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.

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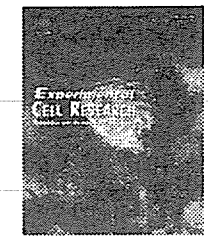

Research Article

Single-cell-derived mesenchymal stem cells overexpressing Csx/Nkx2.5 and GATA4 undergo the stochastic cardiomyogenic fate and behave like transient amplifying cells

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ABSTRACT

Bone marrow-derived stromal cells can give rise to cardiomyocytes as well as adipocytes, osteocytes, and chondrocytes in vitro. The existence of mesenchymal stem cells has been proposed, but it remains unclear if a single-cell-derived stem cell stochastically commits toward a cardiac lineage. By single-cell marking, we performed a follow-up study of individual cells during the differentiation of 9-15c mesenchymal stromal cells derived from bone marrow cells. Three types of cells, i.e., cardiac myoblasts, cardiac progenitors and multipotent stem cells were differentiated from a single cell, implying that cardiomyocytes are generated stochastically from a single-cell-derived stem cell. We also demonstrated that overexpression of Csx/Nkx2.5 and GATA4, precardiac mesodermal transcription factors, enhanced cardiomyogenic differentiation of 9-15c cells, and the frequency of cardiomyogenic differentiation was increased by co-culturing with fetal cardiomyocytes. Single-cell-derived mesenchymal stem cells overexpressing Csx/Nkx2.5 and GATA4 behaved like cardiac transient amplifying cells, and still retained their plasticity in vivo.

Introduction

Cell-based therapy is a novel therapeutic strategy, based on the concept of the cell-mediated restoration of damaged or diseased tissue. Candidate cell sources include embryonic stem (ES) cells, hematopoietic stem cells (HSCs), neural stem cells (NSCs), mesenchymal stem cells (MSCs) [1], and so on. Clinical trials with MSCs have been performed in patients with graft-versus-host disease through immunomodulatory effects [2], and osteogenesis imperfecta [3,4], and MSCs are expected to be one of the most available cells. The source of MSCs includes bone marrow [5], adipose tissue [6], umbilical cord [7] and placenta [8].

Bone marrow-derived stromal cells [9] can differentiate into mesenchymal progenitors, including osteoblasts [10], chondroblasts [11], skeletal myoblasts [12], adipoblasts [13],

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and neurons [14,15] when placed in appropriate in vitro and in vivo environments. We have shown that bone marrow-derived stromal cells are also able to differentiate into cardiomyocytes in vitro and in vivo [13,14,16,17]. However, the characteristics of the cells that can differentiate into cardiomyocytes are poorly understood, and how the progeny of multipotent cells adopt one fate among several possible fates remains a fundamental question.

Hematopoietic stem cells are defined as cells that are capable of self-renewal to maintain a long-term supply of progeny and are capable of differentiating into multiple hematopoietic lineages [18]. Retroviral labeling of individual cells is one of the useful clonal assays to monitor lineage commitment at the single cell level [16,17,19]. At present, several models have been proposed in which hematopoietic lineage determination is driven intrinsically [20], extrinsically [21], or both [22]. We therefore performed retroviral labeling experiments of bone marrow-derived stromal cells to investigate whether cardiomyocytes are generated from committed cardiac precursor cells or uncommitted stem cells.

In the present study, we provide evidence that cardiomyocytes are stochastically differentiated from MSCs, and we demonstrate that forced expression of cardiomyocyte-specific transcription factors, i.e., Csx/Nkx2.5 and GATA4, destined these MSCs to a cardiomyocytic lineage.

Materials and methods

Cell culture

9-15c cells were used as a source of uncommitted stem cells in this study [23,24]. 9-15c cells are available through one of the cell banks (JHSF cell bank: http://www.jhsf.or.jp/English/index_gc.html; RIKEN cell bank: http://www.brc.riken.go.jp/lab/cell/english/guide.shtml). 9-15c cells were cultured using methods described previously [25]. The cells were cultured in Iscove's modified Dulbecco's medium (IMDM) supplemented with 20% fetal bovine serum and penicillin (100 μg/ml)/streptomycin (100 μg/ml)/amphotericin B (250 ng/ml) at 33°C with 5% CO_2.

Primary cultures of cardiac myocytes were prepared from the hearts of 16-day-old fetal C3H/HeJ mice (CLEA Japan, Inc., Tokyo, Japan) according to the method of Simpson et al. [26] with minor modifications. In brief, cardiomyocytes were dissociated into single isolated cells by trypsinization and the cells were plated in culture medium (IMDM with 20% fetal bovine serum).

Cloning of Csx/Nkx2.5 and GATA4 cDNAs

The full open reading frames of mouse Csx/Nkx2.5 and GATA4 cDNAs were cloned by RT-PCR from poly(A) RNA obtained from the hearts of fetal mice using the following primers: Csx/Nkx2.5, sense: 5′-TGAAACCTGCGTCGCCACCATGT-3′, antisense: 5′-GGCTCTTTCCCTACCAGGCTCGG-3′; GATA4, sense: 5′-TAGTTCTTGTCTGCCTCGTGCTCA-3′, antisense: 5′-GGCGCTGATTACGCGGTGATTATG-3′. The PCR products were subcloned into pGEM-T vector (Promega). DNA sequencing confirmed that the plasmids contained the full-length fragments of the mouse Csx/Nkx2.5 and GATA4 coding regions.

Retroviral transduction

The retroviral vectors pCLNCX (Imgenex), pCLPCX and pCLHCX were used. pCLPCX was constructed from pCLNCX by replacing the neomycin resistance gene with a puromycin resistance gene (pPUR; CLONTECH). pCLHCX was constructed from pCLNCX by replacing the neomycin resistance gene with a hygromycin resistance gene (pcDNA3.1/Hygro(+); Invitrogen). Fragments containing the EGFP, Csx/Nkx2.5, and GATA4 genes were cloned into pCLNCX, pCLPCX, or pCLHCX. Each of these DNAs and pCMV-Eco (kindly provided by Nikunj Somia) were transfected into the producer cells (293 gag pol; kindly provided by Nikunj Somia) using TransFast (Promega). Two days after the transfection, the culture supernatant was filtered through a 0.45-μm filter. 9-15c cells were treated with viruses and hexadimethine bromide (polybrene) (Sigma) (8 μg/ml) for 4–6 h. To generate stably expressing cells, 9-15c cells were cultured in the presence of 300 μg/ml G418, 300 ng/ml puromycin or 300 μg/ml hygromycin. The mixtures of drug-resistant clones were used to average the clonal variation of the transfected gene expression.

