Journal Club

Editor's Note: These short, critical reviews of recent papers in the *Journal*, written exclusively by graduate students or postdoctoral fellows, are intended to summarize the important findings of the paper and provide additional insight and commentary. For more information on the format and purpose of the Journal Club, please see http://www.jneurosci.org/misc/ifa_features.shtml.

Differentiation of Mouse Embryonic Stem Cells into Spiral Ganglion Neurons: A Therapeutic Approach to Deafness

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Review of Reyes et al. (http://www.jneurosciorg/cgi/content/full/28/48/12622)

Sensorineural deafness is typically caused by irreversible loss of inner hair cells. Secondary to this inner hair cell loss is the loss of spiral ganglion neurons (SGNs). One possible way of treating this deafness is the use of a cochlear implant. A cochlear implant is a surgically implanted electronic device that replaces lost inner hair cells by transforming sounds into a series of electrical pulses that are applied directly to SGNs along the surface of the cochlea. This technique makes use of the topographical arrangement of the cochlea, with high-frequency sounds coded near the base, and low-frequency sounds at the apex of the cochlea. The electrode of the cochlear implant is in close proximity to auditory neurons which lie along the length of the cochlea.

Obviously, a cochlear implant can only be successful when enough functioning SGNs are present. Experimental strategies to rescue SGNs have been based on treatment with specific neurotrophic growth factors such as ciliary neurotrophic factor (CNTF) and brain-derived neurotrophic factor (BDNF), factors known to promote the embryonic development of cochlear neurons (Staecker et al., 1996; Nakaizumi et al., 2004). Instead of enhancing the survival of SGNs, another approach could be to implant new SGNs using embryonic stem (ES) cells. Hence, research recently conducted by Reyes et al. (2008) aims to transform mouse ES cells into glutamatergic SGNs.

Previous attempts to create SGNs from ES cells in the cochlea have experienced limited success, as the majority of implanted cells differentiated into glia cells, not neurons, despite the addition of neurotrophic factors. Reyes et al. (2008) hypothesized that the addition of a proneural transcription factor along with the neurotrophic factors would increase the number of neurons formed. A likely candidate for such a proneural factor is Neurog1, which is expressed in developing otocysts and is required for SGN differentiation, migration, and survival. Therefore, Reyes et al. (2008) used Neurog1, BDNF, and glial cell line-derived neurotrophic factor (GDNF) to obtain an increased proportion of differentiated cells resembling the SGNs of the auditory nerve.

Reyes et al. (2008) began by creating an inducible Neurog1 ES cell line that also expressed enhanced green fluorescent protein (eGFP). The ES cells were induced to express Neurog1 and cultured with and without the neurotrophic factors BDNF and GDNF. After 24 h, 72 h, and 5 d in culture, cells were immunostained with antibodies for Neurog1, the neuronal marker TUJ1, vesicular glutamate transporters VGLUT1 and VGLUT2, and glial fibrillary acidic protein (GFAP) to assess the differentiation of the cells. Cells colabeled with TUJ1 and VGLUT1/2 were deemed to be glutamatergic neurons, like SGNs. In addition, quantitative rt-PCR was used to examine the expression of neurotrophic factor receptors, Neurog1, and three Neurog1 targets that are typically upregulated during SGN differentiation: Brn3a, GATA3, and NeuroD1.

After 24 h *in vitro*, cells induced to express Neurog1 displayed a typical ES morphology but many with small extensions. After 72 h, Neurog1-expressing cells showed robust neuronal differentiation, whereas no neuronal differentiation was observed in cells in which Neurog1 expression was not induced. Although neuronal differentiation occurred in both Neurog1-expressing groups, a significantly higher percentage of cells expressed neuronal markers when exposed to neurotrophic factors (~69 vs ~54%). In both neuronal populations, a similar percentage of cells (~67%) expressed VGlut1/2.

