UNIVERSITY OF CALIFORNIA, SAN DIEGO

Loss of R-spondin2 Leads to Excess Hair Cell Development in the Mammalian Cochlea

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by

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The thesis of Andrew Thomas Yatteau is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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ABSTRACT OF THE THESIS

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Recent studies have demonstrated the importance of R-spondins, a novel family of secreted proteins, in canonical Wnt signaling and FGF signaling, two pathways that play significant roles in the development of the cochlea, the hearing organ. Cochlear development requires several events including growth, proliferation and cell fate specification. Furthermore, normal cellular patterning in the cochlea, the organ of Corti,

is crucial for auditory function as any patterning defects result in hearing deficit. R-spondins (*Rspo*) consist of four members in mammals encoded by separate genes. To begin to investigate a possible role in cochlear development, the expression of all *Rspo* members was assayed at the time of mechanosensory hair cell differentiation. *Rspo2* and *Rspo3* were detected by PCR but not *Rspo1* or *Rspo4*. The role of *Rspo2* in the development of the mouse cochlea was investigated in this study by examining the *Rspo2*$^{-/-}$ mice. Within the organ of Corti, a single row of inner hair cells and three rows of outer hair cells extend along the basal-to-apical axis of the cochlea. Every sensory hair cell is separated from the next by an intervening non-sensory supporting cell resulting in an invariant mosaic. In the *Rspo2* mutant cochleae, there was an extra row of outer hair cells along with an extra row of support cells in the apical half of the cochlea. These data suggest that *R-spondin2* is involved in cell fate determination in the mammalian cochlea.

INTRODUCTION

Development of the mammalian cochlea

All structures of the inner ear arise from the otic placode, a bilateral thickening of the surface ectoderm in regions adjacent to the developing hindbrain. Further development and invagination of the otic placode leads to the formation of the otocyst. In mice, the cochlear duct begins to develop as a ventral protrusion of the otocyst at embryonic day 11 (E11) (Kelley 2006). By E12 the growing cochlea begins to spiral, and continues coiling throughout development until around E19 or postnatal day 0 (P0) when it reaches its mature shape with approximately 1.75 turns. Beginning at around E13, mitosis ceases in a region between the modiolar 50% and strial 25% of the cochlear duct and becomes the 'zone of non proliferation' (Chen and Segil, 1999), also known as the prosensory domain. This region marks the prospective organ of Corti (OC), the narrow track of sensory epithelium which extends along the floor of the cochlea. Terminal mitoses occur in a wave that progresses from the apex to the base with the last, most basal cells becoming post-mitotic around E13 (Ruben 1967). Cellular differentiation is first observed at E14 when developing sensory cells can be identified in the mid-basal region, and differentiation continues toward both the apex and base of the cochlea.

In the developed cochlea, auditory perception is mediated through the OC. The organ is lined with cells of two basic categories: mechanosensory hair cells, which are the primary transducers of sound waves, and non-sensory supporting cells, which provide structural and physiological support to the hair cells. Both classes of cells have varying phenotypes

and functions, and the arrangement and patterning of these cells along the organ of Corti is crucial for normal hearing, as many hearing-related disorders are caused by abnormal cell patterning in the OC. Mechanosensory hair cells are typified by stereocilliary bundles on the lumenal surface, and are arranged in a pattern of ordered rows that extend along the basal-to-apical axis of the cochlea. A single row of inner hair cells (IHC's) runs along the medial, or modiolar, edge, while three rows of outer hair cells (OHC's) extend along the lateral, or strial, edge of the organ of Corti. The IHC's are separated from the OHC's by the tunnel of Corti, a triangular fluid-filled space that is formed by two rows of highly specialized supporting cells known as pillar cells. Pillar cells are further classified as inner pillar cells (IPC's) and outer pillar cells (OPC's), the former being associated with the IHC's and the latter with the first row of OHC's. Each OHC is separated from neighboring OHC's in the same and adjacent rows by a lumenal projection from a Deiters' cell, which sits beneath the hair cell. Each IHC is separated from adjacent IHC's by the lumenal projection of an inner phalangeal support cell sitting beneath the hair cell. IHC's are also separated by single border cells which contact the medial edges of the hair cells. Other cells which may be considered support cells are the Hensen cells and Claudius cells, which are found on the lateral-most edge of the organ of Corti.

