Noggin induces human bone marrow-derived mesenchymal stem cells to differentiate into neural and photoreceptor cells

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The present study was undertaken to explore the effect of noggin on neuronal differentiating potential of human bone marrow-derived mesenchymal stem cells (hBMMSCs) in vitro so as to provide a means of alleviating retinal degeneration. A green fluorescent protein-tagged noggin gene was transferred into adult hBMMSCs or induce hBMMSCs with classical inducer, epidermal growth factor (EGF). Neurons were observed as early as 48 h after transduction of hBMMSCs with a noggin adenoviral vector. Differentiation peaked by 10 days in culture, and these differentiated cells expressed multiple markers including rhodopsin (18.4 ± 1.5% of cells), chx10 (4.8 ± 0.6%), nestin (4.2 ± 0.8%), and Nrl (3.7 ± 0.4%), as verified by immunofluorescence staining. Noggin-transduced cells produced more photoreceptor cells than non-transduced cells, suggesting that noggin has the ability to induce hBMMSCs to trans-differentiate into photoreceptor cells. In contrast, induction with EGF for 10 days led to lower levels of rhodopsin and chx10, and undetectable levels of Nrl and Nestin. These findings suggested noggin-transduced hBMMSCs produced more photoreceptor cells than EGF-induced cells. It is suggested that the present protocol has application in cell replacement therapy for patients suffering from photoreceptor cell loss.

Keywords: Epidermal growth factor, Mesenchymal stem cells, Noggin, Photoreceptor, Trans-differentiation

Retinal degeneration (RD) is characterized by photoreceptor cell degeneration, resulting in visual loss.[1-3]. Human bone marrow-derived mesenchymal stem cells (hBMMSCs) are easily isolated and can self-renew, proliferate, and differentiate into multiple lineages, including neurons, in vitro[4-8]. Thus, hBMMSCs represent a promising source of stem cells for autologous treatments of neurodegenerative diseases.

A wide range of chemical induction treatments and growth factors have been used to induce the trans-differentiation of BMSCs into neural-like cells in vitro[9-12]. However, it has also been suggested that the induction of morphological changes and immunoreactivity for neural markers in cultured mesenchymal stem cells (MSCs) might be associated with cellular toxicity, cell shrinkage, or cytoskeletal changes[13], and that the differentiation efficiency is unstable[6,8,10]. Novel strategies that cause minimal cellular stress therefore need to be developed.

Noggin is a 32-kDa glycoprotein neural inducer secreted by the Spemann organizer in Xenopus embryos, and has been shown to be important for the dorsal development of embryos[14,15]. Noggin participates in neurogenesis by antagonizing bone morphogenetic protein 2/4[16-19]. Kohyama et al.[20] used noggin in conjunction with other growth factors to generate neurons from subclones of bone marrow cultures obtained from adult female C3H/He mice. Lamba et al.[21] also demonstrated that noggin combined with other factors induced human embryonic stem cells to differentiate into retinal neurons. These studies indicate that noggin induction provides a promising means of driving hBMMSCs' trans-differentiation into neurons and photoreceptor cells.

In this study, we transferred an adeno viral-mediated noggin gene into hBMMSCs and examined the infection efficiency of the adenovirus, as well as the subsequent morphological changes and trans-differentiation potential. The potential of noggin to induce trans-differentiation into neurons and photoreceptor cells was compared with that of the known inducer, epidermal growth factor (EGF).

Materials and Methods

Human bone marrow-derived mesenchymal stem cells (hBMMSCs) were a kind gift from Dr Dai...
Primers for the noggin gene were designed from established GenBank sequences (NC_000077): 5’CCCGGGCTTTATGG CTACTTC 3’ and 5’TGCACAGACTTGGA TTCACA 3’. The cDNA was synthesized and amplified using ReverTra Ace-a and SYBR Green Realtime PCR Master Mix-Plus (Toyobo Nunohiki, Japan) under the following conditions: reverse transcription at 42°C for 10 min, 30°C for 20 min, 99°C for 5 min, 4°C for 5 min, initial PCR activation at 95°C for 5 min followed by 30 cycles of denaturation at 94°C for 30 sec, annealing at 53°C for 30 sec, and extension at 72°C for 30 sec. A final extension step at 72°C for 10 min was included before samples were cooled to 4°C.

To confirm the noggin protein level, hBMMSCs were fixed with 4% paraformaldehyde for 40 min at room temperature, washed three times in PBS and incubated in Tris-buffered saline and 0.3% H2O2. The cells were then incubated with rabbit anti-human noggin IgG (0.5 µg µl−1; ADI, San Antonio, USA) for 24 h at 4°C, followed by biotinylated goat anti-rabbit secondary antibody (1:200) and avidin-biotin peroxidase complex (1:200) for 2 h at room temperature.

