\* Corresponding author: Abolghasem Esmaeili, Ph.D.,

Cell and Molecular Biology

Biology, School of Sciences,

E-mail: aesmaeili@sci.ui.ac.ir Received: 22 Aug 2013

Division. Department of

University of Isfahan,

Tel: +98 311 732490

Fax: +98 311 732456

Accepted: 28 Oct 2013

Isfahan, Iran

# Messenger RNA Expression Patterns of Neurotrophins during Transdifferentiation of Stem Cells from Human-Exfoliated Deciduous Teeth into Neural-like Cells

Abolghasem Esmaeili<sup>1\*</sup>, Sedigheh Alifarja<sup>1</sup>, Nosrat Nourbakhsh<sup>2,3</sup>, and Ardeshir Talebi<sup>4</sup>

1. Cell and Molecular Biology Division, Department of Biology, School of Sciences, University of Isfahan, Isfahan, Iran

- 2. School of Dentistry, Isfahan University of Medical Sciences, Isfahan, Iran
- 3. Torabinejad Dental Research Center, School of Dentistry, Isfahan University of Medical Sciences, Isfahan, Iran
- 4. Department of Pathology, School of Medicine, Isfahan University of Medical Sciences, Isfahan, Iran

#### Abstract

Background: Stem cells from Human Exfoliated Deciduous teeth (SHED) have the capability to differentiate into neural cells. Neurotrophins including Nerve Growth Factor (NGF), Brain-Derived Neurotrophic Factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4 (NT-4) have neurogenesis, neurotrophic, or neuroprotective effects and are expressed in developing teeth. The aim of this study was to measure quantitative changes in mRNA expression levels of neurotrophins in neural-like cells differentiated from dental pulp stem cells.

Methods: Isolated total RNA from SHED, dental pulp and neural-like cells (n=3) were transcribed into cDNA. Then real time PCR was done. Expression levels of mRNA for NGF, BDNF, NT-3, and NT-4 genes were compared in these three cells.

Results: In neural like cells, BDNF mRNA increased (372.1±113.5) significantly (p<0.01) after differentiation. NGF mRNA increased to more than 266 times the dental pulp level after differentiation. A similar pattern was seen for the expression of NT3 after differentiation. NT4 mRNA enhancement was 1344 $\pm$ 630.8 and 30.7±7.9 fold in neural like cells and SHED cells, respectively. Results show alterations with different degrees and direction in neurotrophins mRNA expression levels in these cells.

Conclusion: Our results suggest that neurotrophins dental pulp cells, SHED cells and neural like cells derived from SHED cells produce neurotrophic factors. Since the large amounts of neurotrophins are expressed in SHED and neural like cells they may have important role in survival and differentiation of dental pulp stem cells and obtained information may lead to a novel method for tooth regeneration.

Avicenna / Med Biotech 2014; 6(1): 21-26

Keywords: Cell differentiation, Neurotrophin, Real-time PCR, Survival

### Introduction

Regeneration ability of the adult mammalian Central Nervous System (CNS) is limited after injury or disease. Currently, effective strategies are required for treating patients suffering from CNS injuries or diseases. Strat-

egies such as incorporating the sustained release of neurotrophic factors, gene therapy and cell therapy have been suggested for their ability to overcome this limited regeneration, but may face clinical limitations such as vol-



21



ume restrictions and the inability of donor cells to interact with the host tissue  $^{1,2}$ .

The isolation and characterization of neural stem/progenitor cells from different tissues with subsequent direct cell transplantation of these cells into the damaged brain is one important approach for cell therapy. This is because of multipotential ability of these cells to renew themselves and give rise to neurons, astrocytes and oligodendrocytes.

Neural stem/progenitor stem cells have been isolated from various tissues, including bone marrow, neural tissue, skin, retina, and dental epithelium <sup>3-8</sup>. Incidentally, autologous stem/progenitor cells can be collected from dental tissue including Dental Pulp Stem Cells (DPSCs), SHED, Periodontal Ligament Stem Cells (PDLSCs), Stem Cells from Apical Papilla (SCAP), and Dental Follicle Progenitor Cells (DFPCs) <sup>9</sup>. Achieving SHED is easy and convenient, with little or no trauma. They have the capability to differentiate into osteoblasts, odontoblasts, endothelial cells, adipocytes, and neural cells <sup>10-13</sup>.

