

In Vitro Differentiation of Human Bone Marrow Mesenchymal Stem Cells to Hair Cells Using Growth Factors

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Abstract

Objective: In this study, we attempted to differentiate human bone marrow-derived mesenchymal stem cells (hBMSCs) to auditory hair cells using growth factors. **Methods:** Retinoic acid (RA), basic fibroblast growth factor (bFGF), and epidermal growth factor (EGF) were added to hBMSCs cell culture medium. The cells were evaluated morphologically and the expression of SOX2, POU4F3, MYO7A, and Calretinin at mRNA level and ATOH1 mRNA and protein expression. **Results:** After treatment with the growth factors, the morphology of the cells did not change, but evaluation of gene expression at the mRNA level increased the expression of the ATOH1, SOX2, and POU4F3 markers. Growth factors increased the expression of ATOH1 at the protein level. The expression of calretinin showed decreased and MYO7A no significant change in expression. **Conclusion:** hBMSCs have the potential to differentiate to hair cell-like using the RA, bFGF, and EGF.

Keywords: bone marrow mesenchymal stem cells, hair cell, growth factor.

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INTRODUCTION

The sensorineural hearing loss is one of the most common disabilities worldwide¹. It is often believed that deafness occurs in humans due to the loss or impairment of the function of the hair cells in the cochlea of the ear, when hair cells cannot be regenerated spontaneously; in this case, the treatment of patients with severe to deep deafness is comparatively more difficult². Nowadays, cochlear implants are the only way to restore auditory function in patients with deep deafness. However, despite the best results, normal hearing will not be fully restored³. Therefore, an alternative method is needed to treat profound deafness. Deafness occurs in humans when the hair cells are damaged due to disease or trauma, noise or age. In fish amphibians, and birds but not mammals, it is possible to replace damaged hair cells^{4,5}. Owing to their importance in the functioning of the inner ear, hair cells have attracted more attention. Hair cells are essential for converting sound stimuli to neural signals. The hair cells are the main components of the inner ear that facilitates mechano-electrical transmission to understand the sound⁶. These cells are highly differentiated and have many special characteristics, which make it difficult to regenerate them. Practical methods have not yet been offered for the regeneration of the hair cells. In early 2000, cell transplantation, techniques focused on the reconstruction of the hair cells. However, the migration of the transplanted stem cells to the damaged sensory epithelium of the inner ear occurs rarely⁷.

Stem cell therapy

Although it is important to study embryonic stem cells, there are serious concerns about malignancies associated with these cells. Generation of induced pluripotent stem cell (iPSC) from adult or somatic cells is associated with genetical changes. Genomic changes are a risk factor for mutagenesis and the development of malignant cell transformation⁸. Genetic defects in adult or somatic cell-derived iPSCs not only cause tumorigenicity, but also lead to impairment of differentiation. Further

research is required for the clinical use of these cells⁹. Mesenchymal stem cells are the first candidate for cell therapy, because these cells are located at the target organ, and therefore, have been potential to replace the damaged cells. In addition to the ability to differentiate to functional cell types, these cells release a wide spectrum of differentiating growth factors, cytokines, chemokines, microvesicles or exosomes that facilitated the lateral transmission of organ-protecting signals, and have potent anti-inflammatory immunomodulatory properties to improve the proliferation of endogenous stem cells¹⁰. They are consequently used to repair the tissues.

BMSCs in the treatment of sensorineural hearing loss

BMSCs have been used in a variety of regenerative medical fields¹¹. The use of BMSCs in the inner ear has been studied (Table 1). The fate of autologous BMSCs after intra cochlear transplantation was also investigated. Autologous BMSCs in the cochlea were observed to differentiate to neurons¹². In addition, the transplanted BMSCs migrate to different parts of the cochlea. Consistent findings have been reported regarding the transplantation of the BMSCs¹³. The transplanted BMSCs migrated widely into the spiral ligament, which was used to regenerate the specific fibrocytes in the spiral ligament. These findings suggest that BMSCs can be used for the treatment of sensorineural hearing loss due to the loss of ligand-specific fibrocytes¹⁴. When the source of the cell does not exist in the inner ear, induction of differentiation, for example, induction of differentiation from stem cells to cochlear hair cells is a useful method to restore hair cells through regeneration (Table 1).