Cardiomyogenic induction

To induce differentiation, cells were initially plated at a density of 2×10^4 cells/ml. The cells were treated with 3 μM 5-azacytidine (Sigma) for 24 h the next day. In some experiments, PDGF-BB (Peprotech) and retinoic acid (Sigma) were added to the culture dish coated with fibronectin (BD Biosciences) to give a final concentration of 10 ng/ml and 1 nM, respectively, for 6 days. Total number of beating cells was estimated under phase contrast microscopy.

RT-PCR

Total RNA was extracted from adult mouse hearts, skeletal muscles and cultured cells with an RNeasy kit (QIAGEN), and cDNA was made using the SuperScript First-strand Synthesis System (Invitrogen) from 1 μg of total RNA. First-strand cDNA was diluted 20 fold and 1 μl of cDNA was used for each PCR reaction. The following primer sets for cardiomyocyte-associated genes were used: atrial natriuretic peptide (ANP), sense: 5′-TTCCTCGTCTTGGCCTTTTGG-3′, antisense: 5′-GCTGGATCTTCGTAGGCTCCG-3′; cardiac troponin I (cTnI), sense: 5′-GATCCTGTTCTCTGCCTCTGGA-3′, antisense: 5′-TCATCCACTTTGTCCACCCGAG-3′; fast troponin I (fTnI), sense: 5′-GAAGCGCAACAGGGCCATCACG-3′, antisense: 5′-CCACGTCACGCAGGTCCGGTTC-3′; Csx/Nkx2.5, sense: 5′-TGGCGTCTGGGGACCTGTCTG-3′, antisense: 5′-GAGTCTGGTCCTGCCGCTGTC-3′; GATA4, sense: 5′-TACATGGCCGACGTGGGAGCA-3′, antisense: 5′-TGGAGTTACCGCTGGAGGCAC-3′; exogeneous GATA4, sense: 5′-CCAGAAAACGGAAGCCCAAGAA-3′ (the sequence derived from mouse GATA4 gene), antisense: 5′-GCTTGCCAAACCTACAGGTGGG-3′ (the sequence derived from pCLPCX vector); adiponectin, sense: 5′-CTGAAGAGCTAGCTCCTGCTTTG-3′, antisense: 5′-GAAGAGAACGGCCTTGTCCTTC-3′; glyceraldehyde-

3-phosphate dehydrogenase (G3PDH), sense: 5′-CCCATCAC-CATCTTCCAGGAGC-3′, antisense: 5′-TTCACCACCTTCTT-GATGTCATCATA-3′. G3PDH was used as an internal control. PCR was performed with TaKaRa Ex-Taq (TAKARA SHUZO CO., LTD) for 30–35 cycles, with each cycle consisting of 94°C for 1 min, 61–68°C for 1 min, and 72°C for 2 min, with an additional 7 min incubation at 72°C after completion of the final cycle.

RT-PCR samples were electrophoresed through agarose gels and stained with ethidium bromide and visualized through a UV light digital imaging system. Densities of electrophoresis bands were analyzed using ScnImage software (Scion Corporation).

Western blot analyses

Western blots were performed using whole-cell extracts according to the standard protocol [27]. Aliquots (30 μg) of whole-cell extracts were electrophoresed in SDS-polyacrylamide gels and transferred onto Immobilon-P polyvinylidene difluoride membrane (Millipore) by electroblotting. After treatment in blocking buffer, membranes were sequentially probed with the antibodies against Nkx2.5 (sc-8697, Santa Cruz) or Gata4 (sc-9053, Santa Cruz), and then with HRP-conjugated anti-goat or rabbit IgG. The bands were revealed using the ECL Plus standard protocol (Amersham Pharmacia Biotechnology).

Cellular transplantation

Following priming by 5-azacytidine for 24 h, the cells were cultured for an additional 3 days. Then the cells were harvested with 0.05% trypsin and 0.25 mM EDTA, and suspended as single cells at a concentration of 1×10^5 cells/μl with PBS. The cell viability in suspension, determined by 0.05% erythrosine dye exclusion, was 90% to 95%. After general anesthesia of the recipient mice by an intraperitoneal injection of 0.05 mg/g body weight pentobarbitone, cell transplantation was performed into the quadrant muscles of syngeneic adult recipient C3H/HeJ mice (CLEA Japan, Inc., Tokyo, Japan), aged 8 to 10 weeks old at a dose of 1×10^6 and 1×10^8 cells per mouse. All animals received humane care in compliance with the "Principles of Laboratory Animal Care" formulated by Keio University School of Medicine and the National Research Institute for Child Health and Development, and the experimental procedures were approved by the Laboratory Animal Care and Use Committee of Keio University School of Medicine.

Histological analyses

Tissues were fixed in 10% neutral buffered formalin and embedded in paraffin. Tissue sections (6 μm) were mounted on poly-L-lysine-coated slides. After deparaffinization with xylene, tissues were rinsed in acetone or ethanol. Slides were incubated in 0.3% H_2O_2 for 30 min. After washing in PBS, tissues were preblocked for 30 min with 5% normal swine serum. They were incubated overnight at 4°C with mouse monoclonal antibody against recombinant GFP (CLONTECH Laboratories, Inc.) diluted 1:500. After rinsing in PBS, the slides were incubated with horseradish peroxidase-conjugated swine anti-mouse immunoglobulin diluted 1:100 with 1% BSA in PBS, and washed in cold PBS. Staining was developed using a solution containing DAB and 0.01% H_2O_2 in 0.05 M Tris–HCl buffer, pH 6.7. Slides were counterstained with hematoxylin. Slices with positive signals for EGFP were further stained

