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Inner Ear Development and Advances in Inner Ear Organoid Formation

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ABSTRACT

The inner ear houses the cochlea which contains hair cells responsible for the transduction of sound waves. The process of forming these hair cells responsible for hearing is a complicated process consisting of signaling factors that allow ectoderm to form into the otic placode, the otic vesicle, and prosensory cells such as hair cells. Loss of these hair cells contributes to deafness and hearing loss throughout life, and regeneration of these cells may serve as a therapeutic agent for those with irreversible damage. This review looks at the development of the otic placode and hair cells and the possibility of regenerating hair cells from stem cell populations.

KEYWORDS: inner ear, hearing, regeneration

INTRODUCTION

The ear consists of three major sections, the external or outer ear, the middle ear, and the inner ear, all of which are necessary for the sense of hearing. However, it is the inner ear that transduces the physical vibrations of sound waves into electrical pulses that can be interpreted by the brain. The inner ear consists of the cochlea responsible for the sense of hearing, and the vestibular apparatus and semicircular canals, both contributing to balance and detection of body movement (Torres & Giraldez, 1998). The inner ear begins with the formation of the otic placode from the pre-placodal region from which all cranial placodes are formed (Schlosser, 2006). A cranial placode is a thickened patch of ectoderm that will invaginate to form a sensory structure (Schlosser, 2006). The otic placode is induced by signal from the adjacent hindbrain and, at the same time, invaginates to become the otic cup and otic vesicle once closed (Birol et al., 2016). At this time, the cells can be separated into three groups which will further differentiate to make up all the cells of the inner ear. Cells with neural fate will diverge to form the cochleovestibular ganglion, cells of non-sensory fate will develop into structural components of the inner ear, and cells with sensory fate, termed prosensory cells, will diverge to form the hair cells and supporting cells of the cochlea (Wu & Kelley, 2012). Both induction of the otic placode and differentiation into specific cell types require a multitude of signals which will be discussed in this review. Many signaling pathways and transcription factors are involved in this development including the Wingless/Integrated pathway (Wnt), the Notch pathway, Fibroblast Growth Factors (FGFs), and the transcription factor Atoh1, In short, Wnt, Notch, and FGF signaling play major roles in the induction of the otic placode while Atoh1, Notch and FGF signaling allow the prosensory cells to differentiate into hair cells (Abello et al., 2010; Li et al., 2018; Liu et al., 2012; Ohyama et al., 2006; Vendrell et al., 2000; Yamashita et al., 2018) . Most work studying cell signaling factors use knockout mice or use morpholinos which bind to mRNA to prevent the mRNA transcript from being translated (Maroon et al., 2002).

This knowledge is especially significant for those with hearing loss since human regeneration of hair cells, unlike other vertebrate taxa, is not possible without medical intervention (Kniss et al., 2016). In recent years, stem cells have been researched as a potential for regenerative medicine to combat diabetic nephropathy, autoimmune diseases, and pancreatic diseases (Liu et al., 2020). Sensorineural hearing loss is caused by the gradual loss of outer hair cells in the cochlea and can be due to loud noise, pathogens, genetic mutations, aging, drugs, and trauma (Huh et al., 2012; van der Valk et al., 2021). Current treatment for hearing loss is often through hearing aids and cochlear implants. However, if hair cells could be regenerated either in vivo or in vitro and then transplanted into the cochlea, hearing could theoretically be restored. The formation of hair cells has been approached using human induced pluripotent stem cells, mouse induced pluripotent and embryonic stems cells, and cochlea progenitor cells (Chang et al., 2020; Chen et al., 2018; Peng et al., 2021; Ronaghi et al., 2014). While it is important that these cells resemble the morphology of hair cells, they must also be capable of transducing signals if hearing is to be restored. It is necessary for cells to have the capability to transmit signals through synapses which has been achieved in some hair cells derived from

human induced pluripotent stem cells (Chen et al., 2018). Overall, the differentiation into hair cells is dependent upon similar signaling pathways used during normal development such as Atoh1 and Notch (Munnamalai et al., 2012; Puligilla & Kelley, 2017). Future research is necessary for regeneration of hair cells in vivo or to produce functional hair cells in vitro that can be implanted into the cochlea of those with hearing loss. This review will first look at the development of the otic placode, the embryonic precursor of the ear, and the development of the hair cells of the inner ear, the cells which transduce mechanical stimulation into a neurologic stimulus for interpretation by the brain. Then, the review will discuss the recent advances made in the formation of inner ear organoids and the potential for organoid and hair cell therapy.

