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Original Article

Upregulation of Neuroprogenitor and Neural Markers via Enforced miR-124 and Growth Factor Treatment

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Previous studies have shown that miR-124 plays an important role in the development of auditory neurons, which are degenerated in the sensorineural hearing loss. However, whether the combined use of miR-124 and growth factors can increase the expression of neural related markers in human dental pulp stem cells has been remained unknown so far. In this study, human dental pulp stem cells were transfected with miR-124 following treatment with brain-derived neurotrophic factor or epidermal growth factor/basic fibroblast growth factor. The expression of some neural related markers (nestin, *SOX2*, β-tubulin III, *MAP2*, and peripherin) was analyzed in two groups by qRT-PCR or immunofluorescence. Cellular treatment resulted in morphological changes including neurosphere-like colonies formation. Nestin and *SOX2* were up-regulated, and *MAP2* and peripherin were down-regulated in dental pulp stem cells transfected by miR-124 following treatment with brain-derived neurotrophic factor. Replacement of brain-derived neurotrophic factor with epidermal growth factor/ basic fibroblast growth factor resulted in the up-regulation of *nestin*, *MAP2*, peripherin, and β-tubulin III and down-regulation of *SOX2*. The expression of SOX2 and nestin was also confirmed by immunofluorescence. The combination of miR-124 and growth factors would provide a promising starting point for upregulating the neural progenitor markers in human dental pulp stem cells.

Key words: Spiral ganglion neuron, miR-124, dental pulp stem cells, brain-derived neurotrophic factor, auditory neuropathy.

Auditory neuropathy is one of the most common forms of hearing loss in human representing a severe social and health problem which cannot be well-treated by replacement therapy. Currently, cell therapy is one of the most promising therapeutic hopes for replacement of spiral ganglion neuron (SGN) and efforts have been made to produce auditory nerves (1). The choice of

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an efficient strategy and appropriate cell type to differentiate into this target cell is a key issue to succeed in cell therapy of sensorineural hearing loss (2). Previously, growth factors and neurotrophic agents such as brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT3), sonic hedgehog (SHH), basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), and overexpression of transcription factors involved in the development of spiral ganglion neurons (SGNs) toward auditory neurons have been tested *in vitro*. A wide range of cell sources including embryonic stem cells, induced pluripotent stem cell (iPSCs), and adult stem cells have been used in previous reports for differentiating towards auditory nerves *in vitro* and /or *in vivo* (3–8). Among tissues that can be used to isolate stem cells, dental pulp is an attractive source with desirable features of autologous stem cell. Availability, good proportion of ectomesenchymal stem cells derived from the neural crest, noninvasive access, expression of pluripotency markers including SSEA-4, NANOG, OCT-4, and no ethical issues and immunological concerns for using these cells lead to their promising capacity for neuronal differentiation and repair (9–13). MicroRNAs (miRNAs) are a class of non-coding small RNAs that regulate gene expression through degradation of mRNAs or inhibition of translation after transcription. Studies have shown that miRNAs play essential roles in many of the processes including cell fate determination in the inner ear (14,15). In the present study, for the first time, we developed an approach that combines the application of two factors involved in the development of SGNs (i.e., growth factors and miRNAs). We conducted the present study based on transfection of the above-mentioned stem cells with miR-124 following their culture in the presence of BDNF or EGF/bFGF. Given the essential role of miRNAs in inner ear development, particularly eight-fold higher expression level of miR-124 in the cochlea (16,17), we tested whether transient alteration of miR-124 level combined with either BDNF or EGF/bFGF in dental pulp stem cells (DPSCs), could increase the expression of neuroprogenitor (nestin, *SOX2*) and neural markers (β-tubulin III, peripherin, and *MAP2*).

Materials and methods

Cell culturing and treatment with growth or neurotrophic factors

DPSCs were donated by the Royan Institute for Biotechnology (Isfahan, Iran). Approximately 3×10^5 DPSCs per well were cultured in 6-well plates containing DMEM high glucose supplemented with 10% of FBS and 1% of Pen/Strep. After 24 h, two different protocols including BDNF or EGF/bFGF treatments were tested on DPSCs grown as monolayer cultures. Briefly, the medium was replaced completely by high glucose DMEM supplemented either with N2 (1%)/ B27 (2%)/ BDNF (10 ng/ml) or EGF (20 ng/ml) / bFGF (20 ng/ml) in the first and the second group, respectively. The treated DPSCs were followed for 3 days carefully.

