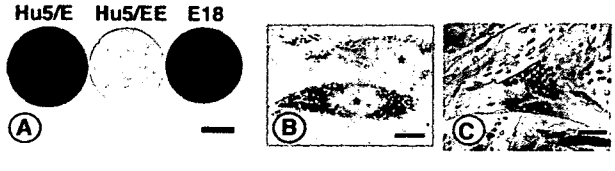


Fig. 5. Multipotentiality of immortalized human myogenic cell clone E18. (A) Hu5/E, Hu5/EE, and clone E18 cells were cultured for 6 days in serum-containing medium supplemented with BMP2 (500 ng/ml) plus βGP (10 mM). The cells were then stained with Alizarin Red S. Whole 35 mm dishes are shown. Scale bar, 10 mm. (B) E18 cells were cultured for 5 days in serum-containing medium supplemented with γ-linolenic acid (200 μM). Numerous lipid droplets were stained with oil red O. Asterisks represent nuclei. Scale bar, 20 μm. (C) E18 cells were cultured for 6 days under myogenic differentiation-inducing conditions. MHC was detected by immunostaining with a horseradish peroxidase reaction product (brown). Nuclei were detected by staining with hematoxylin (blue). Scale bar, 50 μm.

In conclusion, we succeeded in establishing an immortalized human myogenic cell line that preserves the multipotentiality that the parental primary human myogenic cell retains. E18 would provide a useful culture system for analysis of the characteristics, differentiation mechanisms, and species-specific characteristics of human myogenic cells, giving information for both basic biological research and therapeutic approaches.

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Original Article

# Immortalization of Epstein–Barr virus-negative human B lymphocytes with minimal chromosomal instability

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**The genes required for immortalization of human B cells infected by Epstein–Barr virus are multiple, and the precise mechanism of this process remains to be elucidated. In the present study HPV16 E6 and E7 were retrovirally transduced into human primary B cells stimulated by CD40–CD40L interaction, thereby establishing an Epstein–Barr virus negative immortalized human B cell line, which continued to proliferate for more than 2 years (100 population doublings). The established cell line had a high telomerase activity from the beginning of the culture period, and no shortening of the telomere length was observed. A chromosomal analysis revealed that a large portion of the HPV16E6E7 transduced cells had retained a normal karyotype. Similar to human epithelial cells, human B lymphocytes seem to require two steps for immortalization, namely, the inactivation of the p16/Rb pathway and the activation of telomerase, the latter that can be induced by the CD40–CD40L interaction. Furthermore, using this system, it is possible to analyze the role of individual genes in human B lymphocyte immortalization without the influence of a pre-existing Epstein–Barr virus genome.**

**Key words:** CD40–CD40L interaction, Epstein–Barr virus, HPV16 E6E7, human B cells, immortalization, telomerase

Normal cells cease to grow after a limited number of cell divisions when cultured *in vitro*.[1] This phenomenon is called replicative or cellular senescence, and senescence is now widely accepted as one of the important mechanisms in tumor suppression.[2] It has been suggested that senescence can be classified into two types. One is induced by telomere shortening. The telomere length is maintained by telomerase. Generally, somatic cells are devoid of any expression of telomerase reverse transcriptase (TERT), a key component of telomerase, and thus their telomere length becomes shortened after cell division. The other type is associated with the activation of the p16/Rb pathway or the p53 pathway by various types of extrinsic stress such as *in vitro* culture, although the exact activation mechanism remains to be elucidated.[3] The p16/Rb pathway or the p53 pathway is often abolished in tumor cells, and the activation of these pathways leads to the inhibition of cyclin-dependent kinase activity, thus resulting in cell cycle arrest or apoptosis.[4]

Overcoming senescence is called immortalization.[5,6] Because mouse cells have extremely long telomeres, murine cells tend to be spontaneously immortalized. In contrast, human primary cells are hardly ever spontaneously immortalized,[3,7] and some genetic manipulations are thus necessary to establish immortalized human cells. Human foreskin fibroblasts, retinal pigment cells[8] and, according to a most recent report, human fetal mesenchymal stem cells can be immortalized by the introduction of human TERT (hTERT) alone.[9] In contrast, both hTERT expression and the inactivation of the p16/Rb pathway are required for the immortalization of other fibroblasts and epithelial cells such as human mammary gland epithelial cells (HMEC) and human foreskin keratinocytes. E6 and E7 oncoproteins of human papillomavirus (HPV) type 16 or 18 can cooperatively immortalize HMEC, in that E7 directly binds and inactivates Rb, and E6 induces expression of hTERT.[10] Recently, several molecular mechanisms have been proposed for the induction of hTERT expression by E6.[11,12] In some other cells, a direct introduction of hTERT itself, instead of E6, is required for immortalization, probably due to the inefficient activation of endogenous hTERT expression by E6 in those cells.[13,14] SV40 T antigen, which inactivates both p53 and Rb, has also been used to expand the lifespan of human cells.[15]

Human B lymphocytes infected by Epstein–Barr virus (EBV) are called B-lymphoblastoid cell lines (LCL), which have long believed to be immortalized.[5] However, recent studies have suggested that LCL derived from normal

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individuals eventually reach senescence due to a shortening of their telomeres. Whether EBV immortalizes or transforms human B lymphocytes remains an unresolved and confusing problem.[16] Furthermore, at least five proteins of EBV are required for immortalization or transformation, while even more are involved in this process.[17–21] As a result, the exact molecular mechanisms involved in the immortalization of B cells remain to be clarified. Recently, normal somatic cells have been shown to have only a slight degree of telomerase activity.[22] In contrast, lymphocytes contain a high degree of telomerase activity, while also showing an elongation of telomere length during their proliferation, for example in the normal germinal center.[23–25] We therefore speculated that the steps required for human B lymphocyte immortalization may be different from those of other human cells such as HMEC.

