

# Increased Mobilization of c-kit<sup>+</sup> Sca-1<sup>+</sup> Lin<sup>-</sup> (KSL) Cells and Colony-Forming Units in Spleen (CFU-S) Following De Novo Formation of a Stem Cell Niche Depends on Dynamic, But Not Stable, Membranous Ossification

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Stem cells are thought to inhabit in a unique microenvironment, known as “niche,” in which they undergo asymmetric cell divisions that results in reproducing both stem cells and progenies to maintain various tissues throughout life. The cells of osteoblastic lineage have been identified as a key participant in regulating the number of hematopoietic stem cells (HSCs). HSCs receive their regulatory messages from the microenvironment in the bone marrow. This would account for a reason why the localization of hematopoiesis is usually restricted in the bone marrow. To clarify the above possibility we employed a cell implantation-based strategy with a unique osteoblast cell line (KUSA-A1) derived from a C3H/He mouse. The implantation of KUSA-A 1 cells resulted in the generation of ectopic bones in the subcutaneous tissues of the athymic BALB/c nu/nu mice. Subsequently the mice obtained a greater amount of the bone marrow than normal mice, and they showed an increased number of HSCs. These results indicate that the newly generated osteoblasts-derived ectopic bones are responsible for the increase in the number of the HSC population. Furthermore, the increased number of HSCs directly correlates with both the magnitude of dynamic osteogenic process and the size of the newly generated bone or “niche.” *J. Cell. Physiol.* 208: 188–194, 2006. © 2006 Wiley-Liss, Inc.

Stem cell with potential for self-renewal and multi-lineage differentiation can be identified in various self-renewing tissues, including epidermis, intestinal epithelium, and testis, and hematopoietic stem cells (HSCs) are also capable of both self-renewal and multipotency (Ikehara, 2000; Weissman, 2000). The most important experimental evidence for the existence of such cells is the ability of a single bone-marrow-derived cell to reconstitute long-term hematopoiesis in lethally irradiated recipients (Till and McCulloch, 1961; Siminovitch et al., 1963; Till et al., 1964; Matsuzaki et al., 2004). Molecular markers that characterize transplantable cells with stem cell potential and allow their selective purification have been identified, and this achievement has been important to progress both applied and basic science (Spangrude et al., 1988; Goodell et al., 1996). As an example, CD34<sup>-</sup>, c-kit<sup>+</sup>, Sca-1<sup>+</sup>, and Lin<sup>-</sup> cells have been identified as the most primitive HSCs (Osawa et al., 1996).

Stem-cell fate decisions in the developing embryo are governed by complex interplays between cell-autonomous signals and stimuli from the surrounding tissues. Stem cells are thought to inhabit in a unique microenvironment, known as “niche,” in which they undergo asymmetric divisions that generate both stem cells and progenies to maintain the tissue throughout life (Dzierzak et al., 1998; Matsuzaki et al., 2004). HSCs migrate from the yolk sac to the liver during early development, and they ultimately settle in the bone marrow and spleen of the adult. The bone marrow and spleen serve as the microenvironment that supports the

HSCs via cytokines, membrane-bound molecules, and gap junctions. And the classical experiment on HSC-colony formation by Till and McCulloch (1961) showed that reconstitution of hematopoiesis takes place only in hematopoietic organs. The niche hypothesis was first proposed by Schöfield, 1978, and it is supported by the evidence that HSCs have been successfully maintained in co-culture systems with marrow-derived stromal cells in vitro. Steel mice (Sl/Sl) have a mutation at the Sl locus, and spleen colonies cannot be produced in the mice when transplanted with normal marrow cells.

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Steel mice have a defect in the hematopoietic micro-environment, or the niche, where marrow stromal cells constitute (Harrison and Russell, 1972).

Bone marrow stromal cells are capable of differentiating into adipocytes, endothelial cells, chondrocytes, and osteoblasts (Pittenger et al., 1999). They are also capable of transdifferentiating into cardiomyocytes, skeletal myocytes, and neurons when exposed to inducers in vitro and in vivo (Umezawa et al., 1992; Makino et al., 1999; Kohyama et al., 2001; Takeda et al., 2004; Mori et al., 2005; Terai et al., 2005). Previous studies on the role of stromal cells in supporting HSCs have mainly been based on in vitro culture. The trabecular area of cancellous bone is the primary site of HSCs. They arise next to the inner surface of bone, and then migrate towards the blood vessels at the center of the bone marrow cavity as they mature. Since the 1970s, efforts to characterize the HSC niche have been focusing on developing systems in vitro that mimic some of the features of stem cell–niche interactions in vivo, and single clones of stromal cells have been found to be capable of supporting HSC self-renewal and differentiation in vivo (Okada et al., 1991, 1992). Osteoblastic marrow stromal cells are a regulatory component of the HSC niche in vivo that influences stem cell function, and some stromal cell clones are part of the bone-forming ‘osteoblastic’ lineage, which is consistent with a notion that osteoblasts may be a component of the HSC niche in vivo (Lord, 1990; Yoshimoto et al., 2003).

In the present study, we demonstrate that KUSA-A1 osteoblasts, whose number has been increased by local injection into the tissues, support an increase in the 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. We provide in vivo evidence that shows an extra osteogenic process independent from that in the normal bone affects the reproduction of stem cells.

## MATERIALS AND METHODS

### Mice and their major histocompatibility complex (MHC) Class I

BALB/c nu/nu (H-2d), BALB/c (H-2d), C57BL/6N (H-2b), and C3H/He (H-2k) mice were obtained from Clea Japan Inc (Tokyo, Japan).

### Cell lines and cell culture

KUSA-A1 cells, that was derived from a C3H/He mouse, were maintained in the M061101 medium (okada@med-shirotori.co.jp, MED SHIROTORI Co., Ltd., Tokyo) on 100 mm culture dishes (Falcon 3003; Becton Dickinson Labware, Bedford, MA) at 37°C under a humidified atmosphere of 5% CO<sub>2</sub>. ST-2 cells were obtained from the RIKEN cell bank, Japan, and were maintained in RPMI 1640 (Invitrogen Corporation, Auckland, New Zealand) supplemented with 10% FCS and 10<sup>-5</sup>M of 2-ME (GIBCO BRL) at 37°C under a humidified atmosphere of 5% CO<sub>2</sub> in air.

### Cell transplantation

Freshly scraped confluent cells (5 × 10<sup>6</sup>) were subcutaneously implanted into BALB/c nu/nu mice (Clea). These animals were sacrificed by cervical dislocation between 3 and 10 weeks after implantation.

### Antibodies

Phycoerythrin (PE)-conjugated antibodies to CD4, CD8, CD3, B220, Mac-1, Gr-1, and Tre119 (Pharmingen, San Diego, CA), fluorescein isothiocyanate (FITC)-conjugated antibody to CD34, H-2k (Pharmingen), allophycocyanin (APC)-conju-

gated antibody to c-kit (Pharmingen), Sca-1 biotinylated antibody (Pharmingen), and antibody to CD16/32 (Fc III/II receptor; 1: 100; Fcblock; Pharmingen) were used for flow cytometric analysis.

### Flow cytometric analysis

The monoclonal antibodies (mAbs) were either biotinylated or fluoresceinated. Biotinylated mAbs were detected with streptavidin-conjugated Red 613 (Invitrogen Corporation). Cells were incubated for 30 min on ice with CD16/32 (Fc III/II receptor; 1: 100; Fcblock) before staining with the first antibody. Cells were stained with the first antibody, incubated for 30 min on ice, and then washed twice with washing buffer. The secondary antibody was added, and after incubating the cells for 30 min on ice, they were washed twice with washing buffer and suspended in washing buffer. KUSA-A1 cell suspensions were prepared from monolayer cultures by exposure to trypsin (0.02% for 3 min at 37°C), followed by two washes in cold PBS plus 2% FCS and 0.01% sodium azide. After staining with a series of monoclonal antibodies according to manufacturer's protocol, cells were analyzed by fluorescence-activated cell sorter (FACS) with the FACS vantage system (Becton Dickinson, San Jose, CA).

### Colony-forming unit in spleen (CFU-S) assay of hematopoietic cells obtained from ectopic bone

Freshly scraped confluent KUSA-A1 cells (5 × 10<sup>6</sup>) were subcutaneously implanted into BALB/c nu/nu mice (Clea). Hematopoietic cells were obtained from ectopic bone marrow generated by KUSA-A1 cells, and were assayed for CFU-S. Bone marrow cells (5 × 10<sup>6</sup>) were implanted into lethally irradiated BALB/c mice, and the number of colonies (Day 12 CFU-S) was counted 12 days after transplantation. Day 12 CFU-S including erythrocytic, granulocytic, megakaryocytic, and lymphocytic lineages are derived from multipotent HSCs and are more potent in terms of repopulating ability than day 8 CFU-S.