Transdifferentiation for hair cell regeneration

Transdifferentiation is defined as the conversion of one differentiated cell type into another²². Transdifferentiation may be achieved in several ways by using extracellular growth factors, individual transcription factors, or combinations of the two. Research showed that BMSCs differentiate into mesodermal cell types and

Table 1. Studies of BMSCs-based therapies for treatment of hearing loss¹⁵⁻²¹.

Study Model	Main results and mechanisms of action of BM-MSCs in hearing loss repair	Ref
Guinea pig	Results show the potential of NI-hBM-MSCs to give rise to replace the lost cochlear cells in hearing loss mammals.	15
Rat	Migration of MSCs into the cochlea was accompanied by the expression of BDNF, systemic delivery of BM-MSCs may be good option for the stem cell treatment of hearing loss.	16
Mice	MSCs engraftment to the cochlea and differentiated into type II and V fibrocyte-like cells, BM-MSCs successfully transplanted into the cochlea of mice and differentiated into fibrocyte-like.	17
Guinea pig	BDNF gene modified BM- MSC could maintain expression for at least 28 days after transplantation into cochlea of drug deafened guinea pigs.	18
Rat	Transplanted BM-MSCs actually invaded the injured area and contributed to the structural reorganization of the injured cochlea, BM-MSCs transplantation improves incomplete hearing recovery.	14
Mice	Demonstrated the homing capability of BM-MSCs to the deafened cochlea, and these cells displayed mature hematopoietic properties, upregulation of SDF-1 expression, mobilize, migrate MSCs to the site of injury.	19
Mongolian gerbil	Survival of transplanted MSCs into the modiolus of the cochlea may result in regeneration of damaged SGNs, undifferentiated BM-MSCs were able to survive in the modiolus both in the control cochlea and the ouabain-treated cochlea.	20
Guinea pig	ABR results showed mild hearing recovery after transplantation. neural differentiated BM-MSCs are able to restore damaged SGNs and decrease hearing thresholds in an AN model.	21

Neural-induced human mesenchymal stem cells (NI-hMSCs), Brain-derived neurotrophic factor (BDNF), sensorineural hearing loss (SNHL), Auditory-neuropathy (AN), Spiral ganglion neurons (SGNs)

also reprogram to transdifferentiate into endodermal and ectodermal cell types²³. Transdifferentiation is a useful method to regenerate hair cells. However, the method that have so far been reported only to manipulate one factor, and hair cell restoration is also ineffective. Therefore, an examination of the more effective combination of regenerative agents is needed to achieve the regeneration of the hair cell for clinical uses²⁴. Recently, quantitative factors have been manipulated to induce transdifferentiation of the cochlear hair cells, and the induced hair cells are not exactly similar to physiological cells. This indicates that a single factor is not sufficient and several factors should be used for regenerating hair cells. It is important for use of re-programming factors to achieve complete regeneration of the hair cells. Using growth factors and the ATOH1 transcription factor, miRNAs and inhibiting the Notch signal is good option to achieve this goal. The use of growth factors to differentiate stem cells to the hair cells is important. In natural development, the signaling of growth factors is very essential to the development of the inner ear²⁵.

MATERIALS AND METHODS

Human BMSCs were purchased from the bank of BMSCs of the Cell Therapy Center of the Royan Institute (Tehran, Iran) and cultured in the Dulbecco's Modified Eagle's (DMEM) medium containing 15% fetal bovine serum (FBS) and 1% streptomycin and penicillin. The medium was replaced every three days, and the third passage were used to differentiate into hair cells. To differentiate hBMSCs to hair cell precursors (Neurosphere), hBMSCs were first cultured on medium DMEM/F12 containing 20 ng/ml of epidermal growth factor (EGF) (Invitrogen) and 10 ng/ml of basic fibroblast growth factor (bFGF) (Invitrogen) for four days. Then, for differentiating to hair cell the neurospheres grown in DMEM/F12 medium containing EGF and retinoic acid (RA) for 12 days (Table 2). The cells were kept for 12 days on a medium containing 20 ng/ml of EGF and 1 μ M of RA in serum-free DMEM-F12. The culture medium was replaced every four days.