The development of sensory tissue is dependent on the specification of prosensory domains early on in development in which cells become committed to developing into either sensory cells or the associated supporting cells. There are a number of important developmental factors that are thought to play a role in the specification of most prosensory domains, though the specific roles and interactions of many of these factors in

the specification of the cochlear prosensory domain remain to be fully elucidated. Activation of the notch signaling pathway, which includes *Jag1* (a ligand of notch receptors), and the notch modulator *Lunatic Fringe* (*Lfng*), has been shown to be necessary for specification of prosensory domains in the cochlea (Kelley 2007). The HMG-box transcription factor *Sox2* is a likely target of the Notch pathway, and has also been shown to be necessary for formation of the prosensory domain (Kiernan et al. 2005). Though the expression pattern of *Sox2* is consistent with prosensory domain formation, it is not sufficient to induce hair cell development on its own (Dabdoub et al., 2008). Once the prosensory domain has been specified, the individual prosensory cells must now be directed to develop into all of the specialized cell types found in the OC. Cell differentiation in the OC occurs in a basal-to-apical gradient with the earliest differentiation at the base at around E14 and progressing toward the apex until approximately E16.5 when differentiation is complete (Kelley 2007). It is currently thought that the first step in the specification of cell fate in the OC is the expression of *Atoh1*, a basic helix-loop-helix (bHLH) transcription factor. *Atoh1* expression begins between E12.5 and E14 (Kelley 2007) as a relatively broad stripe at the basal end of the cochlea and progresses toward the apex (Lanford et al., 2000; Woods et al., 2004). Though *Atoh1* is expressed throughout the cochlear prosensory domain early on, as development continues individual cells can be identified as having increased levels of *Atoh1* expression. By E16 cells strongly expressing *Atoh1* can be identified as developing hair cells, while intervening *Atoh1*-negative cells will develop as support cells (Kelley 2007). *Atoh1* has been shown to be both necessary and sufficient for hair cell formation

(Bermingham et al., 1999; Zheng and Gao 2000). It has also been demonstrated that the presence of hair cells produced by transient ectopic expression of *Atoh1* in cochlear non-sensory epithelial cells is sufficient to induce neighboring cells to develop as support cells (Woods et al., 2004). *Atoh1* is thus regarded as a key hair cell commitment factor that will initiate a sequence of molecular events that lead to a prosensory cell developing as a hair cell.

Two groups of molecules, inhibitors of differentiation and DNA binding (Ids) as well as Notch members, have been shown to play a role in limiting *Atoh1* expression and directing a developing prosensory cell to differentiate as a supporting cell rather than a hair cell. Ids are structurally similar to *Atoh1* in that they both contain the helix-loop-helix domain (HLH), yet *Ids* lack the basic domain found in *Atoh1* (reviewed in Norton, 2000; Perk et al., 2005). Basic helix-loop-helix transcription factors generally regulate transcription by dimerizing with ubiquitously expressed bHLH E-proteins via their common bHLH domains, and then bind specific DNA sequences via their basic domain. The Ids, lacking the basic domain, therefore act as competitive inhibitors for the HLH domain of the E-proteins and antagonize the activity of the bHLH transcription factors. Ids 1, 2 and 3 are expressed throughout the cochlea between E12 and E14 (Jen et al., 1996; Jones et al., 2006), though by E16 all three are down-regulated in developing hair cells. The decrease in Id expression in these cells corresponds with the increase in *Atoh1* expression that also occurs at this time in developing hair cells (Jones et al., 2006). Notch1 is also broadly expressed throughout the cochlear prosensory domain (Lanford et al., 1999). Yet as a prosensory cell begins to develop as a hair cell, at around E14 it up-

regulates expression of two notch ligands, *Jag*2 and *Dll1*, again seen in a basal-to-apical gradient. *Jag2* and *Dll1* act on cells adjacent to the developing hair cells to increase the *Notch1* activation as well as the expression of two notch target genes, *HES1* and *HES5*. The cells that express *HES1* and *HES5* will develop as supporting cells. This pattern of expression and cell differentiation is consistent with a role for notch signaling in lateral inhibition; developing hair cells prevent their neighbors from developing as hair cells as well and induce the differentiation of support cells.