### Soft X ray system

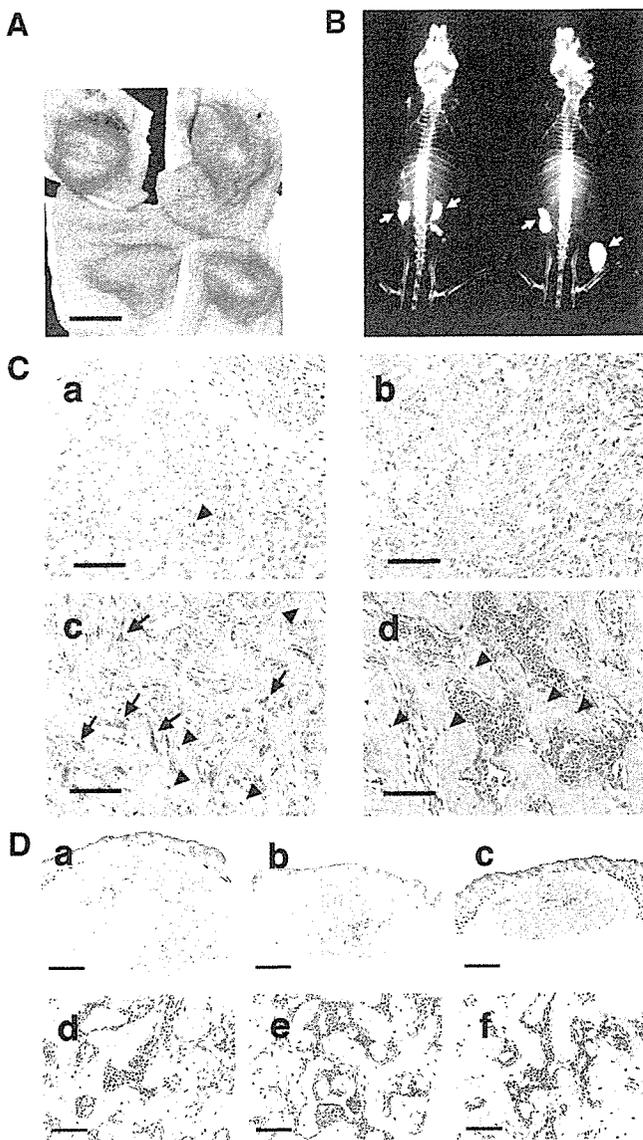
BALB nu/nu mice were examined by whole body soft X-ray radiography at 25.0 kV and 3.0 mA for 10 sec (SRO-IM50, Sofron, Tokyo) with X-ray RX film (Fuji Photofilm GmbH, Düsseldorf, Germany).

## RESULTS

### Induction of hematopoiesis by KUSA-A1 cells

When KUSA-A1 cells were implanted into the subcutaneous tissue, solid hard masses (Fig. 1A) were detected 5 weeks later as electron-dense nodules by soft X-ray analysis (Fig. 1B) at all implantation sites, that is, in the dermal tissue right beneath the cutaneous muscle. Histological examination revealed that the implanted cells survived, and some of them showed mitotic figures (Fig. 1C(a)). At 2 weeks following implantation, an osteogenic matrix was formed in the interstitium, but its matrix formation was still scanty (Fig. 1C(b)). And marked formation of capillary vessels containing erythrocytes in their lumen was observed. At 3 weeks, dense immature bone trabeculae with prominent vascular formation and osteoclast induction were seen (Fig. 1C(c)). By 4 weeks, mature bone trabeculae and sinusoids formed (Fig. 1C(d)), and there were mature granulocytic cells in the marrow space. Hematopoiesis began by 3–5 weeks after implantation.

To determine whether the size of the bone generated by KUSA-A1 cells depends on the implanted cell number, we implanted different numbers of KUSA-A1 cells into subcutaneous tissue (Fig. 1D). The results showed that bone size was clearly depending on the number of cells implanted. Nevertheless, hematopoiesis occurred regardless of the number of cells implanted and bone size.



**Fig. 1.** Time course analysis of hematopoietic induction by KUSA-A1-induced membranous osteogenesis. KUSA-A1 cells were implanted into the subcutaneous tissue of BALB/c nu/nu mice at a density of  $2 \times 10^7$  cells/200  $\mu$ l. **A:** Macroscopic view of bone formation at 5 weeks after KUSA-A1 cell injection. **B:** Soft X-ray image of a bone nodule formed by KUSA-A1 cells 5 weeks after implantation. **C:** Histopathological examination of induction of hematopoiesis and bone formation at 1 (**a**), 2 (**b**), 3 (**c**), and 4 (**d**) weeks after KUSA-A1 cell implantation. The mitotic figure of the implanted cell is indicated by an arrowhead (**a**). Note that numerous osteoclasts (**c**, arrows) as well as osteoblasts and osteocytes (**c**, arrowheads) were detected at 3 weeks after implantation. Mature osteocytes were observed at 4 weeks (**d**, arrowheads). Hematoxylin and eosin stain. Scale bars: 10 mm (**A**), 230  $\mu$ m (**C–F**). **D:** Correlation between the number of cells implanted and the size of the bone nodules. Microscopic view of the KUSA-A1 bone 5 weeks after implantation of  $2 \times 10^7$  (**a**, **d**),  $1 \times 10^7$  (**b**, **e**), or  $5 \times 10^6$  (**c**, **f**) KUSA-A1 cells into subcutaneous tissue. Hematoxylin and eosin stain. Scale bars: 2 mm (**a–c**), 250  $\mu$ m (**d**), 300  $\mu$ m (**e**), 250  $\mu$ m (**f**).

#### Expression of major histocompatibility antigen (MHC) after implantation

Marrow stromal cells have been reported to be immunologically tolerant, probably due to lack of transplantation antigen expression. To determine whether KUSA-A1 cells are tolerant when implanted into an allogeneic host, KUSA-A1 cells, which are C3H/He mouse origin, were implanted into BALB/c mice (Fig. 2). Time-course analysis clearly revealed that all of

the cells were rejected and formed no bone, but numerous foreign body giant cells were observed (Fig. 2C,D), suggesting that KUSA-A1 cells are immunogenic in our experimental setting.

To determine alterations in MHC antigens after implantation, flow cytometric analysis was performed on KUSA-A1 cells (Fig. 2E, open peaks) and cultured mesenchymal cells obtained from KUSA-A1-induced ectopic bone (Fig. 2E, closed peaks) in BALB/c nu/nu mice. The KUSA-A1 cells started to express one of the MHC antigens, H-2k, after implantation into BALB/c nu/nu mice, but expression of Sca-1 was downregulated. Expression of Lin (CD3, CD4, CD8, B220, Gr-1, Mac-1, and Ter119), c-kit, and CD34 remained unchanged after implantation.

#### MHC expression of the hematopoietic cells in the KUSA-A1 cell-induced bone

Morphological analysis showed that hematopoiesis took place in the KUSA-A1-induced ectopic bone (Fig. 3A–E). Megakaryocytes (arrows in Fig. 3D), erythrocytes (Fig. 3D,E), and granulocytes (Fig. 3D,E) were detected as well as osteoblasts (arrows in Fig. 3E) and mature osteocytes (arrowheads in Fig. 4E). The hematopoietic cells isolated from the KUSA-A1 cell-induced ectopic bone expressed the H-2d antigen, implying that they were derived from the host cells and had not differentiated from the implanted KUSA-A1 cells.

#### Cytokine production by the implanted KUSA-A1 cells may not be attributable to the migration of hematopoietic cells

To determine whether cytokines, that is, interleukin-6, macrophage-colony stimulating factor, stem cell factor, fms-like tyrosine kinase-3 ligand, and thrombopoietin, were produced by the implanted cells and contributed to the hematopoiesis, ELISA analysis was performed on the serum from mice with cell implantation as well as conditioned medium of the KUSA-A1 cultures (Fig. 3F). RT-PCR analysis of cytokine gene expression revealed that the KUSA-A1 cells express CSF-1, thrombopoietin, angiotensinogen, c-kit ligand, leptin, lymphotoxin A and B, IL4, IL5, IL6, IL10, IL12B, IL16, IL17B, IL19, and angiopoietin1 genes (Supplementary Figure 1S) and transcriptome analysis revealed that KUSA-A1 cells express the SDF-1 gene at a high level (a frequency of  $1.1 \times 10^{-3}$ ) (Sharov et al., 2003). However, none of the cytokine levels increased in the serum.

#### Analysis of KSL cells in the femur and the ectopic bone, and CFU-S in the peripheral blood and the ectopic bone

To investigate whether HSCs as well as mature hematopoietic cells migrates into the ectopic bone, the proportion of KSL cells was examined. The proportion was found to be the same, that is, 0.08%, in both the host femur (Fig. 4A) and the KUSA-A1 ectopic bone (Fig. 4B), suggesting that the ectopic bone as well as native bone serves microenvironment for HSCs.

The number of CFU-S were also counted in the host femur, peripheral blood, and KUSA-A1-induced bone marrow (Fig. 4C–E), and were found to account for  $11.2 \pm 0.8/1.0 \times 10^5$  the cells in the KUSA-A1-induced ectopic bone (Fig. 4E, right). By day 12 the CFU-S of the host femur had increased from  $28.3 \pm 6.0$  to  $35.0 \pm 3.4/10^5$  cells (Fig. 4E, left). At day 12 CFU-S were also detected in the peripheral blood from the mice and

