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Stem cells and cell lines from the human auditory organ: applications, hurdles and bottlenecks in the development of regenerative therapies for deafness

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The development of any stem-cell-based therapy (and a potential one for deafness is no exception) relies on the generation of the necessary tools: 'cell drugs' that can be safely manufactured for their clinical application. An increasing body of work has focussed on the identification, in animal models, of potential stem cell sources that could have an application for regenerative therapy in the auditory organ. A still more circumscribed effort – owing to ethical and technical difficulties – aims to obtain the actual potential therapeutic candidates (i.e. stem cells of human origin). A recently isolated population of human fetal auditory stem cells could become an ideal model for some of the challenges lying ahead regarding cochlear stem cell purification, expansion and maintenance.

Hearing loss is a major condition that affects millions of people worldwide. The severity of the problem is such that it is believed to affect more than half the population over the age of 60. In the mammalian cochlea, the two sensory cell types (i.e. the hair cells and the auditory neurons) are only produced during the fetal stages. The capacity to replace damaged sensory cells is lost soon after birth, making ensuing deafness irreversible. Although our knowledge of the mechanisms of hair cell and neuronal development has increased exponentially during the past two decades, a medical treatment for deafness remains elusive. Cochlear implants continue to be the only therapeutic option for the profoundly deaf, but the success of their application relies on the preservation of healthy, functioning sensory neurons. It is not surprising, then, that the recent developments in stem cell technologies are bringing new hopes for the hearing impaired.

These hopes were stimulated by the finding by Li *et al.* [1] that stem cells were still present in the vestibular organs of adult mice. Unfortunately, the adult cochlea does not seem to harbour an equivalent population beyond the early postnatal stages; their number declines sharply after birth, and they are almost gone by the third week of age [2]. In the search for alternative external sources for transplantation, efforts have been made to induce differentiation of auditory progenitors from mouse embryonic stem cells (ESCs) [3,4]. Studies on transplantation have been done with undifferentiated and neuralised mouse ESCs, as well as neural stem cells, but these cell types are less adequate because they are not of otic origin and might not fully resemble the tissue they aim to repair [5–9].

The potential replacement of hair cells with an exogenous source faces formidable challenges, some of which have been summarised recently in several excellent reviews [10–13]. For example, the organ of Corti is a highly organised structure, and the transplanted cells would have to be able to repopulate the epithelium without disrupting its precise architecture. To do that, the transplanted cells will need to graft into a cell layer by disrupting strong tight junctions, which will need to be reconstituted quickly to maintain the sealed nature of the scala media. This space harbours the endolimph, an extracellular fluid with an unusually high content of K^+ that is necessary for the correct functioning of the cochlea.

By contrast, replacing the sensory neurons should not need the surgical invasion into the scala media and such a sophisticated cellular arrangement *in situ*. The replacement of sensory neurons seems a more realistic aim for cell therapy in the ear – achievable, perhaps, in a not-too-distant future while some of the challenges presented by hair cell replacement are solved.

But besides these roadblocks, specific to the application of stem cells to the ear, the field also faces more general problems common to all potential stem cell treatments. Clinical-grade cells would

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have to be produced from a human source, using reliable protocols that would need to be scaled up and systematically applied. In the following paragraphs, I highlight some of the important issues lying ahead and the technical challenges that I believe need addressing to move auditory stem cells closer to a clinical therapy.

Human fetal auditory stem cells: a model for cochlear stem cell biology in humans

Despite the advances made in rodents, until recently hearing research has suffered from the lack of a suitable model to study stem cell biology of the auditory organ in humans. This started to change a couple of years ago when a population of stem cells was identified in the human fetal cochlea [14] and later, a protocol was developed that enabled their isolation and expansion *in vitro* [15]. By culturing dissociated cells from sensory epithelia from 9- to 11-week-old foetuses in a serum-free media supplemented with EGF, IGF1 and bFGF (otic stem cell full media), a homogenous population that expressed stem cell markers such as NESTIN, SOX2, OCT4 and REX1 (among others) was selectively expanded.