R-Spondins

R-spondins are a novel family of secreted proteins that consists of 4 members in mammals, encoded by separate genes, which share an overall 60% homology in sequence and a similar organization of protein domains (Kim et al., 2006). *Rspo3* was the first to be identified – from a fetal brain cDNA library (Chen et al., 2002) - though it was not named until after *Rspo1* was isolated from spinal cord/neuroblastoma cells (Kamata et al., 2004). All *Rspo* proteins contain two furin-like cysteine-rich domains, a thrombospondin type 1 domain (TSP1), an N-terminal signal peptide and a positively charged C-terminal tail which contains a nuclear localization sequence (Kim et al., 2006). The furin-like cysteine-rich domain, so named due to its similarity to the furin family of serine proteases, is the functional domain of R-spondin proteins and has been shown to be necessary and sufficient for the effects of Wnt amplification produced by *Rspos* (Kim et., 2006). Removal of the C-terminus region or TSP1 domain shows little effect on the activation of Wnt/β-catenin signaling by *Rspos* (Kazanskaya et al., 2004).

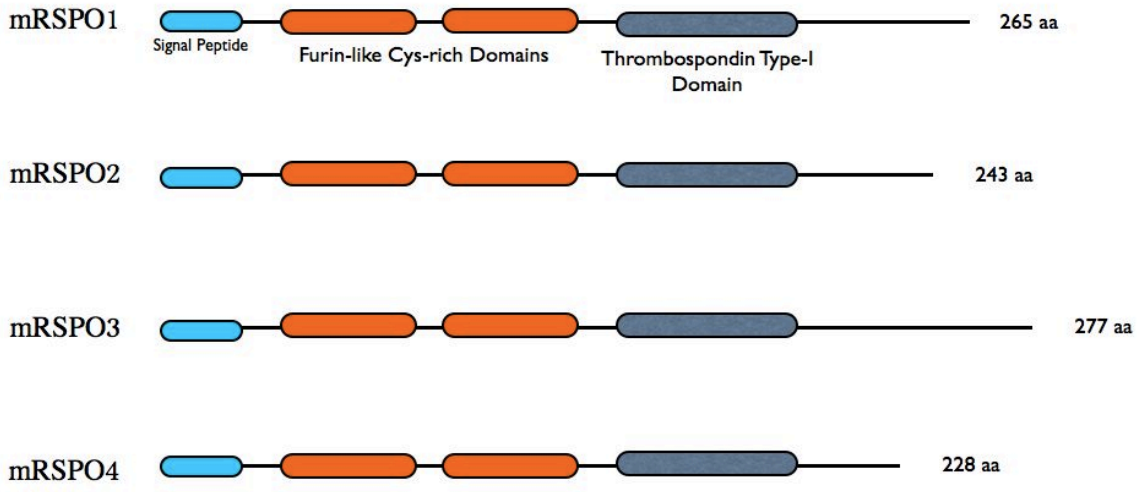

Figure 1: Schematic of R-spondin family members showing the arrangement of protein domains and number of amino acids of each R-spondin.

In a study of R-spondin expression in mouse, all *Rspos* were shown to be expressed in embryonic development, though each with its own unique expression pattern (Nam, Turcotte et al., 2007). The expression patterns overlapped with Wnt ligands, and mutations in *Rspo* genes produced defects similar to those seen in Wnt mutants. Several studies have implicated a close relationship between *Rspos* and the canonical Wnt/β-catenin pathway, showing that *Rspos* are activators of Wnt signaling and β-catenin/TCF-dependent gene expression (Kazanskaya et al., 2004, Kim et al., 2005; Nam et al., 2006). Similarities in Wnt and *Rspo1* expression lead to the investigation of *Rspo1* expression in *Wnt1*$^{-/-}$, *Wnt3*$^{-/-}$, and *Wnt1*$^{-/-}$ and *Wnt3*$^{-/-}$ double-knockout mice. In all three cases the *Rspo1* expression was greatly decreased (Kamata et al., 2004). Subsequently it has been shown that all four members of the *Rspo* family have somewhat similar effects on Wnt

signaling, with *Rspo2* and *Rspo3* being more potent than *Rspo1*, and *Rspo4* showing a minimal effect on β-catenin stabilization within the systems studied (Kim et al., 2008). In addition to playing a role in Wnt signaling, *Rspos* are also implicated in fibroblast growth factor (FGF) signaling and maintenance (Nam et al., 2007; Aoki et al., 2008). Below, I review both the canonical Wnt cascade and the FGF signaling pathway and their known roles in cochlear development.

