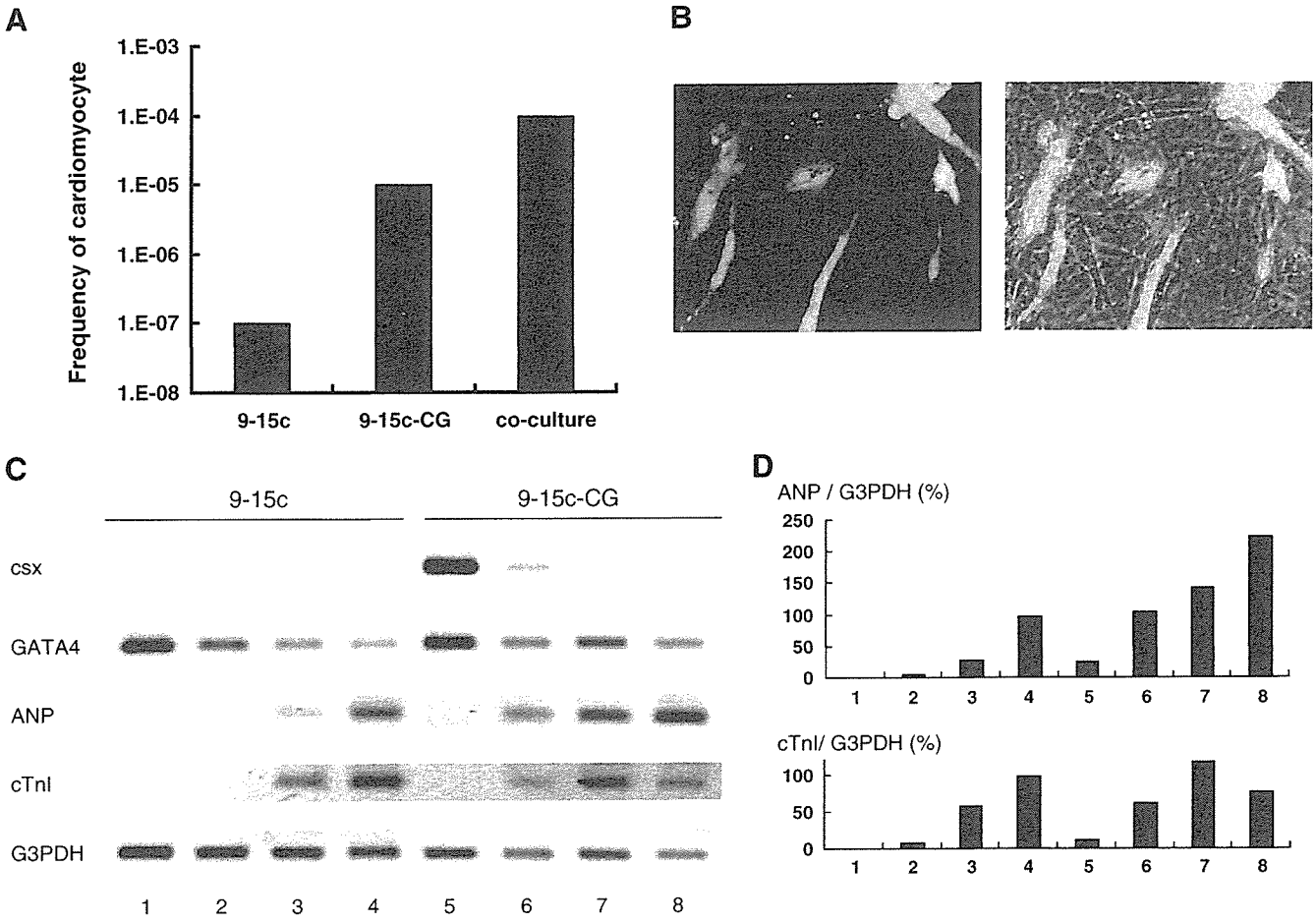

Fig. 5 – Enhancement of cardiomyogenic differentiation of 9-15c cells by co-cultivation with murine fetal cardiomyocytes. A: Frequencies of cardiomyogenic differentiation in 9-15c cells, 9-15c cells overexpressing the Csx and GATA4 genes (9-15c-CG cells), and 9-15c-CG cells co-cultured with murine fetal cardiomyocytes. B: Cardiomyogenic differentiation of EGFP-positive 9-15c-CG cells co-cultured with murine fetal cardiomyocytes. Left: Green fluorescence of EGFP-positive 9-15c-CG cells. Right: Same field visualized by phase-contrast microscopy merged with fluorescence image. C: RT-PCR analysis of the Csx, GATA4, ANP, cTnI and G3PDH genes in 9-15c cells (lanes 1–4) and 9-15c-CG cells (lanes 5–8). 9-15c cells (lane 1) and 9-15c-CG cells (lane 5) were cultured with exposure to 5-azacytidine alone (lanes 2 and 6) or 5-azacytidine and conditioned medium of cardiomyocyte cultures (lanes 3 and 7), or 5-azacytidine, conditioned medium of cardiomyocyte cultures, PDGF, retinoic acid, and fibronectin coating on a dish (lanes 4 and 8) for 4 weeks. D: Ratio mRNA expression level of ANP and cTnI to G3PDH in C. The mRNA level of 9-15c cells (lane 4) was regarded as equal to 100%.

induced by 5-azacytidine or microRNAs, whose key roles in stem cell biology are just emerging [37], also seem to be needed.

Adipogenic 3T3-L1 [38], osteogenic MC3T3-E1 [39], and chondrogenic ATDC5 cells [40] have been isolated from stem cells with a mesenchymal nature. In addition, cardiomyogenic precursors may be obtained from stem cells such as cardiac stem cells, embryonic stem cells, and mesenchymal stem cells. Fetal cardiomyocytes are differentiated cardiomyocytes, but not stem cells that can proliferate in vitro. Recently, cardiac stem cells capable of clonogenically self-renewing have been isolated from the adult heart [41–43]. Some cardiac stem cells also retain plasticity. The retention of plasticity, i.e., the ability to transdifferentiate into skeletal myocytes and endothelium, of 9-15c cells overexpressing Csx/Nkx2.5 and GATA4 supports the idea that these cells may be considered cardiac stem or amplifying cells in terms of differentiation and self-renewal. On the other hand, Csx/Nkx2.5 inhibits