Transfection of hDPSCs with miR-124

At 3 days following treatment, the cells were transfected with miR-124 or scramble control. The transfection complexes were prepared according to the manufacturer's instructions (Invitrogen). Briefly, either miRNA or scramble was mixed with Lipofectamine 2000 reagent (Invitrogen, Massachusetts, USA) and the above mixture was then separately transfected in a final concentration of 24 nM (was selected following the literature search) in to the cells in antibiotic and FBS free high-glucose DMEM medium. Four hours after transfection, the transfection medium was replaced with high-glucose DMEM medium with 10% FBS containing Pen/Strep (1%), and the cells were cultured for 6 h.

RNA isolation, cDNA synthesis, and qRT-PCR

Based on the manufacturer’s instructions, total RNA was extracted from the samples (including transfected BDNF- or EGF/bFGF-treated DPSCs)

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using Trizol reagent (Sigma, MI, USA) and quantified using a NanoDrop spectrophotometer (Thermo Scientific, MA, USA). The transfection efficiency of the miR-124 was evaluated using BONmiR detection kits (Stem Cell Technology Research Center, Tehran, Iran), including a BON-miRmiRNA 1st-Strand cDNA Synthesis Kit (Cat# BON209001) and BON-miR High Specificity miRNA QPCR Core Reagent Kit (Cat#BON 209002). U47 was used as an endogenous control gene in the qRT-PCR. Equal amounts of total isolated RNA (1 µg) per sample were reverse transcribed using a cDNA synthesis kit (Yektatajhizazma [YTA], Tehran, Iran) and were transferred into the qRT-PCR reaction mixture. The transcription levels of nestin β-tubulin III, *MAP2*, and peripherin were evaluated using transcript-specific primers and SYBR® Green PCR Master Mix (Yektatajhizazma [YTA], Tehran, Iran). Specific cycling parameters in the qRT-PCR included an initial denaturation step at 95°C for 3 min, denaturation at 95°C for 15 s, annealing at 58°C for nestin and β-tubulin III, annealing at 60°C for *MAP2*, and annealing at 62°C for peripherin (all for 20 s), followed by an extension step at 72°C for 25 s. The number of cycles was optimized at 40. The primer sequences used were as follows: nestin, (forward) 5'CACCCCTCAGCCCTGACCACT3' and (reverse) 5'CCCTCTATGGCTGTTTCTTTC-TCTACCA3'; *SOX2*, (forward) 5'ATAGCATGG-CGAGCGGGGT3' and (reverse) 5'CTGCGAGC-TGGTCATGGAGTTG3'; β-tubulin III, (forward) 5'CTCAGGGGCCTTTGGACATC3' and (reverse) 5'CAGGCAGTCGCAGTTTTCAC3'; *MAP2*, (forward) 5'CGGAGTAACCAAGAGCCCAGA-AAAG3' and (reverse) 5'GAGTGCCTGGTGTG-CGTGAAGA3'; peripherin, (forward) 5'CACGCT-CCTCATTTGGCTCTTC3; and (reverse) 5'GGC-TCTCGCTCTCAGATTCCTC3'; and 18S rRNA, (forward) 5'GTAACCCGTTGAACCCCATTCG-T3' and (reverse) 5'ACCATCCAATCGGTAGTA-GCGACG3'. The transcription level of 18S rRNA was used as an endogenous control. The $2^{-\Delta\Delta Ct}$ analysis algorithm was used to determine the relative quantification of each sample at the time points analyzed. qRT-PCR reactions were run using a Rotor-Gene 3000 instrument (Corbett Research, Sydney, Australia).Two independent experiments were performed in triplicate samples in each group.

