

Fig.2 Sources and differentiation of mesenchymal stem cells

with considerable fidelity and silenced genes are stably inherited throughout the culture period<sup>49-51</sup>. The demethylating agent induces differentiation by altering the original methylated pattern and reactivating the silenced genes.

## Mesenchymal stem cells (MSC2)

Tissues originating in the mesoderm include blood cells, blood vessels, heart, bone, cartilage, fat, skeletal muscle, tendon, and tissue mesenchyme. Blood cells in bone marrow are the elements that create the concept of stem cells, but bone marrow includes another cell group, i.e., mesenchymal stem cells (MSC2), which possess adherent properties. These cells have the ability to differentiate into a variety of cells and may have an organ maintenance mechanism that serves as back-up. Human mesenchymal stem cells (MSC2) are a useful source of cells for transplantation for several reasons: they have the ability to proliferate and differentiate into mesodermal tissues and they entail no ethical or immunological problems. MSC2 have been studied extensively over the past three decades and numerous independent research groups have successfully isolated them from a variety of sources, most commonly from bone marrow<sup>19,22,52,53</sup>. Yet, in addition to bone marrow, almost all human tissues or organs can be a source of mesenchymal stem cells, since they all have stroma or mesenchyme as well as parenchyma or epithelium.

## Available mesenchymal cell lines and mesenchymal cells in culture

MSC2 have been extracted from fat, muscle, menstrual blood,

endometrium, placenta, umbilical cord, cord blood, skin, and eye (Fig.2). Moreover, the source tissues can be obtained without difficulty from resected tissues at surgery and from birth deliveries (<http://www.nch.go.jp/reproduction/cellbank2.htm> and <http://www.nch.go.jp/reproduction/cells/primary.html>); menstrual blood can be provided from volunteers. The placenta is composed of amniotic membrane, chorionic villi and decidua, each of which can be a source of different types of MSC2. Large numbers of MSC2 can be easily obtained because the placenta is usually provided for research purposes. Menstrual blood also contains a large number of MSC2, although it is usually regarded as waste material.

We have also isolated many specific cell lines from adhering cells of mouse bone marrow (<http://www.nch.go.jp/reproduction/cellbank2.htm>) as follows:

- Multi-potential stem cell line: 9-15c cells (originally KUM2 cells) have multi-potential allowing differentiation into bone, fat, skeletal muscle, and myocardial cells through continued passage;
- Oligo-potential cell lines: KUM9 cells that lose the ability to differentiate to myocardial cells but retain differentiation to bone, fat, and skeletal muscle and NRG cells that lose the capability to differentiate into myocardial cells and skeletal myocytes but retain differentiation to bone and fat;
- Bi-potential cells: KUSA-O cells are capable of differentiating into osteoblasts and adipocytes;
- Precursor cells: KUSA-A1 and H-1/A are osteoblasts and preadipocytes, respectively. Adipogenic 3T3-L1<sup>50</sup>, osteogenic MC3T3-E1<sup>51</sup>, and chondrogenic ATDC5 cells<sup>52</sup> have been isolated from stem cells of a mesenchymal nature.

Focusing on human MSC2 derived from umbilical cord blood (UCBMSC) as an example, isolation, characterization, and differentiation of clonally-expanded UCBMSCs have been reported<sup>59,60</sup>, and UCBMSCs have been found to have multi-potential<sup>61</sup>. Most of the surface markers are the same as those detected in their bone marrow counterparts<sup>42</sup>, with both UCB- and bone marrow-derived cells being positive for CD29, CD44, CD55, and CD59, and negative for CD34 and CD117. Significantly, the differentiation capacity of UCB-derived cells is unaffected during establishment of a plate-adhering population of cells from UCB.

## Life span of MSC1 and MSC2

Marrow stromal cells (MSC1) and mesenchymal stem cells (MSC2) are useful for cell transplantation. However, it is difficult to study and apply them because of their limited life span.

One of the reasons for this is that normal human cells undergo a limited number of cell divisions in culture and then enter a non-dividing state called "senescence"<sup>62,63</sup>. Human cells reach senescence after a limited number of cell replications, and the average number of population doublings (PDs) of marrow-derived mesenchymal stem cells has been found to be about 40<sup>64</sup>, implying that it would be difficult to obtain enough cells to restore the function of a failing human organ. Large numbers of cells must be injected into damaged tissues to restore function in humans, and cells sometimes need to be injected throughout entire organs.

A system that allows human cells to escape senescence by using cell-cycle-associated molecules may be used to obtain sources of material for cell therapy<sup>64,65</sup>. Both inactivation of the Rb/p16INK4a pathway and activation of telomerase are required for immortalization of human epithelial cells, such as mammary epithelial cells and skin keratinocytes. Human papillomavirus E7 can inactivate pRb, and Bmi-1 can repress p16INK4a expression. Inactivation of the p53 pathway is also beneficial, even if not essential, to extension of the life span<sup>66</sup>. Human marrow stromal cell strains with an extended life span can be generated by transduction of combination of TERT, and Bmi-1, E6 or E7<sup>61</sup>. Cells with extended life span grow *in vitro* for over 80 PDs, and their differentiation potential is maintained. Transfection of TERT alone is insufficient to prolong the life span of marrow stromal cells, despite TERT having been reported to extend the life span of cells beyond senescence without affecting their differentiation ability<sup>67</sup>. Human stromal cells transfected with TERT and Bmi-1, E6 or E7 do not transform according to the classical pattern: they do not generate tumors in immunosuppressed mice; they do not form foci *in vitro*; and they stop dividing after confluence. The possibility that gene-transduced stromal cells might become tumorigenic in patients several decades after cell therapy therefore cannot be ruled out. Nevertheless, these gene-modified stromal cells may be used to supply defective enzymes to patients with genetic metabolic diseases, such as neuro-Gaucher disease, Fabry disease, and mucopolysaccharidosis, which have a poor prognosis and are sometimes lethal. The "risk versus benefit" balance is essential when applying these gene-modified cells clinically, and the "risk" or "drawback" in this case is transformation of implanted cells. These marrow stromal cells (MSC1) with prolonged life span also provide a novel model for further study of cancer and stem cell biology.

### Differentiation of mesenchymal stem cells

Retroviral labeling of individual cells is a useful clonal assay

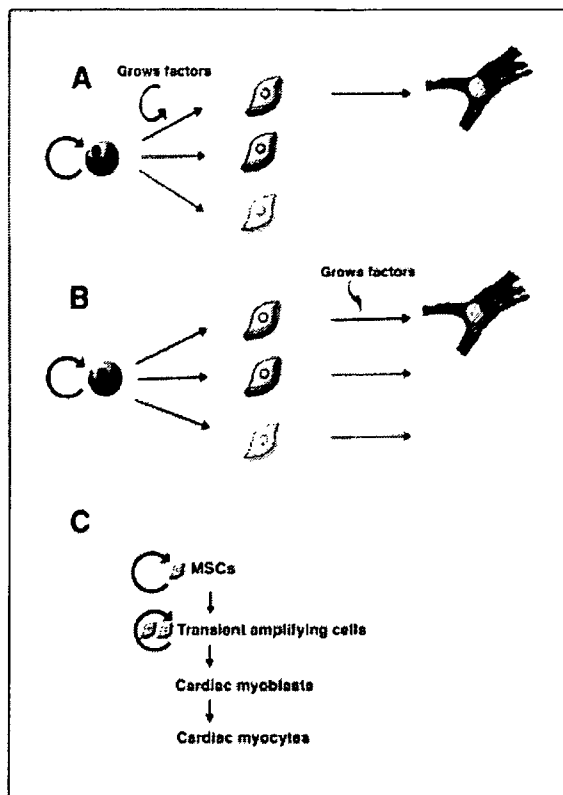


Fig.3 Model of stem cell differentiation  
 A. Deterministic model.  
 B. Stochastic model.  
 C. Differentiation model of mesenchymal stem cells.