It has been demonstrated that neurotrophins including NGF, BDNF, NT-3, and NT-4, (also known as NT-5) have neurogenesis, neurotrophic, or neuroprotective effects on the brain. Neurotrophins are expressed in developing teeth <sup>14-20</sup>. Neurotrophins may have important role in differentiation of SHED into neural-like cells. We compared mRNA expression levels of NGF, BDNF, NT3 and NT4 genes, dental pulp, SHED and neural-like cells differentiated from SHED.

### Materials and Methods

#### Isolation of dental pulp

In this study, 12 normal human exfoliated deciduous incisors were collected from six to nine year-old children under approved guidelines set by Isfahan University of Medical Sciences, Faculty of Dentistry. The pulps were separated from the crown and harvested in a lysis buffer (Qiagen, Hilden, Germany) and stored at -80 % for RNA extraction.

#### Cell culture

SHED were purchased from Royan Institute and subcultured as previously described <sup>21</sup>. Briefly, cells were thawed and resuspended in DMEM/F12 supplemented with 10% FCS. Upon reaching 80-90% confluence, cells were either harvested for phenotypic characterization and differentiation or stored at  $-80^{\circ}C$  for total RNA isolation at later times.

#### Neural Differentiation

Neural differentiation was conducted with protocol published previously <sup>21</sup>. Briefly, SHED at passage 4 were seeded on poly-Llysine (Sigma, P4707)-coated glass cover slips at a concentration of 5000 cells/cm<sup>2</sup> in neurobasal medium containing 1% ITS (Invitrogen, 41400), and cytokines including 100 ng/ml basic fibroblast growth factor (bFGF, Sigma, F0291) for seven days followed by 100 ng/ml bFGF, 10 ng/ml FGF8 (Sigma, F6926), and 100 ng/ml sonic hedgehog (SHH, Sigma, S0191) for an additional seven days. During the first seven days of induction, media were half refreshed and bFGF was added with the full concentration. Following media exchange on day seven, the culture was continued without any media renewal. Differentiated cells were stored at  $-80^{\circ}C$  for total RNA isolation at later times.

#### RNA extraction and reverse transcription (RT)

Total cellular RNA was isolated from frozen tissues using the RNeasy Mini kit (Qiagen). The extracted RNA was dissolved in Diethyl Pyrocarbonate (DEPC)-treated water. The purity and integrity of the extracted RNA were evaluated by optical density measurements (260/280 nm ratios) and by visual observation of samples electrophoresed on agarose gels. Both methods indicated integrity of the extracted RNA with little or no protein contamination. Complementary DNA synthesis reactions were performed using 1  $\mu g$ DNase (Fermentas)-treated total RNA from each sample and cDNA synthesis kit (Fermentas) with random hexamer (Fermentas) priming in a 20  $\mu l$  reaction according to the manufacturer's instructions.

# Real-time PCR

Real-time PCR was performed in the Chromo4 Detection System (BioRad, USA). Briefly, 25 ng of cDNA and gene specific primers were added to SYBR green master kit (Takara, Tokyo, Japan), and subjected to PCR amplification (1 cycle at 95  $\mathcal{C}$  for 10 min, and 45 cycles at 94  $\mathcal{C}$  for 5 s, 59  $\mathcal{C}$  for 20 s and 72  $\mathcal{C}$ for 15 s). All PCR reactions were run in triplicate. Gene-specific primers were designed by Beacon Designer 7.5 software. The primers used for real time PCR are listed in table 1. Expression levels were normalized to that of  $\beta$  actin.

Relative expression data was quantified using  $2^{-\Delta\Delta Ct}$  where  $C_t$  is the cycle threshold. Relative standard curves were generated by plotting the threshold value ( $C_t$ ) versus the log of the amount of total cDNA added to the reaction and used to check the efficiency of primers. Calculation of  $C_t$ , standard curve preparation and quantification of mRNA in the samples were performed by the software provided by Chromo4 (Option 3). All target genes were normalized to the  $\beta$  actin housekeeping gene.

### Statistical analysis

To determine significance, all data were subjected to statistical analysis using a computerized statistics program (GraphPad Prism). One-way ANOVA was used, as indicated in the figure legends, followed by a post hoc test (Tukey) of differences between specific time points. All data were presented as the means  $\pm$ SEM. A level of p<0.05 was considered significant.

### Results

BDNF, NGF, NT3 and NT4 genes showed significant alterations in mRNA expression after differentiation of SHED into neural-like

cells. Figure 1 shows changes in mRNA expression of BDNF, NGF, NT3 and NT4 in dental pulp tissue, SHED and neural-like cells. In each case, the ratio of the obtained data to that expressed in the dental pulp tissue was indicated.