DEVELOPMENT OF INNEAR EAR BY INDUCTION OF THE OTIC PLACODE

The development of the mammalian ear begins with the formation of bilateral otic placodes which are thickenings of the ectoderm and one of multiple cranial sensory placodes on the head. The otic placode then invaginates to form the otic vesicle, also called the otocyst, which is then specified by the hindbrain to develop further into inner ear structures and neural connections allowing operative hearing (Freyer et al., 2011). Fibroblast growth factor 3 (FGF3) has long been associated with the otic placode. When chicken embryos were infected with an HSV-1 variant linked to FGF3, ectopic otic placodes formed in the anterior region of the embryo; however, no placodes formed when injected in the trunk suggesting a competence needed to form the placodes upon contact with FGF3 (Vendrell et al., 2000). In other words, only some cells and tissues are able to respond to the FGF3 signal and are deemed “competent” cells or tissues. These otic placodes in the embryos with ectopic FGF3 transformed into otic vesicles although they differed in size (Vendrell et al., 2000). Because of this, infected embryos often failed to close the otic vesicle due to its increased size; even when the vesicles closed, there was abnormal morphology of the endolymphatic duct which forms from the vesicle (Vendrell et al., 2000). Together, this suggests that FGF3 secreted from the hindbrain is an otic inducer in chickens and that FGF3 influences the size and morphology of the otic vesicle. FGF8 was also found to be important in otic vesicle formation because when morpholinos for both FGF3 and FGF8 were injected, there was a complete loss of the otic vesicle and a reduction in size in a significant number of embryos when compared to injection with a single morpholino (Maroon et al., 2002). In zebrafish, the effects of FGF8 depends on the time of injection. Overexpression of FGF8 at the 1 cell stage results in no otic vesicle and often a complete deficiency of foxi1 and dlx3b expression, which alone negates any formation of otic vesicle even if FGF8 were present and active. After this stage and up to early gastrulation stages, overexpression of FGF8 results in smaller otic vesicles and foxi1 and dlx3b expression domains are present but smaller than controls (Hans et al., 2007). The loss of FGF8 or the overexpression of FGF8 illustrates that there is a sensitive range of FGF8 needed at particular times in development in order for otic vesicle formation to occur properly (Hans et al., 2007; Maroon et al., 2002). Only the domain of Foxi1, not dlx3b, overlaps with the expression of Pax8 and Pax2a (otic fate inducers) which suggests that competence for ectopic induction of the otic placode is dependent on foxi1 only. Additionally, retinoic acid treatment was used on embryos to increase the expression of FGF3 and FGF8 from the hindbrain and FGF-dependent pax8 expression; only foxi1 expression was expanded but dlx3b expression was the same as the untreated embryos (Hans et al., 2007). FGF8 may have this effect through negatively regulating BMP activity in dorsal ectoderm which was found to activate both foxi1 and dlx3b expression (Hans et al., 2007).