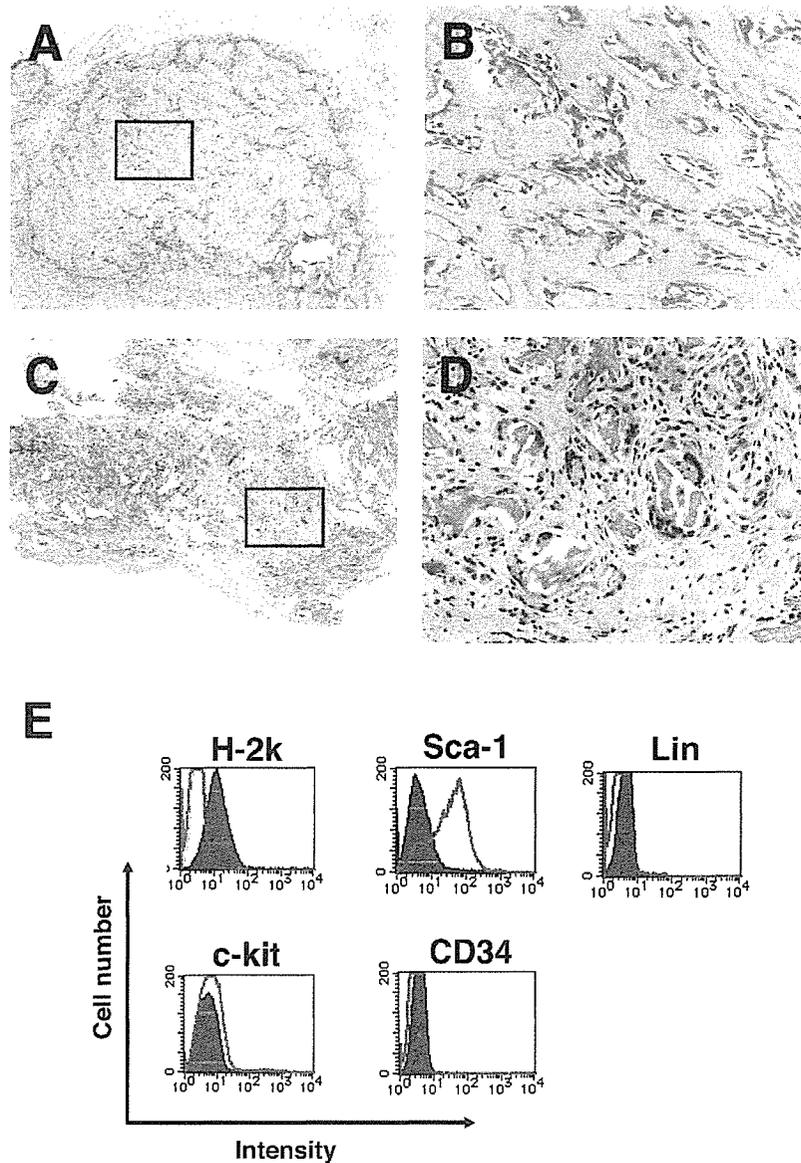


Fig. 2. Rejection of the implanted KUSA-A1 cells in an allogeneic combination. Microscopic view of the generated bone 18 days after the implantation of  $5 \times 10^6$  KUSA-A1 cells, which were derived from C3H/He mice, into the subcutaneous tissue of syngeneic C3H/He mice (A, B) or allogeneic BALB/c 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

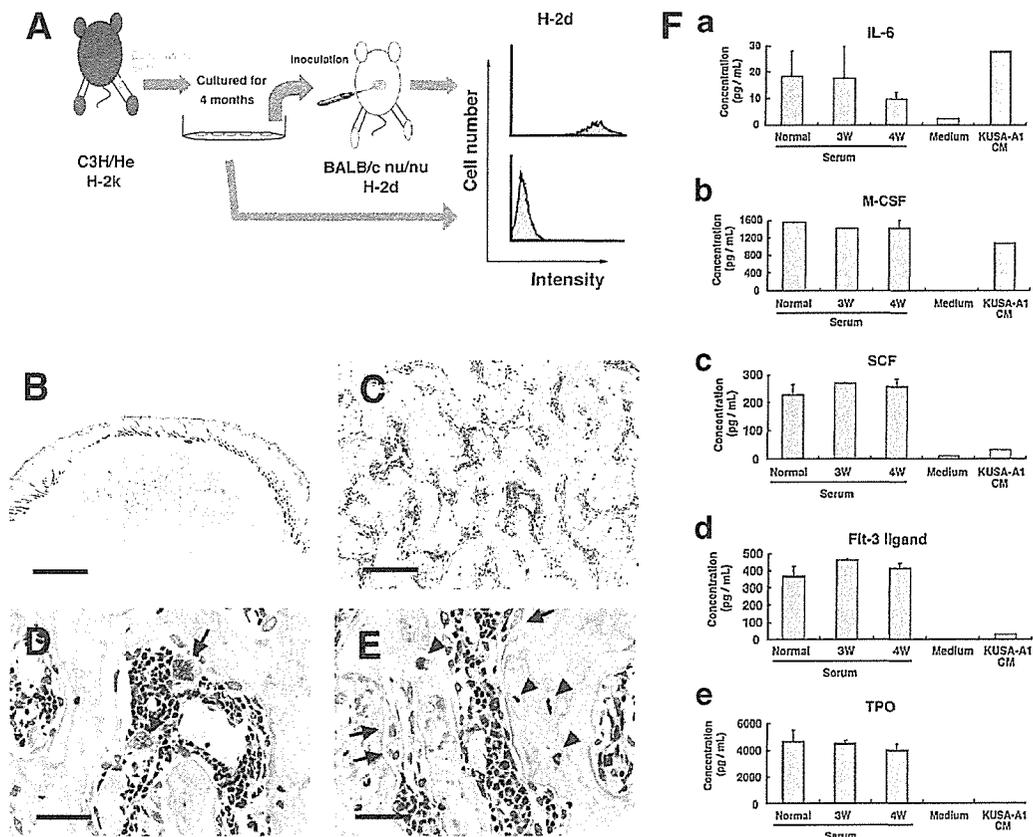


Fig. 3. Hematopoietic cells of host origin in the ectopic bone and serum levels of cytokines after subcutaneous implantation of KUSA-A1 cells. **A**: Flow cytometric analysis of the H-2d antigen in the hematopoietic cells of ectopic bone and KUSA-A1 cells in vitro. The hematopoietic cells in KUSA-A1 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. Tri-lineage 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  $\mu$ m (**C**), 100  $\mu$ m (**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.

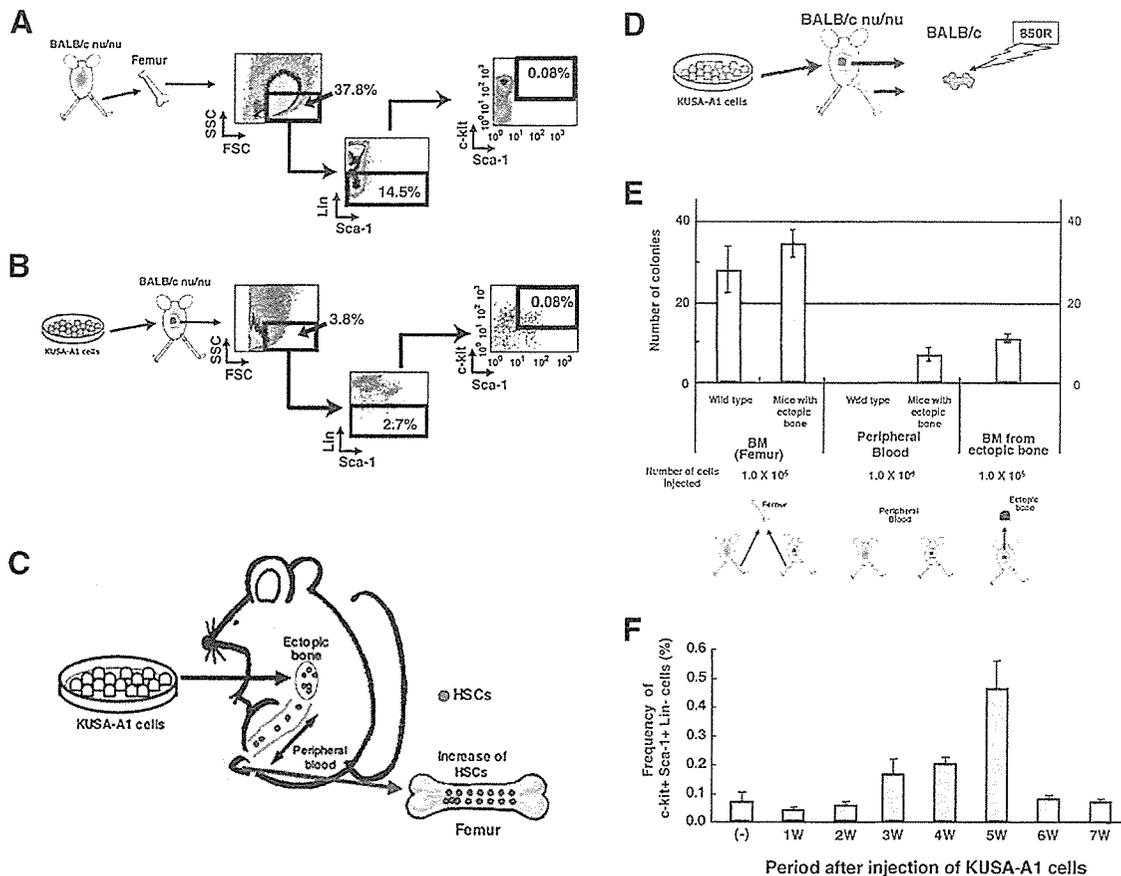


Fig. 4. Mobilization of  $c\text{-kit}^+ \text{Sca-1}^+ \text{Lin}^-$  (KSL) cells in the ectopic bones generated by KUSA-A1 cells. **A** and **B**: Flow cytometric analysis of hematopoietic stem cell markers was performed on hematopoietic cells in the femur of BALB/c nu/nu mice (**A**) and the ectopic 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.

### 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.

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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;  $3\beta$ -HSD I,  $3\beta$ -hydroxysteroid dehydrogenase I;  $17\beta$ -HSD III,  $17\beta$ -hydroxysteroid dehydrogenase III; mMSC, murine MSC; MSC, mesenchymal stem cell; P450arom, cytochrome P450 aromatase; P450c17, cytochrome P450  $17\alpha$ -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  $3\beta$ -hydroxysteroid dehydrogenase I ( $3\beta$ -HSD I) (kindly provided by Dr. A. Payne, Stanford University Medical Center, Stanford, CA), antipig cytochrome P450 17  $\alpha$ -hydroxylase (P450c17) (kindly provided by Dr. D. Hales, University of Illinois at Chicago, Chicago, IL) or anti-GFP (Medical & Biological Laboratories Co., Ltd.) were performed on 10- $\mu$ m 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 *EcoRI*-*StuI* restriction fragment, containing the CYP11A promoter-GFP, was then excised and inserted into *EcoRI* and *SmaI* 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  $\mu$ g) 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,  $3\beta$ -HSD I, P450c17, and  $\beta$ -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),  $3\beta$ -HSD I (kindly provided by Dr. A. Payne), P450c17 (kindly provided by Dr. D. Hales) (23), and  $\beta$ -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),  $3\beta$ -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