Immunostaining

To assess the expression of neuro-progenitor protein markers SOX2 and nestin, both miR-124 transfected and control cells were fixed in 4% paraformaldehyde in PBS at room temperature and washed 3 times with washing buffer (1% BSA in PBS containing 10% normal donkey serum and 0.3% Triton X-100) at room temperature for 45 min. Immunostaining was carried out using a standard protocol of Human/Mouse/Rat Neural Progenitor Cell Marker Antibody Panel (R&D Systems, Canada). The cells were exposed to the primary antibodies (goat anti-SOX2 IgG or mouse monoclonal anti-nestinIgG_{2A}) at the final concentration of 10 µg/ml. After three washing steps, the secondary antibodies (donkey anti-mouse IgG (NorthernLights™) NL493- conjugated antibody or donkey anti-goat NL493-conjugated, Cat#NL009, Cat#NL003) (R&D Systems, Canada) were applied at 1:200 dilution rate in PBS supplemented with 1% BSA. In this experiment, the technical negative controls excluded the primary antibody. The cell nuclei also were stained with DAPI.

Statistics

Statistical analyses of the qRT-PCR data were performed using GraphPad statistical software (GraphPad Software, CA, USA). The relative quantification data were analyzed using the Mann–Whitney test. All p-values <0.05 were considered statistically significant.

Results

DPSCs morphologically changed in presence of BDNF or EGF/bFGF

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DPSCs were donated by the Royan Institute for Biotechnology (Isfahan, Iran). According to characterization data provided by this institute**,** these cells exhibit a high expression of STRO-1, CD146, CD73, CD90, and CD105 (data not shown). They are also positive for collagen type-I but negative for collagen type-III and CD45. The DPSCs also have been shown to be multipotent and capable of differentiating into adipogenic, osteogenic, and chondrogenic lineages. In the first step, the morphology of two groups of treated DPSCs was compared. Photographs of cells demonstrated in great details the temporary events during the neural lineage induction in the presence of BDNF or EGF/bFGF. Both groups formed neurosphere like colonies, however, the groups clearly differed in size and speed of neurosphere formation. These colonies were generated more quickly under the influence of BDNF rather than EGF/bFGF. Furthermore, larger colonies were observed in BDNF-treated cells (Fig.1).

Increased level of miR-124 was observed 6 h post transfection

In order to evaluate the transfection efficiency, miR-124 levels were analyzed 6 h post transfection by qRT-PCR. The results showed that the miR-124 level increased 6 h post-transfection (Fig.2, P= 0.004).

The expression level of nestin is increased 6 h post transfection of miR-124 in the presence of either EGF/bFGF or BDNF

After confirmation of the transfection, the expression levels of neuroprogenitor markers (nestin, SOX2) was evaluated at RNA or protein levels. Nestin is an intermediate filament protein characteristic of neural stem/progenitor cells and it is consistently associated with differentiation towards neural precursor lineages (18,19). According to our previous study, the highest level increase of miR-124 was observed 6 h post transfection and the miR-124 level gradually decreased 16 h, 24 h, and 48 h post- transfection

