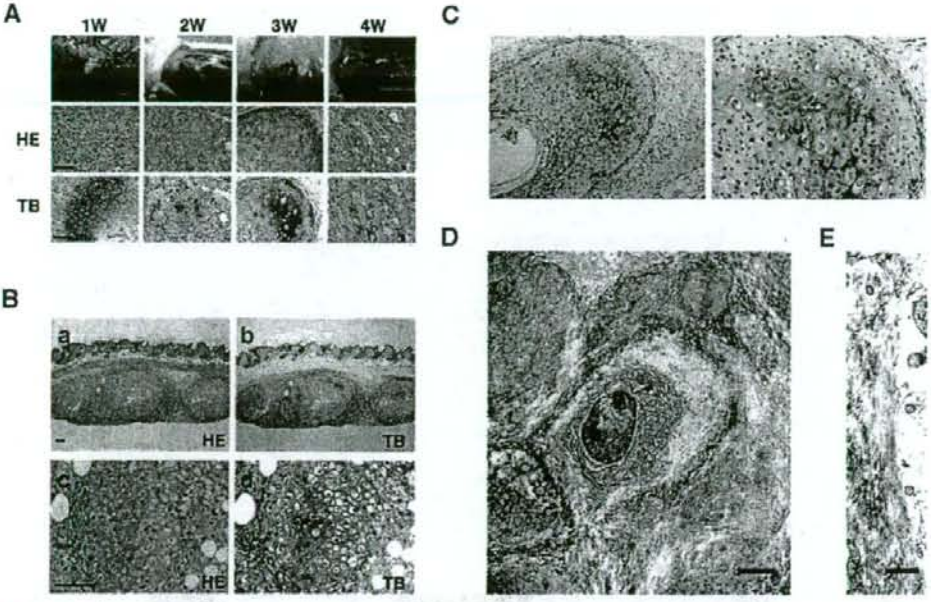

Fig. 6. In vivo chondrogenesis of KUM5 cells. **A:** Macroscopic view (**top**), hematoxylin and eosin stain (HE) (**middle**) and toluidine blue stain (TB) (**bottom**) analysis at 1, 2, 3, and 4 week (w)-cultivation in vivo after direct injection of KUM5 cells. **B:** KUM5 chondrogenic nodules, that were generated after pellet culture for 7 days in the CM supplemented with TGF-β3 and BMP2, were implanted just beneath the cutaneous muscle in the subcutaneous tissue and were cultivated in vivo for 3 weeks. **Panels c** and **d** are higher magnifications of **a** and **b**, respectively. **C:** Expression of chondrocyte-specific collagen type II. The KUM5 chondrogenic nodules were sectioned after 2 week-in vivo cultivation and stained with collagen type II-specific antibody. **D,E:** Ultrastructural analysis (TEM) of KUM5 implants. KUM5 cells were implanted into the subcutaneous tissue of Balb/c nu/nu mice, and the generated cartilage was resected 2 weeks after implantation. Scale bars: 2 mm (A, top row), 100 μm (A, middle and bottom row), 100 μm (B), 2 μm (D), 1 μm (E).

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micromass after the pellet culture into the subcutaneous tissue just beneath the cutaneous muscle (Fig. 7A,B). The OP9 cartilage was formed at 2 and 4 weeks, and abundant metachromatic matrix was observed with the toluidine blue stain. The immunohistochemical analysis shows that OP9 cartilage stains positive for the chondrocyte-specific type II collagen (Fig. 7C).

Sorting of Chondroblasts by Chondrocyte-Specific Cis-Regulatory Element of the Collagen α2(XI) Gene

Although the KUM5 cells used in this study were derived from a single-cell origin or clone, it could be argued that both cells responsive and non-responsive to chondrogenic induction were present [Ko et al., 1990]. In this sense, KUM5 cells might have been a largely heterogeneous cell population. Even cells derived from a single clone have been shown to be heterogeneous in terms of differentiation capacity and stages [Muraglia et al., 2000]. To validate the chondrogenic differentiation observed here, a homogenous population of committed cell obtained after induction should be isolated. Therefore, for the purpose of sorting chondrogenically committed cells, we transfected KUM5 cells with a Venus-expression vector under the control of the Col α2(XI) promoter, analyzed the transfected cells, and collected Venus-positive cells (Fig. 8A–D). The sorted cells were assessed for in vitro (Fig. 9A–F) and in vivo chondrogenesis (Fig. 9G–I). The cells again showed metachromatic chondrogenic micromasses with toluidine blue staining in vitro (Fig. 9B). Direct injection of the cells resulted in the cartilage formation within 1 week and obvious enchondral ossification at the periphery of the cartilage at 4 weeks (Fig. 9G). Again, ultrastructural analysis revealed that KUM5 chondrocytes implanted into the subcutaneous

