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MORPHOLOGICAL DIFFERENTIATION OF TAU–GREEN FLUORESCENT PROTEIN EMBRYONIC STEM CELLS INTO NEURONS AFTER CO-CULTURE WITH AUDITORY BRAIN STEM SLICES

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Abstract—Most types of congenital and acquired hearing loss are caused by loss of sensory hair cells in the inner ear and their respective afferent neurons. Replacement of spiral ganglion neurons (SGN) would therefore be one prioritized step in an attempt to restore sensory neuronal hearing loss. To initiate an SGN repair paradigm we previously transplanted embryonic neuronal tissue and stem cells (SC) into the inner ear *in vivo*. The results illustrated good survival of the implant. One such repair, however, would not have any clinical significance unless central connections from the implanted SGN could be established. For the purpose of evaluating the effects of cell transplantation on cochlear nucleus (CN) neurons we have established organotypic brain stem (BS) cultures containing the CN. At present we have used *in vitro* techniques to study the survival and differentiation of tau–green fluorescent protein (GFP) mouse embryonic stem cells (MESC) as a mono- or co-culture with BS slices. For the co-culture, 300 μm thick auditory BS slices encompassing the CN were prepared from postnatal Sprague–Dawley rats. The slices were propagated using the membrane interface method and the CN neurons labeled with DiI. After 5±2 days in culture a tau-GFP MESC suspension was deposited next to CN in the BS slice. Following deposition the MESC migrated towards the CN. One and two weeks after transplantation the co-cultures were fixed and immunostained with antibodies raised against neuroprogenitor, neuronal, glial and synaptic vesicle protein markers. Our experiments with the tau-GFP MESC and auditory BS co-cultures show a significant MESC survival but also differentiation into neuronal cells. The findings illustrate the significance of SC and auditory BS co-cultures regarding survival, migration, neuronal differentiation and connections. © 2009 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: embryonic stem cells, organotypic culture, cochlear nucleus, vestibulocochlear nerve, neuronal differentiation, cochlear implant.

Partial or complete loss of hearing ability is a seriously disabling condition that affects millions of people around the globe. Age, genetic abnormalities and environmental factors (e.g. noise and ototoxic drugs) are major causes of hearing impairment (Holley, 2002). Due to the limited regenerative potential of mammalian hair cells most types of congenital and acquired hearing disorders arise from the damage or loss of these cells (Ogata et al., 1999; Zheng et al., 1999). Since the hearing organ only partially can be replaced by today's hearing aids significant interest is directed towards strategies to replace hearing function, e.g. by inducing regeneration of the damaged hair cells and their associated neurons. Embryonic stem cells (ESC) have the capacity for self-renewal and ability to differentiate into any cell type (Doetschman et al., 1985) and are promising candidates for the development of cellular and genetic therapies for many disorders, including hearing deficiencies. It is shown that ESC are capable of differentiating into hair cells (Li et al., 2003; Rivolta et al., 2006; Matsumoto et al., 2008). In addition, several recent studies have suggested that exogenous stem cells (SC) and endogenous inner ear progenitor cells can be used for replacement of auditory neurons (Rask-Andersen et al., 2005; Corrales et al., 2006; Martinez-Monedero and Edge, 2007; Altschuler et al., 2008; Martinez-Monedero et al., 2008). Due to their differentiation capabilities into hair cells and auditory neurons it is suggested that SC therapies can become clinically feasible in hearing disorders.