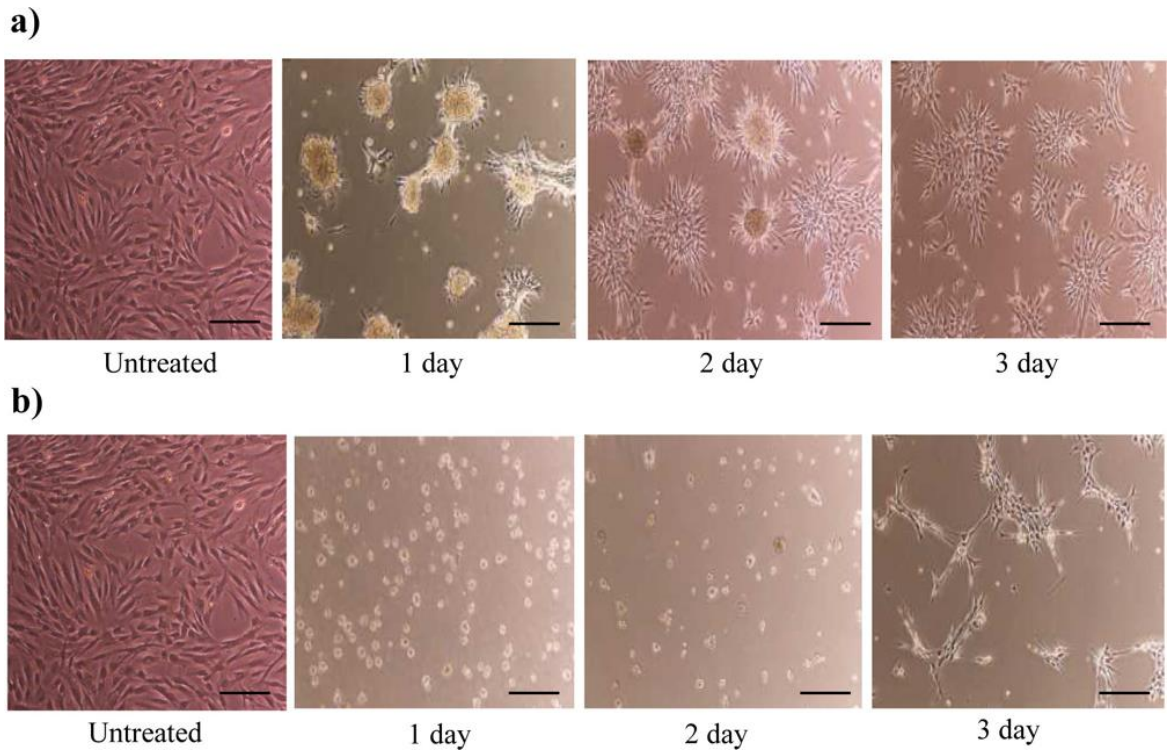

Fig. 1. Morphological changes of DPSCs, three days following treatment with BDNF (a) or EGF/bFGF (b). Samples were viewed under a light microscope (Nikon ECLIPSE TS100, US) at 4X magnification (scale bar; 50 µm).

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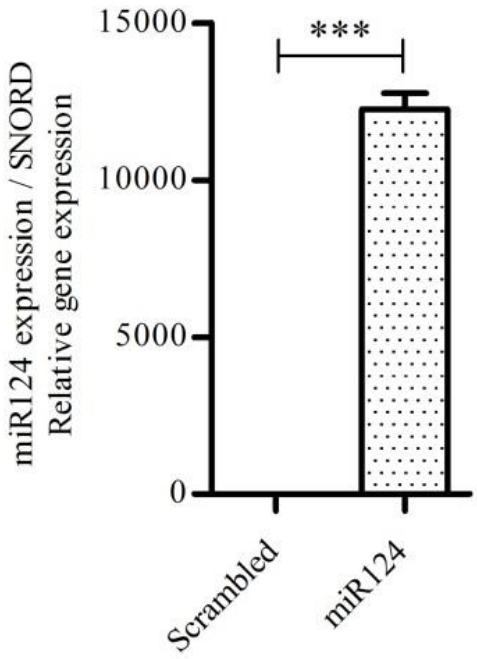

Fig. 2. The qRT-PCR analysis of *miR-124* level in the transfected DPSCs 6 h after transfection. Data showing the increased miR-124 level in 6 h post transfection compared with scrambled control (*P* = 0.004). Data were normalized to expression levels of U47 in scrambled control (transfection efficiency of each group including BDNF-treated or EGF/bFGF-treated have the same results).

(20). Therefore, the qRT-PCR analyzes of neuroprogenitor and neural markers were evaluated at the same time (6 h). Results demonstrated increased mRNA level of nestin in transfected hDPSC in both treatment groups described above, 6 h post transfection. Also, the results showed that *SOX2* was upregulated in the presence of BDNF (P = 0.005), however, DPSCs treated with EGF/bFGF exhibited *SOX2* down-regulation (P= 0.0007) (Fig.3 a, b). We also looked at the protein expression of SOX2 and nestin via immunofluorescence in transfected hDPSC which confirmed the results (Fig. 4).

Increased level of miR-124 6 h post transfection affects the expression of *MAP2* and peripherin but not β- tubulin III

We investigated the expression of the neuron markersβ-tubulin III and *MAP2* at the RNA level. DPSCs transfected with miR-124 had higher levels of these markers 6 h post transfection in EGF/bFGF treated group in comparison with the control cells. However, there were no significant differences observed on the expression levels of β-tubulin III in the transfected and control samples grown in the presence of BDNF. *MAP2* was also down-regulated in this group. In order to investigate whether the intracellular events after miR-124 transfection have induced the expression of auditory neuron lineage, the expression of peripherin was also examined. The results showed that the expression of peripherin was up-regulated in EGF/bFGF treated group (P= 0.0007) and down-regulated in BDNF treated group (P= 0.001) (Fig.3c, d, e).