to monitor lineage commitment at the single cell level. At present, several models have been proposed in which hematopoietic lineage determination is driven intrinsically<sup>68</sup>, extrinsically<sup>69</sup>, or both<sup>70</sup>. The issue of the mechanism and the extent of cellular differentiation that occurs when stem cells begin to differentiate is the area of furthest advanced research. Two models have been proposed: a deterministic model, in which differentiation is governed by the microenvironment (including growth factors and cytokines), and a stochastic model, in which differentiation, self-replication and the direction of differentiation emerge somewhat randomly (Fig.3A,B). The different models arise from different conceptions of mesenchymal stem cells. The mesenchymal stem cell (MSC2) line is stochastically committed toward the cardiac lineage, and following this commitment, they proliferate as transient amplifying cells and differentiate into cardiac myocytes (Fig.3C).

Considering stem cell transplant as a therapy, when mature cells arising from hematopoietic stem cells are needed, as in marrow transplant, there are no problems attending cellular dif-

ferentiation. However, in the case of cells that serve to originate cells of several different organs, as in the case of mesenchymal stem cells, there is a possibility for differentiation to cells not needed in the treatment. Ectopic tissue may therefore emerge from implanted mesenchymal stem cells, especially where the buffering system from a given site is lost and the stem cells begin to differentiate randomly into cells differing from the implanted site, thereby creating unwanted ectopic tissue.

### Conclusion

Mesenchymal stem cells can be isolated from bone marrow by standardized techniques and expanded in culture through many generations, while retaining their capacity to differentiate along set pathways when exposed to appropriate conditions. This property opens up therapeutic opportunities for the treatment of lesions in mesenchymal tissues, and protocols have been devised for the treatment of defects in articular cartilage<sup>71</sup>, bone<sup>72</sup>, tendon<sup>73</sup>, and meniscus<sup>34</sup> and for bone marrow stromal recovery<sup>75</sup> and osteogenesis imperfecta<sup>76</sup>.

In this context, we prefer to use the word "stroma" rather than "mesenchymal stem cells" for accuracy and to avoid confusion. In the field of hematopoiesis, marrow stroma were originally treated as "second class citizens"<sup>77</sup>, and represented a niche field. Today, marrow stroma are a "major player" in regenerative medicine and stem cell biology and are no longer viewed as a peripheral field of research. In addition, there is also a rapidly growing body of research into the biology and potential use of true "mesenchymal stem cells" derived from other human tissues, which are showing significant promise for future therapy, reparation or regeneration of human tissues and organs.

Clearly, this field is in its relative infancy, our understanding is at present limited but the potential benefits are great. We should perhaps, therefore, remember that the unexpected and unrivalled potential of MSCs to differentiate into a wide variety of cells represents a gift not a privilege and, with respect to the two MSCs, we should recognise and welcome their role in medicine with the words "with great power comes great responsibility".

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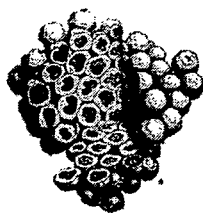
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Minoru Tomita, Taisuke Mori, Kazuichi Maruyama, Tasneem Zahir, Matthew Ward, Akihiro Umezawa and Michael J. Young

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## A Comparison of Neural Differentiation and Retinal Transplantation with Bone Marrow-Derived Cells and Retinal Progenitor Cells

MINORU TOMITA,<sup>a</sup> TAISUKE MORI,<sup>b,c</sup> KAZUICHI MARUYAMA,<sup>a</sup> TASNEEM ZAHIR,<sup>a</sup> MATTHEW WARD,<sup>a</sup> AKIHIRO UMEZAWA,<sup>b</sup> MICHAEL J. YOUNG<sup>a</sup>

<sup>a</sup>The Schepens Eye Research Institute, Department of Ophthalmology, Harvard Medical School, Boston, Massachusetts, USA; <sup>b</sup>Department of Reproductive Biology and Pathology, National Research Institute for Child Health and Development, Tokyo, Japan; <sup>c</sup>Department of Pathology, Keio University, Tokyo, Japan

**Key Words.** Bone marrow stromal cells • Microglia • Retinal stem cells • Retinal transplantation • Neural differentiation

### ABSTRACT

Retinal progenitor cells (RPCs) are immature precursors that can differentiate into retinal neurons, including photoreceptors. Recently, it has been reported that bone marrow-derived cells may also be capable of differentiation into cells of central nervous system lineage, including retinal neurons. We compared these two cell types to evaluate their potential as a source of cells for retinal transplantation. Marrow stromal cells (MSCs) and macrophages were isolated from enhanced green fluorescence protein mice. MSCs were cultured with brain-derived neurotrophic factor, nerve growth factor, and basic fibroblast growth factor to induce neuronal differentiation. RPCs were cultured under the same conditions or with 10% fetal bovine serum. Neuronal marker expression was examined and compared between MSCs and RPCs. MSCs, macrophages, and RPCs were also cultured

with explanted retinas from rhodopsin knockout mice to study their potential for retinal integration. MSCs expressed neuronal and retina-specific markers by reverse transcription-polymerase chain reaction and immunocytochemistry. Both types of cells migrated into retinal explants and expressed neurofilament 200, glial fibrillary acidic protein, protein kinase C- $\alpha$ , and recoverin. RPCs expressed rhodopsin, a photoreceptor marker we never detected in MSCs. A majority of bone marrow derived-macrophages differentiated into cells that resembled microglia, rather than neural cells, in the explanted retina. This study shows that RPCs are likely to be a preferred cell type for retinal transplantation studies, compared with MSCs. However, MSCs may remain an attractive candidate for autologous transplantation. *STEM CELLS* 2006;24:2270–2278

### INTRODUCTION

Marrow stromal cells (MSCs) are a population of multipotent mesenchymal stem cells distinct from hematopoietic stem cells. MSCs were originally reported to contribute to the microenvironment of bone marrow and to be necessary for the proliferation of hematopoietic stem cells [1]. It has recently been shown that MSCs can differentiate into various cell lineages, including bone [2, 3], muscle [4], fat [5], cartilage [6], cardiomyocytes [7–9], and hepatocytes [10]. Recently, some studies claimed that MSCs could differentiate cells expressing markers of neurons and glia in vitro [11–17]. MSCs also have the capacity to migrate into the uninjured [18] and diseased brain [19, 20] and spinal cord [21, 22]. Interestingly, studies show that MSCs differentiate into cells expressing markers of photoreceptors and glia in the retina [23, 24].