the myogenic differentiation of C2C12 cells and promotes neuronal differentiation [44]. This unexpected effect of Csx/Nkx2.5 may be due to differential effects of the gene in different cell types, or of transient versus constitutive expression of the infected gene; dependency of the differentiated phenotypes on the gene expression period is observed for the Notch gene [45,46] and noggin gene [47].

Cell transplantation has been attempted to improve cardiac function in severe heart failure; MSCs have been transplanted to functionally restore damaged or diseased tissue in animal models, and mononuclear cells or myoblasts have been injected into ischemic hearts clinically. MSCs are capable of differentiating into many types of cells, and 'cardiomyogenic master genes' are able to enhance the commitment or determine the path to cardiomyogenic differentiation of these MSCs. The stemness of MSCs determined by single-cell

marking in this study needs to be taken into consideration when we are considering mesenchymal stem cell-based therapy: we should pay attention to the possible unexpected differentiation of donor MSCs such as osteogenesis or chondrogenesis in the implanted heart.

In conclusion, we demonstrated that cardiomyocytes were stochastically differentiated from MSCs and that forced expression of Csx/Nkx2.5 and GATA4 enhanced the commitment or determined the path to cardiogenic differentiation of these MSCs. Our findings suggest that single-cell-derived MSCs overexpressing Csx/Nkx2.5 and GATA4 behave like cardiac transient amplifying cells and that Csx/Nkx2.5 and GATA4 could be interesting target molecules for enhancing cardiogenesis of MSCs.

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Review Article

Two MSCs: Marrow stromal cells and mesenchymal stem cells

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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 multi-purpose 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[6], and bone marrow[6-8] 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[10] 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).

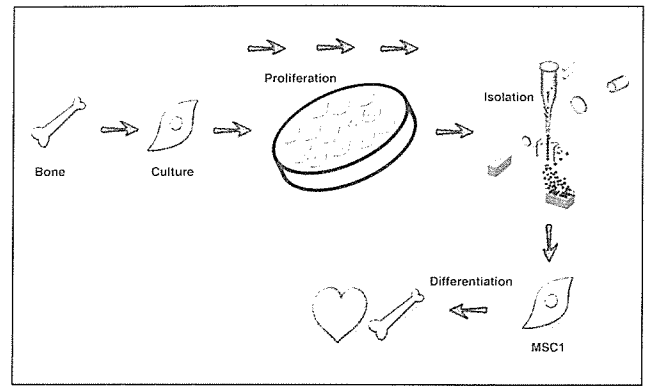

Fig.1 Development and differentiation of mesenchymal stem cells derived from bone marrow

