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Induction of neuronal-like phenotype in human mesenchymal stem cells by overexpression of Neurogenin1 and treatment with neurotrophins

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ABSTRACT

Aim of the study: The induced expression of the transcription factors neurogenin1 (Neurog1) or neuronal differentiation 1 (NeuroD1) has previously been shown to initiate neuronal differentiation in embryonic stem cells (ESC). Human bone marrow-derived mesenchymal stem cells (hBMSCs) are ethically non-controversial stem cells. However, they are not pluripotent. In cochlear implantation, regeneration or replacement of lost spiral ganglion neurons may be a measure for the improvement of implant function. Thus, the aim of the study was to investigate whether the expression of Neurog1 or NeuroD1 is sufficient for induction of neuronal differentiation in hBMSCs.
Materials and methods: Human BMSCs were transduced with lentivirus expressing NeuroD1 or Neuorg1. Transduced cells were then treated with small molecules that enhanced neuronal differentiation. Markers of neuronal differentiation were evaluated.
Results: Using quantitative reverse transcription PCR, the up-regulation of transcription factors expressed by developing primary auditory neurons, such as *BRN3a (POU4F1)* and *GATA3*, was quantified after induction of Neurog-1 expression. In addition, the expression of the receptor *NTRK2* was induced by treatment with its specific ligand BDNF. The induction of expression of the vesicular glutamate transporter 1 was identified on gene and protein level. NeuroD1 seemed not sufficient to induce and maintain neuronal differentiation.
Conclusions: Induction of neuronal differentiation by overexpression of Neurog1 initiated important steps for the development of glutamatergic neurons such as the spiral ganglion neurons. However, it seems not sufficient to maintain the glutamatergic spiral ganglion neuron-like phenotype.

1. Introduction

Severe sensorineural hearing loss as a consequence of damage of inner hair cells (Lawner et al., 1997) is treated with cochlear implantation (CI). This device, consisting of an electrode array that bypasses damaged and lost hair cells, directly activates the first auditory neurons in the Rosenthal's canal, the so-called spiral ganglion neurons (SGN). Thus, the benefit of CI depends on the number and excitability of SGN (Gantz et al., 1993; Nadol and Merchant, 2001; Seyyedi et al., 2014; Xu et al., 2012). Enhancement of neuronal populations has the potential to improve this therapy.

There are two types of bipolar SGN: Type I SGN innervate inner hair cells (one hair cells per SGN) and are myelinated in the murine system (about 90% of all SGN) (Stöver and Diensthuber, 2011). Type II SGN innervate outer hair cells (several of them per SGN) and are not myelinated (Appler and Goodrich, 2012). SGN are glutamatergic neurons expressing two out of three vesicular glutamate

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receptors, namely VGLUT1 and VGLUT2. These glutamate receptors are the most specific markers for glutamatergic neurons known to date. During embryonic development, a network of transcription factors and soluble factors serves to generate SGN and to provide correct innervation. The main function of the transcription factors is in differentiation of neurons and in determination of their specific nature, the main function of soluble factors is to provide innervations within neuronal networks.

Among the transcription factors, Neurog1 is necessary to commit ectodermal cells within the otic placode to a neuronal fate and the two proneuronal bHLH genes Neurog1 and NeuroD1 are necessary for a proper neuroblast delamination (Ma et al., 1998). However, they are not sufficient to induce the development of neurons demonstrating that, apart from Neurog1, further factors are needed in order to induce the neuronal development in the inner ear (Puligilla et al., 2010). The most critical step in the development of SGN seems to be the expression of *GATA3* and *POU4f1* (also known as Brn3a) (Duncan et al., 2011; Huang et al., 2001; Lawoko-Kerali et al., 2004; Maier et al., 2014). *GATA3* is expressed in auditory but not vestibular ganglion neurons in the mouse at embryonic day 14 (Rivolta and Holley, 1998) and mutations have been linked to syndromal sensorineural deafness (HDR syndrome) (Muroya et al., 2001; Van Esch et al., 2000). It is continuously expressed throughout the development of SGN (Luo et al., 2013). Deletion of *POU4f1* leads to a significant loss of spiral ganglion neurons and hampers their migration (Erkman et al., 1996). Furthermore, the expression of *NTRK3* and parvalbumin is down-regulated, indicating that *POU4f1* is located upstream of these genes (Huang et al., 2001). In addition, it is required for proper growth and migration of inner ear sensory neurons and for target innervation and axon guidance by SGN (Huang et al., 2001).

As to the soluble factors, neurotrophins and their corresponding Trk receptors are essential for the support of the survival of sensory neurons (Fariñas et al., 1996). The knock-out of *BDNF* (ligand of Ntrk2) or *NT3* (ligand of Ntrk3) in mice results in loss of more than 80% of neurons within the spiral ganglion. On the other hand, stimulation with the ligands has been shown to upregulate their ligands.