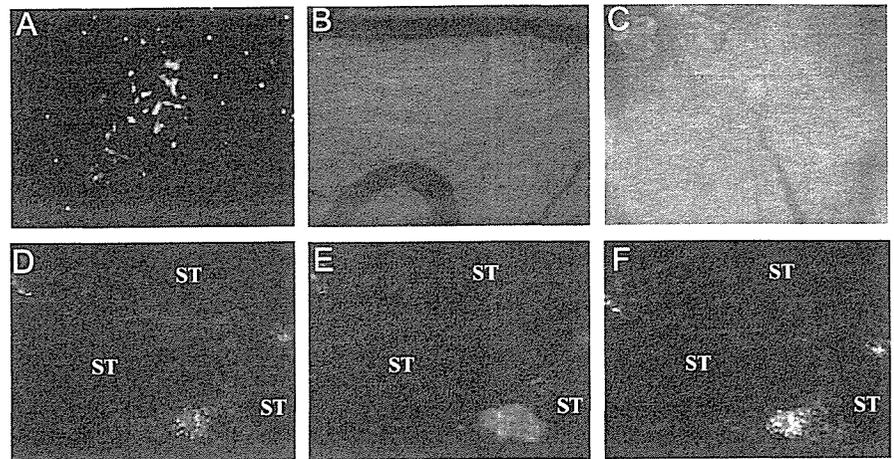
Gene	Sequence	Gene	Sequence
<b>RT-PCR</b>		<b>RT-PCR</b>	
SF-1	F-CGCACAGTCCAGAACAACAAGCA R-CGGTTAGAGAAGGCAGGATAGAG	hHSD3b2	F-CAGTGTGCCAGTCTTCATCT R-AGCAGGAAGCCAATCCAGTA
mStAR	F-GAAGGAAAGCCAGCAGGAGAACG R-CTCTGATGACACCACTCTGCCTC	hP450c17	F-CATGCTGGACACACTGATGC R-GGTTGTATCTCTAAAAATCTGT
mP450scc	F-TTCCGCTTTTCCTTTGAGTCCAT R-GTGTCTCCTTGATGCTGGCTTTC	hHSD17b3	F-GCAGATTTTACAAAAGATGACAT R-TCATGGGCAAGGCAGCCACAGGT
mHSD3b1	F-ACTGCAGGAGGTCAGAGCT R-GCCAGTAACACAGAATACC	hP450c21	F-TGCCTGCCTATTACAAATGT R-GGTGAAGCAAAAAAACCACG
mHSD3b6	F-TCTGGAGGAGATCAGGGTC R-GCCCGTACAACCGAGAATATTT	hP45011 b1	F-ACATTGGTGGCGTGTTCCTC R-GAGACGTGATTAGTTGATGGC
mP450c17	F-AAATAATAAACAACCTGGGAAGGC R-TGGGTGTGGGTGTAATGAGATGG	hP450 11b2	F-TACAGTTTTCTCTACTCG R-AGATGCAAGACTAGTTAATC
mP450c21	F-AGAGGATCCGCTTGGGGCTGC R-GGAGGAATTCCTTATGGATGGC	hP450aro	F-CTGGAAGAATGTATGGACTT R-GATCATTTCCAGCATGTTTT
mP450 11b1	F-TCACCAAATGTATCAAGAATGTGT R-CCATCTGCACATCCTCTTCTCTT	$\beta$ -Actin	F-GGGAATTCGTGCGTGACATTAAG R-TGTGTTGGCGTACAGGTCTTTG
mP450 11b2	F-CCAACAGATGTATCTGGAAGGTGC R-CCATCTGCACATCCTCTTGCCTCA	hIGF-2	F-AGTCGATGCTGGTGTCTTACCTCTT R-TGCGGCAGTTTTGCTCACTCCGATF
mLHR	F-CTCCACCTATCTCCCTGTC R-TCTTTCTTCGGCAAATTCCTG	<b>Real-time PCR</b>	
mACTHR	F-GCTCCAAGGATCATTACTTTCG R-CGCCAGGAGGCTTAACATAAC	mP450scc	F-CCAGTGTCCCATGCTCAAC R-TGCATGGTCCCTCCAGGTCT
GAPDH	F-ACCACAGTCCATGCCATCAC R-TCCACCACCTGTTGCTGA	mHSD3b1	F-TAACAAATTTAACAGCCCTCCTAAGG R-ATCCAGCCATGGTCAACACA
GFP	F-TGACCACCCTGACCTACGGCGT R-GGTAGTGGTTGTCGGGCAGCA	mHSD3b6	F-AAACCATCCTCCACTGTTCTAGCT R-TGGAGATGGTCCAGCCACAAG
mHSD17b3	F-ATTTTACCAGACAAGACATCT R-GGGGTGAGCACCTGAATAATG	mP450c17	F-AGTTTGGCCATCCCGAAGGA R-CTGGTGGTCCATTCATTT
mP450aro	F-TCAATACCAGTCCCTGGCTA R-GTATGCACTGATTCACGTTT	mHSD17b3	F-TGGGACAATGGCAGTGTAT R-GCCAACTCAAATGAATAGGCTTTC
hStAR	F-GAGAGTCAGCAGGACAATGG R-CTGGTTGATGATGCTCTTGG	$\beta$ -Actin	F-CAACCGTGAAGATGACCAGATC R-AGTCCATCACAAATGCTGTGGTAC
hP450scc	F-TAGTGTCTCCTTGATGCTGG 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,  $3\beta$ -HSD types I and VI, and LH receptor (Fig. 2H, lane SCC+). LH receptor and  $3\beta$ -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-P450<sub>scc</sub> (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

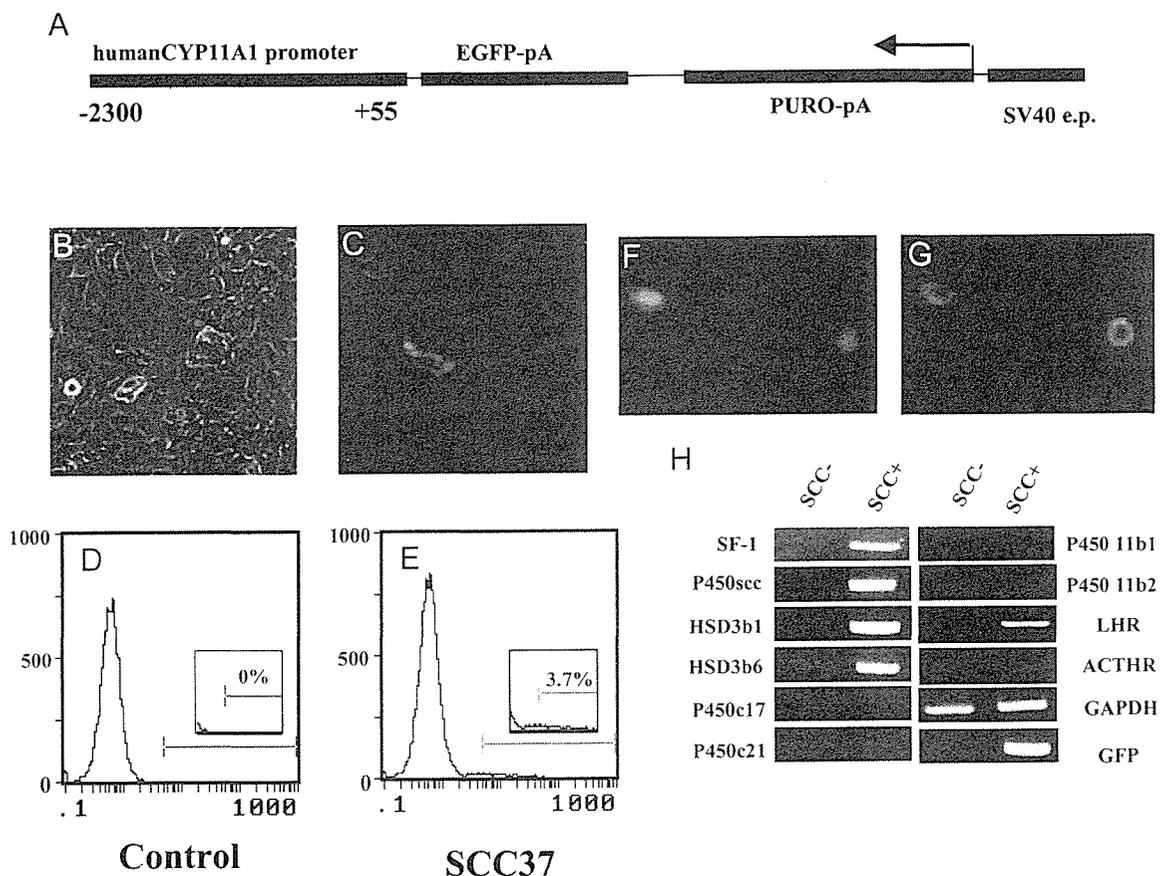


FIG. 2. Spontaneous differentiation of KUM9 into steroidogenic cells. A, Schematic representation of the SCC-reporter gene (SCC-GFP). The SCC-GFP reporter plasmid contains the 2300-bp upstream sequence of the human CYP11A1 gene and the puromycin-*N*-acetyltransferase gene (PURO-pA) driven by the Simian 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-P450<sub>scc</sub> antibody (G). H, SCC-GFP-positive (SCC+) and negative (SCC-) populations were sorted and analyzed for various marker genes by RT-PCR.