Confluent hBMMSC cultures were obtained and passaged three times, then trypsinized with 0.05% trypsin-EDTA (Gibco, Rockville, MD) to form single cell suspension. hBMMSCs were transferred to 24-well plates containing MSC culture medium with EGF (100 ng ml−1)12. Cells were cultured for an additional 10 days, and processed in the same manner as the transduced cells.

Cells were fixed as above, washed, and incubated with 5% (w/v) BSA, 10% FBS (v/v), and 0.1% (v/v) Triton X-100 in PBS for 1 h at room temperature. Cells were then incubated with anti-chx10 (1:100; Boster), anti-glial fibrillary acid protein (GFAP, 1:100; Zhongshan, Beijing, China), anti-rhodopsin (opsin, 1:500; Sigma, Missouri, USA), anti-neural retina leucine zipper (Nrl, 1:300; Santa Cruz Biotech., Santa Cruz, USA), and anti-nestin (1:300, Boster) in 1% BSA and 0.1% Triton 100 for 24 h at 4°C. After washing, sections were incubated with the corresponding secondary antibodies at 1:200 dilutions for 2 h at room temperature, followed by the Cy3 conjugated avidin-biotin complex for 0.5 h at 37°C.

Statistical analysis was performed using SPSS 12.0 software. The data were expressed as the mean ± standard error of mean (SEM). Differences between groups were determined by a one-way analysis of
variance (ANOVA) followed by a Scheffe post hoc test for multiple comparisons. P values ≤ 0.05 were considered statistically significant.

Results

When hBMMSCs were cultured in control medium for 10 days, most of the cells remained largely undifferentiated (Fig. 1B). Expression of GFP in infected hBMMSCs was initially checked after 48 h, and it reached peak level after 10 days. Quantification in five transduction experiments showed that with a MOI of 1, 15.5 ± 1.1% of hBMMSCs were positive for GFP expression after 10 days of the initial vector addition. This increased to 22.7 ± 1.9% with a MOI of 10 and reached a maximum of 69.4 ± 1.9% with a MOI of 50 (Fig. 1C-E). MOIs over 100 can cause serious pathologies in the cultures and an obvious decline in cell viability. An MOI of 50 was, therefore, used for infection in this study.

The presence of noggin mRNA was confirmed in hBMMSCs and HEK293 cells after the fourth passage using RT-PCR. Noggin fragments were not observed in the control cells infected with the pAdTrack-GFP vector (Fig. 1F). Using an MOI of 50, immunocytochemical staining showed the presence of noggin-positive cells in hBMMSC cultures 48 h post-infection with pAdTrack-GFP-noggin (Fig. 1G), and their density had increased significantly by day 10 (Fig. 1H). Thus, pAdTrack-GFP-noggin effectively maintained GFP and, more importantly, noggin expression over extended periods of time and subsequent passages.

After transduction with pAdTrack-GFP-noggin, hBMMSCs showed gradual but dramatic changes in morphology that in many ways resembled neuronal cell development in vivo. Few differentiated cells were observed after 48 h of post-infection, but differentiated cells were increased at day 4, showing relatively few, simple processes. These differentiated neurons at day 10 presented at least three main presumptive cell classes based on the range of dendritic shapes. The first cell class, representing approximately 25% of the differentiated cells, was characterized by small to medium size somata and numerous dendritic branches forming a comparatively compact dendritic arbor (Fig. 2A). The second cell class, representing approximately 33% of cells, was typified by comparatively small bipolar cell bodies with a small number (2–4) of apical cell processes (Fig. 2B), suggestive of a type of retinal photoreceptor. The third cell class, representing approximately 10% of cells, generally had medium to large somata with numerous processes and comparatively large and diffuse dendritic arbors (Fig. 2F). In addition to these three broad classes, there were various intermediate dendritic shapes and variations. In contrast, hBMMSCs infected with the control vector (pAdTrack-GFP) maintained an hBMMSC morphology throughout the culture period, identical to hBMMSCs cultured in control medium.

At day 10, many of the cells were still at a stage of active neurite growth. This was indicated by the presence of numerous presumptive dendritic ‘spines’ or growth processes (Fig. 3C). In some cases there was clear evidence of active presumptive growth cones (Fig. 3D). The active growth process in Fig. 3C indicated a form of directed growth and non-specific growth patterns. However, qualitatively some cells appeared to possess the same number of ‘spines’. For example, the cells were seen having small spines and possibly less active growth (Fig. 3A, B) that indicated a more mature stage of development. Some cells had well developed arbor without spines (see also Fig. 2G-J; Fig. 3E). These results suggested that some cells in the culture were maturing at the same rate and might be specific to particular phenotypes. The cells were rarely encountered and had an extensive growth of process arising from the soma (Fig. 3F), however, the growth appeared to be relatively non-orientated that suggested appearance of an immature oligodendrocyte.