Several stem cell lines were established that retained expression of these stem cell markers and remained proliferative for several months. When cells were grown under defined culture conditions and passaged using a non-enzymatic protocol, the cells remained undifferentiated, growing as an adherent monolayer and displaying an epithelial-like morphology. When they were passaged using trypsin, however, neuronal differentiation was readily induced. Cells grew processes and elongated. After 24–36 hours, they displayed the characteristic bipolar morphology of spiral ganglion neurons. The differentiation process was further supported by exogenous factors such as Shh, NT3 and BDNF, as measured by the expression of the neuronal markers *NEUROGEN1*, *BRN3A*, b-TUBULIN III and NEUROFILAMENT 200. Moreover, five to seven days after inducing differentiation, bipolar cells displayed potassium delayed rectifiers and voltage-gated sodium currents. These findings are important because some neurons obtained from mouse embryonic stem cells have failed to express sodium channels [16]. By contrast, culture in the presence of RA and EGF favoured the differentiation into hair-cell-like phenotypes by inducing the expression of *ATOH1* and *BRN3C*, as well as MYOSIN VIIA and PARVALBUMIN. Furthermore, these cells showed a rearrangement of the actin cytoskeleton, resembling the cuticular plate, and expressed the inward rectifier K^+ current (I_{K1}), the slow decay and voltage activation range of which closely resembled those recorded in pre-hearing mouse cochlear hair cells [17]. Besides I_{K1}, hair-cell-like cells also showed a small outward I_K and a sustained inward Ca^{2+} current. This correlates with the expression of the Cav1.3 subunit by cells under 'hair cell conditions', confirmed by RT-PCR. In summary, these cells are an excellent system to study human ear differentiation and, as a biotechnological tool, allowed to define the conditions needed to induce differentiation into neurons and hair cells.

Cell isolation and expansion: crucial bottlenecks in the large-scale production of clinical-grade cells

The translation of any experimental stem cell approach into a real clinical therapy requires the development of standards and quality control processes that are appropriate for the very strict scrutiny imposed by the regulatory bodies. Clinical production of cells will need to adhere to good manufacturing practices to ensure the delivery of a 'cell drug' that is safe, reproducible and efficient. For this, all parts of the process have to be optimised and well defined. To achieve this final goal, is necessary to develop the tools that will facilitate the scaling up of a controlled production process.

Candidates for cell surface markers

An early, important element to address is how to purify the relevant cells in an efficient and non-invasive manner. To prospectively isolate cells from complex tissues or mixtures, whether the cochlea or a mixed population induced from pluripotent cells, we need suitable markers. An ideal approach would be to use cell surface markers to enable their purification by fluorescent automated cell sorting. Other fields, such as haematology, have developed quickly and haematopoietic stem cells have advanced into clinical application because of the availability of well-defined surface markers. These have made possible the specific isolation and purification of progenitors for different lineages and the monitoring of their differentiation [18]. In the auditory field, we completely lack these tools. The use of cell-specific regulatory elements driving reporter proteins such as GFP has been applied to the successful isolation of supporting cells from the postnatal mouse cochlea [19]. Although highly useful for research in animal models, this approach requires genetic modifications of the target cell (or the generation of transgenic mutants, in the case of animal models), which makes it either undesirable or totally unfeasible for clinical applications in humans. The elucidation of the transcriptome of the human auditory stem cells should yield strong candidates of surface molecules to screen with antibodies, as has been done with glial precursors [20]. This targeted, informed approach should be more advantageous than the random screening of available antibodies.

A potentially useful strategy for their purification could be the isolation, by flow cytometry, of 'side populations' based on the ability of certain stem-cell-like cells to exclude Hoechst dye. This method was successfully used by Savary *et al.* [21] to isolate a population of supporting cells from the mouse cochlea that retained progenitor properties. It is important to note that human fetal auditory stem cells (hFASCs) express the ABCG2 transporter, which is believed to be the molecule responsible for the 'side population' phenotype. Because it is a functional assay, however, it is often difficult to standardise, and different laboratories have obtained very dissimilar results while working in other systems [22].

Signalling pathways that control stemness, cell growth and viability

Another relevant feature to establish in the progression towards a clinical therapy is the identification of signalling cascades that would control proliferation, survival and maintenance of the undifferentiated phenotype. Interrogating gene array data could highlight potential pathways that can then be targeted with growth factors or small chemical compounds. For example, the role of PI3K/AKT, MAPK/ERK and NFκβ signalling in the preservation of human embryonic stem cell pluripotency and viability was detected by transcriptional profiling [23], and a similar approach has shown the importance of PDGF, TGF-beta and FGF signalling for the growth of mesenchymal stem cells [24]. Identifying important signalling pathways that control cell growth, stemness and

viability will help in the design of improved culture media that will facilitate their expansion.