Discussion

The present study is the first to reveal that culture regimes including a temporal increase in the level of miR-124 in BDNF- or EGF/bFGF- treated DPSCs, affect the expression of some neural progenitors and neural markers (Fig.5). Previous studies have shown that miR-124 may play an important role in the development of auditory neurons, which are degenerated in the sensorineural hearing loss. However, whether *miR-124* can

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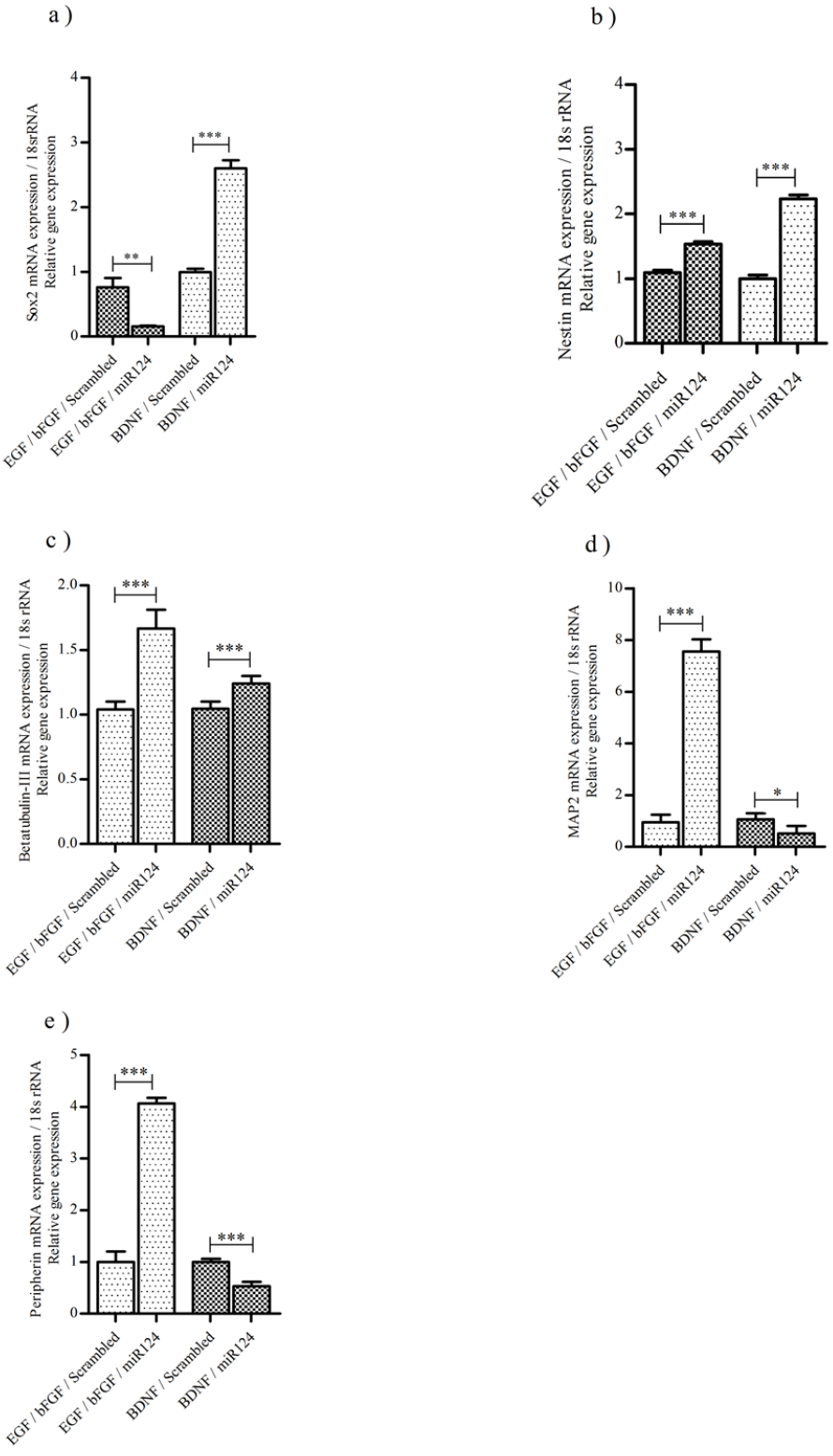