Pax2 is of interest relative to otic induction because it is highly expressed in the ear and other places where there is outward growth (Schaefer et al., 2018). When Pax2 morpholinos were used, Gata3 expression, an early marker for otic tissue, was eradicated but Eya1, Sox2, and Sox3, other early otic markers, were not affected (Christophorou et al., 2010). Therefore, it is likely that Pax2 alone does not determine otic tissue but plays a role with other factors (Christophorou et al., 2010). Pax 2 also plays a role in the shape of placode cells. In cells with loss of Pax2 expression, mutants did not maintain their columnar morphology nor their N-cadherin and NCAM (neural cell adhesion molecule) needed for cell adhesion; this suggests that Pax2 regulates apical cell adhesion molecules which are required for cell elongation and eventually invagination of the otic placode to become the otic vesicle (Christophorou et al., 2010). The tissue field that expresses Pax2 will be divided into otic placode and epidermis (Ohyama et al., 2006). When Pax2+ cells were exposed to canonical Wnt signaling, the cells differentiated into otic placode, but they differentiated into epidermis when not exposed to the Wnt cascade (Ohyama et al., 2006). Moreover, in beta-catenin knockout chicken embryos, Foxi2, which is present in epidermis near the otic placode, expanded to cover the area where the otic placode would normally form (Ohyama et al., 2006). On the other hand, when beta-catenin was overactivated, there was a thickened placode at the expense of less epidermal tissue and increased markers for early ear development such as Pax8 and dlx5 discussed previously (Ohyama et al., 2006).

Notch may also play a role in placode formation that may or may not be linked to the Wnt and beta-catenin pathway.

Signaling molecule	Function
FGF3	Induces otic placode formation in competent tissue only
FGF8	Stimulates otic vesicle formation if present in critical window and in the correct amount
Foxi1	Ectopic induction of otic placode
Pax 2	Influences Gata3 marker and influences shape of placode
Wnt cascade	Induces Pax2-exposed cells to become otic placode instead of epidermis
Notch1	Plays a role in thickening of the placode by refining the area established by the Wnt cascade

Table 1. *Main Signaling Molecules for Otic Placode Induction*

When Notch1 was activated there was a thickening of the placode-like epithelium which expressed Pax8 even though expansion of Pax2 was moderate, a result that could be due to the different fibroblast growth factors that regulate them (Jayasena et al., 2008). When Notch1 was removed or a Notch inhibitor was used, the otic placode was significantly smaller, but not as small as when Wnt signaling is blocked, suggesting that both signaling pathways play a role in determining the size of the otic placode (Jayasena et al., 2008). Both pathways can independently regulate the Pax8, Foxi2, and Jag1 (genes necessary for defining the field that will be used for differentiation into placode and epidermis) but only Wnt signaling regulates the otic-placode specific genes such as Gbx2, Sox9, and Hmx3 which suggests that Wnt signaling is essential for the formation of the otic placode while Notch signaling only enhances expression of these genes (Jayasena et al., 2008). In other words, Notch serves to refine the Wnt gradient that differentiates the Pax2 field: the cells will differentiate into epidermis when Wnt signaling is below threshold, and the otic placode is induced when Wnt signaling is above threshold (Jayasena et al., 2008). Wnt may also cooperate and influence fibroblast growth factors. Spry genes code for an antagonist of tyrosine kinase receptors, including FGFs (Wright et al., 2015). When Spry mutant mice were used, there was an increase in FGF activity, an increase in Wnt signaling activity, and a larger otic placode (Wright et al., 2015). This also supports the claim made by Ohyama that induction of the otic placode and the path of differentiation away from epidermis requires a threshold of Wnt signaling. When FGF signaling is enhanced through low levels of Spry gene expression, Wnt reporter genes were seen in larger areas and resulted in a larger otic placode (Wright et al., 2015). This suggests that FGF normally works upstream of Wnt in order to activate the Wnt signaling pathway (Wright et al., 2015).

DEVELOPMENT OF HAIR CELLS

The loss of hair cells (HCs) in the inner ear is a direct contributor to hearing loss and loss of balance. In mammals, these cells cannot be replaced when lost even though other vertebrates, including zebrafish, have this ability (Kniss et al., 2016). When treated with the transcription factor Atoh1, non-sensory supporting cells of the inner ear were driven to resemble neonatal hair cells by gradually decreasing expression of supporting cell genes and gaining hair cell unique characteristics (Yamashita et al., 2018). This may be due to Atoh1's ability to induce Sox2 expression, a transcription factor known for its role in inducing otic progenitor cells into prosensory cells, even in non-prosensory regions (Puligilla & Kelley, 2017). However, Atoh1 may not be enough to solely convert supporting cells into hair cells depending on the supporting cell type (Liu et al., 2012). With additional manipulation, these cells can become responsive to Atoh1 by adding p27, GATA3, or hPOU4F3 which suggests that some cells require additional factors to become competent (Walters et al., 2017). In another study, adult mice supporting cells were induced by Atoh-1 to form hair cell-like cells, which showed similar properties as hair cells such as potassium ion currents and similar developmental stages, giving an insight into how hair cells are developed and knowledge that can be used for regeneration in humans (Kong et al., 2020).