The two major clinical subtypes of retinal degeneration (RD) are retinitis pigmentosa and age-related macular degeneration. A hallmark of these diseases is photoreceptor cell degeneration, resulting in visual loss. No effective restorative treatment exists for either RD subtype. Previously, we reported that brain-derived progenitor cells can migrate and differentiate into cells expressing markers of mature neurons and glia when grafted to the retina of mice and rats with RD [25–29]. Despite incorporation into the host retina and morphological similarities to various retinal cell types, the transplanted cells failed to express retina-specific markers in each of these studies. Recently, the transplantation of stem and progenitor cells isolated from retina has shown promise as a strategy for photoreceptor replacement [26, 28, 30–32]. Many mammalian tissues, including the retina, contain stem or progenitor cells that can be

Correspondence: Minoru Tomita, M.D., Ph.D., The Schepens Eye Research Institute, Department of Ophthalmology, Harvard Medical School, 20 Staniford St., Boston, Massachusetts 02114, USA. Telephone: 1-617-912-7419; Fax: 1-617-912-0101; e-mail: Minoru Tomita: tomita@vision.eri.harvard.edu; or Michael J. Young, Ph.D.: e-mail: mikey@vision.eri.harvard.edu Received October 12, 2005; accepted for publication May 23, 2006. © AlphaMed Press 1066-5099/2006/\$20.00/0 doi: 10.1634/stemcells.2005-0507.



isolated, propagated, and grafted into animal models of RD [26, 32]. The goal of retinal transplantation is the replacement of dead or diseased host cells with healthy, functional donor cells. In the present study, we investigated whether MSCs could effectively differentiate into retinal cells by using a cocktail of brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), and basic fibroblast growth factor (bFGF), which (as we previously reported) induces MSC differentiation into neurons [17]. Because there are reports of the differentiation of microglial cells into neurons [33] and bone marrow-derived macrophages into brain microglia [34, 35], we examined the differentiation of macrophages when grafted into the retina. Here, we compared the potential of retinal progenitor cells (RPCs) and MSCs for use in retinal transplantation studies.

## MATERIALS AND METHODS

### Experimental Animals

All experiments were performed in adherence with the ARVO (Association for Research in Vision and Ophthalmology) Statement for the Use of Animals in Ophthalmic and Vision Research and with the Schepens Eye Research Institute Animal Care and Use Committee (Boston, MA). Rhodopsin knockout mice (*rho*<sup>-/-</sup> mice; C57/Bl6 background, provided by Peter Humphries, University of Dublin, Trinity College, Dublin, Ireland) and postnatal day 1 (P1) enhanced green fluorescence protein (EGFP) mice (C57BL/6 background; Dr. Masaru Okabe, University of Osaka, Osaka, Japan) were euthanized by CO<sub>2</sub> gas.

### Isolation of MSCs and Macrophages

Humeri, femurs, and tibias were obtained from P1 EGFP mice and divided into small pieces. These small pieces were cultured in Dulbecco's modified Eagle's medium (DMEM)/F-12 with 10% fetal bovine serum (FBS), and the nonadherent cells were removed by replacement of the media. After approximately 2 weeks, the adherent cells became confluent and were incubated with trypsin for 3 minutes and removed from the flask. All cell cultures were maintained at 37°C, 5% CO<sub>2</sub>.

After two or three passages, bone marrow-derived adherent cells were incubated with trypsin for 3 minutes to generate a single-cell suspension. Cells ( $1 \times 10^6$ ) were labeled with phycoerythrin-conjugated antibody against CD11b (1:50, marker for macrophages; BD Biosciences PharMingen, San Diego, <http://www.bdbiosciences.com>) and Cy-5-conjugated antibody against CD45 (1:50, marker for hematopoietic cells; BD Biosciences PharMingen). To isolate MSCs (CD45<sup>-</sup>, CD11b<sup>-</sup>) and macrophages (CD45<sup>+</sup>, CD11b<sup>+</sup>) from bone marrow-derived adherent cells, cell sorting was performed (data not shown). After sorting, the isolated MSCs and macrophages were cultured in 20% FBS for 2–3 days and then used for the subsequent experiments.

### RPC Line

RPCs harvested from the retina of P1 EGFP mice were isolated and maintained in culture as previously described [32]. Briefly, retinas were surgically removed. The tissue was finely minced with two scalpel blades (no. 10), these whole retina homogenates were incubated in 0.1% collagenase, and a single-cell suspension was obtained. Dissociated cells were then cultured in

DMEM/F-12 supplemented with B27 (Invitrogen, Carlsbad, CA, <http://www.invitrogen.com>) and 20 ng/ml of epidermal growth factor (EGF). The neurospheres that were generated could in turn be dissociated and subcultured to generate new spheres [26, 32].

### Neural Differentiation and Characterization of MSCs

To examine the differentiation of GFP-expressing MSCs in vitro, MSCs were incubated with trypsin for 3 minutes to generate a single-cell suspension. Cells ( $1 \times 10^3$ ) were plated on eight-well poly(D-lysine)/laminin-coated chamber slides (BD Biosciences, San Jose, CA, <http://www.bdbiosciences.com>) in DMEM/F-12 medium supplemented with 25 ng/ml BDNF (R&D Systems, Minneapolis, <http://www.rndsystems.com>), 40 ng/ml NGF (R&D Systems), and 20 ng/ml bFGF (R&D Systems) and were fixed with 4% paraformaldehyde (PFA) at 2 weeks after plating. The cells were blocked in 1% bovine serum albumin (Sigma-Aldrich, St. Louis, <http://www.sigmaaldrich.com>) + 0.2% Triton-100 (Sigma-Aldrich) and then incubated for 2 hours with primary antibody to Ki67 (1:100, cell proliferation marker; Vector Laboratories, Burlingame, CA, <http://www.vectorlabs.com>), nestin (1:1, immature neuronal marker; Developmental Studies Hybridoma Bank, Iowa City, IA, <http://www.uiowa.edu/~dshbwww/>), glial fibrillary acidic protein (GFAP) (1:50, astrocyte marker, Dako), MAP-2 (1:500, neuronal markers; Sigma-Aldrich), anti-protein kinase C (PKC)- $\alpha$  (1:200, bipolar cell marker; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, <http://www.scbt.com>), 2D4 rhodopsin (1:500, rod photoreceptor marker; kind gift of Dr. R. Molday, University of British Columbia, Vancouver, BC, Canada), and recoverin antibodies (1:1,000, photoreceptor and bipolar cell marker; Chemicon International, Temecula, CA, <http://www.chemicon.com>). After rinsing in phosphate-buffered saline (PBS [0.1 M]), samples were incubated in Cy3-conjugated species-specific IgG (1:800) for 1 hour. Samples were rinsed again and then coverslipped in polyvinyl alcohol-1,4-diazabicyclo (2.2.2) octane (PVA-Dabco) with 4',6-diamidino-2-phenylindole (DAPI) and viewed under fluorescent illumination. As a control, the untreated MSCs were fixed with 4% PFA and labeled with the same antibodies.

### Differentiation and Characterization of RPCs

To examine the differentiation of GFP-expressing RPCs in vitro, RPC spheres were incubated with trypsin for 1 minute to generate a single-cell suspension. In two separate experiments, cells ( $1 \times 10^3$ ) were plated on eight-well poly(D-lysine)/laminin-coated chamber slides (BD Biosciences) in DMEM/F-12 medium supplemented either with 10% FBS or with BDNF, NGF, and bFGF (the same growth factors used in MSCs differentiation experiments [17]) and were then fixed with 4% PFA at 1 day and 2 weeks after plating. The cells were then reacted and prepared with the antibodies described for MSCs.

### Morphometry of Differentiated Cells

In each of the three culture conditions (MSCs with BDNF, NGF, and bFGF; RPCs with 10% FBS; and RPCs with BDNF, NGF, and bFGF), quantitative morphometry was performed by counting positive cells from a total cell number of at least 200 cells per well in randomly selected wells, selected based on DAPI

labeling ( $n = 5$ ). In this counting study, cells ( $1 \times 10^3$ ) were plated on eight-well poly(D-lysine)/laminin-coated chamber slides (BD Biosciences). Five of eight wells were randomly chosen (by a masked observer), and all cells in the wells were counted. Nestin-positive cells from RPCs were counted at day 1, and MSCs and RPCs positive for other markers were counted after 2 weeks of treatment.