### Alterations in BDNF expression after differentiation

BDNF expression was altered after differentiation (Figure 1A). One-way ANOVA indicated significant alteration after differentiation. In SHED, BDNF mRNA increased 107.4±34.35 fold which was not significant (p>0.05) (Figure 1A). In neural-like cells, BDNF mRNA began to increase (372.1± 113.5) after differentiation. Enhancement at these cells was significant (p<0.01) (Figure 1A). When BDNF expression levels of SHED were compared with neural-like cells, there was a significant difference between them (p<0.05) (Figure 1A).

### Alterations in NGF expression after differentiation

In contrast, NGF mRNA (Figure 1B) was higher than dental pulp in both SHED and neural-like cells. The greatest alterations were seen in neural-like cells where NGF mRNA increase was 266 times higher than the dental pulp level after differentiation. SHED showed a 130.8±31.28 fold increase in mRNA (Figure 1B). In neural-like cells, the mRNA expression level of NGF was significantly (p< 0.05) more than the SHED level.

### Alterations in NT3 expression after differentiation

A similar pattern was seen for the expression of NT3 after differentiation (Figure 1C). NT3 mRNA significantly (p<0.001) increased by 1375±259.6 and 338.8±82.13 fold in neural like cells and SHED, respectively.

Name	Forward Primer (5'-3')	Reverse Primer (5'-3')	Prod. Length (bp)	Ann. T (° <i>C</i> )
BDNF	GGATGAGGACCAGAAAGT	AGCAGAAAGAGAAGAGGAG	117	60
NGF	CCATCCCATCTTCCACAG	CTCTCCCAACACCATCAC	115	59
NT3	CGGAGCATAAGAGTCACC	CCTGGCTTCCTTACATCG	179	61
NT4	GCTGATAACGCTGAGGAAG	CACATAGGACTGCTTGGC	105	62
β Actin	CACACCTTCTACAATGAGC	ATAGCACAGCCTGGATAG	160	59

Table 1. Primers and the reaction conditions of RT-PCR

Avicenna Journal of Medical Biotechnology, Vol. 6, No. 1, January-March 2014

23



Figure 1. Real time PCR analysis of BDNF, NGF, NT3 and NT4 genes after differentiation of SHED into neural-like cells. All of these neurotrophins increased after differentiation. DP=dental pulp tissue, SHED=Stem cells from human-exfoliated deciduous teeth, NC=neural like cells. Error bars show mean $\pm$ SD. For each experiment, n=12 for each gene, \*\*\*p<0.001, \*\*p<0.01, \*p<0.05 in comparison to dental pulp tissue and ###p<0.001, ##p<0.01, #p<0.05 in comparison to SHED

# Alterations in NT4 expression after differentiation

NT4 mRNA significantly (p<0.05) increased in neural-like cells when compared with both dental pulp tissue and SHED. NT4 mRNA enhancement was  $1344\pm630.8$  and  $30.7\pm7.9$  fold in neural-like cells and SHED, respectively (Figure 1D).

#### Discussion

We analyzed the expression of the mRNA for BDNF, NGF, NT3 and NT4 genes after differentiation of SHED into neural-like cells. In the current study, SHED were differentiated towards neural phenotype with a previously described protocol <sup>21</sup>. Real-time PCR allowed us to obtain quantitative data before and after differentiation with respect to the dental pulp tissue. Each of the BDNF, NGF, NT3 and NT4 genes appeared to be unique in its pattern of alterations after differentiation. To our knowledge, this is the first time that mRNA expression levels after differentiation of SHED into neural-like cells for BDNF, NGF, NT3 and NT4 genes were shown.

We supposed that when SHED differentiated into neural cells, neurotrophins mRNA expression levels may change. Neurotrophic factors and their receptors were expressed during tooth development. This implies their important functions in odontogenic processes. Neurotrophic factors derived from dental pulp play an important role in orchestrating the dental pulp innervations <sup>22</sup>. This suggests that these neurotrophic factors might be involved in the innervation of dental structures. The rich expression of neurotrophic factors in developing dental tissues proposes that developing, or possibly adult, dental tissue might be used as an allograft source of trophic support for diseases of the nervous system such Alzheimer's disease <sup>23</sup>. Neurotrophic factors derived from dental pulp may also act as targetderived neurotrophic factors in the trigeminal system. These factors promote the survival of dopamine (DA) neuron and provide neuroprotection for DA neurons. Some dental pulp cells also differentiate into cells with neuronal characteristics <sup>24</sup>.