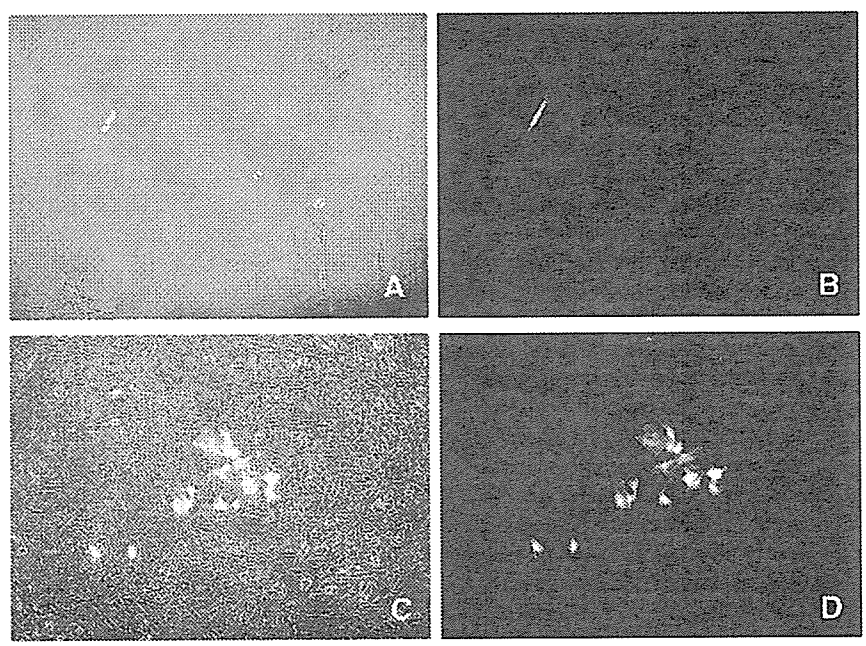

Fig. 1 – Single cell marking by infection of retrovirus carrying EGFP. Phase contrast photomicrograph (A, C) and fluorescent photomicrograph (B, D) of 9-15c cells 1 day (A, B) or 7 days (C, D) after infection with retroviruses carrying EGFP. EGFP-positive single cell-derived cells were clustered.

with anti-CD31 (PECAM-1) antibody (M-20, Santa Cruz Biotechnology, Inc, California, USA).

Frozen sections (6 μm) of the samples were used to detect the donor cells and the differentiation status by examination under a fluorescence microscope. After fixation with acetone and blocking with PBS containing 5% rabbit serum, anti-CD31 or anti-desmin (Bio-Science Products AG, Switzerland) antibodies was used as the first antibody, and rat anti-mouse IgG antibody conjugated with tetramethylrhodamine isothiocyanate (T4280, Sigma, Missouri, USA) and goat anti-mouse IgG antibody conjugated with rhodamine (M116, Leinco Technology, Inc., MO, USA) were used as the second antibody, respectively.

Results

Single-cell marking of 9-15c cells

9-15c cells are mesenchymal stem cells [23,24] capable of differentiating into cardiomyocytes in vitro with the use of 5-azacytidine. To determine if cardiomyocytes were generated from committed cardiac precursor cells or uncommitted stem cells during the differentiation of 9-15c cells, we carried out a single-cell marking experiment. Following retrovirus-mediated EGFP gene infection, a single EGFP-labeled cell could be detected at Day 1 after infection (Figs. 1A, B). The fate of

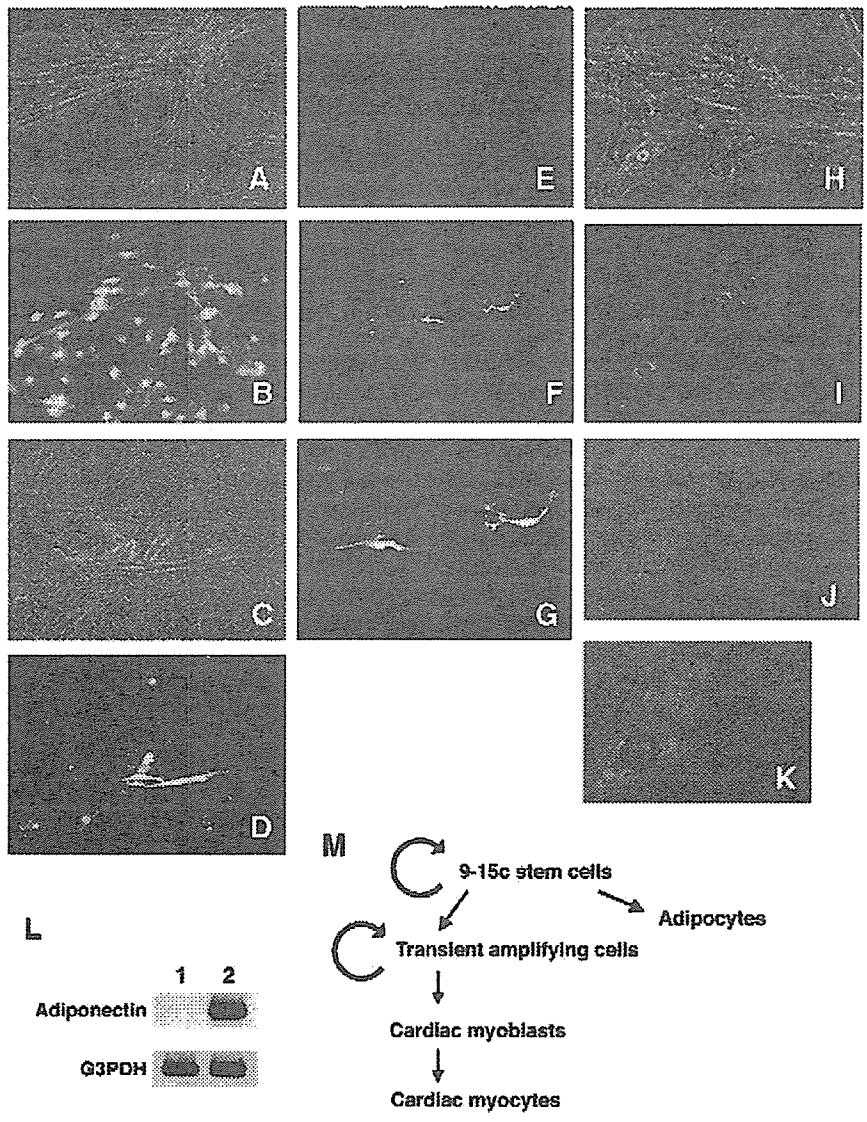

Fig. 2 – Bipotency, i.e., cardiomyogenic and adipogenic differentiation, of single cell-derived cells. Single-cell-derived 9-15c cells marked by EGFP exhibited cardiomyogenic and adipogenic differentiation after exposure to 5-azacytidine. (A–B) Cardiomyogenic and undifferentiated EGFP-marked, single-cell-derived 9-15c cells; (C–G) Cardiomyogenic differentiation of EGFP-marked, single-cell-derived 9-15c cells; (H–J) Cardiomyogenic and adipogenic differentiation of EGFP-marked, single-cell-derived 9-15c cells. (A, C, E, H, J) Phase contrast photomicrographs; (B, D, F, G, I) fluorescent photomicrographs. (K) Enlargement of the panel J. (L) RT-PCR analysis of the adiponectin and G3PDH genes in 9-15c cells at the growing phase without any treatment (lane 1) and 4 weeks after exposure to 5-azacytidine (lane 2). (M) Scheme of 9-15c cell differentiation.