### Reverse Transcription-Polymerase Chain Reaction Analysis of MSCs

For reverse transcription-polymerase chain reaction (RT-PCR) analysis, total RNA was extracted using TRIzol (Invitrogen) from MSCs grown in the presence or absence of BDNF, NGF, and bFGF in poly(D-lysine)/laminin-coated culture dishes (BD Biosciences) and from P1 EGFP mice retina for a positive control. First-strand cDNA was prepared from total RNA by reverse transcriptase using oligo(dT) primers. To detect nestin,  $\beta$ -tubulin class III (BT-III; neuronal marker), Map2, GFAP, PKC- $\alpha$ , recoverin, and rhodopsin, primers were used as described in Table 1.

### Retinal Organ Culture

Retinal organ culture was performed as previously described [36–38] with minor modifications. Briefly, eyes were enucleated from rhodopsin knockout ( $\rho^{-/-}$ ) mice and transferred to ice-cold Hanks' balanced salt solution (Invitrogen). The retinas were separated from the retinal pigment epithelium and placed onto Millicell-CM membrane culture inserts (diameter 30 mm, pore size 0.4  $\mu$ m; Millipore Corporation, Billerica, MA, <http://www.millipore.com>) with the ganglion cell layer downward. The inserts with neural retina were placed in six-well plates containing approximately 1 ml/well of medium containing DMEM/F-12 supplemented with B27 neural supplement (Invitrogen), 2 mM L-glutamine (Sigma-Aldrich), 2,000 U of nystatin (Invitrogen), and 100  $\mu$ l/ml penicillin-streptomycin (Sigma-Aldrich). Organ cultures were maintained at 37°C, 5% CO<sub>2</sub> and fed every 2–3 days.

### Explant Coculture

The host retinas were explanted from  $\rho^{-/-}$  mice (4–8 weeks of age). Cell suspensions (1  $\mu$ l,  $5 \times 10^3$  cells/ $\mu$ l) containing (a) RPCs ( $n = 12$ ); (b) MSCs with ( $n = 12$ ) or without ( $n = 6$ )

pretreatment with BDNF, NGF, and bFGF for 1 week; and (c) macrophages ( $n = 6$ ) were added to the retinas using a pipette immediately after isolation of recipient retinas. We placed the grafted cells onto the surface of retinal explants using a 200- $\mu$ l pipette. The cells were spread out over the entire surface of the explant, confirmed by viewing under fluorescent illumination. The explanted retinas were cultured for 1 week.

### Tissue Preparation

After 1 week in explant coculture, the explanted retinas were fixed with 4% PFA, followed by cryoprotection with 20% sucrose. The retinas were sectioned at 12  $\mu$ m on a cryostat. Sections were stained with neurofilament (NF) 200 (1:1,000, neuronal marker; Sigma-Aldrich), GFAP, PKC- $\alpha$ , recoverin, and rhodopsin antibodies as described above. After fixation with PFA and sucrose, some whole-mount retinas were stained with biotin-*Griffonia simplicifolia* (GS)-lectin (5  $\mu$ g/ml, microglia and macrophages marker; Sigma-Aldrich) for 15 minutes and NF200 antibody for 2 hours. After rinsing in PBS, samples were respectively incubated in Cy3-conjugated streptavidin (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, <http://www.jacksonimmuno.com>) and Cy3-conjugated species-specific IgG (1:800) for 1 hour. Samples were rinsed again and then coverslipped in PVA-Dabco and viewed under fluorescent illumination.

## RESULTS

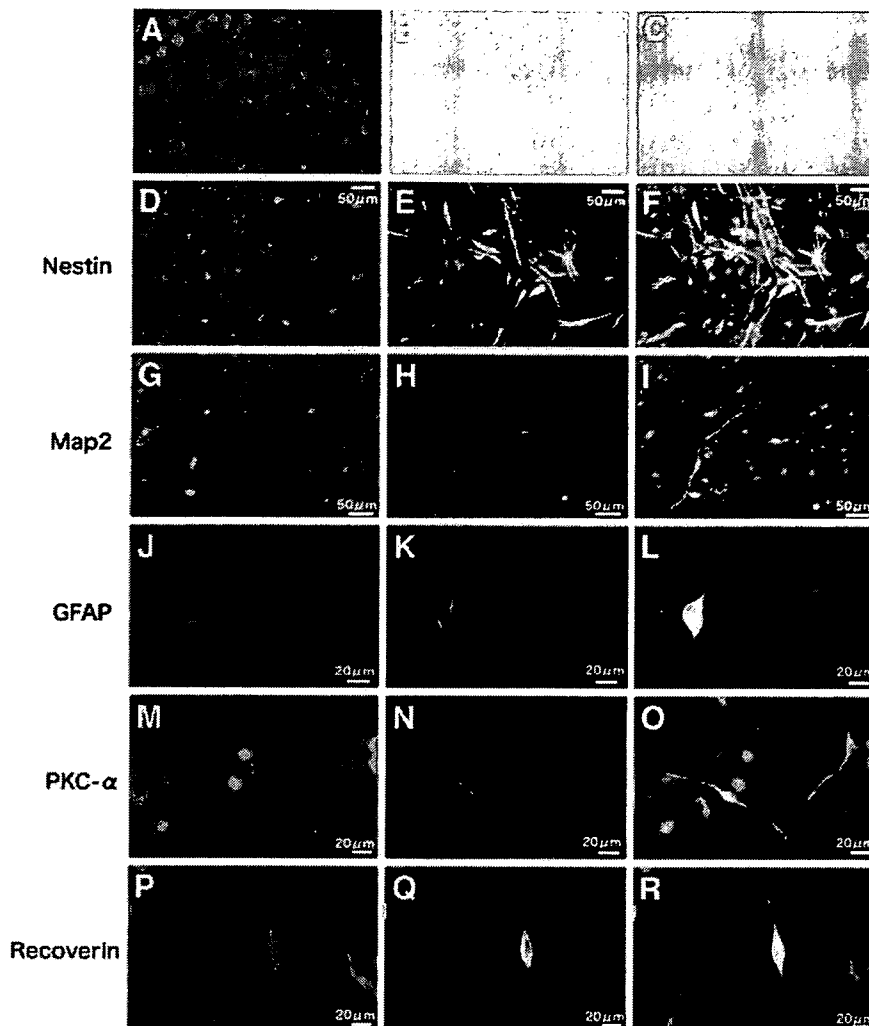
### Characterization of MSCs

When grown on conventional substrates in media supplemented with 10% FBS, GFP-transgenic MSCs exhibited high levels of endogenous green fluorescence (Fig. 1A). The untreated MSCs did not express nestin, Map2, GFAP, PKC- $\alpha$ , recoverin, or rhodopsin (data not shown). To examine differentiation in vitro, medium without 10% FBS was supplemented with BDNF, NGF, and bFGF. After 2 weeks of culture under differentiation conditions, MSCs differentiated into cells with neuronal morphologies and neurite-like processes (Fig. 1B) and also formed spheres (Fig. 1C). Subpopulations of MSCs expressed nestin (Fig. 1D–1F), Map2 (Fig. 1G–1I), GFAP (Fig. 1J–1L), PKC- $\alpha$  (Fig. 1M–1O), and recoverin (Fig. 1P–1R). These markers are consistent, although not conclusive, with differentiation into