Real-time qRT-PCR to evaluate the expression of genes

To isolate total RNA from the cells, TRIzol Reagent (Ambion) was used based on the manufacturer's protocol. RNA samples were measured using a spectrophotometer (NanoDrop®, Thermo Fisher Scientific) and evaluated qualitatively and quantitatively. Two microgram of total RNA used to synthesize cDNA using a kit (Thermo scientific-K1622). The thermal conditions consisted of a denaturation of 3 min at 95°C followed by 40 cycles at 95°C

Table 2. Media for inducing differentiation into neurospheres and hair cells.

Neurosphere medium	day	HC medium	day
DMEM: F12		DMEM: F12	
bFGF 20 ng/mL	4	20 ng/ml EGF	12
EGF 10 ng/mL		1 μ M RA	
1% Antibiotic-antimycotic		1% Antibiotic-antimycotic	

for 15s and 72°C for 25s. The primers used for each of the studied genes are shown in Table 3. Relative quantities were calculated using the 2^{-($\Delta\Delta$ Ct)} method. 18S used as internal controls for mRNA quantification. A t-test was used to investigate the difference in the expression of mRNA between the treated and control samples in the Real-time qRT-PCR method. The difference between control and treated groups was considered statistically significant if p < 0.05 (Figures 1 and 2).

Western blotting

Total cell protein was extracted using urea lysis buffer (8 M) and approximately 30 μ g of total protein were fractionated by 12% SDS-PAGE and transferred to a PVDF

Table 3. Primers used in Real-time qRT-PCR for mRNAs.

Name	Sequence
POU4F3 forward	5'- AGTCTCTCACTCTCTCGCACAA- 3'
POU4F3 reverse	5'- GCTGTTCTTCTCTCGGTAGGC- 3'
MYO7A forward	5'- GTGGTGAAGCTCTGCGACT- 3'
MYO7A reverse	5'- GCATAGGCTTGATGTGCGTT- 3'
Calretinin forward	5'- CGCAGACGGAATGGGTATATTG- 3'
Calretinin reverse	5'- ATCATACTTCTGCATGAACTCCTT- 3'
ATOH1 forward	5'- CCCCTCCAGCAAACAGGTG- 3'
ATOH1 reverse	5'- ACGGGATAACATTGCGCAGC- 3'
SOX2 forward	5'- GGGGAAAGTAGTTTGCTGCC- 3'
SOX2 reverse	5'- CGCCGCCGATGATTGTTATT- 3'
18S forward	5'- GTAACCCGTTGAACCCATTTCGT- 3'
18S reverse	5'- ACCATCCAATCGGTAGTAGCGACG- 3'

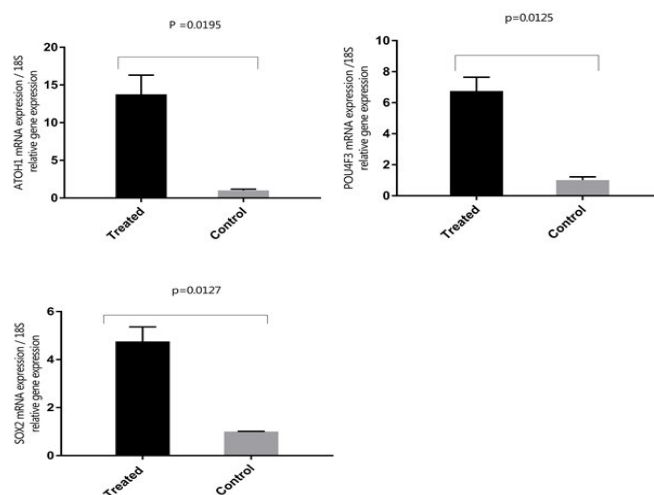


Figure 1. Real-time qRT-PCR showed significant upregulation of ATOH1, SOX-2, POU4F3 mRNAs in the treated cells as compared with control cells.