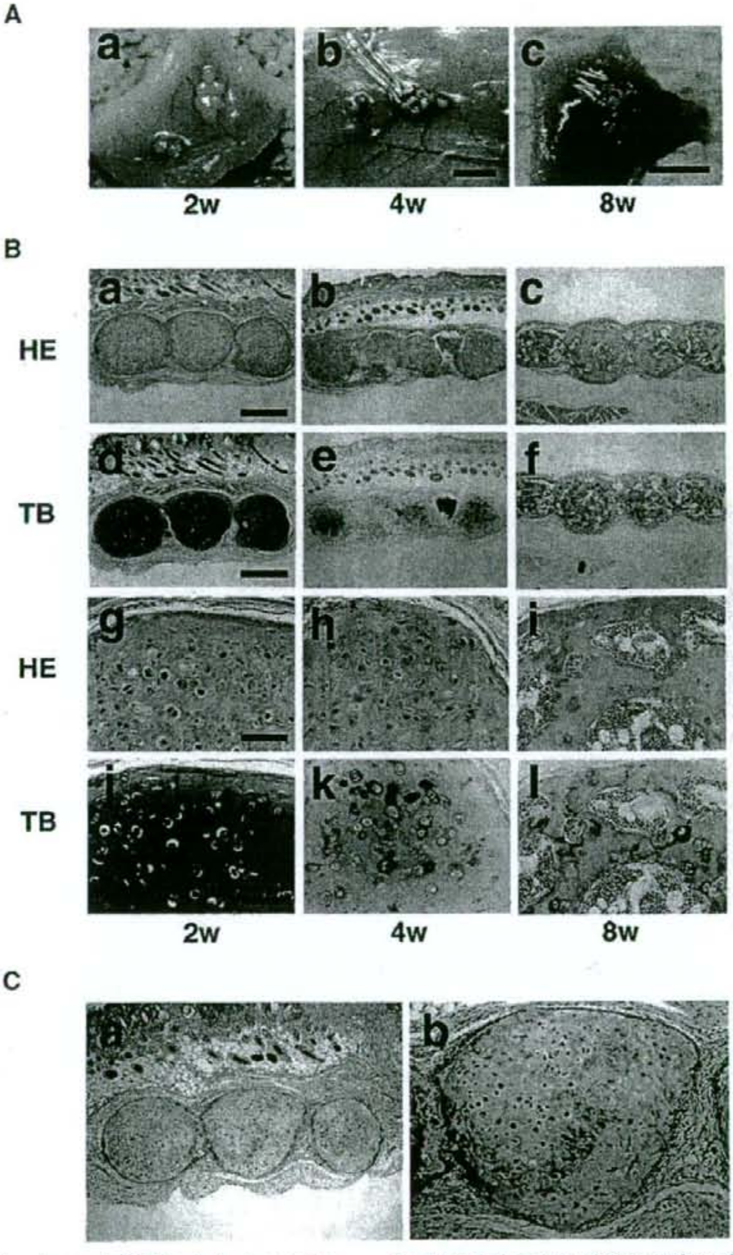

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Fig. 7. In vivo chondrogenesis of OP9 cells. In vivo chondrogenesis was examined by implantation of OP9 chondrogenic nodules. OP9 chondrogenic nodules, which were generated after pellet culture for 7 days in the CM supplemented with TGF-β3 and BMP2, were implanted just beneath the cutaneous muscle in the subcutaneous tissue and were cultivated in vivo for the number of weeks indicated. **A**: Macroscopic view of OP9 cartilage after 2 (a), 4 (b), and 8 (c)-week-in vivo cultivation. **B**: Histological analysis of OP9 cartilage after 2 (**a,d,g,j**), 4 (**b,e,h,k**), and 8 (**c,f,i,l**)-week-in vivo cultivation. (a,b,c,g,h,i), HE stain; (d,e,f,j,k,l), TB stain. **Panels g–l** are higher magnifications of **a–f**, respectively. **C**: Immunohistochemical analysis of the in vivo OP9 chondrogenic nodules. The OP9 chondrogenic nodules after 2-week-in vivo cultivation stained positive for collagen type II. Scale bars: 2 mm (A), 500 μm (Ba–f), 100 μm (Bg–l).