**Table 1. Primers used for reverse transcription-polymerase chain reaction analysis**

| Genes         | Primer sequences (5'–3')   | Product size (bp) | Temperature (°C) |
|---------------|----------------------------|-------------------|------------------|
| Nestin        | F: AACTGGCACACCTCAAGATGT   | 235               | 60               |
|               | R: TCAAGGGTATTAGGCAAGGGG   |                   |                  |
| GFAP          | F: CACGAACGAGTCCCTAGAGC    | 234               | 60               |
|               | R: ATGGTGATGCGGTTTTCTTC    |                   |                  |
| TB-III        | F: ACCTCAACCACCTGGTATCG    | 344               | 60               |
|               | R: TGCTGTTCTTGCTCTGGATG    |                   |                  |
| Map2          | F: CTGGACATCAGCCTCACTCA    | 164               | 60               |
|               | R: AATAGGTGCCCTGTGACCTG    |                   |                  |
| PKC- $\alpha$ | F: CCCATTCCAGAAGGAGATGA    | 212               | 60               |
|               | R: TTCCTGTCAGCAAGCATCAC    |                   |                  |
| Recoverin     | F: ATGGGGAATAGCAAGAGCGG    | 179               | 60               |
|               | R: GAGTCCGGGAAAACTTGGGAATA |                   |                  |
| Rhodopsin     | F: TCACCACCCTCTACACA       | 216               | 60               |
|               | R: TGATCCAGGTGAAGACCACA    |                   |                  |

Abbreviations: bp, base pair; F, forward; GFAP, glial fibrillary acidic protein; PKC, protein kinase C; R, reverse; TB, tubulin.



**Figure 1.** Differentiation and characterization of marrow stromal cell (MSCs) in vitro. Undifferentiated GFP<sup>+</sup> MSCs grown in Dulbecco's modified Eagle's medium with 10% fetal bovine serum, viewed under fluorescein isothiocyanate illumination (A). MSCs cultured in serum-free medium with brain-derived neurotrophic factor, nerve growth factor, and basic fibroblast growth factor for 14 days (B–R). After 2 weeks of culture under differentiation conditions, MSCs morphologically differentiated into neuronal shape and had neuronal processes (B) and also formed spheres (C). Constitutive GFP expression (D, G, J, M, P), antibody/cytokeratin-3 immunoreactivity for nestin (E), Map2 (H), GFAP (K), PKC- $\alpha$  (N), and recoverin (Q), and merged images (F, I, L, O, R). Abbreviations: GFAP, glial fibrillary acidic protein; GFP, green fluorescent protein; PKC, protein kinase C.

retinal neurons. Interestingly, these immunopositive cells also showed morphological evidence suggestive of differentiation into immature photoreceptors, bipolar cell types, glial cells, and neuronal cells (Fig. 1F, 1I, 1L, 1O, 1R). We could not find any rhodopsin-positive cells from treated MSCs.

#### Characterization of RPCs

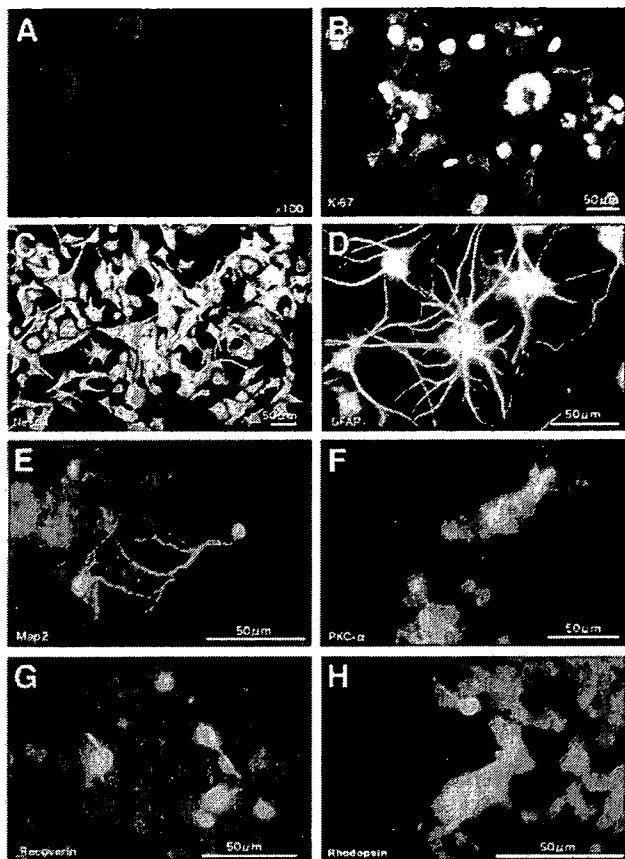
When grown on conventional substrates in medium supplemented with EGF, GFP-transgenic RPCs exhibited high levels of endogenous green fluorescence (Fig. 2A) and maintained an undifferentiated state characterized by ubiquitous Ki67 and nestin immunoreactivity (Fig. 2B, 2C). Cells could be maintained in this state for up to 1 year or 50 passages as neurospheres. To examine differentiation in vitro, medium without EGF was supplemented with 10% FBS. After 2 weeks culture under differentiation conditions, the cells were analyzed immunocytochemically. The number of Ki67<sup>+</sup> cells markedly decreased (data not shown), and subpopulations expressed GFAP (Fig. 2D), Map2 (Fig. 2E), PKC- $\alpha$  (Fig. 2F), recoverin (Fig. 2G), or rhodopsin (Fig. 2H). These markers are consistent with differentiation into rod photoreceptors, bipolar cells, and Muller glia, all of which are known to be born late in retinogenesis. More-

over, these immunopositive cells also showed morphological evidence suggestive of immature photoreceptor differentiation, as well as of other retinal cell types (Fig. 2D–2H).

#### Quantitative Evaluation of Differentiated Cell Numbers: MSCs Versus RPCs

To examine the optimal source of cells for retinal transplantation, quantitative evaluation of differentiation into neuronal and retinal cells was carried out using cell counting as previously described [39].

After 2 weeks of BDNF, NGF, and bFGF treatment, the percentages of surviving MSCs expressing nestin, Map2, GFAP, PKC- $\alpha$ , and recoverin were 5.55%, 3.27%, 1.42%, 3.97%, and 13.9%, respectively. The percentages of nestin-, Map2-, GFAP-, PKC- $\alpha$ -, recoverin-, and rhodopsin-positive cells from RPCs treated with 10% FBS were 90.5%, 15.2%, 64.4%, 12.9%, 23.6%, and 3.17%, respectively. The rates of nestin-, Map2-, GFAP-, PKC- $\alpha$ -, recoverin-, and rhodopsin-positive cells from RPCs treated with BDNF, NGF, and bFGF were 89.2%, 29.4%, 10.9%, 28.2%, 22.3%, and 2.25%, respectively (Fig. 3A).