In conclusion, the embryonic development of glutamatergic neurons in general and of SGN in particular is incompletely understood. Nevertheless, in experimental otology, cell-based therapies rapidly emerge envisioning to replace degenerated SGN. Thus, neuronal differentiation of stem cells is one of the major goals. Transient expression of Neurog1 in embryonic stem cells (ESCs) has been shown to induce a neuronal fate (Reyes et al., 2008). Additional treatment with brain-derived neurotrophic factor (BDNF) and glial cell line-derived neurotrophic factor (GDNF) led to an increase in neuronal differentiation and to the development of glutamatergic neuron-like cells (Reyes et al., 2008). However, advancement of ESCs towards clinical application is not feasible. Due to ethical and technical considerations, mesenchymal stem cells (MSCs) seem more promising in terms of clinical application. They are easily accessible from various sources such as bone marrow, adipose tissue and skin, they can rapidly be isolated and expanded in vitro, and they are immunologically inert (Seo and Cho, 2012). In addition, they secrete trophic factors that exert neuroprotection (Hao et al., 2014a, b; Sadan et al., 2009; Seo and Cho, 2012) and modulate inflammation (Lotfinejad et al., 2014; Ohtaki et al., 2008; Rhijn et al., 2014; Tan et al., 2014). Most importantly, they have been widely used in clinical studies (Trounson et al., 2011). Even MSC isolated from more exotic tissue such as nasal mucosa (Bas et al., 2014) or Wharton's jelly (Mellott et al., 2015) can be advanced towards an inner ear phenotype. Overexpression of Neurog1 improved the therapeutic efficacy of human bone marrow-derived MSCs (hBMSCs) transplanted for the treatment of brain ischemia in rodents (Kim et al., 2008). Moreover, improved neurological functions of rats with traumatic brain injury were observed after transplantation of MSCs transfected with a BDNF gene (Yuana et al., 2013). Improved function was also observed in a rat model for sub-acute spinal cord injury after treatment with neurally induced bone marrow derived mesenchymal stem cells (Yazdani et al., 2012). Thus, neuronal differentiation seems not only desirable for cell replacement therapies but might also present an option for improving the therapeutic efficacy of mesenchymal stem cells.

The protocol utilized by Reyes and his colleagues has not been tested in hBMSCs. The aim of the present study was therefore to investigate whether overexpression of Neurog1 or Neuronal differentiation factor 1 (NeuroD1) followed by treatment with BDNF, NT3 and retinoic acid (RA; RA can drive cells to adopt a neuronal phenotype, especially when combined with neurotrophic factors (Jones-Villeneuve, 1982; Takahashi et al., 1999)) in hBMSCs is sufficient to induce the developmental path of auditory neurons by up-regulation of the key transcription factors GATA3 and POU4F1 resulting in the generation and the preservation of a glutamatergic phenotype in vitro.

2. Material and methods

2.1. Ethics statement

Human bone marrow was obtained during routine orthopedic procedures from otherwise healthy donors after approval of the institutional ethical committee of Hannover Medical School. Written informed consent was obtained from all donors. All personal information apart from age and gender was deleted.

2.2. Isolation and cultivation of human MSCs

Human BMSCs were prepared from fresh bone marrow aspirates (Schäck et al., 2013a,b). Briefly, heparin-containing bone marrow diluted with phosphate buffered saline (PBS) was carefully layered onto a density gradient of Biocoll (Biochrom AG, Berlin, Germany, $\rho = 1.077$ g/ml) and centrifuged for 30 min at 500*g*. The mononuclear cells located at the interface were seeded in cell culture flasks. The MSC medium consisted of DMEM FG 0415 (Biochrom, Berlin, Germany) with 10% (v/v) fetal bovine serum (FBS; not heat-inactivated) (Thermo Fisher Scientific, Schwerte, Germany), 20 mM HEPES, 1% (100 U/ml/100 μg/ml) penicillin/streptomycin (Biochrom AG, Berlin, Germany), and 2 ng/ml human recombinant FGF2 (PeproTech, Hamburg, Germany). The cells were cultured at 37 °C with 5% CO_2 at 85% humidity and washed regularly. The cells were passaged at a density of around 70% by the use of 0.025% Trypsin-EDTA solution (Biochrom AG, Berlin, Germany) and seeded at a density of $2*10^3$ cells per cm^2.

2.3. Lentiviral vector construction

At the time when the present study was initiated, cDNA material from an appropriate human source was not available in our lab. However, since previous unpublished results have proven that murine factors showed a biological effect in the human system and vice versa, we decided to perform the clonings of *Neurog1* and *NeuroD1* from murine cDNA sources. The coding sequence of murine *NeuroD1* was amplified by polymerase chain reaction (PCR) at 58.6 °C from RNA isolated from mouse brain and reverse transcribed into cDNA with the following primers: NeuroD1_fwd 5′- TATA**GGATCC**GCCACCATGaccaaatcatacagcgagagcg (Bam HI cloning site in bold, Kozak translation initiation sequence underlined) and NeuroD1_rev 5′-TATA**GTCGAC**CTAAGCGTAATCTGGAACATCGTATGGGTAATCGT GAAAGATGGCATTAAGCTGGGC (Sal I cloning site in bold, HA epitope tag sequence underlined). The coding sequence of murine

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