retrovirally tagged 9-15c cells could be traced by monitoring EGFP throughout the differentiation process after exposure to 5-azacytidine. Seven days later, the EGFP-positive, single-cell-derived cells were clustered (Figs. 1C, D). Four weeks after 5-azacytidine treatment, the EGFP-positive cells were examined for differentiated phenotypes. We identified beating cells as cardiomyocytes and oil-red-positive cells as adipocytes. Three kinds of cell populations were observed: a) a cell population in which cardiomyocytes and undifferentiated stem cells were EGFP-positive (Figs. 2A, B); b) a cell population in which all the EGFP-positive cells were cardiomyocytes (Figs. 2C–G); c) a cell population in which cardiomyocytes, adipocytes and undifferentiated stem cells were EGFP-positive (Figs. 2H–K). RT-PCR analysis shows that these cells express adiponectin (Fig. 2L), suggesting the presence of adipocytes among the differentiated population. These results imply that cardiomyocytes are generated from uncommitted stem cells (Fig. 2M).

9-15c multipotent cells were preferentially destined to generate cardiomyocytes by forced expression of transcription factors Csx/Nkx2.5 and GATA4

In order to elucidate the roles of Csx/Nkx2.5 and GATA4 in 9-15c cell differentiation, we infected 9-15c cells with retroviruses carrying Csx/Nkx2.5 and GATA4. We detected Csx/Nkx2.5 and GATA4 gene expression in the infected cell by RT-PCR and Western blotting (Figs. 3A and B). GATA4 gene was originally expressed in 9-15c; we detected the GATA4 transgene with specific primers, but not the endogenous GATA4 gene (Fig. 3A).

Four weeks after the induction of differentiation by 5-azacytidine treatment, we examined the efficiency of cardiomyogenic differentiation or the expression of cardiomyogenic markers. The expression of the ANP and cTnI genes was up-regulated in 9-15c cells overexpressing Csx/Nkx2.5 and GATA4 (9-15c-CG cells) compared to the uninfected 9-15c cells (Fig. 3C, lanes 5 and 9). When 9-15c-CG cells were treated with PDGF and retinoic acid on dishes coated with fibronectin in addition to 5-azacytidine, the expression of the ANP and cTnI gene was further up-regulated (Fig. 3C, lane 10).

Cell implantation into immunodeficient mice

To investigate whether 9-15c-CG cells differentiate in vivo, the cells treated with 10 μM 5-azacytidine for 24 h were injected into immunodeficient mice (Figs. 4A–F). The donor cells clearly formed striated muscles without a branched structure as well as undifferentiated cells 81 days after implantation. The implanted 9-15c-CG cells clearly expressed desmin (Fig. 4G). The grafted cells also generated neovascularization near the injected site 1 month after injection; the EGFP-positive donor cells could be identified as the endothelium of these vessels (Fig. 4H). Immunohistochemistry with an antibody against CD31, a marker for endothelium, confirmed that the donor cells of the newly formed vessels had differentiated into endothelium (Fig. 4Hb). Engrafted donor cells appeared to maintain the characteristics of stem cells, that is, they continued to produce progeny, i.e., differentiated endothelial cells in this case.

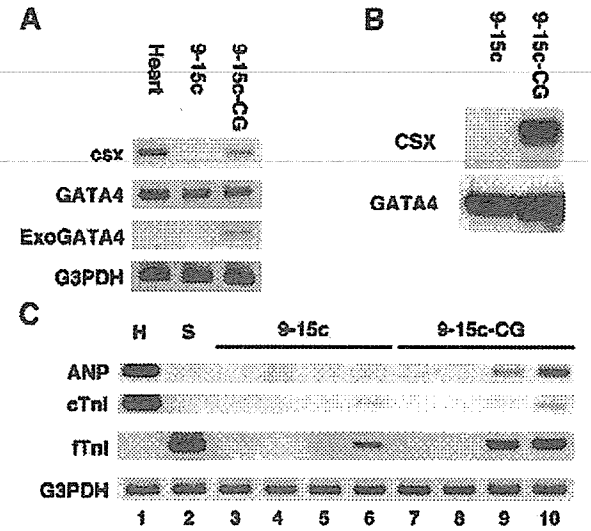

Fig. 3 – Expression of cardiomyocyte-specific or associated genes in 9-15c cells. A: RT-PCR analysis of the Csx, GATA4, exogeneous GATA4 and G3PDH genes (from top to bottom) in adult mouse heart, 9-15c cells and 9-15c cells overexpressing the Csx and GATA4 genes (9-15c-CG cells). B: Western blotting analysis of the Csx and GATA4 proteins in 9-15c cells and 9-15c-CG cells. C: RT-PCR analysis of the ANP, cTnI, and G3PDH genes (from top to bottom) in 9-15c cells (lanes 3–6) and 9-15c-CG cells (lanes 7–10). 9-15c cells (lane 3) and 9-15c-CG cells (lane 7) were cultured without any treatment (lanes 4 and 8) or with exposure to 5-azacytidine alone (lanes 5 and 9), or 5-azacytidine, PDGF, retinoic acid, and fibronectin coating on a dish (lanes 6 and 10) for 4 weeks. Heart (lane 1: H) and skeletal muscle (lane 2: S) served as controls.