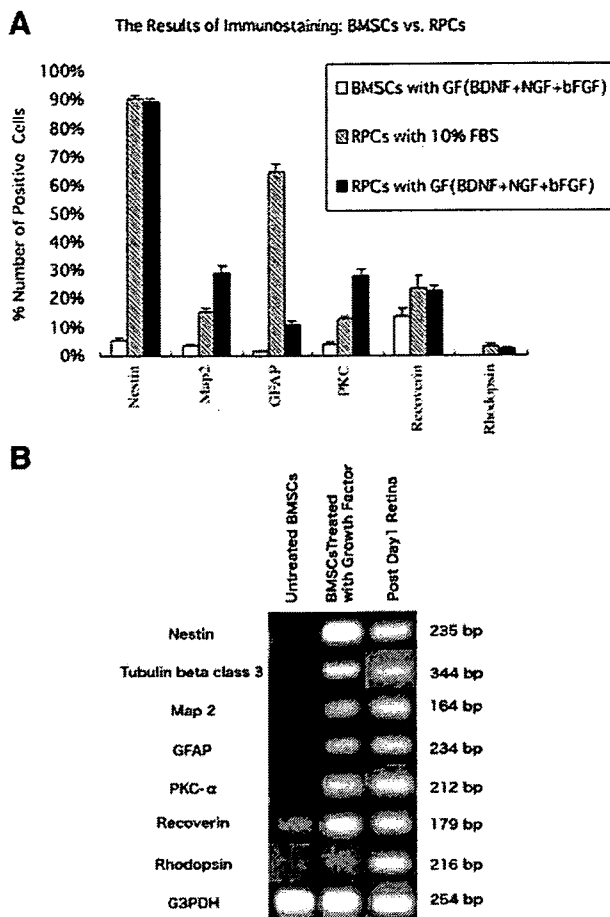
**Figure 2.** Differentiation and characterization of retinal progenitor cell (RPCs) in vitro. RPCs formed green fluorescent protein-positive neurospheres (A). RPCs cultured in the absence of epidermal growth factor and in the presence of 10% fetal bovine serum for 1 (B, C) or 14 (D–H) days. The cells were stained for Ki67 (B), nestin (C), GFAP (D), Map2 (E), PKC- $\alpha$  (F), recoverin (G), and rhodopsin (H). Abbreviations: GFAP, glial fibrillary acidic protein; MSC, marrow stromal cell; PKC, protein kinase C.

**RT-PCR Analysis of BDNF, NGF, and bFGF Treatment**

Semiquantitative RT-PCR analysis was carried out to determine the effect of BDNF, NGF, and bFGF on MSCs (Fig. 3B). MSCs without treatment showed only weak recoverin expression. (MSCs without treatment did not express nestin, BT-III, Map2, GFAP, PKC- $\alpha$ , or rhodopsin.) After 2 weeks of BDNF, NGF, and bFGF treatment, MSCs expressed nestin, BT-III, Map2, GFAP, PKC- $\alpha$ , and recoverin. Rhodopsin expression was not found. Recoverin expression was increased in treated MSCs.

**Macrophages Differentiated into Microglia After Coculture with Explanted Retinas**

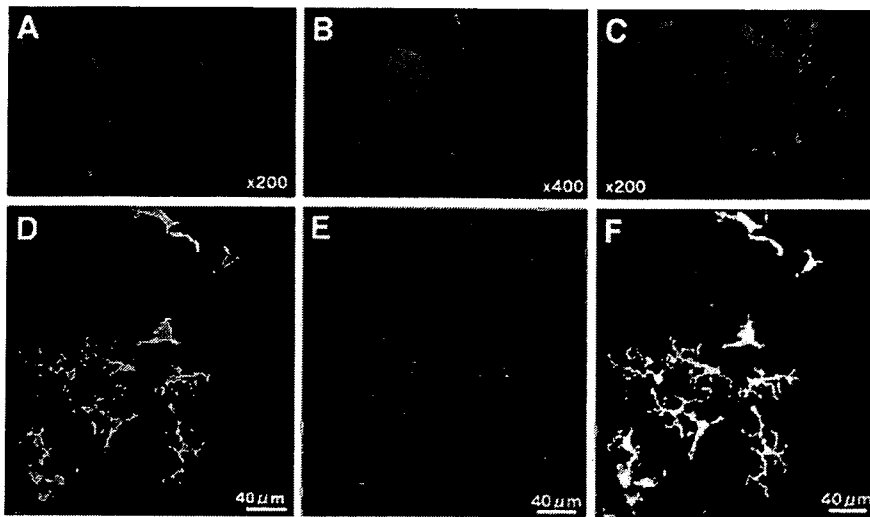
After coculture with explanted rho-/- mouse retinas, macrophages were viewed by fluorescent illumination at 3 and 7 days. Macrophages migrated into the retina and assumed morphology very reminiscent of microglial cells (Fig. 4A–4C). The cocultured macrophages also expressed GS-lectin, a marker of microglia (Fig. 4D–4F). There was no evidence of neuronal differentiation upon immunocytochemical and morphological analyses (data not shown).



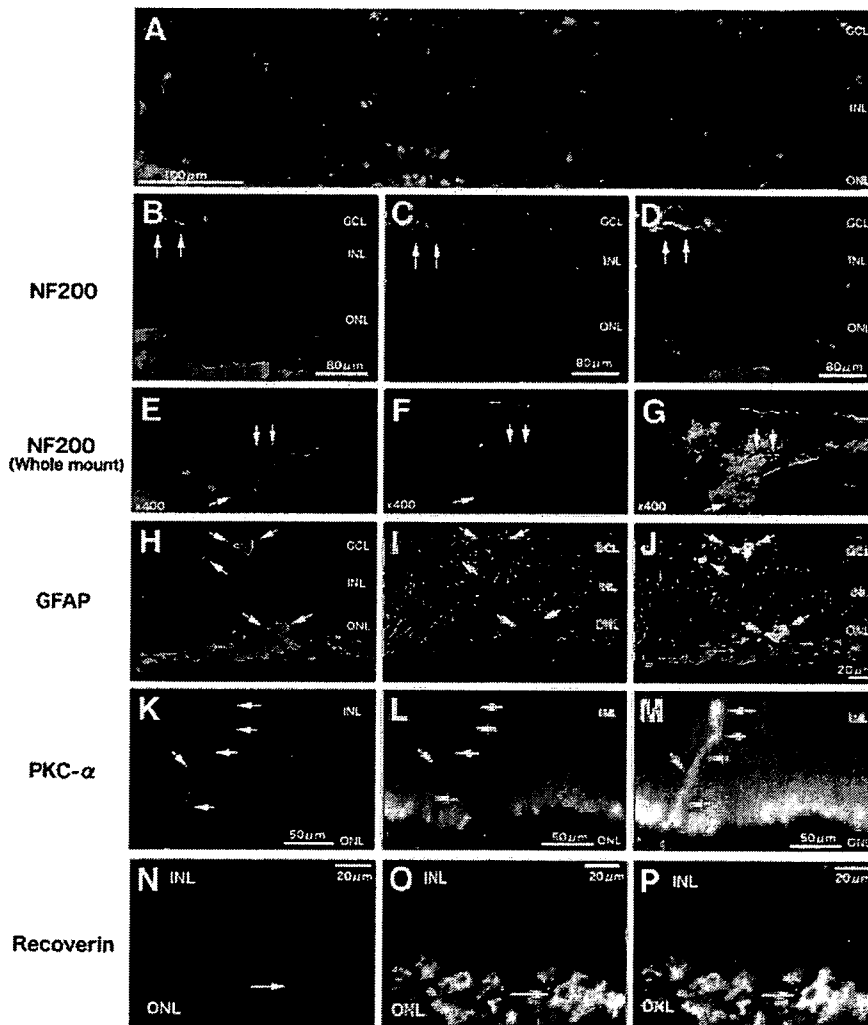
**Figure 3.** Comparison of BMSCs and RPCs. (A): The number of cells differentiated into retinal cells: comparison of marrow stromal cell (MSCs) and RPCs. In this study, nestin-positive cells were counted at day 1, and other markers cells were counted at 2 weeks after treatment. (B): Effect of BDNF, NGF, and bFGF on transcription of retinal cell markers. Semiquantitative reverse transcription-polymerase chain reaction analysis was carried out to determine the effect of BDNF, NGF, and bFGF on MSCs. MSCs without treatment showed only weak recoverin expression. (MSCs without treatment did not express nestin, BT-III, Map2, GFAP, PKC- $\alpha$ , and rhodopsin completely.) After 2 weeks of BDNF, NGF, and bFGF treatment, treated MSCs expressed nestin, BT-III, Map2, GFAP, PKC- $\alpha$ , and recoverin; however, rhodopsin expression was not found. Recoverin expression was increased in treated MSCs. Abbreviations: BDNF, brain-derived neurotrophic factor; bFGF, basic fibroblast growth factor; BMSC, bone marrow stromal cell; bp, base pair; BT-III,  $\beta$ -tubulin class III; FBS, fetal bovine serum; GF, growth factor; GFAP, glial fibrillary acidic protein; NGF, nerve growth factor; PKC, protein kinase C; RPC, retinal progenitor cell.