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[37]. 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[45]: 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[25,26]. 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

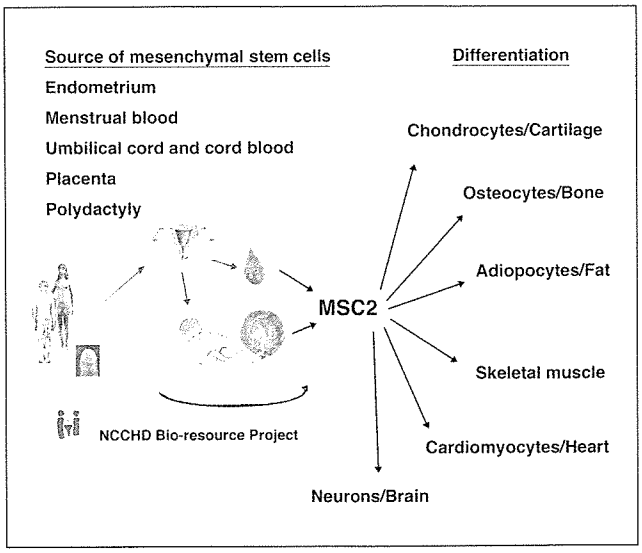

Fig.2 Sources and differentiation of mesenchymal stem cells

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[19,22,52-55]. 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[56], osteogenic MC3T3-E1[57], and chondrogenic ATDC5 cells[58] 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[59,60], and UCBMSCs have been found to have multipotential[61]. 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).

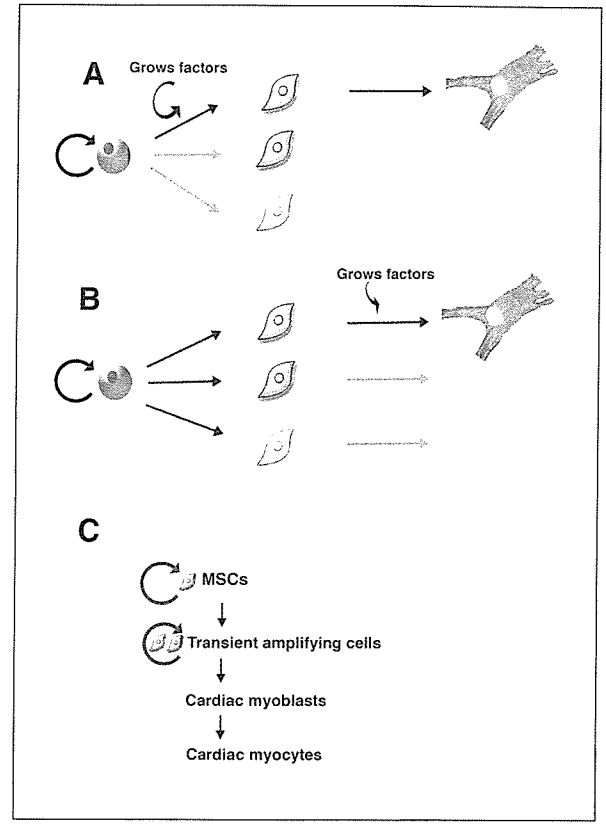

Fig.3 Model of stem cell differentiation
A. Deterministic model.
B. Stochastic model.
C. Differentiation model of mesenchymal stem cells.

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 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[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.