The canonical Wnt/β-catenin pathway and its role in cochlear development

The canonical Wnt signaling pathway includes Wnt ligands which bind to receptor complexes on the surface of target cells. The receptor complexes are composed of a Frizzled (FZD) receptor and low-density lipoprotein receptor-related protein (LRP5/6) co-receptor at the cell surface. Once a Wnt protein binds the FZD/LRP complex, the signal is transduced through the cell by several proteins. At a resting state, the concentration of β-catenin is kept relatively low; a complex containing glycogen synthase 3β (GSK3β), adenomatous polyopsis coli (APC) and Axin target β-catenin for degradation by the proteasome. Binding of the FZD/LRP complex by a Wnt ligand causes the recruitment of Disheveled (DVL), a cytosolic protein which disrupts the Axin/APC/ GSK3 complex and allows the accumulation of β-catenin within the cytoplasm and nucleus. β-catenin within the nucleus then interacts with transcription factors such as the lymphoid enhancer-binding factor 1 / T cell-specific transcription factor (LEF/TCF) to affect transcription of target genes. A potent class of Wnt signaling inhibitors is the Dickkopf (DKK) proteins, which bind LRP with high affinity. By binding the LRP

receptors, DKK makes it unavailable for reception of Wnt ligands, and promotes the internalization of the receptor (Logan & Nusse 2004, review). *Rspo*s are believed to activate the Wnt pathway at the level of the ligand-receptor binding. Rspo1 has been shown to be a high-affinity ligand of LRP6 that strongly synergizes with Wnt3A to induce the accumulation of β-catenin (Wei et al., 2007; Binnerts et al., 2007). It is thought that all *Rspos* amplify the Wnt pathway through a common mechanism in which *Rspos* reduce the internalization of LRP6 by blocking the activity of the Wnt inhibitor DKK1 and are therefore important regulators of the amount of LRP6 present on the cell surface (Kim et al., 2008).

The Wnt pathway plays an important role early on in ear development with specification of the prosensory patch. In mouse, it was shown with a TCF/Lef-lacZ transgenic reporter that a subset of *Pax2* expressing cells in the cranial ectoderm become active in Wnt signaling from E8.25, with the highest levels of reporter activity near the neural tube, and decreasing activity in the lateral regions of ectoderm (Ohyama et al., 2006). Wnt signaling was disrupted in developing mice in the presumptive otic placode using a *Pax2*-Cre transgene system in which *Pax2*$^{+}$ cells are lacking β-catenin. In these conditional knockout mice, the otic placode was significantly reduced to about 20% of its normal size, while the cranial epidermis showed an increase in size (Ohyama et al., 2006). Conversely, conditional upregulation of canonical Wnt signaling in *Pax2*$^{+}$ cells was evaluated by using a stabilized form of β-catenin in which exon 3 is deleted by Cre recombinase, eliminating the serine and threonine residues whose phosphorylation mark β-catenin for degradation (Ohyama et al., 2006). In these mice with non-degradable β-

catenin, the size of the otic placode had increased significantly in size, while the cranial epidermis had decreased in size (Ohyama et al., 2006). These experiments provide evidence for a role of Wnt signaling in the formation of the otic placode, though it did not provide any information for a possible role for this pathway in later stages of cochlear development as the β-catenin conditional knockout embryos die by E12. Unpublished data from our lab indicate a continued and similar role for Wnt signaling in cochlear development. Cochlear explant cultures were prepared from E13 embryos, and activation of canonical Wnt signaling resulted in a robust increase in both the size of the prosensory domain, as well as the number of differentiated hair cells. Conversely, attenuation of the pathway led to a decrease in the number of differentiated hair cells in the organ of Corti. The time period during which Wnt signaling had these effects is restricted to before E16, correlating with the period for hair cell commitment.