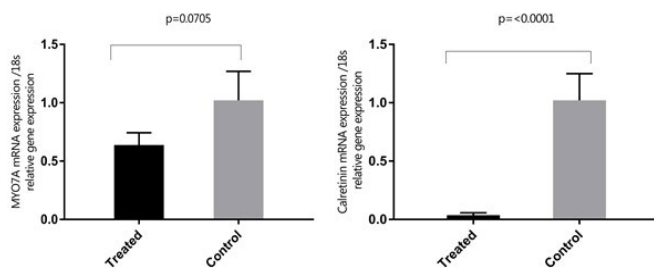


Figure 2: Real-time qRT-PCR results indicate decrease mRNA expression of the calretinin gene and no significant change in expression of mRNA MYO7A on treated cells, as compared with control cells.



Figure 3: Expression of ATOH1 at protein level in treated cells with growth factors and control by using Western blot, it demonstrated overexpression of ATOH1 proteins in the treated cells.

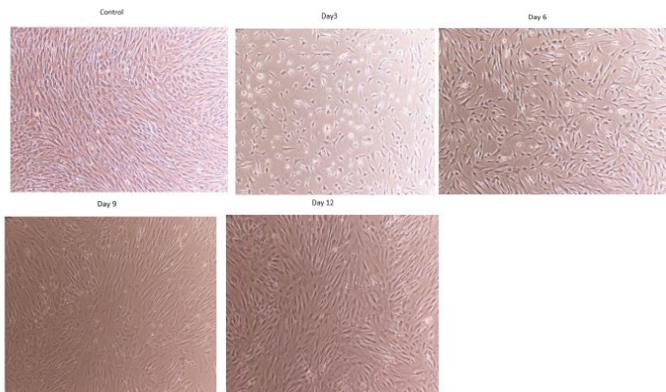


Figure 4. Morphology of hBMSCs at 40X magnifications in basic growth medium (DMEM) and in growth factor-enriched medium (DMEM /F12) at passage 3. After treatment with the growth factor, the morphology of the cells did not change.

membrane. After the transfer, the PVDF membrane was blocked in 3% BSA in TBST at room temperature for 1 hr and then incubated overnight in primary antibody ATOH1 (ab137534) at 1/2000 dilution at 4°C, then incubated in the HRP-conjugated secondary antibody (ab97051) solution for 1 hr at room temperature. The membrane was then incubated with antibody against beta-actin (4970s) to ensure the same loading of proteins in the gel. Finally, bands were visualized using the ECL kit according to the manufacturer's instructions (Figure 3).

RESULTS

Morphological features of hBMSCs cells at passage 3 (P3) grown using two different media were morphologically compared (Figure 4).

DISCUSSION

The differentiation of BMSCs to inner ear hair cells *in vitro* is considered a milestone to understand the regeneration of inner ear hair cells based on stem cells. The induction of hair cell from stem cells will gradually become a reliable method. This method will be used to explain the mechanism of inner ear diseases or to screen for the drug. It is necessary to improve induction, and to provide a method that specifically induces hair cells. There are many problems with the use of *in vivo* induced hair cells. It is impossible to transplant adult stem cells and fit them well into the organ of Cori, and other approaches are needed²⁶. Growth factors are crucial factors in the development of the ear. EGF plays a part in