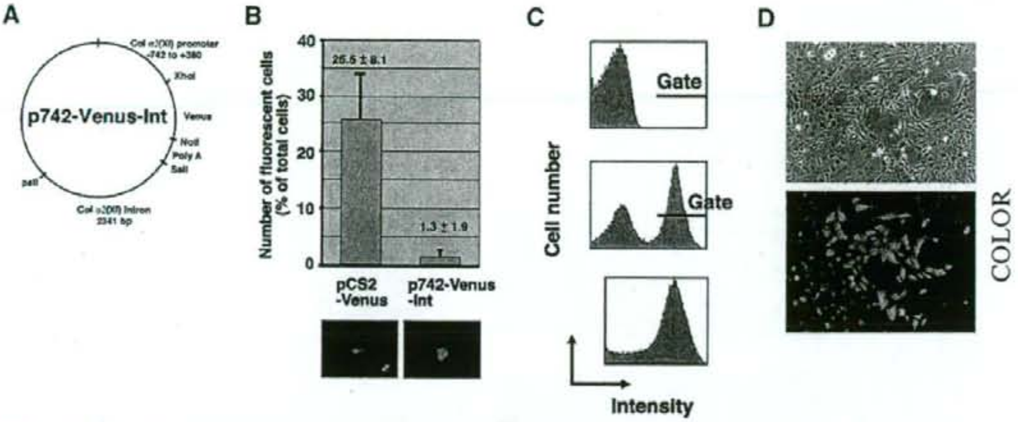

Fig. 8. Isolation of KUM5 chondroblasts using the chondroblast-specific cis-regulatory element. **A:** The p742-Venus-Int plasmid containing the fluorescent Venus gene driven by the cis-regulatory elements of the α2(XI) collagen gene. **B:** The number of fluorescent KUM5 cells (**upper**) after transfection with the p742-Venus-Int plasmid or pCS2-Venus containing the Venus gene driven by the CMV-promoter. Fluorescent photomicrograph of KUM5 cells after the first sorting (**lower**). **C:** Flowcytometric analysis of KUM5 cells after transfection with the p742-Venus-Int plasmid (**top**); The fluorescence-positive cells were sorted, propagated, and analyzed (**middle**). Again, the propagated fluorescence-positive cells were sorted, propagated, and analyzed (**bottom**). The "gate" for sorting is shown by the horizontal bar in the upper and middle panels. More than 80% of cells became positive after the final sorting. **D:** Phase contrast micrograph (upper) and fluorescent photomicrograph (lower) of the finally sorted cells (the lower panel of C).

tissue of nude mice were embedded in the hypertrophic chondrocytes and had abundant endoplasmic reticulum and a small number of mitochondria (Fig. 9H,I). The post-mitotic daughter cells in the cell nest, which are often observed in cartilage, were also detected (Fig. 9I).

DISCUSSION

In this study, we focus on the chondrogenic differentiation in vitro and in vivo using the two cell lines, KUM5 and OP9. The chondrogenic process is determined by the sequential expression of matrix component, and the differential response of differentiating cells to the growth factors may be attributed to the differentiating stages that depend on the expression patterns of the gene set as is the case for hematopoietic cells. The process of the chondrogenic differentiation is influenced by a number of growth factors including TGF-β and/or BMPs. Three isoforms of TGF-β have been known to have the ability to induce the chondrogenic differentiation. Both TGF-β2 and -β3 are more effective than TGF-β1 in promoting chondrogenesis, and TGF-β3 accelerates production of cartilaginous extracellular matrix in differentiating mesenchymal stem cells [Barry et al., 2001].

This study was undertaken to obtain mesenchymal stem cells with chondrogenic potential that retain critical in vivo cell functions, as do mammary gland epithelial cells, skin keratinocytes, and pigmented epithelial cells. To achieve this, we attempted to identify marrow-derived cells with chondrogenic nature and immortality without transformation among the cells obtained by the limiting-dilution method [Umezawa et al., 1992], defining "immortality" simply as indefinite cell division.

OP9 cells are known to serve as a niche or a specific microenvironment for the regulation of self-renewal and differentiation of stem cells [Nakano, 1996], and the question is raised of whether marrow stromal cells or marrow-derived mesenchymal cells with chondrogenic potential are capable of constituting a microenvironment for stem cells. It is inconceivable that cartilage can form a niche for cells in the living body based on structural and morphological considerations; however, a cell with chondrogenic or adipo-chondrogenic potential may serve as a niche not only in the case of OP9 cells but also as a general concept, at least in vitro.

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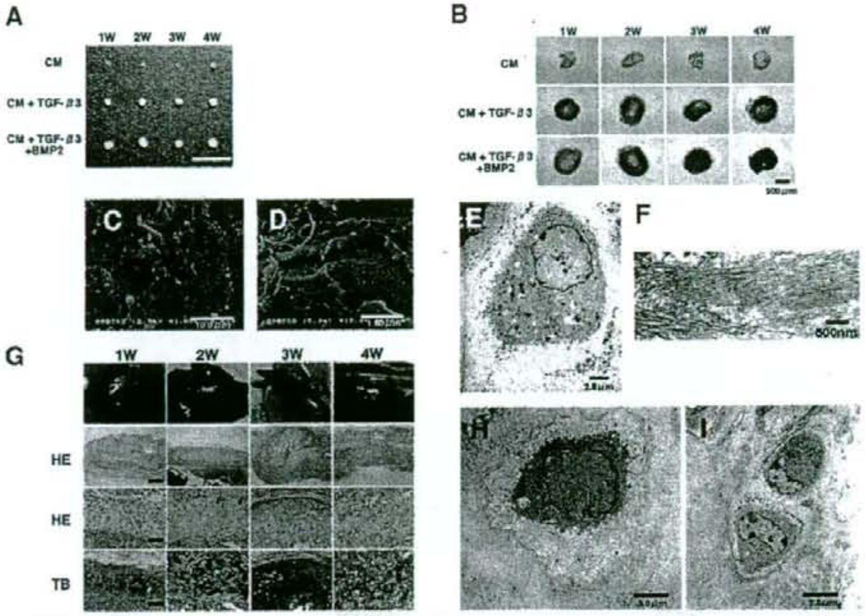