It has been shown that NT-4 regulates proliferation and differentiation of dental epithelium, and promotes the production of enamel matrixes <sup>25</sup>. Results of another study revealed that all neurotrophin mRNAs were detected in embryonic teeth<sup>18</sup>. They also showed the developmentally changing, distinct expression patterns for NGF and NT-3. It has been shown that NGF is involved in the guidance of trigeminal axons to embryonic teeth. Expression of NGF mRNAs in postnatal teeth was correlated with trigeminal axon growth. This may propose a role for NGF in local sprouting and establishment of the final innervation pattern of the dental papilla and dentin.

These results imply that NGF is required for tooth innervation and that other neurotrophins may also have regulatory roles. Furthermore, the expression patterns of NGF, NT-3, and NT-4/5 as well as neurotrophin receptors suggest that the neurotrophin system may also provide non-neuronal functions during tooth development <sup>18</sup>. NGF was also characterized as a potent promoter of mineralization during dentin formation and it also promotes *in vitro* odontoblast differentiation <sup>26</sup>. It has been shown that NT-4 could regulate proliferation and differentiation of the dental epithelium and promote production of the enamel matrix <sup>27</sup>.

In study models, human dental pulp cells expressed a neuronal phenotype and produced the neurotrophic factors NGF, GDNF, and BDNF. Dental pulp cells protected primary neurons in *in vitro* models of Alzheimer's and Parkinson's disease and can be considered as possible candidates for studies on cell-based therapy <sup>28</sup>. Results of a study showed that neurotrophins speed up dentin sialophosphoprotein (DSPP), ALPase, osteopontin (OPN), type I collagen and bone morphogenetic protein-2 (BMP-2) mRNA expressions and calcified substances formation in cultures of HP cells. In addition, NGF stimulated cell proliferation in cultures of these cells. This shows a new role of neurotrophins in differentiating pulp cells into mineralizing cells in addition to the reported function of the peptides. This study also showed that neurotrophins may be candidates for capping agents, which biologically stimulate reparative dentin formation<sup>29</sup>.

#### Conclusion

In general, the results of the current study show the expression of neurotrophins in dental pulp tissue, SHED and neural-like cells differentiated from SHED and the highest level of neurotrophins expression in neurallike cells was observed. Finally, because neurotrophins have neurogenesis, neurotrophic, or neuroprotective effects on the brain, they may have an important role during differentiation of SHED into neural-like cells. Obtained information from this study may lead to a novel method for tooth regeneration.

### Acknowledgement

This study was supported by grants (No; 287112, 287005, 287006) of Dental School, Isfahan University of Medical Sciences.

#### References

- 1. Lindvall O, Kokaia Z. Stem cells in human neurodegenerative disorders-time for clinical translation? J Clin Invest 2010;120(1):29-40.
- 2. Manfredsson FP, Okun MS, Mandel RJ. Gene therapy for neurological disorders: challenges and future prospects for the use of growth factors for the treatment of Parkinson's disease. Curr Gene Ther 2009;9(5):375-388.
- 3. Harada H, Kettunen P, Jung HS, Mustonen T, Wang YA, Thesleff I. Localization of putative stem cells in dental epithelium and their association with Notch and FGF signaling. J Cell Biol 1999;147(1): 105-120.
- 4. Fuchs E, Segre JA. Stem cells: a new lease on life. Cell 2000;100(1):143-155.
- 5. Bianco P, Riminucci M, Gronthos S, Robey PG. Bone marrow stromal stem cells: nature, biology, and potential applications. Stem Cells 2001;19(3): 180-192.
- Bianco P, Robey PG. Stem cells in tissue engineering. Nature 2001;414(6859):118-121.