In the present study we retrovirally transduced HPV16 E6 and E7 simultaneously into CD40L-stimulated human primary B cells, and attempted to gain some insight into the molecular mechanism of immortalization of human B lymphocytes.

## MATERIALS AND METHODS

### Long-term culture of human B lymphocytes

Peripheral mononucleocytes were obtained from the peripheral blood of a healthy donor by Ficoll gradation. Informed consent was obtained from the donor following the ethics standards of Aichi Cancer Center Research Institute. The cells were then cultured on 96 Gy-irradiated CD40 ligand (CD40L)-expressing NIH 3T3 cells (a kind gift from Dr Gordon Freeman, Dana-Farber Cancer Institute, Boston, MA, USA) as previously described.[26] The cells were analyzed by flow cytometry using Cy 5-labeled anti-CD19 antibody (Immunotech, Marseille, France), 1 week after the initiation of culture to confirm that >98% of the cells were CD19-expressing B lymphocytes, and thereafter were cultured further, with slight modification as described previously.[27]

Vector construction and retroviral transduction of HPV16 E6E7, enhanced green fluorescent protein (EGFP) and short hairpin RNA (shRNA) pCLMSCV-neo and pCLMSCV-puro comprise the CMV/LTR fusion promotor, packaging signal Psi+ and the multicloning site from pCLXSN (Imgenex, San Diego, CA, USA), followed by the PGK-neo cassette and 3′ long-terminal repeat of murine stem cell virus from pMSCVneo or pMSCVpuro (Clontech, Palo Alto, CA, USA). The Gateway system (Invitrogen, Carlsbad, CA, USA) was used for subcloning the genes into retroviral vectors. PCLMSCVneo-16E6E7 was constructed by recombining the segment of pCLXSN-16E6E7 containing full-length HPV16 E6 and E7 into the destination vectors as described previously.[13] For EGFP, a segment encoding a fusion protein of hygromycin-resistant gene and EGFP was polymerase chain reaction (PCR) amplified from pHygEGFP (Clontech), and then was inserted into the destination vector pDEST-CLMSCVpuro- as described here. shRNA E6Ri3 (GTATGGAACAACATTAGAA) and E7Ri2 (GAGATACACCTACATTGCA) were designed by selecting a 19-mer nucleotide sequence open reading frame (ORF) of HPV 16 E6 and E7, according to the criteria of Dharmacon siRNA Design Center (http://design.dharmacon.com/rnadesign/). E6Ri3 was chosen from the sequence inside the E6 ORF (122–306 nt), which is excised in two of the three splicing variants of the HPV 16 E6-E7 polycistronic transcript.[28] Sense and antisense oligonucleotides with the shRNA fragments, cohesive restriction sites, the stem loop sequence and the polyT signal were designed as follows: sense, GATCCCC (sense shRNA 19mer) TTCAAGAGA (antisense shRNA 19mer) TTTTTGGAAA; antisense, AGCTTTTCCAAAAA (sense shRNA 19mer) TCTCTTGAA (antisense shRNA 19mer) GGG. A total of 4.5 μmol/L each of sense and antisense oligonucleotides were mixed in 100 mmol/L NaCl and annealed by lowering the temperature from 95°C to 4°C in 2 h, and then were ligated into an entry vector with an H1 promotor region, and then were finally recombined into pCMSCV-puro by the Gateway system. The production of retroviruses has been described.[29] Briefly, retroviral vector and packaging vector encoding viral *gag-pol* gene, and pCL-GALV, which encodes envelope protein from gibbon ape leukemia virus[30] were cotransfected into 293T cells using Trans IT-293 (Mirus, Madison, WI, USA) according to the manufacturer's instructions, and the culture fluid was directly harvested at 48–72 h after transfection or sometimes harvested after centrifuging overnight for concentration. The titers of the recombinant viruses were $>1 \times 10^5$ drug-resistant colony-forming units/mL on HeLa cells. For retroviral infection, $1 \times 10^6$ B cells were placed in a 12-well plate, and centrifuged at 32°C, 1000 *g* for 1 h with 1 mL retroviral supernatant in the presence of 10 μg/mL polybrene. After infection, the cells were drug selected by either 0.8 mg/mL G418 or 0.5 μg/mL puromycin.

### Reverse transcription–polymerase chain reaction and western blot analysis

RNA was extracted from E6 and E7/or EGFP-transduced B cells using RNeasy kit (Qiagen, Hilden, Germany) combined with RNAse-Free DNase Set (Qiagen) according to the manufacturer's instructions. Next, cDNA was synthesized using the random hexamers provided in the SuperScript III First-Strand Synthesis System for reverse transcription–polymerase chain reaction (RT-PCR; Invitrogen). RT-PCR was performed as described previously[31] with the following primers: HPV16 E6-FW, GCAACAGTTACTGCGACGTG; HPV16 E6-RV, GGACACAGTGGCTTTTGACA; HPV16