Fig. 9. In vitro and in vivo chondrogenesis of KUM5 cells sorted according to the activity of the chondrocyte-specific cis-regulatory element. **A,B**: Macroscopic view of the chondrogenic nodules which were generated after pellet culture of the finally sorted KUM5 cells for 1–4 weeks in the CM supplemented with growth factors as indicated (A) and toluidine blue stained section (B). **C–F**: Ultrastructural analysis of the micromasses of KUM5 cells sorted according to the activity of the Col α2(XI) cis-regulatory element (KUM5-Venus) after culturing in the CM supplemented with TGF-β3 for 3 weeks. (C,D), SEM; (E,F), TEM. **G**: In vivo chondrogenesis was examined 1–4 weeks after direct injection of the finally sorted KUM5 cells. From top to bottom: Macroscopic view, scale bars: 2 mm; histological analysis, scale bar: 600 μm, HE stain; histological analysis, scale bar: 120 μm, HE stain; histological analysis, scale bar: 120 μm, TB stain. **H,I**: Ultrastructural analysis (TEM) of the sorted KUM5 cartilage. The sorted KUM5 cells were implanted into the subcutaneous tissue of Balb/c nu/nu mice, and the generated cartilage was resected 2 weeks after implantation. Scale bars: 5 mm (A), 500 μm (B), 2 mm (G, **top row**), 500 μm (G, **2nd row**), 100 μm (G, **3rd** and **bottom row**).

The sequence of enchondral or perichondral ossification by KUM5 and OP9 cells was as follows: deposition of homogeneous matrix surrounding the small nests of the injected cells that subsequently became positive for type II collagen and exhibited metachromasia with toluidine blue staining, trapping them in the secreted homogeneous matrix, and the appearance of small nests of isogenous chondrocytes that probably resulted from repeated cell division. At a later stage, that is, 4–8 weeks after injection, the peripheral region of the generated cartilage became ossified. Importantly, the chondrogenesis by KUM5 and OP9 cells was irreversible and reproducible, and the implanted cells never transformed into malignant cells, formed any abnormal extracellular matrices, or induced any significant inflammatory reactions. It is again noteworthy that the osteogenesis by these two different lines of cells was mediated by chondrogenesis, and it was therefore considered to be chondral ossification. Thus, the unique characteristics of these two cell lines provide an opportunity to analyze the process of enchondral or perichondral ossification in an experimental system in detail.

In fetal life, primary ossification centers form by one of two processes: enchondral ossification or membranous ossification. Enchondral ossification refers to bony replacement of cartilage and is the mode of formation of the long bones. During membranous ossification mesenchymal cells form membranes within which ossification occurs and this is the mode of formation of the scapula and skull and, in part, of the clavicle and pelvis. After birth, bone growth continues by both enchondral and membranous ossification. Further enchondral ossification occurs in the physes and results in continuous longitudinal growth of the long bones until skeletal maturity. KUM5 and OP9 cells were obtained from long bone and calvaria, respectively, and showed enchondral ossification. We have also reported that KUSA-A1 cells form bone by membranous ossification in vivo, and thus we have three different types of cells showing distinctive in vivo characteristics. The process of chondrogenesis or enchondral ossification may also serve as a model for chondromatosis and osteochondromatosis in a joint cavity.

The expression pattern of chondrocyte-specific genes in OP9 and KUM5 cells is different from that in ATDC5 cells, which are a mouse embryonal carcinoma-derived chondrogenic cell line. ATDC5 cells exhibit a multistep differentiation process encompassing the stages from chondrogenesis to enchondral ossification [Shukunami et al., 1996]. Early-phase differentiation is characterized by the expression of type II collagen, followed by induction of the aggrecan gene. Late stage differentiation is characterized by the start of expression of short-chain collagen type X genes. By contrast, marrow-derived mesenchymal stem cells express the aggrecan genes at an early stage and then type II collagen during chondrogenic differentiation [Pittenger et al., 1999]. Surprisingly, gene expression pattern determined by the gene chip analysis was consistent with protein levels of cell surface molecules; this consistency indicates that the expression profiling is valid. Expression of "structural proteins" on Gene Ontology, including the extracellular matrix, was much higher by OP9 and KUM5 cells than by non-chondrogenic cells such as KUSA-A1 osteoblasts, H-1/A preadipocytes, and 9-15c mesenchymal stem cells, implying that the OP9 and KUM5 cells are mainly engaged in synthesizing extracellular matrix.

Can we inhibit enchondral or perichondral ossification after the completion of chondrogenesis? This is a challenge for the future, probably the not-too-distant future. We could not prevent the generated hyaline cartilage from ossifying at present even after selection based on the chondrocyte-specific cis-regulatory element of the collagen α2(XI) gene, probably due to the inability to inhibit vasculogenesis from the neighboring connective tissue. However, these established murine marrow-derived mesenchymal cells with in vivo chondrogenic activity and expression profiles provide a powerful model for studies of chondrogenic differentiation and our further understanding of cartilage regeneration. Bone marrow-derived chondroblasts with chondrogenic potential are useful candidate cell sources in addition to dedifferentiated chondrocytes obtained from cartilage for transplantation in osteoarthritis and rheumatoid arthritis.