Phenotypic characteristics of the noggin-induced hBMMSCs were examined after 10 d in culture using five different antibodies typically used to define cell populations. A pAdTrack-GFP-noggin MOI of 50 produced cultures containing cells labeled with rhodopsin (18.4 ± 1.5%; a photoreceptor-specific cell marker), chx10 (4.8 ± 0.6%; an ocular-specific marker of proliferating retinal progenitor cells), nestin (4.2 ± 0.8%; a neuronal and glial progenitor marker), and Nrl (3.7 ± 0.4%; a rod-specific transcription factor) (Fig. 4). None of the cells showed positive staining for GFAP (an astrocytic cell marker). These results suggested that around 25% of cells differentiated into neurons, neural or glial progenitors, and around 27% of cells became photoreceptor cells, proliferating retinal progenitor cells, or cells expressing a rod-specific transcription factor. In contrast, hBMMSCs infected with the control vector (pAdTrack-GFP) were negative for these specific makers.
Human bone marrow mesenchymal stem cells (hBMMSCs) transduced with pAdTrack-GFP-noggin. (A) A schematic diagram showing the adenoviral expression vector; (B) hBMMSCs in control medium for 10 days, largely undifferentiated; (C), (D) Expression of green fluorescent protein (GFP) in hBMMSCs at multiplicities of infection (MOI) of 10 and 50, respectively, 10 days post-infection; (E) Relationship between the percentage of GFP-positive cells and MOI after 10 days of infection; (F) Reverse transcription-polymerase chain reaction confirmed the presence of the noggin gene (371 bp). Lane 1: marker; lanes 2 and 3: hBMMSCs after 10 days infection and HEK293 cells after 5 days infection; lanes 4 and 5: hBMMSCs and HEK293 cells infected with control vector (pAdTrack-GFP); (G) Noggin-positive hBMMSCs identified in 48 h post-infection cultures; and (H) Intensity of noggin staining increased after 10 days of infection. [Scale bars = 50 μm].
Control hBMMSCs with no treatment showed no morphological changes. When hBMMSCs were induced with EGF for 10 days, a small number of cells developed neuron-like appendages, while others shrank and did not take on a typical neural cell morphology. The ratios of hBMMSCs expressing specific antigens were markedly different from those seen after noggin induction. EGF induction produced only

Fig. 2—Photomicrographs of cultured cells illustrating three main cell classes. (A–D)- Class 1 (small to medium) with a comparatively compact dendritic arbor represented around 25% of cells (scale bar refers to all micrographs); (E)- Class 2 (small polar cell body) represented about 33% of the cells (possibly retinal photoreceptors); (F)- Class 3 cells (medium to large) represented about 10% of cells; and (G–J)-Various intermediate dendritic shapes or variations were seen that resembled neuronal cells.
Fig. 3—Cell processes in culture. (A, B)- Differentiating cells were seen in close contact with neighboring undifferentiated cells (U) and appeared to form numerous contacts (arrows). This arrangement suggests that undifferentiated cells may limit the growth of the differentiating cells, possibly through contact inhibition [inserts show the same cells at lower magnification; scale bars for all micrographs and inserts = 25 μm]; (C)- Many of the cells had numerous presumptive dendritic spines (arrows, see also A and B) along the arbor indicating active growth or spinogenesis; (D)- The cell has a well defined growth cone at the end of a long process indicating active growth and extension; (E)- Some cells assumed a relatively mature morphology and shape. Note that this cell does not have dendritic spines and resembles ventral thalamic cells seen in vivo; and (F)- This cell type has non-orientated diffuse growth pattern of its processes and was seen infrequently.
rhodopsin- or chx10-immunopositive cells, but were negative for Nrl, and Nestin labeling. The differentiation ratios were also significantly lower than those seen in the noggin-transduced cell cultures (rhodopsin, 5.2 ± 0.8% versus 18.4 ± 1.5% (P < 0.01); chx10, 1.2 ± 0.3% versus 4.8 ± 0.6% respectively (P < 0.05)).