The FGF signaling pathway and its role in cochlear development

The fibroblast growth factor (FGF) signaling pathway is highly involved in embryonic development. In mouse and human, 23 different FGF's and 4 FGF receptors (FGFR) have been identified. FGF signaling is implicated at several stages throughout inner ear development with evidence of interaction with Wnt signaling. FGF protein binding at the surface of the target cell induces dimerization of the FGF receptors, activating receptor tyrosine kinases and causing autophosphorylation of the cytoplasmic domains of the receptors. The activation of receptor tyrosine kinases leads to a signal that is transduced through multiple pathways (Boilly et al., 2000). The phosphorylated

tyrosines are also recognized as binding sites by adaptor proteins such as growth factor receptor-bound protein 2 (Grb2) (Boilly et al., 2000). Grb2 binds to the Ras guanine nucleotide-releasing factor sons of sevenless (Sos) via its SH3 domain, and this complex recruits a Ras GTPase in the plasma membrane. Ras is then further activated by an exchange of GDP for GTP facilitated by Sos. This leads to a signaling cascade in which Ras activates the kinase Raf. Once activated, Raf can phosphorylate the protein kinase MEK, which can now act downstream on the serine/threonin-specific kinase ERK - extracellular-signal-regulated kinase - which affects gene transcription levels in the target cell (Goldfarb 2001).

In the mouse inner ear, FGF3 and FGF10 are influential signals that induce otic placode formation. Both factors are expressed near the pre-otic field at the time of otic induction and mice lacking both genes fail to form the ear primordia (Alvarez et al., 2003; Wright et al., 2003). Possible targets of FGF3 and FGF10 include another FGF member, FGF4, as well as *Foxi2* and *Foxi3*, markers of the prospective otic and non-otic ectoderm, respectively (Urness et al., 2010). Another key target of FGF3 and FGF10 is *Wnt8a* expression in the hindbrain, which was significantly reduced or even absent in *FGF3* and *FGF10* double mutants. However, a single copy of *FGF3* is sufficient to regain *Wnt8a* expression (Urness et al., 2010) suggesting that FGF3 plays a more important role in activating *Wnt8a*. In explant cultures, FGF4 strongly induced *Wnt8a*, while FGF3 showed a less frequent induction and FGF10 rarely induced *Wnt8a* (Urness et al., 2010).

Early on in cochlear development, at E13.5, both *FGFR1* and *FGFR2* are expressed in the cochlear duct. *FGFR1* expression is consistent with a role in specifying prosensory domains, while *FGFR2* is confined to non-sensory regions (Pirvola et al., 2002). By E15.5, shortly after the onset of hair cell differentiation, *FGFR1* is expressed in concentrated clusters of cells at the basal cochlea in the region of developing outer hair cells (Hayashi et al., 2010). *FGFR4* expression emerges at this time too, though in the adjacent mesenchyme and not in the epithelial cells. *FGFR3* also begins expression in the cochlea at E15.5, specifically at the base. The expression patterns of *FGFR1* and *FGFR3* overlap at this point, and the *FGFR3*-expressing cells appear to be a subset within the cells that are highly expressing *FGFR1*. The position of these cells adjacent to the *Atoh1*-expressing inner hair cells suggests that the presumptive outer hair cells, pillar cells and Deiters' cells are expressing both *FGFR1* and *FGFR3* at this point. A likely ligand for FGFR1 is FGF20, and inhibition of FGF20 shows a loss of hair cells similar to that seen in *FGFR1*$^{-/-}$ cochleae (Hayashi et al., 2008). While deletion of *FGFR1* causes loss of hair cell development, a targeted deletion of *FGFR3* leads to an increase of hair cells (Colvin et al., 1996; Hayashi et al., 2007; Puligilla et al., 2007). The increase in hair cells was due to the presence of an extra fourth row of outer hair cells in the apical two-thirds of the cochlea, along with extra Deiters' cells in that region, and was accompanied by a decrease in the number of pillar cells. This suggests that FGFR3 plays a role in controlling cell fate decision. A likely ligand for FGFR3 is FGF8 (Hayashi et al., 2007; Jacques et al., 2007), and it is believed that FGF8 released from IHC's acts to induce pillar cell fate in the closest cells, and influence a Deiters' cell fate in those cells greater