It is important to highlight the relevance of studying these events in the right experimental system. Although a lot of information obtained from animal cells has translated well to similar populations of human origin, relevant important differences are found on the behaviour of stem cells, primarily those regarding self-renewal of mouse and human ESCs [25,26]. Some of these differences have been attributed to a problem of 'timing' and the fact that blastocyst-derived human ESCs will 'drift', once *in vitro*, to become comparable to the more developed, epiblast stem cells derived from post-implantation mouse embryos. However, this model of 'cell progression' would still reflect an intrinsic, species-related difference between blastocyst-derived ESCs in their ability to remain pluripotent *in vitro* [27–29].

Information gathered from hFASCs, however, is likely to be applicable to the production of auditory cells from other human sources such as ESCs or iPS cells.

The genetic signature of auditory stem cells

The global analysis of gene expression by using oligonucleotide arrays is an extremely powerful tool for defining the molecular identity of a cell population. A few years ago, it was initially applied to different populations of stem cells aiming to identify a set core of genes that will define 'stemness' across very different types of cells such as embryonic, neural and haematopoietic stem cells of adult and fetal origin [30,31]. These studies were then criticised for producing lists of genes that were too broad and vague [32,33] and did not reflect a true core of stem cell genes and the elusive 'stemness'. Although this criticism was partially appropriate, these experiments were successful in identifying nanog, a gene that was later independently characterised by two different laboratories as having a pivotal role in stem cell behaviour [34,35]. Since then, gene arrays have been applied extensively to studying stem cell populations, and the results have been most useful when the experimental systems are well characterised and the data interrogated by experiment-lead questions. When properly applied, they have, for instance, enabled the identification of signalling cascades involved in the survival and differentiation of human embryonic stem cells [36] and, more recently, the identification of regulatory networks that define different classes of stem cells [37].

In the ear, gene arrays have been used to study the developing cochlea [38], the organ after injury [39,40] and different immortalised mouse cell lines while proliferating and differentiating [41,42]. Their comprehensive use in hearing research has been reviewed recently in Ref. [43].

Screening of small-compound libraries

Small-compound libraries currently available contain hundreds of thousands or even millions of chemicals, targeting different molecules and signalling pathways. The complexity of these huge, highly diverse libraries created through combinatorial chemistry sometimes conspires against the applicability in the search for a specific phenotype (for a review, see Ref. [44]). It is possible, then, to select a more specific approach, in which compounds are synthesised based on key biological motifs used as 'core' scaffolds, and targeting particular sets of molecules or protein families (i.e. kinases, phosphatases, and so on [45]). This target-based approach is feasible when some prior knowledge enables the preselection of 'candidate' signalling cascades that are likely to be relevant for the interested phenotype. By contrast, in a phenotype-based approach, high-throughput screening of unbiased chemical libraries could lead to the identification of new pathways. For example, in Ref. [46], a phenotype-based screening identified pluripotin as a promoter of self-renewal in mES cells. Characterisation of this compound identified it as an inhibitor of Erk1 and RasGAP and identified that the inhibition of these two targets was necessary for the pluripotent phenotype.

Oligonucleotide microarrays and screening of small-compound libraries are not the only high-throughput screening methods that could produce vital results from auditory stem cells. Combinatorial evaluation of synthetic biomaterials uses a library of photopolymerisable material arranged in a microarray format. Using this approach, interaction between cells and the physical surfaces in which they are grown can be studied. In a particular study, the effect of more than 1700 polymers on hESC growth and differentiation was explored [47]. This method could be particularly attractive for exploring potential interactions between stem cells and electrodes, developing their interface and applicability in combination with cochlear implants. Other high-throughput screening methods applicable to stem cell biology are reviewed in Ref. [48].

Concluding remarks

The past few years have produced a series of important developments in the search for a stem-cell-based therapy for deafness. Many challenges lying ahead will need to be overcome if we want to translate this technology into a feasible therapy. But we should not overlook – even at these early stages – the importance of having the right tools for the task. The expression profiling of auditory stem cells isolated from the human fetal cochlea combined with the use of other high-throughput approaches, such as chemical library screening, should allow us to identify crucial markers and signalling pathways vital for their isolation and expansion. The use of hFASCs as a model should enable us to successfully exploit other putative sources with clinical potential, such as ES and induced pluripotent stem cells.

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