Enhancement of cardiomyogenic differentiation by the co-cultivation with cardiomyocytes

We co-cultured EGFP-labeled 9-15c-CG cells with cardiomyocytes of fetal mice in vitro. Four weeks after 5-azacytidine treatment, EGFP-positive beating cardiomyocytes were increased (Figs. 5A, B). To determine whether factors secreted from the cultured cardiomyocytes promoted cardiomyocytic differentiation, 9-15c cells and 9-15c-CG cells were cultured in growth medium supplemented with conditioned medium from cardiomyocyte cultures. The expression of the ANP and cTnI genes was up-regulated in both 9-15c cells and 9-15c-CG cells with exposure to the conditioned medium of cardiomyocyte cultures (Fig. 5C, lanes 3 and 7). Furthermore, treatment with PDGF and retinoic acid, and fibronectin coating on a dish enhanced cardiomyogenic marker expression in both 9-15c cells and 9-15c-CG cells (Fig. 5C, lanes 4 and 8).

Discussion

Different models arise from different conceptions of the MSCs as in hematopoietic stem cells' differentiation [28,29]. A hierarchical model of MSCs has been proposed based on the in vitro differentiation potential of human MSCs as observed

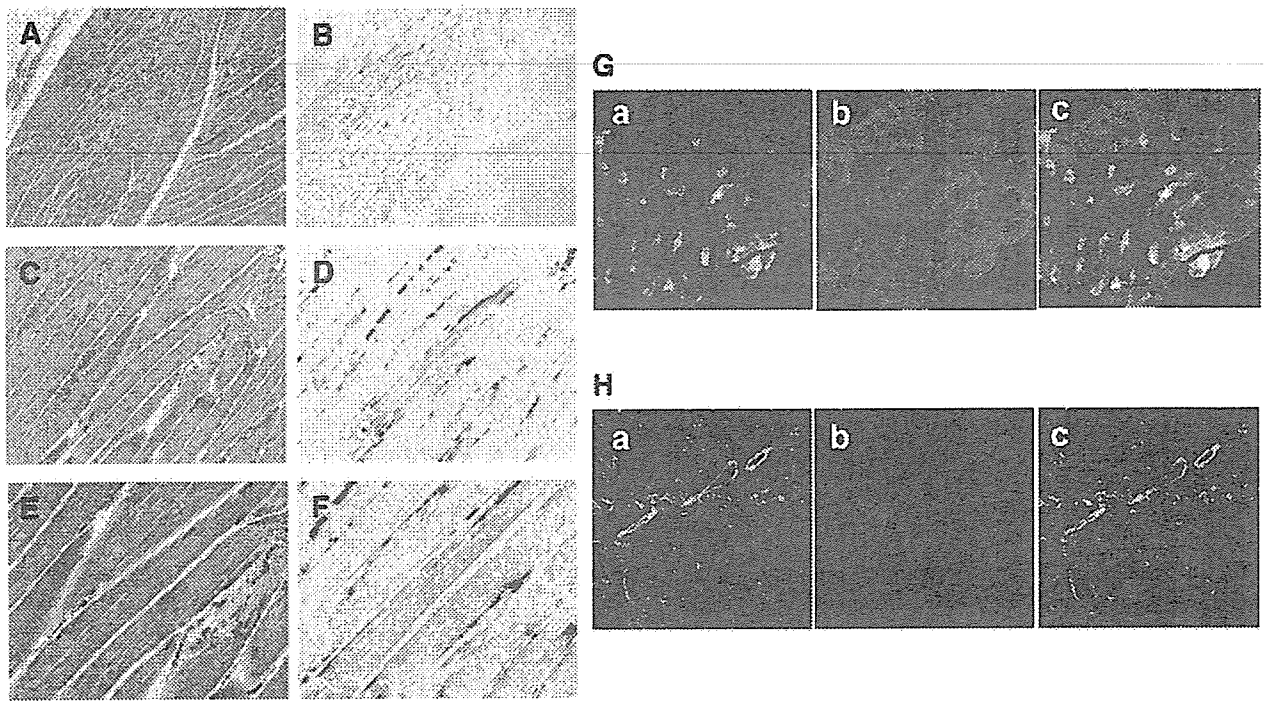

Fig. 4 – Myogenic differentiation of the EGFP-labeled 9-15c-CG cells into the quadriceps femoris muscle. EGFP-labeled 9-15c-CG cells could be recognized morphologically as the skeletal myocytes in the quadriceps femoris muscle 3 months after transplantation (A, C, E: HE staining; B, D, F: immunohistochemistry using anti-GFP antibody). The EGFP-positive donor cells exhibited skeletal myocyte-specific features such as multiple nuclei in the periphery of the cells and striation. Generation of myocytes (G) and endothelial cells (H) by the EGFP-labeled 9-15c cells. The injected donor 9-15c cells labeled with EGFP were detected by green fluorescence. (Ga, Ha) Green fluorescence of EGFP-labeled donor cells. (Gb, Hb) Immunohistochemistry for desmin (Gb, red) or CD31 (Hb, red). (Gc, Hc) The merged images of green fluorescence of injected 9-15c cells and rhodamine of desmin or CD31 clearly demonstrated that 9-15c cells differentiated into myocytes or endothelium. A–F: Longitudinal section; G, H: Cross section.

by clonal analysis [30]. In the present study using single-cell marking, we found that 9-15c cells in culture consisted of a mixture of at least three types of cells, i.e., cardiac myoblasts, cardiac progenitors and multipotent stem cells. Cardiac myoblasts are defined as cells which can differentiate into only cardiac myocytes and have low proliferative potential; cardiac progenitors have proliferative capability and the ability to become cardiomyocytes; multipotent stem cells have both proliferative capability and multipotency. The results obtained in the present study suggest that 9-15c cells are stochastically committed toward the cardiac lineage, and that following this commitment they proliferate as transient amplifying cells and differentiate into cardiac myocytes through the differentiation process, and the hierarchical model applies in the case of 9-15c multipotent cells.

In the present study, we used 5-azacytidine to induce differentiation. 5-azacytidine is a cytosine analog that causes extensive demethylation. The demethylation is attributable to covalent binding of DNA methyltransferase to 5-azacytidine in the DNA [31], with the subsequent reduction of enzyme activity in cells resulting in random loss of methylation at many sites in the genome. Previously, it has been thought that 5-azacytidine activates cardiomyogenic master genes, such as Nkx2.5/Csx, GATA4, and MEF-2C, leading to stochastic transdifferentiation of MSCs into cardiomyocytes [32,33]. This concept is difficult to account for the existence of cardiac progenitors and multipotent stem cells we identified, and we propose two possibilities how 5-azacytidine works. First, treatment of 5-azacytidine modulates heterochromatin remodeling and leads to dedifferentiation of 9-15c cells. Second, 9-15c cells are stochastically committed toward the cardiac lineage, being independent of treatment of 5-azacytidine. At this time we cannot conclude which is feasible, but it is certain cardiomyocytes are not only transdifferentiated by treatment of 5-azacytidine.