than two cell-diameters away through its binding to FGFR3 (Hayashi et al., 2007). FGFR3 was shown to play an important role in the differentiation of pillar cells; overactivation of its signaling by ectopic expression of *FGF8* to cochlear explant cultures (Jacques et al., 2007) or a targeted deletion of *Sprouty2* (Shim et al., 2005), an FGF signaling inhibitor, resulted in an increase in the number of pillar cells. Furthermore, mutated overactive forms of FGFR3 also results in increased numbers of pillar cells (Mansour et al., 2009).

To summarize, R-spondins are a novel family of secreted proteins that have been shown to potentiate Wnt-β-catenin signaling, a pathway that is involved in the development of the cochlea. R-spondins are co-expressed with Wnts and show a positive feedback interaction with Wnts. Though the mechanism of action of R-spondins on Wnts is not well understood, it is strongly evidenced that they interact at the level of ligand-receptor binding, ultimately leading to increased stabilization of β-catenin. In addition to modulation of the Wnt pathway, R-spondins were also shown to interact with the FGF signaling pathway, again a pathway that is key to cochlear development in both prosensory specification and cell differentiation. Studies have shown that the FGF and canonical Wnt pathways interact with each other in multiple feedback loops, and that R-spondins affect components of both pathways.

MATERIALS AND METHODS

Animals

Rspo2$^{-/-}$ were generated by crossing *Rspo2*$^{+/-}$ mice (Yamada et al., 2009). Newborn mice and embryos from ICR/CD1 mice were euthanized according to protocols approved by the Animal Care and Use Committee at the University of California San Diego. Cochleae from embryos and newborn pups were removed, isolated, dissected and processed as either whole mounts or sectioned in a cryostat at a thickness of 12 μm.

PCR

RNA Extraction

Tissue samples for RNA extraction were dissected from mouse in an RNAse-free environment and stored in Ambion RNA later at -20˚C until extraction. Ambion RNAqueous kits were used for isolation of total mRNA. Tissue samples were homogenized with a pestle in cell lysis solution, and the RNA was precipitated with 100% ethanol. The lysate was passed through a filter by centrifugation, washed once with wash solution 1, and twice with solution 2/3. The RNA was eluted using heated elution solution and collected in an RNAse-free tube. The collected RNA was treated with DNAse I (Ambion) at 37˚C for 30 minutes and deactivated at 75˚C for 5 minutes. For large tissue samples such as brain, Invitrogen Trizol reagent was used to extract total mRNA. Tissues were homogenized in a 15 mL tube with a pestle in 2 mL of Trizol. Then 0.4 mL of chloroform was added and the tube was shaken vigorously. The phases were separate by centrifugation at 12,000 g for 15 minutes at 4˚C. The aqueous RNA-

containing phase was collected in a microcentrifuge tube. The RNA was precipitated with 2 mL of 75% ethanol and pelleted by centrifugation at 7,500 g for 5 minutes at 4˚C. The pellet was air-dried and redissolved in distilled water.

cDNA Preparation

Complementary DNA was generated from collected total mRNA using Invitrogen SuperScript III reverse transcriptase. Approximately 500 ng of total mRNA from sample tissues was added to a microcentrifuge tube with dNTP mix and oligo dT primers, brought to a volume of 13 µL with sterile distilled water, and incubated at 65˚C for 5 minutes, then on ice for 1 minute. 4 µL of 5X first-strand buffer, 1 µL of DTT, 1 µL of RNAse inhibitor and 1 µL (200 U) of RT was then added to the tube. The contents were mixed by gentle pipetting up and down, and then incubated at 50˚C for 60 minutes. The reaction was deactivated by heating at 70˚C for 5 minutes.

PCR conditions

BioNeer AccuPower PCR premix was used for PCR reactions. 50ng of template DNA was added to the PCR tube. 0.5 µL each of 100µM sense and anti-sense primer was added to the tube. The volume was brought to 20 µL with distilled water.