ACKNOWLEDGMENTS

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Review Article

Two MSCs: Marrow stromal cells and mesenchymal stem cells

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Department of Reproductive Biology, National Institute for Child Health and Development

Marrow stromal cells (MSC1) are able to generate a series of terminally-differentiated cells *in vitro*. Most experiments are performed with heterogeneous stromal cells obtained by adherence to plastic culture dishes. Since bone marrow-derived stromal cells are purified to a homogeneous population meeting the criteria for non-hematopoietic stem cells, these cells have been termed "mesenchymal stem cells" and have the capability of generating an array of cells. However, "mesenchymal stem cells" (MSC2) are also actual multipurpose cells capable of differentiating into cells of mesoderm-origin regardless of cell sources. MSC2 can be recovered from a variety of other tissues, such as fat, muscle, menstrual blood, endometrium, placenta, umbilical cord, cord blood, skin, and eye. The terms "mesenchymal stem cell" and "marrow stromal cell" have been used interchangeably in emerging literature to describe cells that can be used in regenerative medicine, thereby introducing a degree of confusion. In this review, we re-organize the understanding of the two MSCs, describe their biology and differentiate between the two.

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Key words transdifferentiation, celltherapy, epigenetics, senescence

Introduction

Two MSCs, i.e., marrow stromal cells (MSC1) and mesenchymal stem cells (MSC2), are attracting a great deal of attention, as they represent a valuable source of cells for use in regenerative medicine, as well as offering an excellent model of cell differentiaton in biology. However, confusion exists in the literature due to poor application or misuse of the terms and nomenclature.

In general, mesenchymal stem cells are multi-potential stem cells that can differentiate into a variety of cell types (ref. http://en.wikipedia.org/wiki/Mesenchymal_stem_cell). They have been shown to differentiate, *in vitro* or *in vivo*, into osteoblasts, chondrocytes, myocytes, adipocytes and neuronal cell among others. Mesenchymal stem cells have traditionally been obtained from bone marrow, and have commonly been referred to as "marrow stromal cells"(MSC1).

While the terms "marrow stromal cell" (or "stromal cell") and "mesenchymal stem cell" have frequently been used interchangeably, they are increasingly recognized as separate entities as:

1. Stromal cells (MSC1) are a highly-heterogenous cell population, usually derived from bone marrow, consisting of multiple cell types with different potentials for proliferation and differentiation.

2. Mesenchymal stem cells (MSC2) encompass cells derived from other non-marrow tissues, such as fat, muscle, menstrual blood, endometrium, placenta, umbilical cord, cord blood, skin, and eye.

Bone marrow-derived mesenchymal stem cells or bone marrow stromal cells (MSC1) were discovered by Friedenstein in 1976, who described clonal, plastic-adherent cells from bone marrow that were capable of differentiating into osteoblasts, adipocytes, and chondrocytes. More recently, investigators have demonstrated that mesenchymal stem cells (MSC2) *per se* can be recovered from a variety of adult tissues and have the capacity to differentiate into a variety of specialist cell types. This review describes the recent advances in understanding of the two MSC cells, their biology and ongoing investigation and use.

Somatic stem cells

Somatic stem cells have been identified in hematopoietic[1], hepatic[2], epidermal[3], gastrointestinal[4], neural[5,6], muscle[7], and bone marrow[8-9] tissues. Many researchers have since demonstrated the developmental pluripotency of these cells. Bone marrow-derived stem cells can be transdifferentiated into multilineage cells, such as muscle[9] of mesoderm, lung[10] and liver[10,11] of endoderm, and brain[12-15] and skin[16] of ectoderm. Somatic stem cells are more desirable than embryonic stem (ES) cells for cell therapeutics because of ethical considerations and the possible immunologic rejection of ES cells. Mesenchymal stem cells have become the most popular somatic stem cells in medicine and biology, not least because of their high reproductive capability *in vitro*.

Bone marrow stromal cells (MSC1)

The existence of non-hematopoietic cells in bone marrow was first suggested by Cohnheim about 130 years ago[16]. Bone marrow-derived stromal cells (MSC1) can differentiate into most somatic cells, including osteoblasts, chondrocytes, myoblasts, cardiomyocytes[17-21], and adipocytes, when placed in appropriate *in vitro*[20] and *in vivo* environments[22], and thus are a useful cell source for regenerative medicine[23]. Recent studies suggest that MSC1 can also differentiate into a neuronal lineage[24], and murine bone marrow-derived adult progenitor cells can differentiate into dopaminergic neuronal cells[25,26]. Since the use of MSC1 entails no ethical or immunological problems, and bone marrow aspiration is an established routine procedure, these cells provide a useful and almost routine source of material for transplantation and tissue repair or regeneration (Fig.1).