Csx/Nkx2.5 and GATA4 are two cardiac-enriched transcription factors that are expressed in precardiac mesoderm from the very early developmental stage [34,35]. In the present study, increased frequency of cardiomyogenic differentiation of 9-15c cells was successfully achieved in vitro by forced expression of Csx/Nkx2.5 and GATA4. These results are consistent with a report showing that both Csx/Nkx2.5 and GATA4 are required for the cardiac differentiation of P19CL6 cells derived from embryonic teratocarcinoma cells [36]. Cardiomyogenic differentiation, however, could proceed only after treatment with 5-azacytidine in our experimental setting, implying that Csx/Nkx2.5 and GATA4 are required but not sufficient for cardiac differentiation. Unknown factors

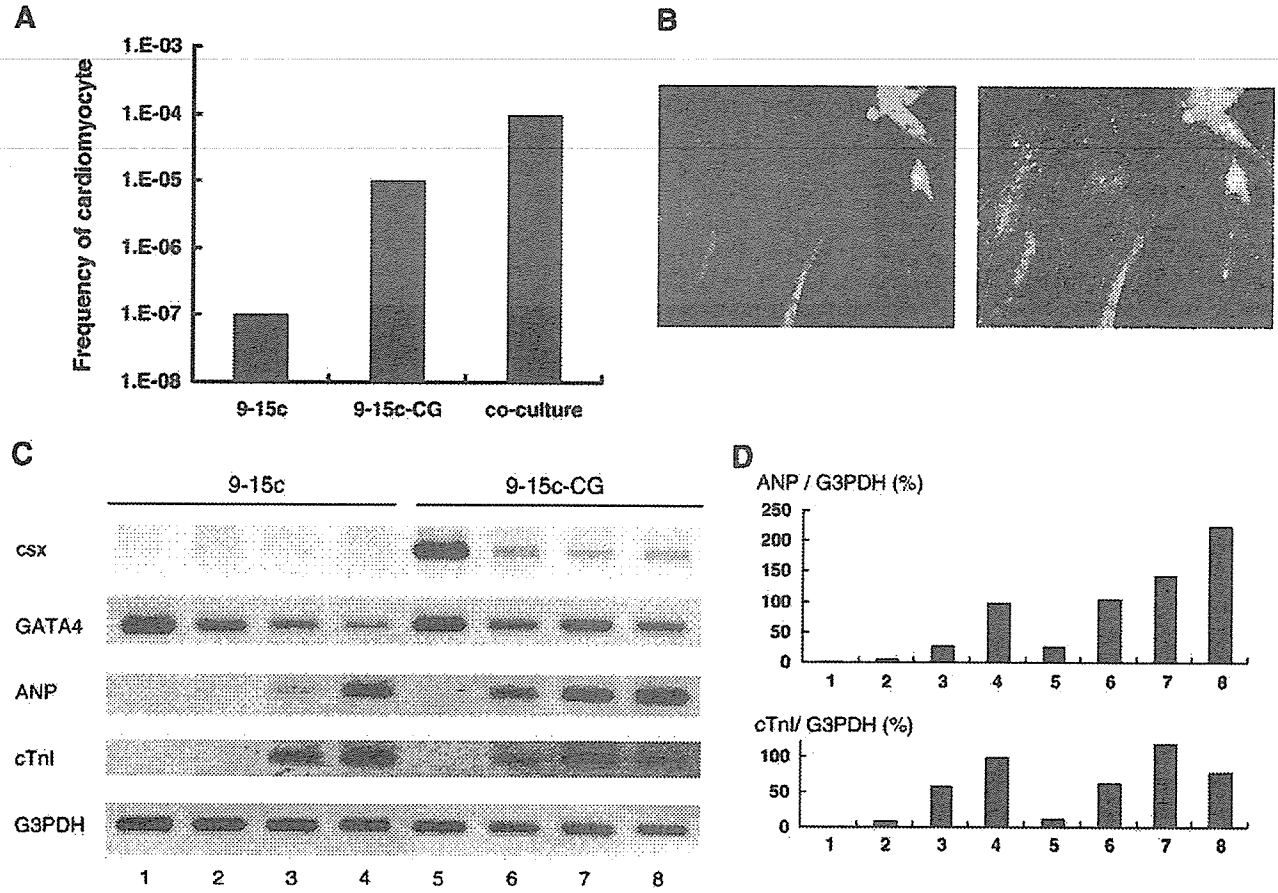

Fig. 5 – Enhancement of cardiomyogenic differentiation of 9-15c cells by co-cultivation with murine fetal cardiomyocytes. A: Frequencies of cardiomyogenic differentiation in 9-15c cells, 9-15c cells overexpressing the Csx and GATA4 genes (9-15c-CG cells), and 9-15c-CG cells co-cultured with murine fetal 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.

Acknowledgments

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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.