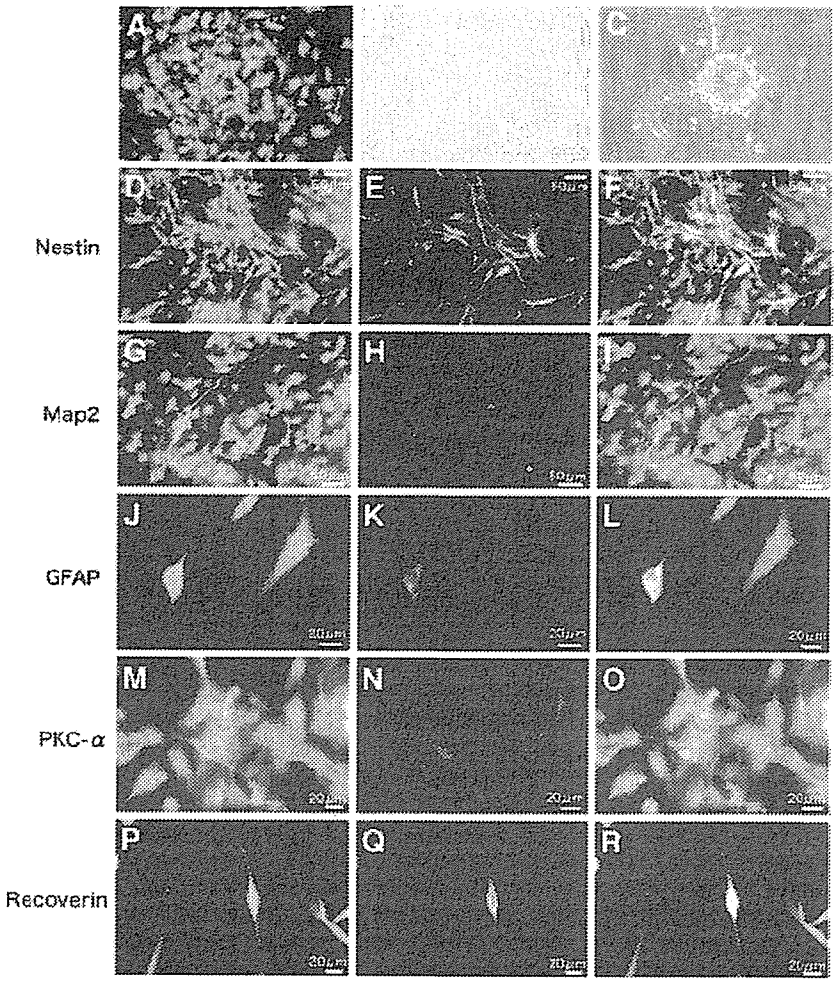

Figure 1. Differentiation and characterization of marrow stromal cell (MSCs) in vitro. Undifferentiated GFP$^+$ MSCs grown in Dulbecco's modified Eagle's medium with 10% fetal bovine serum, viewed under fluorescein isothiocyanate illumination (A). MSCs cultured in serum-free medium with brain-derived 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).

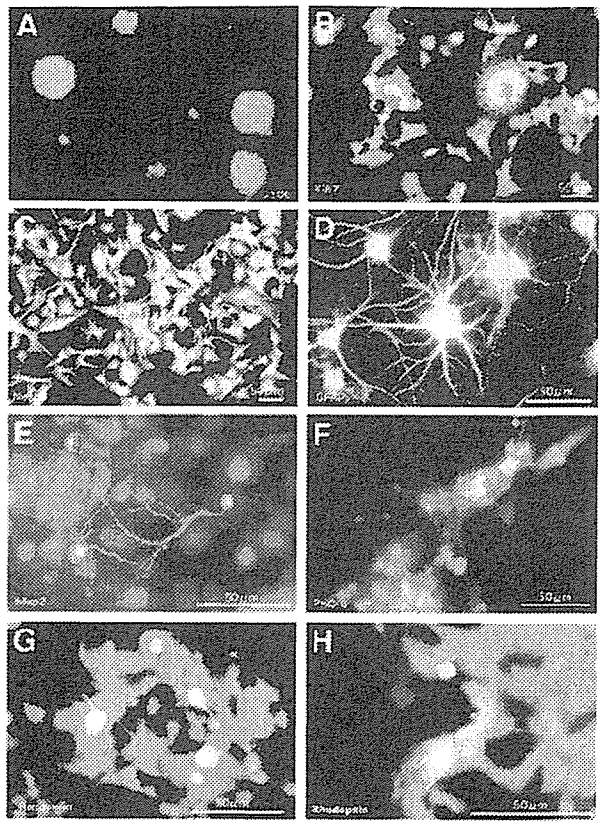

Figure 2. Differentiation and characterization of retinal progenitor cell (RPCs) in vitro. RPCs formed green fluorescent protein-positive neurospheres (A). RPCs cultured in the absence of epidermal growth factor and in the presence of 10% fetal bovine serum for 1 (B, C) or 14 (D–H) days. The cells were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