PCR Program:

Hot Start: 92˚C for 2 minutes

32 Cycles:

92˚C for 30 seconds

58˚C for 30 seconds

72˚C for 40 seconds

Final:

72˚C for 10 minutes

Stored at 4˚C or -20˚C until ran on gel

Primers:

Rspo1

Sense: TGTGAAATGAGCGAGTGGTCC

Antisense: TCTCCCAGATGCTCCAGTTCT

Rspo2

Sense: TTGCATAGAGGCCGCTGCTTT

Antisense: CTGGTCAGAGGATCAGGAATG

Rspo3

Sense: GTACACTGTGAGGCCAGTGAA

Antisense: ATGGCTAGAACACCTGTCCTG

Rspo4

Sense: GTGCCTGATGCCTGGACTGTTGAC

Antisense: CAGGGCCCAGGCGCGATTAGA

GAPDH

Sense: TGAAGGTCGGTGTGAACGGATTTGGC

Antisense: CATGTAGGCCATGAGGTCCACCAC

PCR primers were produced by BIONEER Corporation

Immunohistochemistry

Inner ears were dissected out from mouse and fixed in 4% paraformaldehyde over night at 4˚C with rotation. The cochleae were then opened and the Reisners and tectorial membranes were removed so as to expose the sensory epithelium. The cochleae were left attached to the vestibular system for easy handling until mounted on a microscope slide. Myosin VI primary antibody (Proteus, Rabbit) was used at a concentration of 1:500 in PBST. hProx1 antibody (R&D, goat) was used at a concentration of 1:250 and mProx1 antibody (Covance, rabbit) was used at a concentration of 1:500 in PBS with 0.5% triton (PBST). Each primary antibody was applied overnight in PBST at 4˚C with rotation. Samples were then washed 3 times for 10 minutes each wash in PBST. For fluorescent staining, Invitrogen Alexa fluorescent secondary antibodies were used at a concentration of 1:1000 and incubated for approximately 1 hour. Phalloidin (Invitrogen) was used at 1:100. Samples were then washed in PBST for 5 minutes. Typically DAPI was applied for nuclear labeling at a 1:3000 dilution for 5 minutes. Washed in PBST for 5 minutes, then in PBS for 5 minutes before mounting.

For DAB staining, the Vector Laboratories Vectastain ABC kit was used. Samples were treated with 0.3% hydrogen peroxide for 30 minutes prior to the primary antibodies, washed in PBST, incubated for 20 minutes with normal serum and then treated with primary antibodies overnight. After washing with PBST, samples were incubated with biotinylated secondary antibodies for 30 minutes, washed for 5 minutes and treated with

ABC reagent. After a final wash the samples were reacted with DAB solution until the desired darkness of stain was reached.

RESULTS

Expression of R-spondins in cochlear tissue

We screened for the expression of all four R-spondin members in the cochlea by using RT-PCR from total mRNA of whole cochleae and then PCR with gene-specific primers for each member. RNA was harvested from E15 cochleae as this is the developmental time period of hair cell differentiation. *Rspo2* expression was detected in the cochlea, using forelimb cDNA as a positive control and skin cDNA as a negative control (Nam et al., 2007). *Rspo3* expression was also detected in the cochlea, using kidney cDNA as a positive control and skin cDNA as a negative control (Nam et al., 2007). Neither *Rspo1* nor *Rspo4* were detectable in the cochlea while they were detectable in positive control tissue, kidney and skin respectively (Nam et al 2007). GAPDH was detected in each tissue sample, confirming the quality of the cDNA.

Table 1: Rspo2 and Rspo3 are expressed in the cochlea at age E15.

Gene	Expression in Cochlea	Positive Control	Negative Control
Rspo1	No	Kidney	Skin
Rspo2	Yes	Forelimb	Kidney
Rspo3	Yes	Kidney	Skin
Rspo4	No	Skin	Kidney

Analysis of Rspo2 mutant cochlea

Rspo2$^{-/-}$ mice die immediately after birth and since hearing onset in mice does not occur until postnatal day 14, we were unable to evaluate their hearing ability at any stage.