At 5 d *in vitro*, the Neurog1-expressing cells that were exposed to neurotrophic factors exhibited a much denser neuritic network than those not exposed to these factors. However, in both groups, there was an increase in GFAP-positive cells (30%). So at this time point, only 45% of the Neurog1-expressing cells became neurons without neurotrophic factors, versus 56% of those exposed to neurotrophic factors. Quantitative rt-PCR confirmed the induction of an SGN phenotype of the induced neurons by demonstrating a large

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upregulation of the expression of Brn3a, GATA3, and NeuroD1 at 72 h [Reyes et al. (2008), their Fig. 5].

Next, Reyes et al. (2008) implanted inducible Neurog1 ES cells into the left cochlea of guinea pigs, using the right cochlea as a control. The test animals were systematically deafened with drug injections that resulted in near total loss of both inner and outer hair cells, as well as associated SGN, as confirmed by auditory brainstem responses. The mouse ES cells were injected and then treated for 2 d to induce expression of Neurog1. Control animals did not receive Neurog1 expression induction; however, all animals were equipped with an osmotic pump to deliver infusions of BDNF and GDNF for 27 d.

After 4 weeks, implanted ES cells were found throughout the scala tympani and modiolus of the cochlea [Reyes et al. (2008), their Fig. 6, Fig. 7, Fig. 8]. Undifferentiated cells were still present after 4 weeks, showing a typical small, round morphology; their actual number was not mentioned. The neuronal marker TUJ1-positive ES cells were larger and exhibited a stellate or fusiform morphology, typical of neurons. Actual SGN numbers were calculated by dividing the number of TUJ1/VGLUT1/2-coimmunolabeled cells by the total number of TUJ1-positive cells, i.e., neurons (which were 66% of the implanted ES cells): a mean of 79% VGLUT1/2-expressing neurons was found *in vivo* which is comparable to the 75% found *in vitro*. Animals, in which Neurogl-expression was not induced, had significantly fewer cells that exhibited neuronal phenotypes. In this study, only 1 of the 5 guinea pigs showed a significant proportion (30%) of GFAP-positive glial cells while previous studies with similar experiments all showed a vast majority of glia. Even in the early hours of development, the ES cells in this study resisted differentiation into glia, with the switch happening only after Neurog1 expression, which inhibits gliogenesis, was turned off.

From these results, Reyes et al. (2008) conclude that by mimicking developmental conditions it is possible to increase the number of ES cells that differentiate into SGNs. Their use of transiently expressed Neurog1 gene is indeed a novel and successful improvement of previous protocols. However, their assumption that these neurons were SGN neurons seems premature, since it was mainly based on the expression of a limited number of marker proteins. Whether or not these neurons were truly functional and had electrophysiological properties characteristic for SGNs was unclear because no electrophysiological tests were reported.

In their discussion, Reyes et al. (2008) comment that high percentages of cells differentiate *in vivo* without inciting an immune reaction, also because the implantation site was described as relatively immunoprivileged. But it is not clear what criteria were used to assess actual immune responses and no concrete data were presented. This aspect of the research certainly merits further analysis and could be evaluated using T-cell or B-cell staining. Although ES cells have been considered low immunogenic, eventually upon differentiation they will express MHC proteins, so an immune reaction is to be expected, and immunosuppression will likely be required.

The next essential step before this ES differentiation approach can proceed to the clinic will be to demonstrate that the differentiated cells are functional SGNs, with the proper electrophysiological characteristics and the ability to form a substitute auditory nerve able to transduce signals received in the cochlea to proper circuitries in the auditory cortex. Functional integration will be a landmark in the pursuit of the advancement of cochlear prosthetics for the hearing impaired. For functional integration, the environment in which the ES cells will be implanted should be considered, as previous research suggests that the normal adult cochlea may lack essential components for the long-term survival and integration of implanted cells (Ulfendahl et al., 2007). Finally, a major concern for future clinical application of ES cells in deafness treatment is the possible transformation of undifferentiated ES cells into teratoma-forming cells. In the study by Reyes et al. (2008), the number of undifferentiated ES cells present in the cochlea after implantation was not reported and it is unclear whether teratomas emerged. Teratoma formation by undifferentiated ES cells could be avoided by assuring complete differentiation of ES cells before implantation.

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