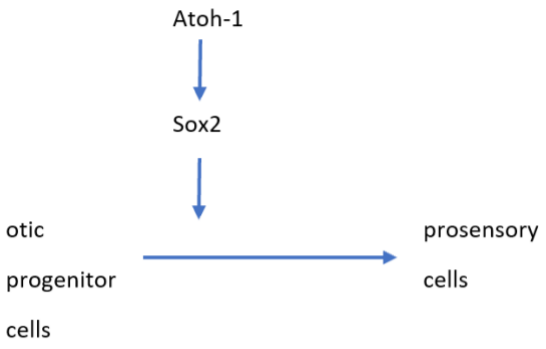

Figure 1. *Role of Atoh-1 in Formation of Prosensory Cells*

Notch activity also appears to be involved in the differentiation of hair cells in the cochlea. Specifically Notch inhibition was shown to influence stereocilia formation, structures essential for converting sound into an electrical signal, in supporting cells and hair cells (Li et al., 2018). Before Atoh1 differentiates cells into hair cells, Notch receptors and the Jag1 ligand determine the sensory regions of the ear which will later diverge into supporting cells and hair cells (Kiernan et al., 2006). Although Sox2 expression initially covers a large area, discrete prosensory patches are developed only where Jag1 and Sox2 reside together because Jag1 works to maintain the Sox2 expression only in these areas (Neves et al., 2011). The knockout of Jag-1 had different effects in the six sensory regions of the ear suggesting that Jag-1 and Notch pathway signaling are partially responsible for patterning the different sensory cell types of the inner ear at least in the initial stages (Kiernan et al., 2006). However, Jag1 is not the only ligand associated with sensory cell differentiation. DI1 competes with Jag1 for Notch receptors and induces higher levels of Notch activity; combination of these two ligands and their opposite regulation, Jag1 regulated by lateral induction and DI1 regulated by lateral inhibition, gives rise to different levels of the functional genes which is activated by Notch signaling (Petrovic et al., 2014).

Notch activity also effects the expression of FGF20, a transcription factor which activates FGFR1 receptors that work to regulate sensory cell formation, as illustrated by the decreased FGF20 expression during notch inhibition and in Jag1 knockouts (Munnamalai et al., 2012). FGF20 has been known to regulate the Hey1 and Hey2 genes, but when Hey1 and Hey2 were both knocked out there was a smaller decrease in the outer hair cells than when FGF20 was knocked out; this suggests that the Hey1 and Hey2 genes aren't the only genes that FGF20 signaling activates in order to differentiate hair cells (Ono et al., 2014; Yang et al., 2019). Recently, the effects of the FGF20 cascade was found to involve both MAPK and PI3K pathways, but when AKT (protein Kinase B) was reinstated hair cell formation was not rescued implying that there are other effects of the pathway (Su et al., 2021). However, the study still illustrated that MAPK and PI3K pathways are activated by the binding of FGF20 to the FGFR1 which eventually impact hair cell differentiation from supporting cells (Su et al., 2021). FGF20 expression continues into the postnatal stage suggesting that it has functions later in sensory cell development (Huh et al., 2012).