Fig. 4—Phenotypic cell markers expressed by transduced human bone marrow mesenchymal stem cells (hBMMSCs). Cultured hBMMSCs transduced with pAdTrack-GFP-noggin (multiplicity of infection of 50) were stained with several different antibodies after 10 days in culture. The micrographs in each row show green fluorescent protein (GFP) fluorescence, antibody staining and merged images. The antibodies used and the associated percentage of labeled cells were-(A)- rhodopsin 18.4 ± 1.5%; (B)- chx10, 4.8 ± 0.6%; (C)- nestin 4.2 ± 0.8%; and (D)- Nrl 3.7 ± 0.4%. [Scale bars = 50 μm].
Discussion

hBMMSCs are potentially exciting candidates for replacement or transplant therapy. Induced BMMSCs have been successfully grafted into the subretinal space or brain where they express neuronal markers without associated neural pathology or teratoma formation. Adenoviral vectors carrying foreign DNA and GFP combined with hBMMSCs as carrier cells can provide an ideal method for treating RD defects. In this study, since hBMMSCs infected with pdmTrack-GFP-noggin could be easily labeled and isolated, this could provide an ideal method for producing large number of labeled hBMMSCs containing high levels of noggin for further research into the effects of noggin on hBMMSCs, both in vivo and in vitro.

It has previously been suggested that neuron-like morphology of hBMMSCs in culture may be due to cell shrinkage, loss of focal contacts, and the disruption of the actin cytoskeleton, which may also be related to a particularly high cell death rate in the cultures. In addition, the localization and expression of neuronal markers in some cases are abnormal. However, we noted that the morphological characteristics of hBMMSCs transduced with pdmTrack-GFP-noggin changed gradually, resembling neuronal cell development in vivo in many respects. Previous studies suggest that neurons follow a fundamental growth strategy, with three principle stages: simple growth followed by a period of exuberance, ending with a dramatic pruning of dendritic and axonal arbors. A high degree of variability between cells with regard to maturation, size, appendage number and complexity at any particular point in time also seem to be common features, even within relatively homogeneous cell populations. Therefore, it was not surprising that noggin-induced cultures also appeared to show a range of morphological maturity, based on intrinsic growth principles. In vivo analysis has shown that the exuberant and pruning stages have a duration of around 15–30 days, which is in accordance with the time-course seen in this study. Phenotypic gene expression started around day 3, and is in agreement with the degree of morphological maturity seen at day 4. Different protocols (i.e., induction and number of passages) and MSCs from different species show a range of time-related changes in morphology and maturity in culture, ranging from 72 h to 4–5 weeks.

The potential of noggin-induced hBMMSCs to trans-differentiate into neurons and photoreceptor cells was evaluated using specific markers. We found that, in hBMMSCs infected with noggin for 10 days, about 20% of the cells assumed a retinal (rhodopsin, Nrl) phenotype, and that at least a further 5% were committed to a neural lineage (neuronal or glial), but had not undergone differentiation (Chx10 and Nestin, neural progenitor markers). None of the cells showed positive staining for GFAP, an astrocytic cell marker. This is consistent with other reports suggesting that noggin induction promotes stem cell differentiation into neurons, and inhibits astrocyte differentiation. To the best of our knowledge, this is the first time that noggin transduction has been reported to promote hBMMSC differentiation into photoreceptor cells. We found that cells genetically modified by a noggin-expressing adenoviral vector produced more obvious photoreceptor cells than non-transduced cells, suggesting that noggin, as a neuronal inducer, has the potential to induce hBMMSCs to trans-differentiation into photoreceptor cells.

Regarding the protocol for trans-differentiating MSCs, Kicic et al. have demonstrated that 20–30% of adult rat CD90+ MSCs induced with activin A, taurine, and EGF expressed photoreceptor-specific markers. Activin A, taurine, and EGF have similar trans-differentiation potentials. Thus, the ratios of hBMMSCs differentiating into neurons and photoreceptors after induction with EGF were compared with those after induction with noggin. We demonstrated that the ratios of differentiated hBMMSCs after induction with EGF for 10 days were markedly different from those seen after noggin induction. EGF induction produced only rhodopsin- (5.2 ± 0.8%) or chx10- (1.2 ± 0.3%) immunopositive cells, and the percentages of differentiated cells were lower than those seen in the noggin-infected cell cultures. This indicated that pdmTrack-GFP-noggin transduction provided a more efficient means of producing photoreceptor cells than EGF induction. Notably, the ratio of hBMMSCs differentiating into photoreceptors after EGF induction was lower than that of adult rat CD90+ MSCs, possibly due to differences in BMMSCs between different species, and the use of different subpopulations of BMMCS.

The aim of these experiments using hBMMSCs is to identify a suitable stem cell/progenitor supply for use in replacement or transplant therapy. The
production of photoreceptor cells in the current study has potentially promising therapeutic implications. However, the long term fate of undifferentiated noggin-transfected cells could pose concerns for future transplantation therapy. Further studies are needed to determine the reason(s) for high proportion of noggin-transfected cells express retinal phenotypic markers, as this may provide a viable methodology for generating cells suitable for cell replacement therapy in patients suffering from photoreceptor loss (e.g., retinitis pigmentosa).

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References