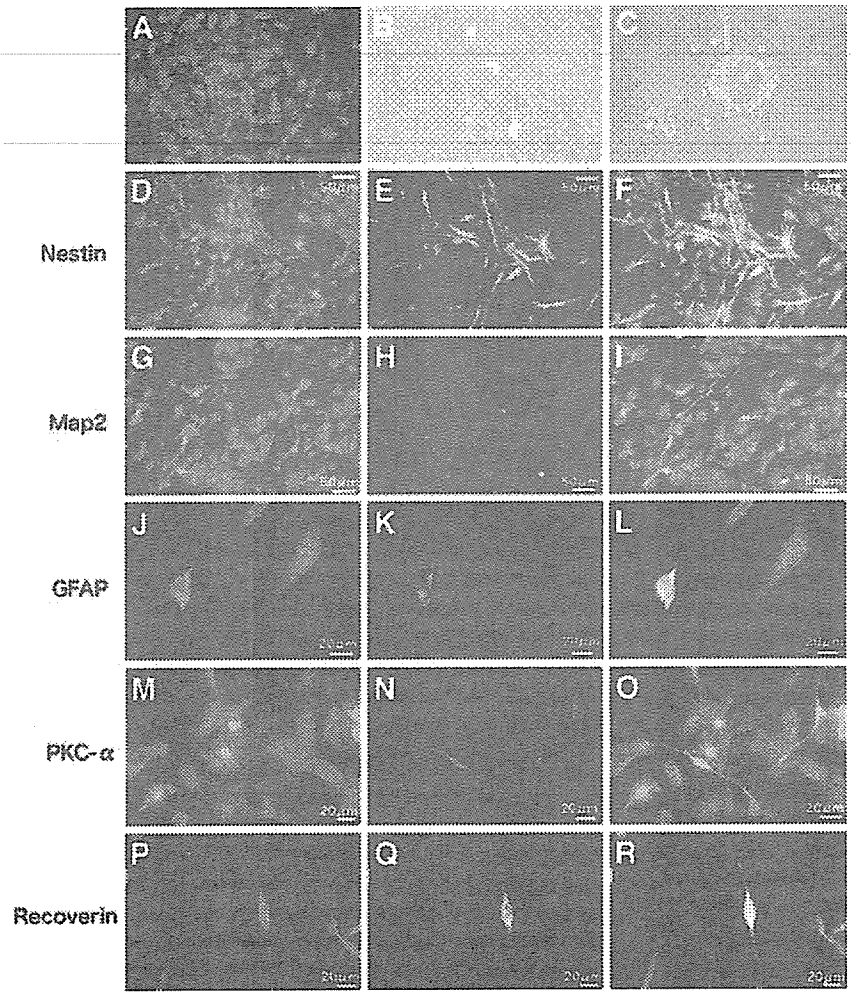

Figure 1. Differentiation and characterization of marrow stromal cell (MSCs) in vitro. Undifferentiated GFP$^+$ MSCs grown in Dulbecco's modified Eagle's medium with 10% fetal bovine serum, viewed under fluorescein isothiocyanate illumination (**A**). MSCs cultured in serum-free medium with brain-derived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

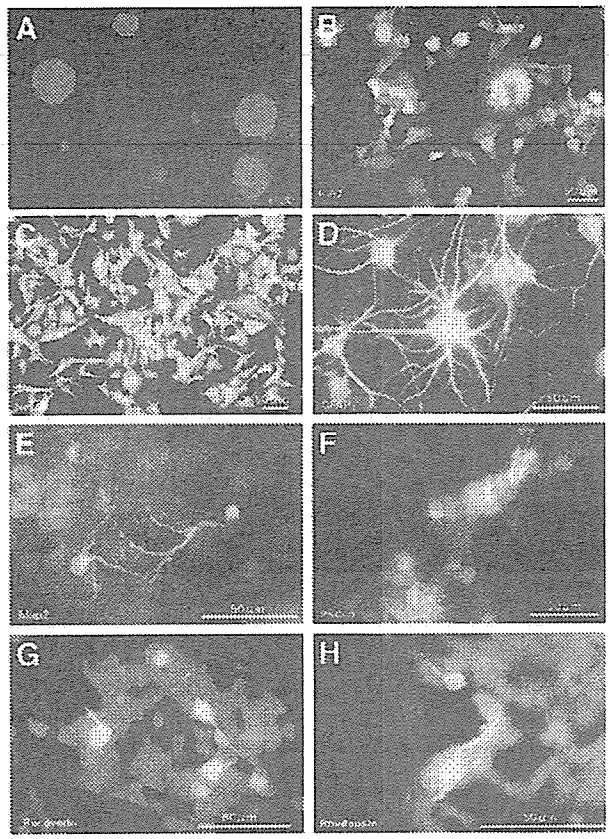

Figure 2. Differentiation and characterization of retinal progenitor cell (RPCs) in vitro. RPCs formed green fluorescent protein-positive neurospheres (**A**). RPCs cultured in the absence of epidermal growth factor and in the presence of 10% fetal bovine serum for 1 (**B, C**) or 14 (**D–H**) days. 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.

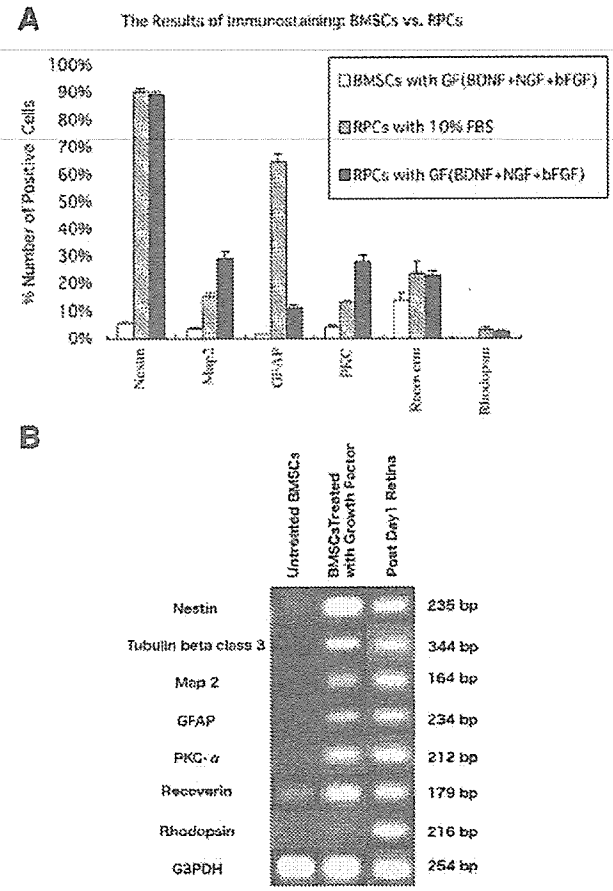

Figure 3. Comparison of BMSCs and RPCs. (**A**): The number of cells differentiated into retinal cells: comparison of marrow stromal cell (MSCs) and RPCs. In this study, nestin-positive cells were counted at day 1, and other markers cells were counted at 2 weeks after treatment. (**B**): Effect of BDNF, 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.