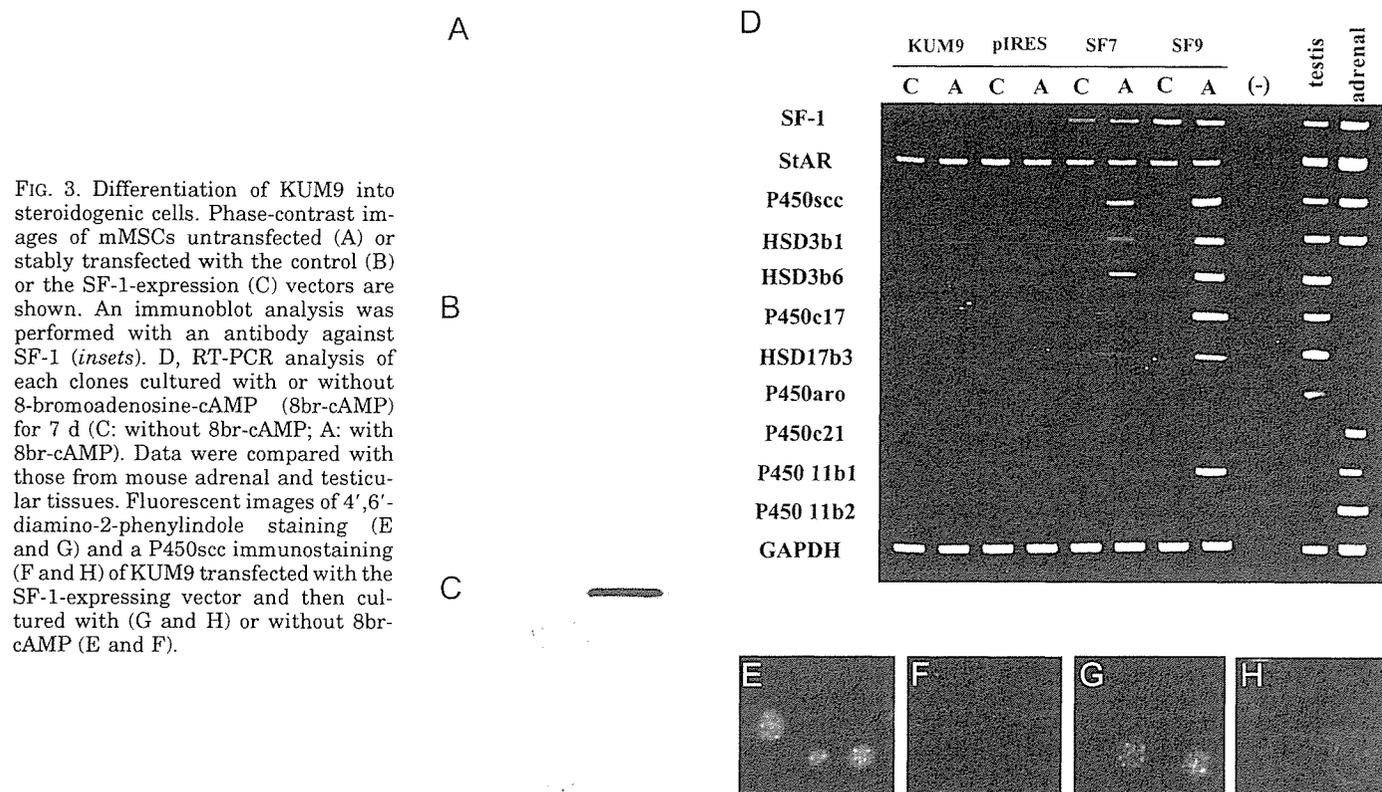


FIG. 3. Differentiation of KUM9 into steroidogenic cells. Phase-contrast images of mMSCs untransfected (A) or stably transfected with the control (B) or the SF-1-expression (C) vectors are shown. An immunoblot analysis was performed with an antibody against SF-1 (insets). D, RT-PCR analysis of each clones 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).

3 $\beta$ -HSD types I and VI were induced 3 d after cAMP treatment (Fig. 4). In the testis, the formation of testosterone is dependent on 3 $\beta$ -HSD activity, and isoform types I and VI have been shown to be expressed in the adult mouse testis (27). P450c17 and 17 $\beta$ -hydroxysteroid dehydrogenase III

(17 $\beta$ -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. 3 $\beta$ -HSD enzymes are essential for the production of progesterone, and P450c17 and 17 $\beta$ -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 $\pm$ 4.25	1.6 $\pm$ 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 $\pm$ 82.5	17.5 $\pm$ 0.20	0.21 $\pm$ 0.11	520 $\pm$ 200	1.56 $\pm$ 0.42

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

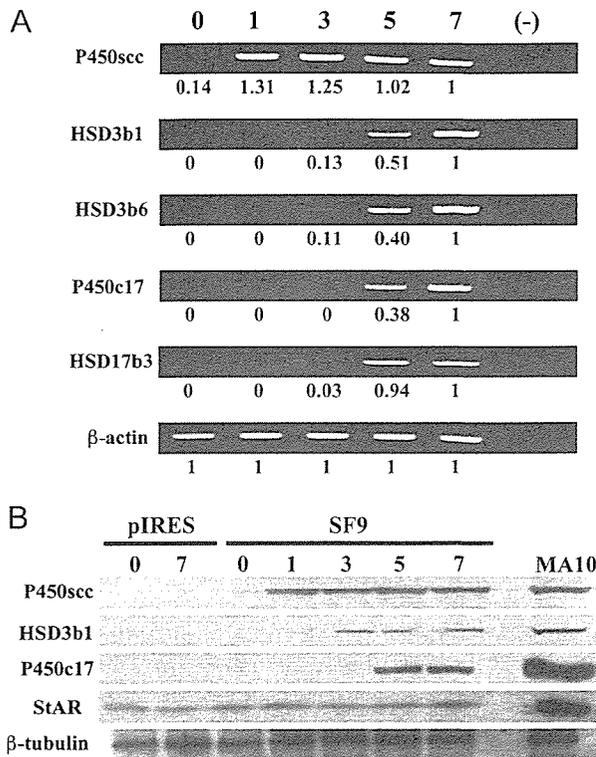


FIG. 4. Time-dependent induction of steroidogenic enzymes by cAMP. KUM9 cells stably transfected with SF-1-expression (SF9) or control (pIRES) vector were cultured and treated with 8-bromoadenosine-cAMP for the indicated times. A, P450scc, 3 $\beta$ -HSD I, 3 $\beta$ -HSD VI, P450c17, and 17 $\beta$ -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, 3 $\beta$ -HSD I, P450c17, and  $\beta$ -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, 3 $\beta$ -HSD II, P450c17, cytochrome P450 steroid 21-hydroxylase (P450c21), cytochrome P450 aromatase (P450arom), and cytochrome P450 steroid 11  $\beta$ -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 treat-

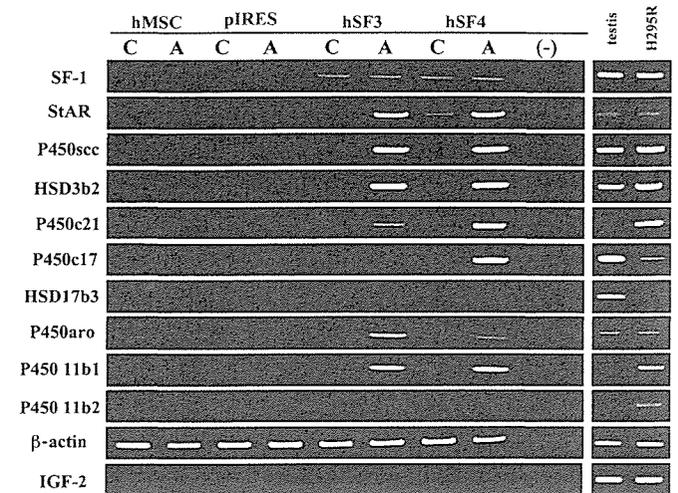


FIG. 5. Induction of steroidogenic enzymes in hMSCs. hMSCs were stably transfected with the control (pIRES) or SF-1-expression (SF3, -4) vector. RT-PCR analysis of each clone was cultured with or without 8-bromoadenosine-cAMP (8br-cAMP) for 7 d (C: without 8br-cAMP; A: with 8br-cAMP). The data were compared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

ment 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).

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.

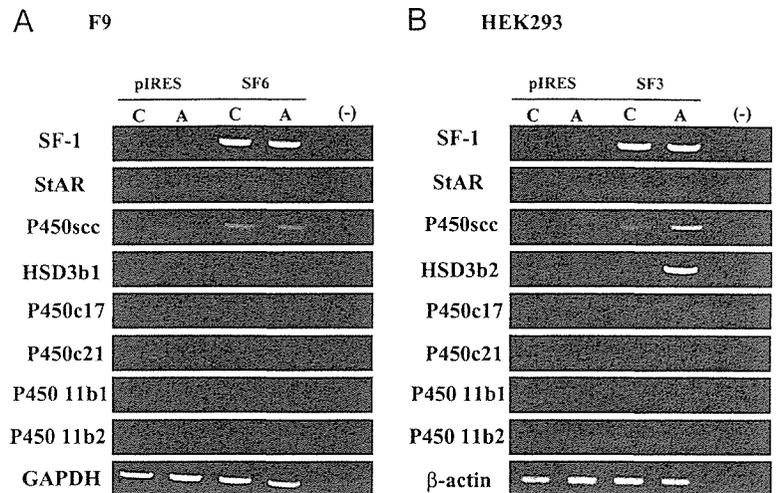
*Stable transfection of SF-1 into cells other than MSCs*

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 3 $\beta$ -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 3 $\beta$ -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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## Complementation of placental defects and embryonic lethality by trophoblast-specific lentiviral gene transfer

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Placental dysfunction underlies many complications during pregnancy, and better understanding of gene function during placental development could have considerable clinical relevance. However, the lack of a facile method for placenta-specific gene manipulation has hampered investigation of placental organogenesis and the treatment of placental dysfunction. We showed previously that transduction of fertilized mouse eggs with lentiviral vectors leads to transgene expression in both the fetus and the placenta<sup>1,2</sup>. Here we report placenta-specific gene incorporation by lentiviral transduction of mouse blastocysts after removal of the zona pellucida. All of the placentas analyzed, but none of the fetuses, were transgenic. Application of this method substantially rescued mice deficient in *Ets2*, *Mapk14* (also known as *p38 $\alpha$* ) and *Mapk1* (also known as *Erk2*) from embryonic lethality caused by placental defects. Ectopic expression of *Mapk11* also complemented *Mapk14* deficiency during placental development.