- 7. Blau HM, Brazelton TR, Weimann JM. The evolving concept of a stem cell: entity or function? Cell 2001;105(7):829-841.
- 8. Blau IW, Basara N, Lentini G, Guenzelmann S, Kirsten D, Schmetzer B, et al. Feasibility and safety of peripheral blood stem cell transplantation from unrelated donors: results of a single-center study. Bone Marrow Transplant 2001;27(1):27-33.
- 9. Huang GTJ, Gronthos S, Shi S. Mesenchymal stem cells derived from dental tissues vs. those from other sources: their biology and role in regenerative medicine. J Dent Res 2009;88(9):792-806.
- Chadipiralla K, Yochim JM, Bahuleyan B, Huang CY, Garcia-Godoy F, Murray PE, et al. Osteogenic differentiation of stem cells derived from human periodontal ligaments and pulp of human exfoliated deciduous teeth. Cell Tissue Res 2010;340(2):323-333.
- Laino G, Carinci F, Graziano A, d'Aquino R, Lanza V, De Rosa A, et al. In vitro bone production using stem cells derived from human dental pulp. J Craniofac Surg 2006;17(3):511-515.
- Miura M, Gronthos S, Zhao M, Lu B, Fisher LW, Robey PG, et al. SHED: stem cells from human exfoliated deciduous teeth. Proc Natl Acad Sci USA 2003;100(10):5807-5812.
- Sakai Y, Yoshiura Y, Nakazawa K. Embryoid body culture of mouse embryonic stem cells using microwell and micropatterned chips. J Biosci Bioeng 2011;111(1):85-91.
- Nosrat CA, Ebendal T, Olson L. Differential expression of brain-derived neurotrophic factor and neurotrophin 3 mRNA in lingual papillae and taste buds indicates roles in gustatory and somatosensory innervation. J Comp Neurol 1996;376(4):587-602.
- 15. Nosrat CA, Tomac A, Lindqvist E, Lindskog S, Humpel C, Stromberg I, et al. Cellular expression of GDNF mRNA suggests multiple functions inside and outside the nervous system. Cell Tissue Res 1996;286(2):191-207.
- Nosrat CA, Fried K, Lindskog S, Olson L. Cellular expression of neurotrophin mRNAs during tooth development. Cell Tissue Res 1997;290(3):569-580.
- 17. Nosrat CA, Fried K, Ebendal T, Olson L. NGF, BD NF, NT3, NT4 and GDNF in tooth development. Eur J Oral Sci 1998;106(Suppl 1):94-99.
- Luukko K, Arumae U, Karavanov A, Moshnyakov M, Sainio K, Sariola H, et al. Neurotrophin mRNA expression in the developing tooth suggests multiple roles in innervation and organogenesis. Dev Dyn 1998;210(2):117-129.

- 19. Luukko K, Sainio K, Sariola H, Saarma M, Thesleff I. Localization of nerve cells in the developing rat tooth. J Dent Res 1997;76(7):1350-1356.
- Luukko K, Suvanto P, Saarma M, Thesleff I. Expression of GDNF and its receptors in developing tooth is developmentally regulated and suggests multiple roles in innervation and organogenesis. Dev Dyn 1997;210(4):463-471.
- Nourbakhsh N, Soleimani M, Taghipour Z, Karbalaie K, Mousavi SB, Talebi A, et al. Induced in vitro differentiation of neural-like cells from human exfoliated deciduous teeth-derived stem cells. Int J Dev Biol 2011;55(2):189-195.
- 22. Nosrat IV, Widenfalk J, Olson L, Nosrat CA. Dental pulp cells produce neurotrophic factors, interact with trigeminal neurons in vitro, and rescue motoneurons after spinal cord injury. Dev Biol 2001; 238(1):120-132.
- Nosrat I, Seiger Ã, Olson L, Nosrat CA. Expression patterns of neurotrophic factor mRNAs in developing human teeth. Cell Tissue Res 2002;310(2):177-187.
- 24. Nosrat IV, Smith CA, Mullally P, Olson L, Nosrat CA. Dental pulp cells provide neurotrophic support for dopaminergic neurons and differentiate into neurons in vitro; implications for tissue engineering and repair in the nervous system. Eur J Neurosci 2004;19(9):2388-2398.
- 25. Iwamoto T, Yamada A, Arakaki M, Sugawara Y, Ono M, Futaki M, et al. Expressions and functions of neurotrophic factors in tooth development. J Oral Biosc 2011;53(1):13-21.
- Arany S, Koyota S, Sugiyama T. Nerve growth factor promotes differentiation of odontoblast-like cells. J Cell Biochem 2009;106:539-545.
- Yoshizaki K, Yamamoto S, Yamada A, Yuasa K, Iwamoto T, Fukumoto E, et al. Neurotrophic factor neurotrophin-4 regulates ameloblastin expression via full-length TrkB. J Biol Chem 2008;283(6): 3385-3391.
- 28. Apel C, Forlenza OV, de Paula VJ, Talib LL, Denecke B, Eduardo CP, et al. The neuroprotective effect of dental pulp cells in models of Alzheimer's and Parkinson's disease. J Neural Transm 2009;116 (1):71-78.
- 29. Mizuno N, Shiba H, Xu W, Inui T, Fujita T, Kajiya M, et al. Effect of neurotrophins on differentiation, calcification and proliferation in cultures of human pulp cells. Cell Biol Int 2007;31(12):1462-1469.