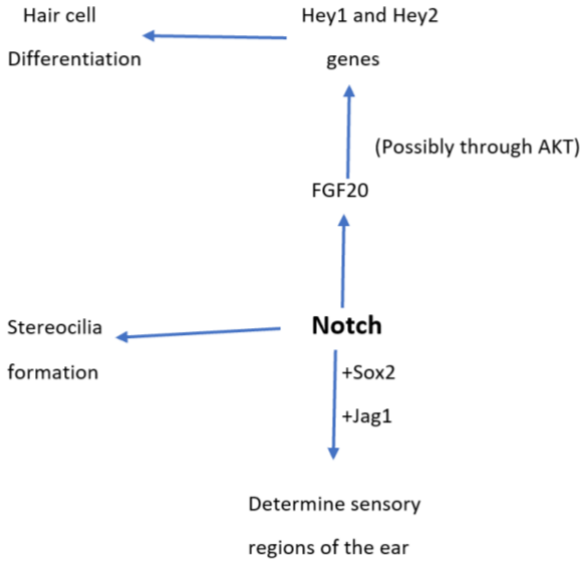

Figure 2. *Many Roles of Notch Signaling in Formation and Differentiation of Hair Cells*

FGFR1 binds with another ligand, currently unknown, in order to determine the prosensory domain, but it is not FGF20 because FGF20 knockouts showed no difference in Sox2 expression representing the prosensory domain (Yang et al., 2019). Interestingly, Sox2 and FGF20 interact to form a temporal buffer region so that specification can occur before differentiation, and the loss of Sox2 and FGF20 resulted in late specification and early differentiation especially in the basal side of the cochlea (Yang et al., 2019). This timing difference may be small, but it leads to altered spatial borders of the cochlea and a large difference in the basal population of outer hair cells and outer supporting cells in the cochlea (even though the apical population and inner cells of the cochlea were altered significantly less) (Yang et al., 2019). Although different than the topic of hair cell formation covered here, FGF signaling, possibly by FGF8 expression, also works to determine the neurogenic domain anterior to the prosensory domain (Abello et al., 2010). FGF proves to be a significant regulator of many steps in ear formation from induction of the otic placode, differentiation of hair cells, and further effects into postnatal periods (Huh et al., 2012; Vendrell et al., 2000; Yang et al., 2019).

DEVELOPMENT OF ORGANOIDS AND GENERATION OF HAIR CELL-LIKE CELLS

Progress made over the past few decades has led to production of inner ear organoids from embryonic and induced pluripotent stem cell populations that have not only aided in developmental research but also have given insight to possible therapeutic stem cell treatments for those with sensorineural hearing loss. In vitro, inner ear organoid formation begins with induction of non-neural ectoderm through BMP activation and TGF-beta inhibition and continues with induction of the pre-placodal region when BMP is inhibited (Koehler et al., 2013). This process appears to promote induction of the sensory tissue and hair cells (Koehler et al., 2013). Although this process is not for use in therapeutics, it provided a significant step in understanding hair cell regeneration and provided basic hair cells to study future therapeutics in vitro. Later, this process was enhanced by using an inhibitor of GSK3, thereby activating beta-catenin and Wnt- associated genes, to increase the number of Pax2+ cells and to increase in the number of vesicles with otic characteristics (DeJonge et al., 2016). Importantly, the correct timing and dosage of the Wnt signaling agonist proved to be essential as researchers discovered a window of optimal Wnt activity, an optimal dosage, and a higher dosage toxic to the cells (DeJonge et al., 2016).

Induced pluripotent stem cells have been of major interest for regeneration of hair cells since they require less invasion than embryonic stem cell recovery and should be more accepted by the host since the stem cell comes from the same individual. One study has been able to produce not only vestibular hair cells but also cochlear hair cells, arguably the most important cells type in terms of treatment for hearing loss (Jeong et al., 2018). More importantly, these cells illustrate stereocilia bundles, which are important for transformation of mechanical sound to electrical pulses and have electric activity suggesting that they not only look like hair cells but can also serve the same function (Jeong et al., 2018). In another study, human urinary cells were transformed into human induced pluripotent stems cells, using OCT-4, Sox-2, KLF-4, and c-MYC, which were then induced to form otic epithelial progenitor cells and then hair-cell like cells (Chen et al., 2018). These cells were then transplanted into a mouse where some of the cells integrated into different parts of the mouse cochlea to form hair cells (Chen et al., 2018). The Myo15A gene was found to be mutated in a family with high rates of deafness (Chen et al., 2018). When induced pluripotent stem cells from those with the mutated gene were genetically corrected, the morphology and conductive function of the hair cell-like cells returned (Chen et al., 2016). A mutation in this gene has been found to contribute to both partial and full deafness in multiple other populations including those in Oman and Morocco making this gene and its activity an important research topic is treatment of hearing loss (Manzoli et al., 2016; Palombo et al., 2017; Salime et al., 2017). In the previous study with the human induced pluripotent stem cells, some of the cells transplanted into the mice also expressed MYO7A, related to Myo15A, signaling that they could form synaptic transmission (Chen et al., 2018).