the inner ear. EGF stimulates cell proliferation in the atrial sensory epithelium. In a study, human embryonic stem cells were cultured as a single layer and synergistically treated with FGF3 and FGF10 to induce the fate of the otic placode in mice. Epithelial cell-like colonies have been reported to differentiate more in an RA- or EGF-enriched medium²⁷. The proliferation and regeneration of stem/stem-like cells in the adult sensory epithelium require the simultaneous release mitogenic EGF or the insulin-like growth factor^{28,29}. RA is a morphogenic agent who acts on the anterior-posterior axis of the ear³⁰. FGFs also are key factors for the development of the organ of Corti. In mice, signaling of fibroblastic growth factors (FGFs) is involved in the early events of induction of auditory vesicles. Specific deletion of FGFR1 results in severe impairment of the development of hair cells and precursor cells³¹. FGF20 is specifically needed to differentiate the side constituents of the cochlea (including outer hair cells and Dieter's cells)³². In patients with low-frequency hearing, who undergo cochlear implantation, the maintenance of the remaining hair cells is essential. Growth factors can prevent hair cell damage or even regenerate hair cells³³. The RA pathway is activated immediately after the loss of hair cells, accelerating the proliferation of cell precursors, and regulating the expression of two key genes, P27kip and SOX2. The results have indicated that RA can be used as an essential signal for the regeneration of hair cells³⁴. In the cochlea of rats fed with all-trans retinoic acid (ATRA), the protection of the organ of corti is more likely to occur than in the cochlea of those feds with oil and saline. ATRA inhibits JNK activation. The results have shown that ATRA has an anti-apoptotic effect on the noise-exposed cochlea³⁵. ATRA increases the expression of Prdx6 and improves hearing loss in noise-induced hearing loss (NIHS). Prdx6 induction may be associated with the rapid treatment of NIHS³⁶. Inhibition of the RA signaling causes a significant reduction on the number of hair cells. The cells in the developing cochlea respond to RA, and RA signals are critical to the normal growth of the organ of corti. After exposure to noise, ATRA can help preserve hearing. Also, when acoustic trauma occurs accidentally, RA has a protective effect. RA is a clinical drug that is used orally and considered to be healthy. Although the findings can also be used for human, further research is needed to determine the safe and effective dose, duration and time of initiation of drug administration for practical application³⁷. The first known gene whose expression is associated with hair cell is a basic helix-loop-helix (bHLH) transcription factor called ATOH1³⁸. During the development process, the expression of ATOH1 is limited to cells that ultimately differentiate into hair cells. The expression of ATOH1 in the bird's cochlea increases during the process of regeneration of the hair cells³⁹. Among the several transcription factors associated to the evolution of hair cells, ATOH1 is the most promising factor for the regeneration of hair cells. Purposeful loss of ATOH1 leads to complete destruction of the hair cells⁴⁰. In order to regenerate hair cell, transport of a specific gene associated with induction of a hair cell, such as ATOH1,

was reported to be effective in mouse⁴¹. An increase in the expression of ATOH1 in the guinea-pig cochlea after drug-induced hair cell damage led to the retrieval of the hair cell function, in addition to repairing the hair cell⁴¹. ATOH1 is a major gene to commit to the fate of the hair cells, but we still do not know how the expression of ATOH1 is induced in transplanted cells *in vivo*, as well as what other genes are needed for the differentiation of the hair cells.

CONCLUSION

Regenerating hair cells with physiological function is considered a challenge for researchers. Using different types of growth factors that are known for regenerating hair cells increases the possibility of obtaining hair cells phenotype. Real-time qRT-PCR analysis demonstrated increased expression of SOX2, which is a critical gene in the development of cochlear hair cells, the receptor cells for hearing. SOX2 is expressed in the neurosensory domain of the otic placode. The real-time qRT-PCR analysis also revealed increased expression of POU4F3, which is a transcription factor known to play an essential role for the survival of hair cells. ATOH1 is a major gene for committing the fate of the hair cell. Analysis of gene and protein expression in our study showed that the expression of ATOH1 increased in the hBMSCs treated with the RA, bFGF, and EGF. However, the expression of calretinin decreased and no significant change in expression levels of mRNA MYO7A. The hBMSCs, which the expression of the ATOH1, SOX2, and POU4F3 marker's increase, can get a new approach to regenerate inner ear sensory hair cells. In this study, we showed that hBMSCs have the potential to differentiate in auditory hair cell-like. Current findings point about the importance to the application of BMSCs in the regenerative medicine for the inner ear. Recent developments in biology and rapid advances in the field of stem cell biology are expected to lead to the resolution of problems that prevent successful mammalian hair cell regeneration.

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Conflict of interest

The authors declare that they have no conflicts of interest.

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