Fig. 3. qRT-PCR analysis of SOX2 (a), Nestin (b), β-tubulin III (c), MAP2 (d), and Peripherin (e) mRNA expression in EGF/bFGF and BDNF treated DPSCs 6 h post transfection. According to (a), SOX2 was down-regulated (P = 0.0007) and up-regulated (P = 0.005) in EGF/bFGF- and BDNF treated DPSCs, respectively. Both treated groups had nestin up-regulation compared with scrambled control (P = 0.004, P = 0.005). Data show no significant alteration of β-tubulin III, 6 h post transfection (P = 0.0014) and down-regulation of MAP2 (P = 0.004) and Peripherin (P = 0.001) in BDNF treated DPSCs. Data were normalized to expression levels of 18s rRNA in scramble control.

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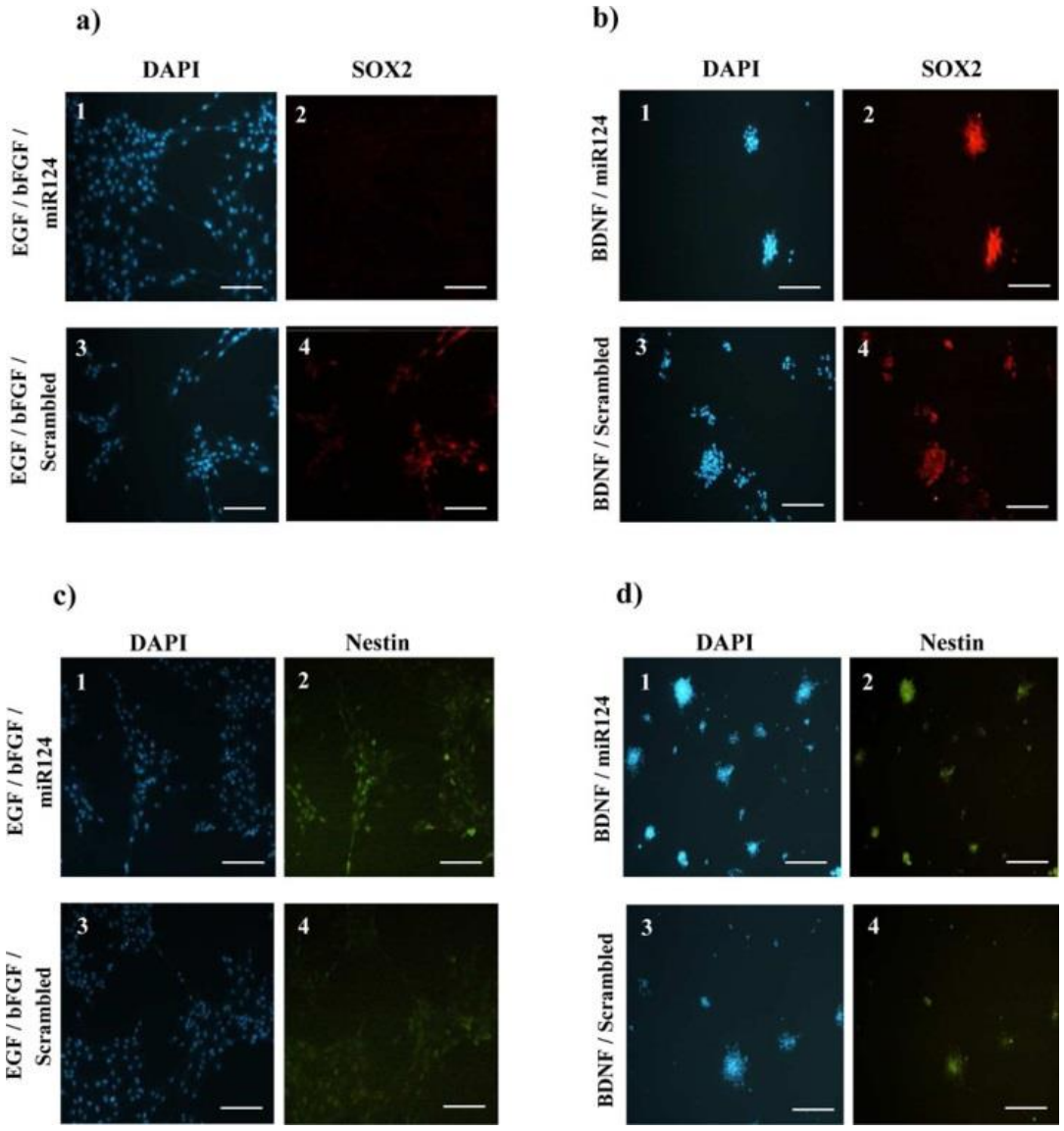