Human embryonic stem cells have also been used to derive otic progenitors and eventually hair-cell like cells (Ronaghi et al., 2014). The cells were specified for ectoderm by using Wnt inhibition by DDK-1 and TGF-beta inhibitor SIS3 to suppress mesoderm and endoderm differentiation. Then, FGF, Wnt, BMP, and Notch pathways were used which were all mentioned earlier as important for otic tissue and hair cell formation (Ronaghi et al., 2014). Upregulation of Pax2 was seen early in the process, as expected, because of its early role in specifying the placode field (Ronaghi et al., 2014). With use of Atoh-1, hair cell markers were increased after differentiation which illustrated the cells' hair cell-like morphology (Ronaghi et al., 2014). However, these cells did not form hair cell bundles which are necessary for proper function and are usually seen in mature hair cells, suggesting that further research is needed to generate fully functional and mature hair cells (Ronaghi et al., 2014). Mouse embryonic stem cells have also been used in research as they are easier to obtain than human embryonic stem cells (Chang et al., 2020). Again, Wnt, FGF, and BMP signaling are used to transform the stem cell eventually into an otic placode that will invaginate and form sensory cells (Longworth-Mills et al., 2016). A recent advancement in this field involves the use of photobiomodulation to push cells to move, multiply, and differentiate due to the light's effect on the metabolism of the cell (Chang et al., 2020). When used on embryonic stem cells, there was a decrease in supporting cell genes at the same time of the light therapy which suggests a causal role (Chang et al., 2020). Photobiomodulation has also been shown to increase Wnt signaling which was shown earlier to be important in otic placode induction (Chang et al., 2020).

Cochlear progenitor cells also provide a possible route for regeneration of hair cells. By using miR-125 on cochlear progenitor cells capable of forming hair cells, cyclin-dependent kinase2 (CDK2) was decreased which inhibits proliferation of progenitor cells (Peng et al., 2021). This suggests that CDK2 plays a role in proliferation of progenitor cells (Peng et al., 2021). Lgr5 and Lgr6 are stem cell markers and Lgr+ cells are more likely to be able to generate hair cells (Zhang et al., 2018). In these Lgr+ cells, genes involved in hair cell formation, such as Hey 2 discussed previously, were highly expressed which

suggests that they have the potential to differentiate into hair cells and should be used to research stem cell therapeutics for hearing loss (Zhang et al., 2018).

CONCLUSION

In closing, the development of the inner ear is a complicated process that involves multiple transcription factors, which affect the production of mRNA, and signaling molecules from adjacent tissues and cells. The research on Atoh-1, a transcription factor, is controversial as some studies have found that non-sensory supporting cells can be induced to form hair cells, and some studies have demonstrated that Atoh-1 is not sufficient to induce differentiation into hair cells (Liu et al., 2012; Yamashita et al., 2018). It is likely that the transformation depends on the type of supporting cell, each subset requiring additional unique transcription factors. This could be a future topic to study that could yield information on how to turn specific types of cells, already in the ear, into hair cells. While many studies have shown that hair cell-like cells have similar morphology as true inner ear hair cells, the cells may not always function in the same way. Therefore, full transducing capability and synapse connections are needed to have a true model that can be used in research and one day used in therapeutics. Researchers could manipulate the timing of the introduction of transcription factors, the amount of incubation, add additional factors, etc, to find the essential pattern of molecules needed to form functional cells. All in all, any future knowledge on the normal development of mammalian hair cells will allow the field of hair cell regeneration to edge closer to producing functional and transplantable inner ear organoids.

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