The placenta, the first organ to be formed during gestation, is responsible for gas and nutrient exchange as well as hormone production to support fetal development. Over the past decade, gene manipulation in the mouse has substantially advanced our understanding of the genetic control of placental development<sup>3,4</sup>. However, in most cases, it is not clear whether phenotypes originate in the placenta, the fetus, or both. Direct injection of gene-therapy vectors into the placenta has resulted in complications such as injury and patchy expression<sup>5,6</sup>, underscoring the need for an efficient, noninvasive approach for gene manipulation exclusively in the placenta.

In the preimplantation embryo, trophoblast cells are the first differentiated cells that can be distinguished from the pluripotent inner cell mass (ICM) and they form the outermost layer of the blastocyst. The trophoblast cell lineage provides the most important cell types for the main structural and functional components of the placenta. To introduce a foreign gene into trophoblast cells, we chose a self-inactivating lentiviral vector based on HIV-1<sup>1,2,7</sup> and transduced preimplantation blastocysts, expecting placenta-specific gene expression (Fig. 1a,b). The strong and ubiquitously expressed CAG

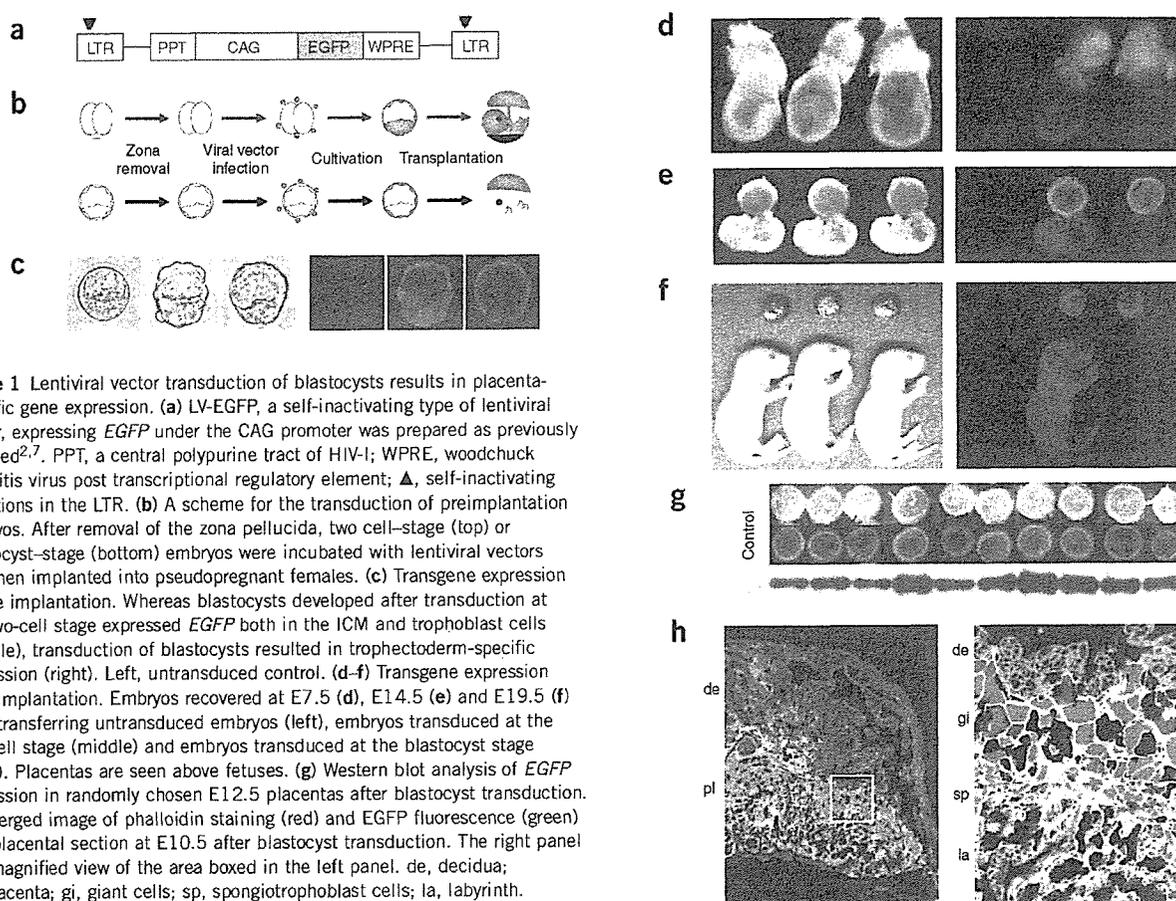
promoter<sup>2</sup> was used in all of the experiments. As the zona pellucida functions as a physical barrier to viral infection, we removed it by treatment with acidic Tyrode's solution<sup>8</sup> before exposing blastocysts for 4 h to a lentiviral vector LV-EGFP at  $1 \times 10^3$  ng p24/ml (p24 is a capsid protein in the HIV virus particle). When blastocysts were transduced with an EGFP-containing construct, fluorescence was observed only in trophoblasts after 6–8 h of incubation, whereas when two-cell- to four-cell-stage embryos were transduced, uniform expression of EGFP in both ICM and trophoblast cells was observed (Fig. 1c). The capacity for transgene expression before implantation suggests the potential of this approach for investigating trophoblast cell function during implantation.

We transplanted the LV-EGFP-transduced embryos into pseudo-pregnant females and determined the efficiency of transgene incorporation by PCR-mediated amplification of genomic DNA prepared from the fetus and placenta at embryonic day (E) 13.5 (Supplementary Table 1 online). Consistent with previously reported efficiencies of the transduction of material spanning the range from fertilized eggs to morula-stage embryos<sup>1,2,9</sup>, 61% (51/83) of the embryos transduced at the two-cell stage showed transgene incorporation in both the fetus and placenta. By contrast, transduction of blastocysts resulted in transgene incorporation exclusively in the placentas of all embryos (81/81). More efficient transduction of trophoblast cells than two-cell stage blastomeres was also supported by an increased range (3–11 versus 1–9) and average number (6.6 versus 4.4) of proviral insertions per cell, as determined by fluorescent *in situ* hybridization analysis (Supplementary Fig. 1 online). Lentiviral vector transduction of preimplantation embryos was not deleterious. The percentages of newborn pups per implanted blastocysts were comparable in the following three groups: nontransduced embryos (38.4%; 56/146), embryos transduced at the two-cell stage as a control (34.0%; 53/156) and embryos transduced at the blastocyst stage (43.3%; 78/180). More importantly, the 78 newborn pups obtained from blastocyst transduction developed to term normally (data not shown).

Whereas genes introduced with certain other retroviruses tend to be silenced, transgenes introduced lentivirally are stably expressed *in vivo*<sup>2,9,10</sup>. As expected, consistent EGFP transgene expression was

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**Figure 1** Lentiviral vector transduction of blastocysts results in placenta-specific gene expression. **(a)** LV-EGFP, a self-inactivating type of lentiviral vector, expressing *EGFP* under the CAG promoter was prepared as previously reported<sup>2,7</sup>. PPT, a central polypurine tract of HIV-1; WPRE, woodchuck hepatitis virus post transcriptional regulatory element;  $\blacktriangle$ , self-inactivating mutations in the LTR. **(b)** A scheme for the transduction of preimplantation embryos. After removal of the zona pellucida, two cell-stage (top) or blastocyst-stage (bottom) embryos were incubated with lentiviral vectors and then implanted into pseudopregnant females. **(c)** Transgene expression before implantation. Whereas blastocysts developed after transduction at the two-cell stage expressed *EGFP* both in the ICM and trophoblast cells (middle), transduction of blastocysts resulted in trophoblast-specific expression (right). Left, untransduced control. **(d–f)** Transgene expression after implantation. Embryos recovered at E7.5 **(d)**, E14.5 **(e)** and E19.5 **(f)** after transferring untransduced embryos (left), embryos transduced at the two-cell stage (middle) and embryos transduced at the blastocyst stage (right). Placentas are seen above fetuses. **(g)** Western blot analysis of *EGFP* expression in randomly chosen E12.5 placentas after blastocyst transduction. **(h)** Merged image of phalloidin staining (red) and *EGFP* fluorescence (green) of a placental section at E10.5 after blastocyst transduction. The right panel is a magnified view of the area boxed in the left panel. de, decidua; pl, placenta; gi, giant cells; sp, spongiotrophoblast cells; la, labyrinth.

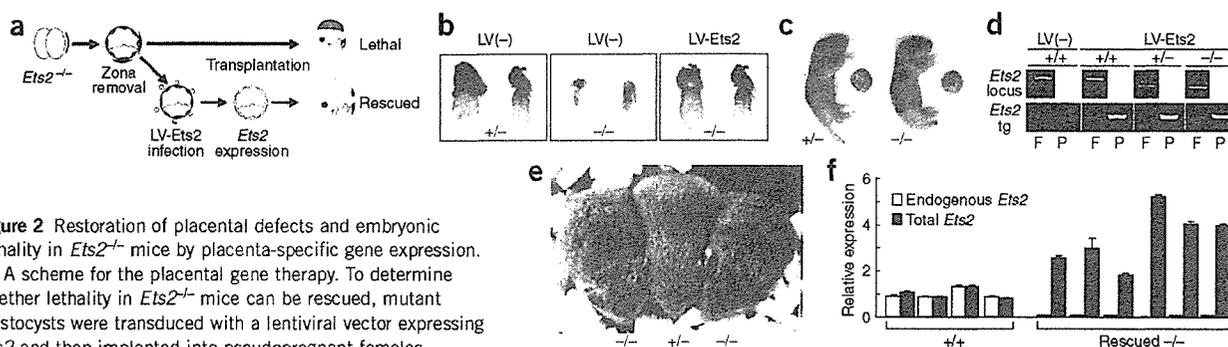
confirmed throughout pregnancy by fluorescence and western blot analysis (**Fig. 1d–h**). In the transgenic embryos developed by lentiviral vector transduction at the two-cell stage, *EGFP* expression and fluorescence in both placenta and fetus were ubiquitous. However, when blastocysts were transduced, green fluorescence was restricted to the placenta. Histology revealed uniform and ubiquitous expression of *EGFP* in all three major layers of the placenta: the labyrinth, spongiotrophoblast and giant cells (**Fig. 1h**).