The loss of hair cells in the mammalian cochlea is followed by a secondary degeneration of auditory neurons (Webster and Webster, 1981; Zappia and Altschuler, 1989; Dodson and Mohuiddin, 2000; McFadden et al., 2004). These spiral ganglion neurons (SGN) are target neurons for cochlear implants (CI) which have rendered a tremendous success in regaining hearing for profoundly deaf patients. Today more than 100,000 patients in the world have a CI and the implantation rate is progressive. The number of functioning auditory neurons is essential for the

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Abbreviations: BS, brain stem; CI, cochlear implants; CM-DiI, chloromethylbenzamido derivative of 1,1′-dioctadecyl-6, 6′-di-(4-sulfophenyl)-3, 3, 3′, 3′-tetramethylindocarbocyanine perchlorate; CN, cochlear nucleus; DAPI, 4′,6-diamindino-2-phenylindole dihydrochloride; DMEM, Dulbecco's modified Eagle's medium; EDTA, ethylenediaminetetraacetic acid; ESC, embryonic stem cells; FBS, fetal bovine serum; GDNF, glial derived neurotrophic factor; GFAP, glial fibrillary acidic protein; GFP, green fluorescent protein; HBSS, Hanks' balanced salt solution for further dissection; IR, immunoreactive; LIF, leukemia inhibitory factor; MEF, mouse embryonic fibroblasts; MESC, mouse embryonic stem cells; NT−3, neurotrophin-3; PBS, phosphate-buffered saline; PFA, paraformaldehyde; RT, room temperature; SC, stem cells; SD, Sprague–Dawley; SGN, spiral ganglion neuron; SV2, synaptic vesicle protein 2; Tuj1, class III β-tubulin.

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function of the CI. It would therefore be of a great clinical significance if SC differentiation could not only be directed towards forming new auditory neurons but also render newly formed connections with the cochlear nucleus (CN) in the medulla. In case of an auditory neuronal deficit such newly formed connections could improve the functional significance of a CI.

The generation of ESC-derived neural cells is a very well-studied example of SC differentiation (Brustle et al., 1997, 1999). Further, it was previously shown that ESC-derived neurons and astrocytes could become functionally integrated into hippocampal slice cultures (Benninger et al., 2003; Scheffler et al., 2003). We have previously shown *in vivo* that implanted mouse embryonic stem cells (MESC) can survive, differentiate into neurons and migrate into the CN following transplantation to the vestibulocochlear nerve (Hu et al., 2004a; Regala et al., 2005). Moreover, in order to explore the neuronal mechanisms in the CN following MESC implantation we have developed an *in vitro* co-culture model where various types of cells can be transplanted to the auditory nerve on the brain stem (BS) slice (Thonabulsombat et al., 2007; Glavaski-Joksimovic et al., 2008). By using morphology and immunohistochemistry we have illustrated that auditory BS slices can survive very well in *vitro* for more than five weeks.

In the present study we have explored the effect of auditory BS slice tissue on MESC differentiation. In order to further elucidate the differentiation stage at which SC should be delivered *in vivo* in the future (Coleman et al., 2007a) we have searched for interactions between implanted undifferentiated ESC and auditory BS neurons. During a two week co-culture period implant survival, migration and differentiation of the MESC were explored. Our findings illustrated that the monocultured MESC differentiated into a higher degree of neurons than glial cells. However a significantly higher percentage of MESC in co-culture with BS differentiated into neurons as compared to monoculture. The findings illustrate a stimulatory effect from the auditory BS on neuronal differentiation of tau–green fluorescent protein (GFP) MESC. Exploration of interactions between the co-cultured implant and host was evaluated from expression of the synaptic vesicle protein 2 (SV2), which is a membrane glycoprotein specifically localized to secretory vesicles in neurons and endocrine cells (Bajjalieh et al., 1992). It was previously shown that the isoform SV2A is expressed ubiquitously throughout the rat brain at varying levels while the other isoform, SV2B, has a more limited distribution with varying degree of co-expression with SV2A (Bajjalieh et al., 1994). Further, in the cochlea it has been shown that SV2 is expressed in neuronal structures surrounding the basolateral surface of inner and outer hair cells (Layton et al., 2005). In the present study we observed punctuated SV2 staining in tau-GFP MESC in contact with BS in the slice preparation suggesting formation of synaptic connections between MESC and CN neurons.