Fig. 4. Immunofluorescence analysis of the expression of SOX2 and Nestin in EGF/bFGF treated DPSCs (a, c) and BDNF treated DPSCs (b, d) 6 h after transfection with miR-124 (2) versus scrambled miR (4). Samples were viewed under an epi-fl microscope (Nikon AZ100, US) at 2X magnification (scale bar; 25 µm).

promote the expression of neuroprogenitor and neural markers in DPSCs, has not been investigated so far. Following our previous study which examined the effects of the temporary increased level of miR-124 alone on the expression of some neuroprogenitor and neural markers (20), here we attempted to enhance the effects of miR-124 on the expression of neuroprogenitor and neural markers by treating DPSCs with either BDNF or EGF/bFGF before transfection with miR-124. We previously observed no morphological changes in transfected DPSCs. Here, when DPSCs were exposed to growth factors (BDNF or EGF/bFGF), neurosphere- like morphologies were progressively developed. We monitored and compared the morphological changes of BDNF-treated DPSCs with those treated with EGF/bFGF using microscopy in 3 days after treatment (before transfection with miR-124). Under both conditions (in the presence of BDNF or EGF/bFGF), morphological similarities were observed between the cultured DPSCs and neurosp- heres. DPSCs showed morphological changes from spindle/ fibroblast shape into neurosphere-like body. How-

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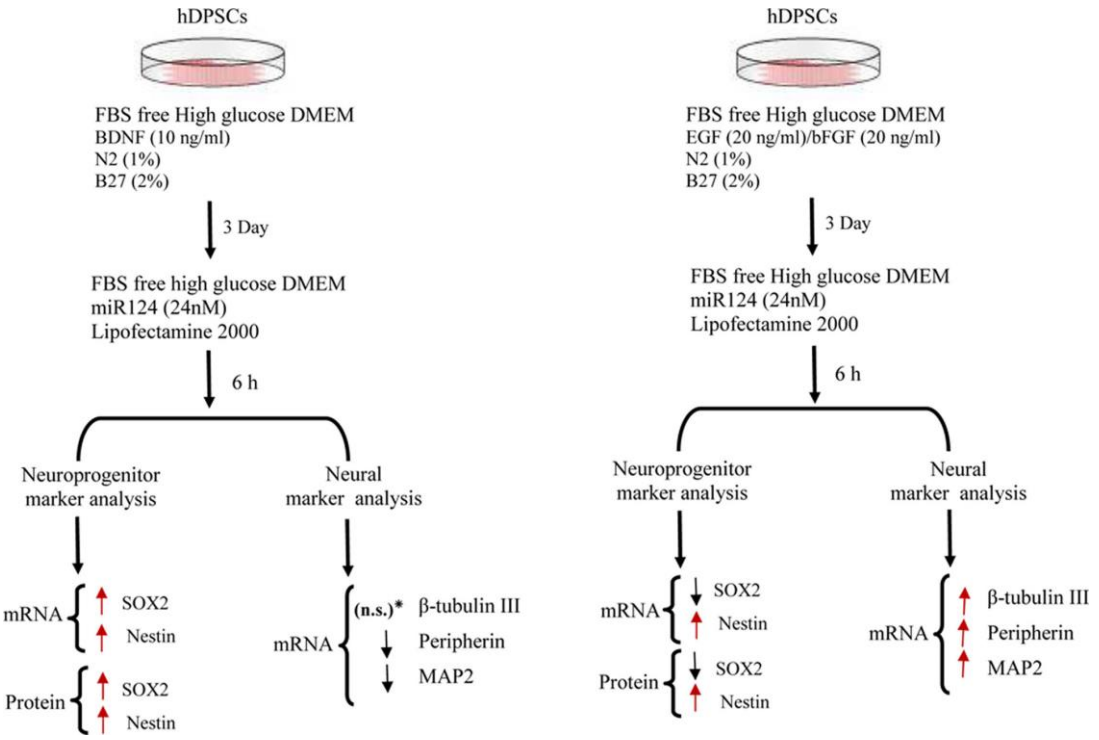