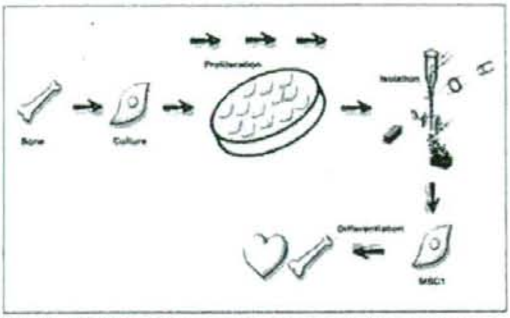

Fig.1 Development and differentiation of mesenchymal stem cells derived from bone marrow

1) Osteogenesis

KUSA-A1 cells, a murine marrow stromal cell line, are capable of generating mature bone *in vivo*[27]. They are a unique, mature osteoblast cell line and serve as a very suitable model for *in vivo* osteogenesis. Bone forms in subcutaneous tissue after subcutaneous injection of the cells into mice. The osteogenesis by KUSA-A1 is not mediated by chondrogenesis and thus is considered to be membranous ossification. Follow-up study on the fate of bone by immortalized osteoblasts shows that the ectopically-generated bone keeps its size and shape for 12 months[21]. Furthermore, the implanted cells do not metastasize like tumor cells. These unique characteristics of KUSA-A1 cells provide an opportunity to analyze the process of membranous ossification in detail.

2) Chondrogenesis

Chondrocytes differentiate from mesenchymal cells during embryonic development[28] and the phenotype of the differentiated chondrocyte is characterized by the synthesis, deposition, and maintenance of cartilage-specific extracellular matrix molecules, including type II collagen and aggrecan[29-31]. The phenotype of differentiated chondrocytes is rapidly lost since it is unstable in culture[32-35]. This process is referred to as "dedifferentiation" and is a major impediment to use of mass cell populations for therapy or tissue engineering of damaged cartilage. When isolated chondrocytes are cultured in a monolayer at low density, the typical round chondrocytes morphologically transform into flattened fibroblast-like cells, with profound changes in biochemical and genetic characteristics, including reduced synthesis of type II collagen and cartilage proteins[36]. When cultured three-dimensionally in a scaffold such as agarose, collagen, and alginate, redifferentiated chondrocytes re-express the chondrocytic differentiation phenotype.

KUM5 mesenchymal cells, a MSC1 line, generate hyaline cartilage *in vivo* and exhibit endochondral ossification at a later stage after implantation[17]. OP9 cells, another MSC1 line, derived from macrophage colony-stimulating factor-deficient osteopetrotic mice, and also known to be niche-constituting cells for hematopoietic stem cells, express chondrocyte-specific or -associated genes, such as type II collagen β1, Sox9, and cartilage oligomeric matrix protein at an extremely high level, as do KUM5 cells. OP9 micromasses exposed to TGF-β3 and BMP2 form type II collagen-positive hyaline cartilage within two weeks *in vivo*. The unique characteristics of KUM5 and OP9 cells provide an opportunity to analyze the process of endochondral ossification.

3) Cardiomyogenesis

It has been generally accepted that cardiac myocytes are unable to divide once cell proliferation ceases shortly after birth in the mammalian heart, because mitotic figures have not been detected in myocytes[38]. Cardiomyocytes induce DNA synthesis *in vivo* and *in vitro*[39,40]. Adult hearts often exhibit a polypoid structure, which results from stochastic accumulation of mutations as cells pass through cell-cycle checkpoints[41]. Bone marrow-derived stromal cells (MSC1) are able to differentiate into cardiomyocytes *in vitro* and *in vivo*[19,20,42,43] and a hierarchical model has been proposed for this *in vitro* cardiomyogenic differentiation. MSC1 in culture include a mixture of at least three types of cells, i.e., cardiac myoblasts, cardiac progenitors and multi-potential stem cells, and a follow-up study of individual cells suggests that commitment of a single-cell-derived stem cell toward a cardiac lineage is stochastic[44]. Furthermore, MSC1 over-expressing well-known master transcription factors, i.e., Csx/Nkx2.5 and GATA4, unavoidably undergo cardiomyogenic fate and behave like transient amplifying cells. MSC1 also transdifferentiate into cardiomyocytes in response to humoral factors, such as demethylation of the genome, in addition to environmental factors (See the chapter "Epigenetic modifier as a differentiating inducer".

4) Neurogenesis

MSC1 can exhibit neural differentiation when exposed to demethylating agents[14]: the cells differentiating into three types of neural cells, i.e., neurons, astrocytes, and oligodendrocytes. With exposure to basic fibroblast growth factor, nerve growth factor, and brain-derived neurotrophic factor, the transdifferentiation of human stromal cells is limited to neurons[14]. The change in gene expression during differentiation is global and drastic[15]: the differentiated cells no longer exhibit the profile of stromal cells or the biphenotypic pattern of neuronal and stromal cells. Osteoblasts capable of intra-membranous ossification are likely to differentiate into neuronal lineages, but adipocytes do not[14]. Interestingly, the cranio-facial membranous bones develop from the neural crest, which is of ectodermal origin. Development naturally progresses from neural crest cells to terminally-differentiated osteoblasts[46]. The finding of *in vitro* differentiation from mesoderm- to ectoderm-derived cells is thus the opposite of the developmental process, i.e., from ectoderm- to mesoderm-derived cells. Converting differentiated osteoblasts or MSC1 to neuronal cells, a key future task for any cell-based therapy, would thus oppose the usual direction of cell differentiation. This can now be achieved by exposing stromal cells to neurotrophic factors, at least *in vitro*.