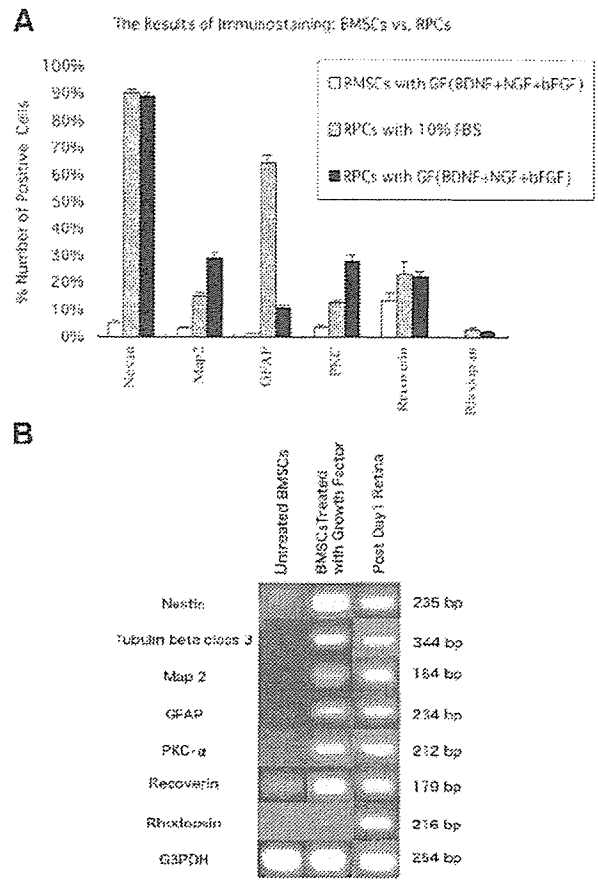

Figure 3. Comparison of BMSCs and RPCs. (**A**): The number of cells differentiated into retinal cells: comparison of marrow stromal cell (MSCs) and RPCs. In this study, nestin-positive cells were counted at day 1, and other markers cells were counted at 2 weeks after treatment. (**B**): Effect of BDNF, 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-α, and rhodopsin completely.) After 2 weeks of BDNF, NGF, and bFGF treatment, treated MSCs expressed nestin, BT-III, Map2, GFAP, PKC-α, 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, β-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.

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-α, or rhodopsin.) After 2 weeks of BDNF, NGF, and bFGF treatment, MSCs expressed nestin, BT-III, Map2, GFAP, PKC-α, 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).

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-α (Fig. 5K–5M), and recoverin (Fig.

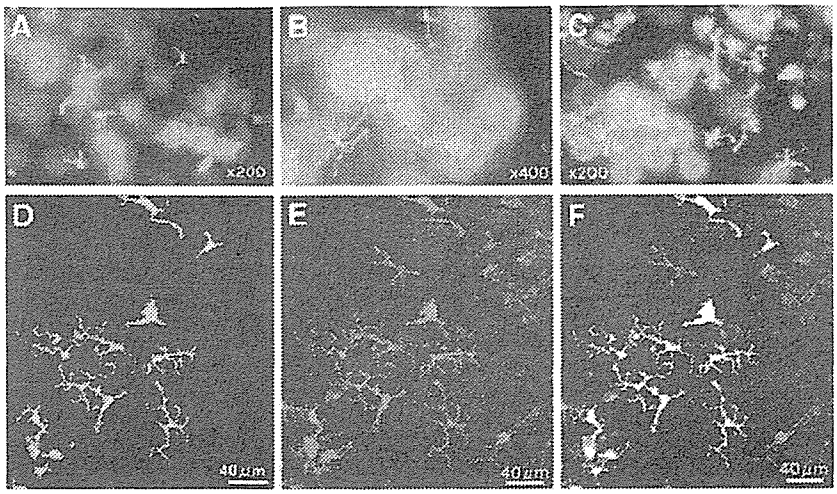

Figure 4. Macrophages differentiated into microglia after transplantation to explanted retinas. Rho−/− mice retina at 3 (A) and 7 (B, C) days. Macrophages migrated into retina and morphologically changed their shape to that resembling microglia (A–C). Confocal (D–F) images seen at 1 week after grafting;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