Fig. 5. Schematic representation of treatments outcomes.

ever, the morphological changes were clear in BDNF-treated DPSCs in comparison with EGF/bFGF-treated cells. Also, a comparison of treated cells in these conditions with respect to the previous published data showed a clear increase in the expression of neuroprogenitor or neural markers. β-tubulin III is a microtubule element of the tubulin family that is known as a neural specific marker, and peripherin is the type III intermediate neurofilament which marks mature type II primary auditory neurons (21,22). MAP2 is a member of the microtubule-associated protein family thought to be involved in microtubule assembly, which is a required step in neurogenesis (23). Our previous data showed that the expression of nestin and β-tubulin III was upregulated in the absence of BDNF or EGF/bFGF at mRNA or protein levels. SOX2 is a transcription factor of the SRY-related HMG box family that is expressed in neuroprogenitors, and is involved in neural commitment and self-renewal (24–26).The results of the present study showed that after transfection with miR-124, up-regulation of neuroprogenitor markers including nestin and *SOX2* was clearer in BDNF-treated DPSCs in comparison with EGF/bFGF- treated DPSCs. Also, peripherin, β-tubulin III and *MAP2* were up-regulated post transfection with miR-124 in EGF/bFGF-treated DPSCs which suggests the promotion of neuronal marker expression. bFGF induces neuronal differentiation via signaling pathways that require FGFR-1, MAPK/ERK, and AP-1 transcription factor (27). Moreover, EGF interacts with bFGF to regulate the proliferation of neural progenitors. BDNF and NT-3 (important neurotrophins during the embryonic development of auditory neurons) are secreted from the organ of corti and cochlear nucleus (28). According to the results, it seems that the temporary increase of miR-124 level provide an excellent starting point for up-regulating neuroprogenitor or neural markers if the level of growth factors increased in DPSCs. Undoubtedly, further investigations are necessary to determine whether optimization of this strategy during follow-up over long time, can lead to

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promote differentiation of SGNs from DPSCs. In recent years, attempts have been devoted to generate SGNs using various protocols and cell sources. The strategies and approaches included co-culturing, application of growth and neurotrophic factors, genetic manipulation, and induction of transcription factors inside stem cells to increase the expression of neuron-related markers (3,5,29–33). In a study, the possibility of generating functionally active neurosensory progenitors using media containing EGF, bFGF, and small peptide Y27632 was demonstrated (34). A study conducted by Alonso et al. showed that growth factors induced the differentiation of bone marrow-derived MSCs into nestin- and *SOX2*-expressing neural progenitors (35). Jiang et al. transfected mouse inner ear neural stem cells with miR-124 and observed alterations in the expression of tropomyosin receptor kinase B and *Cdc42* (36). Our findings demonstrated that after growth factor treatment (including BDNF or EGF/bFGF), the expression of neuroprogenitor and neuronal markers was affected by miR-124 in DPSCs. In conclusion, differentiation potential of DPSCs towards auditory neuron will open a new hope for applications of these cells in regenerative medicine of sensorineural hearing loss. The miRNAs and growth factors would provide a promising starting point for promoting the expression of neuropr-ogenitor or neural markers. According to the presnt results, it seems that DPSCs have a good potential to generate neuroprogenitor- or neural marker expressing cells for differentiating toward SGNs under appropriate conditions.

Acknowledgments

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Conflicts of interest

The authors declare that there are no conflicts of interest.

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