Dopaminergic neuron-associated genes, such as nurr1 and wnt-5a, are induced at an extremely high level in the neuronally-differentiated stromal cells. Wnt5a and nurr1 are involved in the differentiation of mid-brain precursors into dopaminergic neurons[35,36]. It is quite significant that dopaminergic neurons can be generated from MSC1, since they are one of the key targets for regenerative medicine.

Epigenetic modifier as a differentiating inducer

The demethylating agent, 5-azacytidine, is a cytosine analog that has a remarkable effect on transdifferentiation of cells and has been shown to induce differentiation of stromal cells into cardiomyocytes, skeletal myocytes, adipocytes, and chondrocytes[19,42,47]. The effect of this low-molecular substance is not surprising, since it is incorporated into DNA and has been shown to cause extensive demethylation. The demethylation is attributable to covalent binding of DNA methyltransferase to 5-azacytidine in the DNA[48], with subsequent reduction of enzyme activity in cells resulting in dilution-out and random loss of methylation at many sites in the genome. This may, in turn, account for the reactivation of cardiomyogenic "master" genes, such as MEF-2C, GATA4, dHAND, and Csx/Nkx2.5, leading to stochastic transdifferentiation of MSC1 into cardiomyocytes. Use of 5-azacytidine is beneficial, but since it may have drawbacks, i.e., gene activation leading to oncogenesis and undesired differentiation, care must be exercised before using it to induce cells to differentiate into target phenotypes. Immortalized cells, including marrow stromal cells, have specific patterns of DNA methylation. The established methylation pattern of cells is maintained

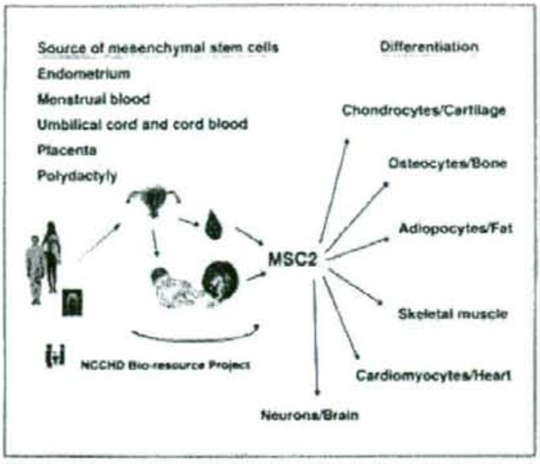

Fig.2 Sources and differentiation of mesenchymal stem cells

with considerable fidelity and silenced genes are stably inherited throughout the culture period[49-51]. 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[19,22,32,55]. 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:

a. 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;
b. 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;
c. Bi-potential cells: KUSA-O cells are capable of differentiating into osteoblasts and adiopocytes;
d. Precursor cells: KUSA-A1 and H-1/A are osteoblasts and preadipocytes, respectively. Adipogenic 3T3-L1[36], osteogenic MC3T3-E1[37], and chondrogenic ATDC5 cells[38] 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[39,40], and UCBMSCs have been found to have multipotential[41]. Most of the surface markers are the same as those detected in their bone marrow counterparts[42], 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"[62,63]. 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[42], 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[64,65]. 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[66]. 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[45]. 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[67]. 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 to monitor lineage commitment at the single cell level. At present, several models have been proposed in which hematopoietic lineage determination is driven intrinsically[68], extrinsically[69], or both[70]. 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).

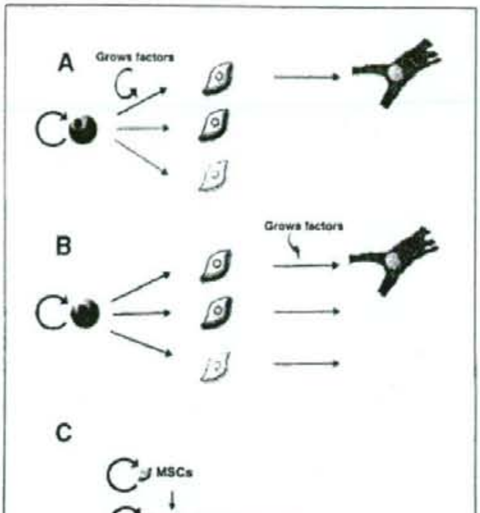

Fig.3 Model of stem cell differentiation

A. Deterministic model.

B. Stochastic model.

C. Differentiation model of mesenchymal stem cells.

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 differentiation. 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[71], bone[72], tendon[73], and meniscus[74] and for bone marrow stromal recovery[75] and osteogenesis imperfecta[76].