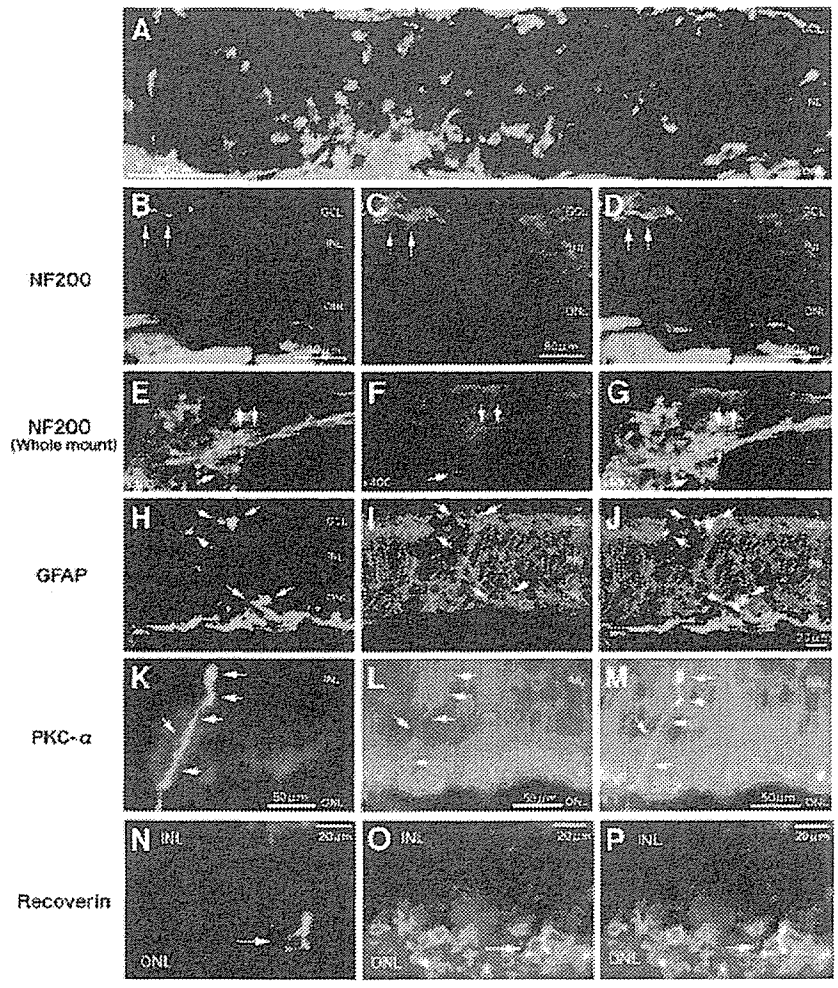

Figure 5. Migration and differentiation of pretreated marrow stromal cell (MSCs) into explanted retinas of rho−/− mice. A large number of MSCs migrated into explanted retinas of rho−/− mice (A). Epi-fluorescent (K–P) and confocal (B–J) images of the expression of neural and photoreceptor markers by pretreated MSCs that were grafted onto explanted retinas from rho−/− 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

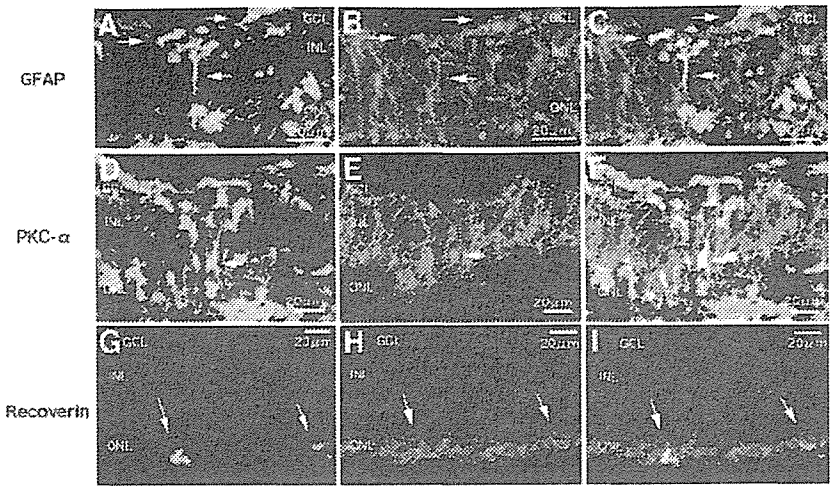

Figure 6. Migration and differentiation of pretreated retinal progenitor cells (RPCs) into explanted retinas of rho−/− mice. Confocal images of the expression of neural and photoreceptor markers by RPCs grafting to explanted retinas of rho−/− mice, seen at 1 week after grafting; constitutive green fluorescent protein expression (A, D, G), antibody/cytokeratin-3 immunoreactivity for GFAP (B), PKC-α (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$^+$ donor cells coexpressed a number of markers indicative of phenotypic maturation, including GFAP (Fig. 6A–6C), PKC-α (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-α, and recoverin by immunocytochemistry and RT-PCR. In the explanted retina, pretreated MSCs showed differentiation into retinal cells expressing NF200, GFAP, PKC-α, 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, neural 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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