EXPERIMENTAL PROCEDURES

Animals

Pregnant Sprague–Dawley rats (SD; $n=6$) were obtained from Scanbur (Stockholm, Sweden). The animals were maintained through parturition in individual cages under standard colony conditions, with food and water available *ad libitum*. Postnatal rats (P 12–P 14, $n=50$) were used for preparing the host tissue slices. Wild-type mouse C57BL6 (Scanbur) embryos were used as donor animals for fibroblast culture preparation (E 13−E 15, $n=4$). All animal-related procedures were conducted in accordance with local ethical guidelines and approved animal care protocol (approval N 329/07 and 330/07).

Tau-GFP MESC cultures

The tau-GFP MESC used in this study were generously provided by Dr. John Mason, Genes and Development Group, Department of Biomedical Sciences, University of Edinburgh, Edinburgh, UK. In Dr. Mason's laboratory a linearized tau-GFP expression construct pTP6 had been introduced in the MESC by electroporation (Pratt et al., 2000). In this construct the tau-GFP fusion protein and puromycin resistance genes were linked by an internal ribosome entry site and were under the control of a powerful promoter active in all cell types including those of the CNS. In our laboratory tau-GFP MESC were propagated and maintained as previously described (Evans and Kaufman, 1981; Wobus et al., 1984; Wernig et al., 2002). Briefly, MESC were co-cultured with mitotically inactivate mouse embryonic fibroblasts (MEF) in 5% CO_2 at 37 °C. MESC culture medium consisted of Dulbecco's modified Eagle's medium (DMEM with L-glutamine, glucose, and without sodium pyruvate; GIBCO Invitrogen Corporation, Grand Island, NY, USA) supplemented with 15% fetal bovine serum (FBS; GIBCO Invitrogen Corporation), 0.5% penicillin/streptomycin (GIBCO Invitrogen Corporation), 1% minimal essential medium (MEM)—nonessential amino acids (GIBCO Invitrogen Corporation), 2 mM glutamine (GIBCO Invitrogen Corporation), 0.1 mM β-mercaptoethanol (Sigma, St. Louis, MO, USA), and 10^3 U/ml leukemia inhibitory factor (LIF, Chemicon, Hofheim, Germany). For fibroblast cultures, 13–15 day old mouse embryos were trypsinized and cultivated in MEF medium containing DMEM, 10% FBS, 1% penicillin/streptomycin, and 2 mM glutamine (all obtained from GIBCO Invitrogen Corporation). Flasks of fibroblasts were incubated in 5% CO_2 at 37 °C until growth was confluent. Cells subcultivated two or three times were mitotically inactivated by treatment with mitomycin C (10 μg/ml, Sigma) for 2 hours at 37 °C, seeded onto plastic Petri dishes (Falcon, Becton Dickinson Labware, Franklin Lakes, NJ, USA) and used as feeder layers. Control cultures could never grow after mitomycin C treatment. Tau-GFP MESC were passaged by dispersion after treatment with 0.025% trypsin–EDTA buffer (GIBCO Invitrogen Corporation) on a fresh feeder layer every 2–3 days. Immediately prior to plating MESC on feeder plates, MEF medium was replaced with fresh MESC medium.

For induction of neural differentiation undifferentiated tau-GFP cells were dissociated and plated on round coverslips coated with poly-D-lysine and laminin (BD Biosciences, Bedford, MA, USA) placed in four-well culture plates (Nunc, Roskilde, Denmark). Tau-GFP MESC were plated at the density of $0.5-1.5\times10^4/cm^2$ in MESC medium without LIF. The medium was changed every 2–3 days in 5% CO_2 at 37 °C during the entire 7 and 14 day survival period respectively.

Interface organotypic slice cultures

The SD postnatal rats (P 12–P 14) were deeply anesthetized i.m. using ketamine (Pfizer AB, Täby, Sweden, 4 mg/100 g BW) and xylazine (Bayer AG, Leverkusen, Germany, 1 mg/100 g BW), sacrificed by cervical transection and the skulls opened longitudinally along the midline. After removal of the posterior skull the