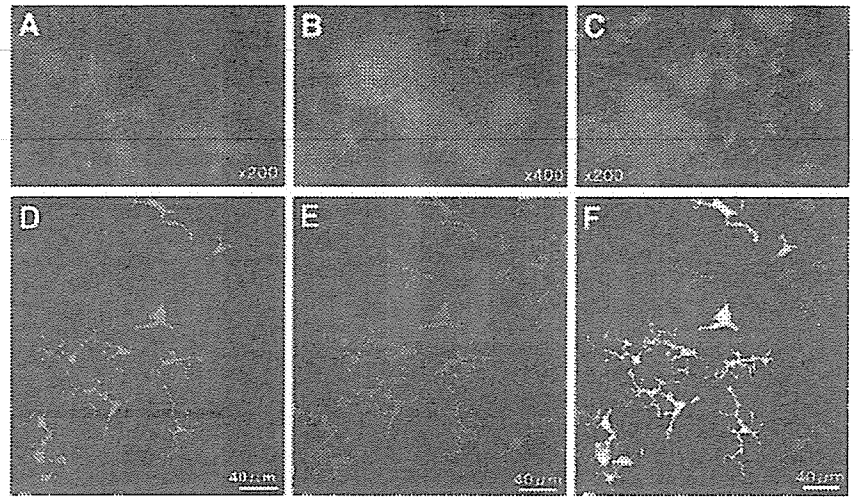

Figure 4. Macrophages differentiated into microglia after transplantation to explanted retinas. Rho−/− mice retina at 3 (**A**) and 7 (**B, C**) days. Macrophages migrated into retina and morphologically changed their shape to that resembling microglia (**A–C**). Confocal (**D–F**) images seen at 1 week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

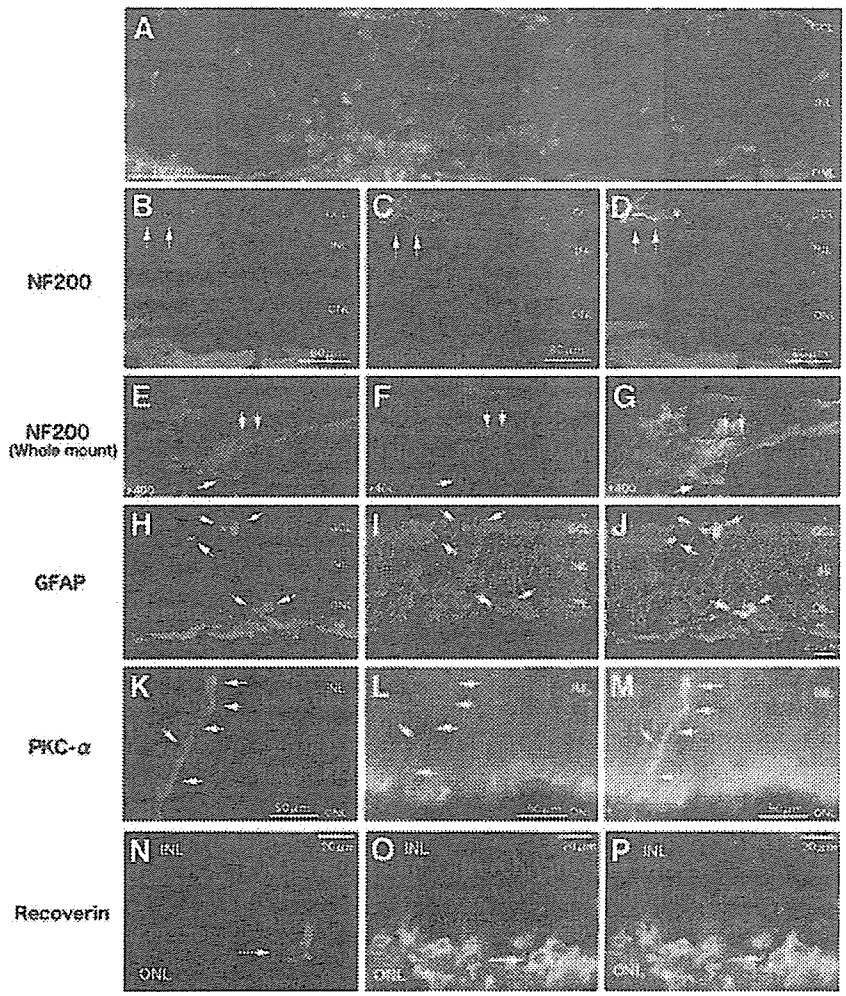

Figure 5. Migration and differentiation of pretreated marrow stromal cell (MSCs) into explanted retinas of rho−/− mice. A large number of MSCs migrated into explanted retinas of rho−/− mice (**A**). Epi-fluorescent (**K–P**) and confocal (**B–J**) images of the expression of neural and photoreceptor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

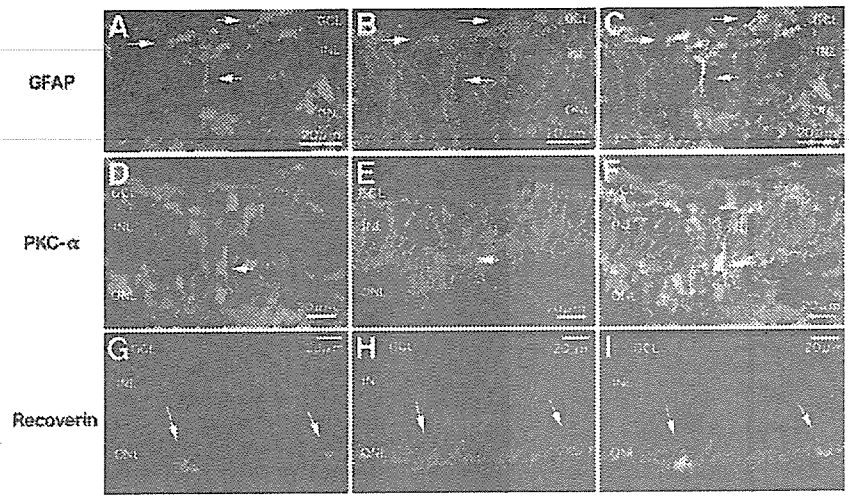

Figure 6. Migration and differentiation of pretreated retinal progenitor cells (RPCs) into explanted retinas of rho−/− mice. Confocal images of the expression of neural and photoreceptor markers by RPCs grafting to explanted retinas of rho−/− mice. seen at 1 week after grafting; constitutive green fluorescent 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.

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DISCLOSURES

The authors indicate no potential conflicts of interest.

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