It has been reported that the direct injection of lentiviral vectors into the blastocoel of rhesus monkeys resulted in chimeric transgene expression in all three placentas analyzed<sup>11</sup>. Our replication of this approach in mice confirmed the uneven transduction of placentas (**Supplementary Fig. 2a** online), possibly owing to limited transduction of trophoblast cells as polar trophoblast cells underlying the ICM are not accessible from the blastocoel. By the onset of implantation, mural trophoblast cells cease to proliferate, whereas polar trophoblast cells remain mitotic. Moreover, the transduction was not limited in placentas after blastocoel injection, at least in mice. We observed transgene expression in 5 of 58 fetuses examined (**Supplementary Fig. 2b** online), indicating that this method did not enable placenta-specific transduction. This is consistent with a report that 65% of the murine pups were transgenic after blastocoel injection of retroviral vector-producing cells<sup>12</sup>. On the contrary, incubation of blastocysts in the presence of lentivirus after zona pellucida removal transduced both mural and polar trophoblast cells and resulted in even transgene expression throughout the placenta without generating transgenic

pups. Our method is also less damaging to embryos, more cost effective and technically less demanding.

To determine whether trophoblast cell-specific gene manipulation might complement genetic aberrations of the placenta, we chose mice deficient in *Ets2* (ref. 13), *Mapk1* (refs. 14,15) and *Mapk14* (refs. 16–18) from over 100 mutant animals with placental defects that cause embryonic lethality<sup>3,4</sup>. *Ets2* is a member of the Ets family of transcription factors that mediate transcriptional responses of many signaling pathways and is highly expressed in a subset of cells in the trophoblast and extra-embryonic ectoderm during early embryogenesis. Targeted disruption of *Ets2* results in abnormal, cone-shaped, yolk-sac envelopes, much less trophoblastic tissue and abnormal placentation, leading to embryonic lethality by E8.5 (ref. 13). Placental defects in *Ets2*<sup>-/-</sup> can be rescued by aggregating a mutant eight-cell embryo with a wild-type tetraploid embryo (tetraploid complementation).

We prepared a lentiviral vector expressing *Ets2*, LV-*Ets2* and transduced blastocysts from *Ets2*<sup>+/-</sup> intercrosses (**Fig. 2a**). Transducing blastocysts with LV-*Ets2* restored placentation and embryonic development in *Ets2*<sup>-/-</sup> embryos (**Fig. 2b**), yielding 24 *Ets2*<sup>-/-</sup> pups from 154 transplantation attempts (**Fig. 2c** and **Table 1**). At birth, the average body weights of rescued *Ets2*<sup>-/-</sup> newborn pups were comparable to those of wild-type pups. Average placental weight was slightly lower than in the wild type, but no overt abnormalities were observed in either newborn pups or placentas (**Supplementary Fig. 3** online). PCR analysis confirmed placenta-specific gene incorporation in the



**Figure 2** Restoration of placental defects and embryonic lethality in *Ets2*<sup>-/-</sup> mice by placenta-specific gene expression. (a) A scheme for the placental gene therapy. To determine whether lethality in *Ets2*<sup>-/-</sup> mice can be rescued, mutant blastocysts were transduced with a lentiviral vector expressing *Ets2* and then implanted into pseudopregnant females. (b) E7.5 embryos after LV-Ets2 treatment. (c) Live *Ets2*<sup>-/-</sup> pups obtained by LV-Ets2 treatment. (d) Fetal (F) and placental (P) tissue subjected to PCR analysis for transgene detection. The lentiviral vector transgene was detected only in the placenta. tg, transgene. (e) Rescued *Ets2*<sup>-/-</sup> mice with wavy hair. (f) Relative amounts of *Ets2* mRNA determined by quantitative real-time RT-PCR. Amounts of endogenous and total *Ets2* mRNA were normalized to the  $\beta$ -actin control and relative amounts to the mean amounts of endogenous *Ets2* mRNA in +/+ mice were plotted.

rescued *Ets2*<sup>-/-</sup> pups (Fig. 2d). The rescued *Ets2*<sup>-/-</sup> pups developed wavy hair but were otherwise normal (Fig. 2e) and fertile, as originally reported<sup>13</sup>. Thus, restoration of placental function by lentiviral vector-mediated transgene expression was as efficient as tetraploid embryo complementation, an approach that has proven difficult and inefficient for many nonrodents<sup>19,20</sup>. Therefore, prospects for applying lentiviral vector-mediated gene transfer to species currently recalcitrant to tetraploid embryo complementation seem promising. Fewer -/- fetuses were rescued than anticipated from Mendelian segregation. When we compared the amounts of endogenous and transgenic *Ets2* mRNA by quantitative real-time RT-PCR, all of the rescued -/- placentas analyzed expressed more *Ets2* mRNA than endogenous *Ets2* mRNA in +/+ placentas (Fig. 2f). This may imply that a critical level of *Ets2* expression is required to rescue the lethal phenotype and that some of the transduced -/- placentas do not attain this threshold. As the timing and cell specificity of transgene expression may also reduce the frequency of mutant complementation, the use of endogenous promoters might improve the efficiency. As far as we know, this is the first case of gene therapy-mediated correction of placental dysfunction that normally leads to pregnancy loss. The rescue of *Mapk1*<sup>-/-</sup> mice from embryonic death by LV-Mapk1 transduction confirmed the effectiveness of this method (Table 1).

We next tried to rescue *Mapk14*-deficient animals that die at midgestation because of impaired placentation<sup>16-18</sup>. The p38 mitogen-activated protein kinase (MAPK) family comprises four members—*Mapk14* (refs. 21,22), *Mapk11* (ref. 23), *Mapk12* (ref. 24) and *Mapk13* (ref. 25) (also known as *p38 $\alpha$* , *p38 $\beta$* , *p38 $\gamma$*  and *p38 $\delta$* , respectively)—and plays important roles in cell proliferation, differentiation and survival<sup>26</sup>. Approximately 75% amino acid identity, as well as overlaps in the upstream and downstream pathways suggest functional redundancy between *Mapk14* and *Mapk11*. On the other hand, differences in their signaling pathways as well as their different patterns of tissue expression may reflect specific functions *in vivo*<sup>23,26,27</sup>. Interestingly, *Mapk11* cDNA was first isolated from a placental cDNA library, but later shown to be poorly expressed in placenta<sup>25</sup>. To determine whether *Mapk14* has a unique function in the placenta, the effect of ectopically expressed *Mapk11* was examined in *Mapk14*-deficient mice.

Blastocysts from *Mapk14*<sup>+/-</sup> intercrosses were transduced with the lentiviral vectors LV-Mapk14 or LV-Mapk11, expressing either *Mapk14* or *Mapk11*, respectively. Histological analysis showed full recovery of the thickness of the labyrinthine layer, with normal

vasculogenesis in *Mapk14*<sup>-/-</sup> placenta treated with LV-Mapk14 (Fig. 3a). Complementation of the placental defects was associated with full restoration of normal fetal development. Notably, ectopic expression of *Mapk11* could also complement *Mapk14* function *in vivo*: live *Mapk14*<sup>-/-</sup> pups were obtained with the same efficiency (16/106) as upon LV-Mapk14 transduction (29/180) (Fig. 3a-c and Table 1). The normal appearance and comparable average weights of pups and placentas also imply successful substitution of *Mapk14* with *Mapk11* (Fig. 3b,d). Overexpression of *Mapk14* or *Mapk11* in placenta did not seem to be deleterious because +/+ and +/- littermates that developed from transduced blastocysts seemed normal. Elucidation of the common cascade between *Mapk14* and *Mapk11* may facilitate identification of an essential signaling pathway(s) for normal placentation.

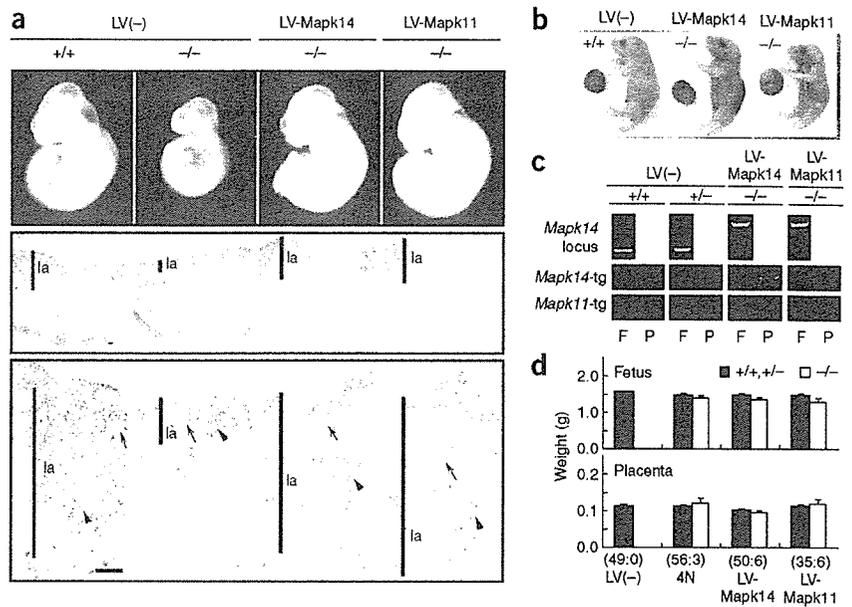
Our demonstration that lentiviral vector-transduction of blastocysts after zona pellucida removal ensures trophoblast cell-specific gene expression addresses the need for a placenta-specific gene expression system for elucidating the molecular basis of placental development and function. Combination of this approach with technologies such as RNAi, Cre/loxP and Tet On/Off systems will provide versatile systems to elucidate mechanisms of implantation and placentation. Certain human genetic defects known to cause placental dysfunction and/or

**Table 1** Genotypes of newborn pups after lentiviral vector transduction

Deficient gene	Treatment	Genotypes of newborn pups			Total
		+/+	+/-	-/-	
<i>Ets2</i>	(-)	19	37	0	56
	LV-EGFP	14	30	0	44
	LV-Ets2	45	84	24	154
<i>Mapk1</i>	(-)	17	33	0	50
	4 N	4	8	2	14
	LV-Mapk1	14	30	7	51
<i>Mapk14</i>	(-)	16	33	0	49
	4 N	19	37	3	59
	LV-Mapk14	51	100	29	180
	LV-Mapk11	31	59	16	106

Preimplantation embryos collected from heterozygous intercrosses were transduced with lentiviral vectors at blastocyst stage and implanted into pseudopregnant females. Tetraploid complementation (4N) was performed as described previously<sup>8</sup>.