**Migration and Differentiation of MSCs**

At 1 week in coculture, MSCs with and without pretreatment of BDNF, NGF, and bFGF migrated into explanted rho-/- retina (Fig. 5A). MSCs without pretreatment did not show morphological or immunocytochemical evidence of neural differentiation (data not shown). On the other hand, pretreated MSCs showed morphological and immunocytochemical evidence of neuronal differentiation. Pretreated MSCs migrated into explanted retinas (Fig. 5A) and expressed NF200 (Fig. 5B–5G), GFAP (Fig. 5H–5J), PKC- $\alpha$  (Fig. 5K–5M), and recoverin (Fig.



**Figure 4.** Macrophages differentiated into microglia after transplantation to explanted retinas. Rho<sup>-/-</sup> mice retina at 3 (A) and 7 (B, C) days. Macrophages migrated into retina and morphologically changed their shape to that resembling microglia (A–C). Confocal (D–F) images seen at 1 week after grafting; constitutive green fluorescent protein expression (D), macrophage/microglia antibody/cytokeratin-3 immunoreactivity (E), and merged images (F).

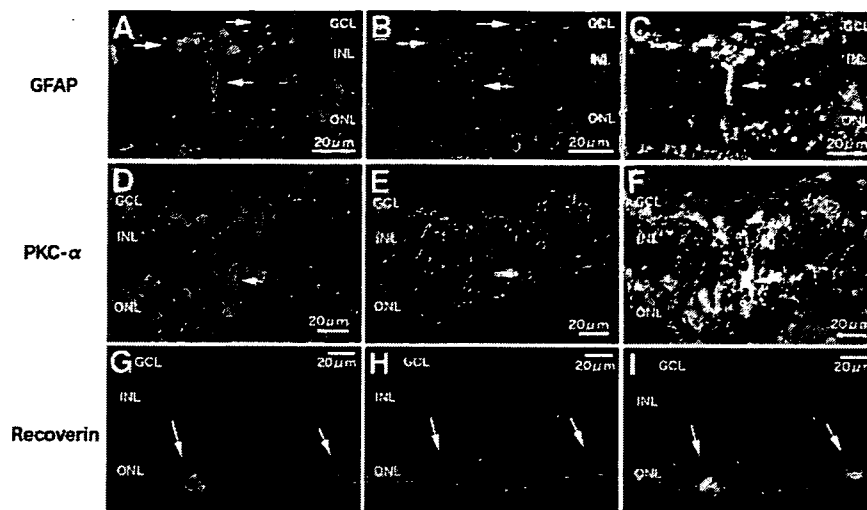


**Figure 5.** Migration and differentiation of pretreated marrow stromal cell (MSCs) into explanted retinas of rho<sup>-/-</sup> mice. A large number of MSCs migrated into explanted retinas of rho<sup>-/-</sup> mice (A). Epi-fluorescent (K–P) and confocal (B–J) images of the expression of neural and photoreceptor markers by pretreated MSCs that were grafted onto explanted retinas from rho<sup>-/-</sup> mice, seen at 1 week after grafting; constitutive green fluorescent protein expression (B, E, H, K, N), antibody/cytokeratin-3 immunoreactivity for NF200 (C, F) (whole mount), GFAP (I), PKC-α (L), recoverin (O), and merged images (D, G, J, M, P). Abbreviations: GCL, ganglion cell layer; GFAP, glial fibrillary acidic protein; INL, inner nuclear layer; NF, neurofilament; ONL, outer nuclear layer; PKC, protein kinase C.

5N–5P). We also found morphological evidence of neuronal differentiation (Fig. 5B–5P). However, we could not find any rhodopsin-positive cells among coculture, pretreated MSCs.

#### Migration and Differentiation of RPCs

At 1 week in coculture, RPCs migrated into all retinal lamina adjacent to the graft after addition to the outer retina and showed



**Figure 6.** Migration and differentiation of pretreated retinal progenitor cells (RPCs) into explanted retinas of  $\rho^{-/-}$  mice. Confocal images of the expression of neural and photoreceptor markers by RPCs grafting to explanted retinas of  $\rho^{-/-}$  mice, seen at 1 week after grafting; constitutive green fluorescent protein expression (A, D, G), antibody/cytokeratin-3 immunoreactivity for GFAP (B), PKC- $\alpha$  (E), recoverin (H), and merged images (C, F, I). Abbreviations: GCL, ganglion cell layer; GFAP, glial fibrillary acidic protein; INL, inner nuclear layer; MSC, marrow stromal cell; ONL, outer nuclear layer; PKC, protein kinase C.

morphological evidence of neuronal differentiation (Fig. 6D–6I). GFP<sup>+</sup> donor cells coexpressed a number of markers indicative of phenotypic maturation, including GFAP (Fig. 6A–6C), PKC- $\alpha$  (Fig. 6D–6F), and recoverin (Fig. 6G–6I). In the  $\rho^{-/-}$  mice, the rod marker rhodopsin was not detected in either grafted RPCs or the host outer nuclear layer.

#### DISCUSSION

The results presented here demonstrate that MSCs treated with BDNF, NGF, and bFGF can differentiate into retinal cells expressing Map2, BT-III, GFAP, PKC- $\alpha$ , and recoverin by immunocytochemistry and RT-PCR. In the explanted retina, pretreated MSCs showed differentiation into retinal cells expressing NF200, GFAP, PKC- $\alpha$ , and recoverin, although nonpretreated MSCs did not show any evidence of differentiation into retinal cells. This shows that treatment with growth factors (as in our previous report [17]) is very important for neural induction of MSCs. Moreover, our data show that using growth factors promoted neuronal differentiation over glial differentiation in RPCs (Fig. 3A). In the present study, RPCs clearly showed a higher level of differentiation into retinal cells compared with MSCs. Induced MSCs expressed neuronal and glial markers and morphologically differentiated into neuron- and glia-like cells; however, RPCs showed better morphological differentiation and also expressed rhodopsin (Figs. 1, 2). Although a subpopulation of MSCs differentiated morphologically into neuronal-like cells and expressed neuronal markers, the majority remained undifferentiated both in terms of morphology and marker expression during the time course examined. The lack of rhodopsin expression *in vivo* and *in vitro* by MSCs may be an impediment to their use in photoreceptor replacement. One must be cognizant of the fact that the absence of evidence is not evidence of absence. The lack of differentiation *in vitro* indicates that the optimal conditions have yet to be determined. This is especially true in the case of RPC photoreceptor differentiation, which we have shown to be dependent upon specific conditions *in vivo*. The fact that RPCs failed to express rhodopsin after migration into explants is not surprising, considering that our previous studies found no evidence for rhodopsin among RPCs transplanted to  $\rho^{-/-}$

mice *in vivo* [32]. The same study showed that RPCs expressed rhodopsin in another mouse strain with RD, the C3H mouse [32].