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"[77], 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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A Comparison of Neural Differentiation and Retinal Transplantation with Bone Marrow-Derived Cells and Retinal Progenitor Cells

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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-α, 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

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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−/− 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_2 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_2.

After two or three passages, bone marrow-derived adherent cells were incubated with trypsin for 3 minutes to generate a single-cell suspension. Cells (1×10^6) 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 ($CD45^-$, $CD11b^-$) and macrophages ($CD45^+$, $CD11b^+$) 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 (1×10^3) 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)-α (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 (1×10^3) 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 (1×10^3) 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, β-tubulin class III (BT-III; neuronal marker), Map2, GFAP, PKC-α, 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 µm; 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 µl/ml penicillin-streptomycin (Sigma-Aldrich). Organ cultures were maintained at 37°C, 5% CO_2 and fed every 2–3 days.

Explant Coculture

The host retinas were explanted from rho−/− mice (4–8 weeks of age). Cell suspensions (1 µl, 5×10^3 cells/µl) 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-µl 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 µm on a cryostat. Sections were stained with neurofilament (NF) 200 (1:1,000, neuronal marker; Sigma-Aldrich), GFAP, PKC-α, 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 µg/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-α, 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-α (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 R: TCAAGGGTATTAGGCAAGGGG	235	60
GFAP	F: CACGAACGAGTCCCTAGAGC R: ATGGTGATGCGGTTTTCTTC	234	60
TB-III	F: ACCTCAACCACCTGGTATCG R: TGCTGTTCTTGCTCTGGATG	344	60
Map2	F: CTGGACATCAGCCTCACTCA R:AATAGGTGCCCTGTGACCTG	164	60
PKC-α	F: CCCATTCCAGAAGGAGATGA R: TTCCTGTCAGCAAGCATCAC	212	60
Recoverin	F: ATGGGGAATAGCAAGAGCGG R: GAGTCCGGGAAAAACTTGGAATA	179	60
Rhodopsin	F: TCACCACCACCCTCTACACA R: TGATCCAGGTGAAGACCACA	216	60

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

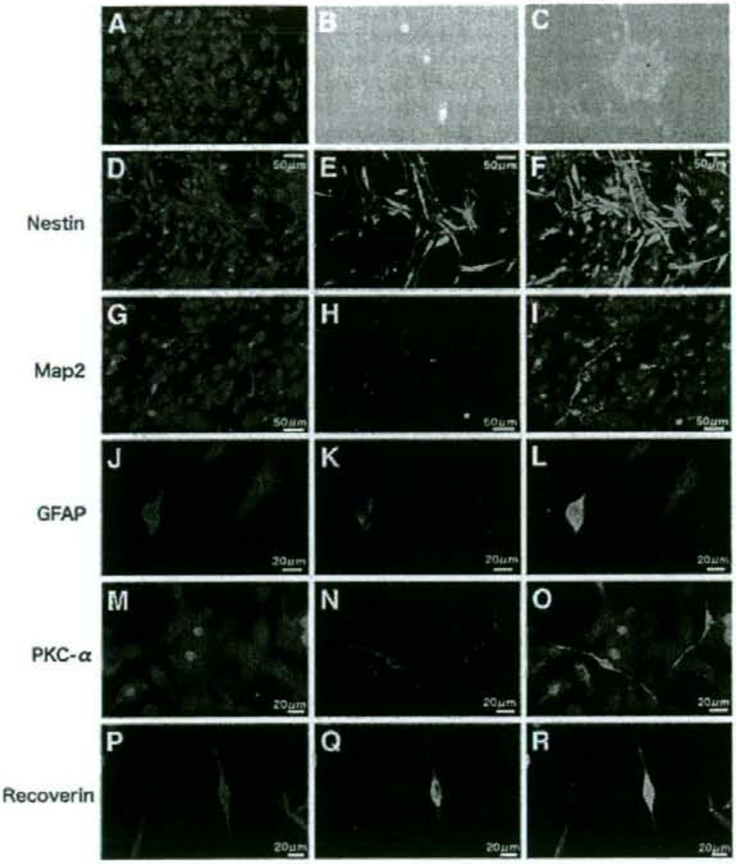

Figure 1. Differentiation and characterization of marrow stromal cell (MSCs) in vitro. Undifferentiated GFP$^+$ MSCs grown in Dulbecco's modified Eagle's medium with 10% fetal bovine serum, viewed under fluorescein isothiocyanate illumination **(A)**. MSCs cultured in serum-free medium with brain-derived 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-α **(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$^+$ cells markedly decreased (data not shown), and subpopulations expressed GFAP (Fig. 2D), Map2 (Fig. 2E), PKC-α (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. Moreover, 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-α, and recoverin were 5.55%, 3.27%, 1.42%, 3.97%, and 13.9%, respectively. The percentages of nestin-, Map2-, GFAP-, PKC-α-, 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-α-, 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).