**Figure 3** Functional complementation of *Mapk14* with *Mapk11* during placentation. (a) Embryos collected at E10.5 after LV-*Mapk14* or LV-*Mapk11* transduction at the blastocyst stage. (Top) Fetuses. (Middle and bottom) Placental sections stained with hematoxylin and eosin observed at 4 × magnification (middle) or at 20 × magnification (bottom). Vertical bars labeled la mark the labyrinthine layer. Fetal vessels filled with nucleated erythrocytes are indicated by arrowheads and maternal blood vessels containing enucleated erythrocytes are indicated by arrows. (b) Live *Mapk14*<sup>-/-</sup> newborn pups obtained by LV-*Mapk14* or LV-*Mapk11* treatment. (c) Fetal (F) and placental (P) tissue collected at birth subjected to PCR analysis for transgene detection. The lentiviral vector transgene was detected only in the placenta. (d) Average body and placenta weight with standard error at birth after LV-vector treatment or tetraploid complementation (4N). There were no significant differences between wild-type (+/+ and +/-, black bars) and rescued -/- embryos (white bars). Numbers of mice examined are indicated in parentheses.



complications during pregnancy<sup>28,29</sup> can be diagnosed by isolating and examining a blastomere from preimplantation embryos (preimplantation genetic diagnosis)<sup>30</sup>. Our gene manipulation method may be used to explore novel assisted reproduction technologies to overcome defects in implantation and placental function.

## METHODS

**Animals.** *Ets2*- or *Mapk1*-deficient mice used were prepared as previously reported<sup>13,14</sup>. *Mapk14*-deficient mice were generated by inserting a gene trap cassette between exon 10 and 11 (ref. 13 and unpublished data). Mice were genotyped by 40 cycles of PCR amplification from genomic DNA with the following primers; *EGFP-F* (5'-accatggtgagcaaggcgag-3') and *EGFP-R* (5'-tcacctgagccttcttct-3') for the *EGFP* transgene, *Ets2-1* (5'-cgtccctactgagtgta cagcg-3'), *Ets2-2* (5'-tgctttggtaaataggagcactg-3'), and *Ets2-3* (5'-aatgacaagacg ctggcg-3') for the *Ets2* locus, *Ets2-tg-F* (5'-gcggatccgggacccgcgcatctctc-3') and *Ets2-tg-R* (5'-gcctcgaggtctttggagcaagtgggtcag-3') for the *Ets2* transgene, *Mapk14-1* (5'-tagatacagagccatcagaccacca-3'), *Mapk14-2* (5'-tgaatggtgtagcatagc tggg-3'), and *Mapk14-3* (5'-atctggagcaagagcatcag-3') for the *Mapk14* locus, *Mapk14-tg-F* (5'-gatgtcgcagagagggccacgttctac-3') and *Mapk14-tg-R* (5'-gatattc catgagaaggtctccc-3') for the *Mapk14* transgene, *Mapk11-tg-F* (5'-gcgtctagac tctgtggatgatgctgggc-3') and *Mapk11-tg-R* (5'-gcctcgagtcctcactgctcaatttcag gtc-3') for the *Mapk11* transgene, and *Mapk1-1* (5'-taactgggtcagcacagtgat gtc-3'), *Mapk1-2* (5'-tcagaattgatctgtcttcaagacttg-3'), and *Mapk1-3* (5'-atgtat gctatacgaagtattaggc-3') for the *Mapk1* locus. All animal experiments were approved by the Animal Care and Use Committee of the Research Institute for Microbial Diseases, Osaka University.

**Lentiviral vector production.** HIV-I based, self-inactivating, lentiviral vector plasmid pLV-EGFP was constructed by replacing the PGK promoter with the CAG promoter fragment in pRRLsin-hPGK-EGFP<sup>27</sup>. Other constructs such as pLV-*Ets2*, pLV-*Mapk14*, pLV-*Mapk11* and pLV-*Mapk1* were generated by replacing the *EGFP* fragment of pLV-EGFP with PCR-amplified murine cDNAs. VSV-G pseudotyped lentiviral vectors were generated as described<sup>7</sup>. Briefly, 293T cells were transfected with the pLV plasmid, packaging plasmid and VSV-G-expressing plasmid by the calcium phosphate method. Lentiviral vectors were harvested at 2 and 3 days after transfection, and then concentrated 1,000 times by ultracentrifugation (50,000g, 2 h × 2 times). After resuspension with HBSS buffer, lentiviral vector concentration was determined by measuring p24 gag antigen by ELISA (Retrotek, Zeptomatrix).

**Transduction of preimplantation embryo.** Wild-type or heterozygous mutant female mice with B6D2F1 background were superovulated by intraperitoneal injection of pregnant mare's serum gonadotropin (5 units) followed by human chorionic gonadotropin (5 units) 48 h later and then mated with wild-type or heterozygous mutant males respectively. Two cell-stage embryos were collected from the females at 1.5 days after copulation, and then incubated for 2 days to obtain blastocysts. Prior to lentiviral vector transduction, the zona pellucida was removed by treatment with acidic Tyrode's solution<sup>8</sup>. Zona pellucida-free embryos were incubated individually in 5 μl of medium containing lentiviral vector (1 × 10<sup>3</sup> ng p24/ml) for 2 days for two-cells or 4 h for blastocysts. For blastocoele injection, lentiviral vector (1 × 10<sup>4</sup> ng p24/ml) was injected into blastocoele using an Eppendorf Transjector 5246 micromanipulator<sup>11</sup>. Transduced blastocysts were observed under a fluorescence microscope (KEYENCE, BZ-8000) or implanted into pseudopregnant females. Blastocyst images were obtained by computer-assisted Z-stack and haze reduction.

**Fluorescence in situ hybridization analysis (FISH).** pLV-EGFP plasmid DNA was labeled with digoxigenin-11-dUTP by nick translation for use as a probe. Slides were denatured for 2 min in 70% formamide/2 × SSC at 70 °C and dehydrated. The probe was denatured for 10 min in at 75 °C and applied to the slides. After hybridization, the slides were washed in 50% formamide/2 × SSC at 37 °C and 1 × SSC at 20 °C for 20 min each. Detection of the probe signals was performed with Cy3-labeled anti-digoxigenin. The FISH images were captured under a Leica DMRA2 microscope with the CW4000 FISH application program of Leica Microsystems Imaging Solution Ltd.

**Tetraploid complementation.** Tetraploid embryo and aggregation chimera were prepared as described previously<sup>8</sup>. In brief, B6D2F1 two cell-stage embryos were placed in fusion buffer and electrofusion was performed by applying 140 V for 50 μs after aligning embryos between the electrodes. A wild-type tetraploid four-cell embryo and a mutant diploid eight-cell embryo were aggregated and then the developed blastocysts were implanted into pseudopregnant females.

**Histological examination.** Placentas were fixed in 4% paraformaldehyde (PFA) for 12 h, then rinsed with PBS for 1 h, dehydrated in acetone for 1 h and embedded in glycol methacrylate (Technovit 8100; Heraeus Kulzer). Sections were prepared at a thickness of 5 μm and stained with hematoxylin and eosin. For the fluorescence staining, 10 μm frozen sections were prepared after PFA fixation. The sections were stained with TRITC-phalloidin (Sigma) to visualize actin distribution and observed with a confocal imaging system (FV1000MP, Olympus).

**Quantitative real-time RT-PCR.** Total RNA prepared from placenta with ISOGEN (Nippon gene) was treated with DNase (TURBO DNA-free, Ambion), then 40 ng of each RNA sample was reverse transcribed with Superscript III RT (Invitrogen). One twentieth of each cDNA sample was subjected to quantitative RT-PCR analysis with an ABI PRISM 7900HT (Applied Biosystems) using SYBR Green PCR Master Mix (Applied Biosystems). To prepare a standard curve, serial dilutions (1:1, 1:5, 1:25, 1:125, 1:625) of wild-type cDNA was included in each set of reactions. The relative expression level of each target gene mRNA was then normalized to the amount of  $\beta$ -actin control. Each reaction was performed in triplicate and data are presented as the mean plus s.d. Primer sets used were as follows: 5'-aagtgtgacgttgacatccg-3' and 5'-gatccacatctgctggaagg-3' for  $\beta$ -actin, 5'-ggcgtccagcctgatacaga-3' and 5'-gtggaagagcgctggatgaaag-3' for internal *Ets2*, 5'-gcagtgatgacccaagcctt-3' and 5'-ggctgctgcatccatgg-3' for total *Ets2*.

Note: Supplementary information is available on the Nature Biotechnology website.

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#### AUTHOR CONTRIBUTIONS

Y.O. performed research, analyzed data and wrote the manuscript; Y.U. performed research; A.I. performed tetraploid complementation; T.S.F. performed quantitative real-time RT-PCR; H.N. performed histological analysis; K.K. performed histological analysis; A.M. performed histological analysis and provided useful discussions; M.O. provided p38 $\alpha$  mutant mice; Y.M. provided useful reagents; M.O. provided p38 $\alpha$  and *Mapk1* mutant mice and useful discussions; R.G.O. provided *Ets2* mutant mice and useful discussions; M.O. provided useful discussions and wrote manuscript; M.I. designed, coordinated and performed research, analyzed data and wrote manuscript.

#### COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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