As with previous studies in the brain [34, 35], our results showed that macrophages migrated into explanted retina and appeared to differentiate into microglia. Although a previous report showed that microglia have potential for neuronal differentiation [33], we did not find evidence of differentiation into neuronal or glial cells in our explant study. Further studies will be needed to determine the neuronal potential of macrophages and microglia.

From a clinical perspective, MSCs are a good source for stem cell transplantation. Bone marrow cell transplantation is already an approved therapy for some kinds of hematological diseases and has the advantage of the possibility of autologous cell transplantation. Moreover, because recent reports have shown that MSCs have the capacity to modulate allogeneic cellular immunity [40, 41], MSCs may be useful for allogeneic transplantation.

Cell fusion has recently been proposed as the underlying explanation for the apparent plasticity and “transdifferentiation” of stem cells, including MSCs. This raises questions about the mechanisms of transdifferentiation *in vitro* and *in vivo* [42, 43]. Evidence against cell fusion has begun to mount; recent studies reported that MSCs can undergo transdifferentiation into various organ cell types, including neurons, without fusion [10, 44, 45]. We believe that our results cannot be attributed to cell fusion; this study shows that MSC differentiation into post-mitotic neuronal and retinal cells occurred in a controlled culture environment. Recent studies have shown that MSCs have a potential of transdifferentiation as cultured MSCs express mesodermal, endodermal, ectodermal, and germline genes, suggesting the potential to differentiate into all these cell types [46–48]. Moreover, our previous study [17], using the same methods for neuronal induction as this study, showed neuroectodermal induction, neuronal differentiation, and calcium uptake in response to a depolarizing stimulus from human MSCs. It has also been reported that neuroectodermal induction and electrophysiological characteristics of midbrain dopaminergic, serotonergic, and GABA-ergic neurons arise from treated MSCs [16].

## CONCLUSION

The present study shows that RPCs have clear advantages over MSCs in potential retinal transplantation applications. First, no evidence was found for MSC differentiation into rod photoreceptors. Second, RPCs showed more complete differentiation into retinal cell subtypes than did MSCs, and this occurred at a significantly higher rate. Finally, we have previously reported that neuronal progenitor cells (NPCs) have inherent immune privilege, suggesting increased resistance of allogeneic NPC grafts to host rejection [49, 50]. Such findings suggest the possibility that RPCs possess immune privilege properties as well. MSCs also have significant therapeutic potential in transplantation medicine because they can be readily obtained through a well-established clinical procedure. They are relatively easy to isolate and expand

for autologous transplantation without the need for immunosuppression or the risk of rejection. In this comparison study, we submit that RPCs possess significant advantages for differentiation into retinal cells compared with MSCs.

## ACKNOWLEDGMENTS

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## DISCLOSURES

The authors indicate no potential conflicts of interest.

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**A Comparison of Neural Differentiation and Retinal Transplantation with Bone Marrow-Derived Cells and Retinal Progenitor Cells**

Minoru Tomita, Taisuke Mori, Kazuichi Maruyama, Tasneem Zahir, Matthew Ward, Akihiro Umezawa and Michael J. Young

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## 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

KAZUNARI NAGAYOSHI,<sup>1,2</sup> HIROYUKI OHKAWA,<sup>2</sup> KEIGO YOROZU,<sup>2</sup> MASATO HIGUCHI,<sup>2</sup> SAYUMI HIGASHI,<sup>2</sup> NAOKI KUBOTA,<sup>2</sup> HIROYASU FUKUI,<sup>2</sup> NOBUO IMAI,<sup>2</sup> SATOSHI GOJO,<sup>3</sup> JUN-ICHI HATA,<sup>1</sup> YOSHIRO KOBAYASHI,<sup>4</sup> AND AKIHIRO UMEZAWA<sup>1\*</sup>

<sup>1</sup>Department of Reproductive Biology and Pathology, National Research Institute for Child Health and Development, Tokyo, Japan

<sup>2</sup>Chugai Pharmaceutical Co., LTD, Fuji Gotemba Research Institute, Gotemba, Shizuoka, Japan

<sup>3</sup>Department of Cardiovascular Surgery, Saitama Medical Center, Kawagoe, Japan

<sup>4</sup>Department of Biomolecular Science, Faculty of Science, Toho University, Chiba, Japan

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 multilineage 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 Schönfield, 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/Sld) 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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\*Correspondence to: Akihiro Umezawa, Department of Reproductive Biology and Pathology, National Research Institute for Child Health and Development, 2-10-1 Okura, Setagaya-ku, Tokyo 157-8535, Japan. E-mail: [umezawa@1985.jukuin.keio.ac.jp](mailto:umezawa@1985.jukuin.keio.ac.jp)

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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 biotininate 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>5</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

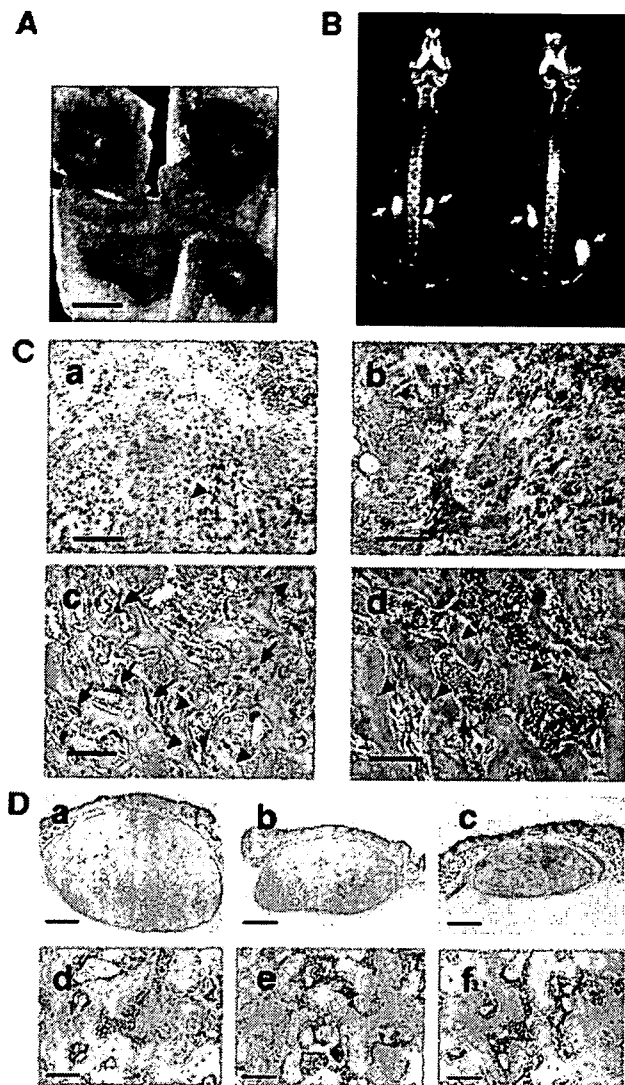
BALB nu/nu mice were examined by whole body soft X-ray radiography at 25.0 kV and 3.0 mA for 10 sec (SRO-iM50, Sofron, Tokyo) with X-ray RX film (Fuji Photofilm GmbH, Düsseldorf, Germany).

## 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  $5 \times 10^5$  (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