Supernumerary hair cells are present in Rspo2$^{-/-}$ *mice*

There was no difference in the total length of the cochlea between *WT*, *Rspo2*$^{+/-}$ or *Rspo2*$^{-/-}$ cochleae. Examination of hair cells in WT and *Rspo2*$^{+/-}$ cochleae, using the hair cell specific marker Myosin 6, indicated that there was no difference between wild type and heterozygotes in terms of hair cell number and patterning. However, in the apical region of *Rspo2*$^{-/-}$ cochleae there was a recognizable phenotype in the number of outer hair cells. Whereas wild type cochleae, as well as *Rspo2*$^{+/-}$ cochleae, have an invariant mosaic of 3 outer hair cells to every inner hair cell (Figure 2A and C), in *Rspo2*$^{-/-}$ cochleae there was an extra row of outer hair cells (Figure 2B and D). This phenotype was easily noticed in the more apical regions of the cochlea as the fourth row is consistently present from the mid-apex, where it appeared to begin, up to the most apical areas of developed hair cells.

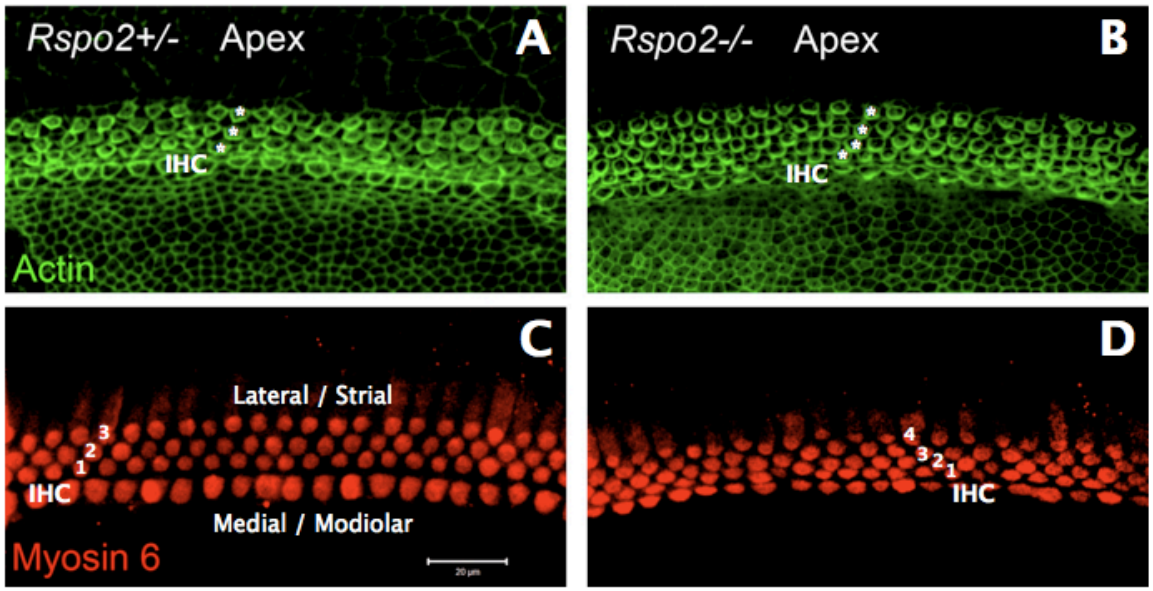

Figure 2: Apical regions of E18 *Rspo2*$^{-/-}$ cochleae contain an extra row of outer hair cells. Actin staining in green marks the membranes of all cells in the plane while Myosin 6 staining in red shows only the hair cells. A single row of inner hair cells (IHC) is indicated in all panels. Outer hair cells are marked with an asterisk in panels A and B, and with numbers in panels C (1-3) and D (1-4). Scale bar in C is 20μm applies to A-D.

In order to quantify the increase in hair cells in the *Rspo2*$^{-/-}$ mice, inner and outer hair cells, stained with the hair cell-specific marker Myosin 6 (Myo6), were counted in 100 μm segments at specific positions - 25%, 50% and 75%, determined as a percent of the total length along the basal-to-apical axis of the cochlea starting from the base. There was no significant difference in the number of hair cells when comparing WT cochleae and *Rspo2*$^{+/-}$ cochleae, and the heterozygous cochleae were used as a control for comparison with the *Rspo2*$^{-/-}$ cochleae.

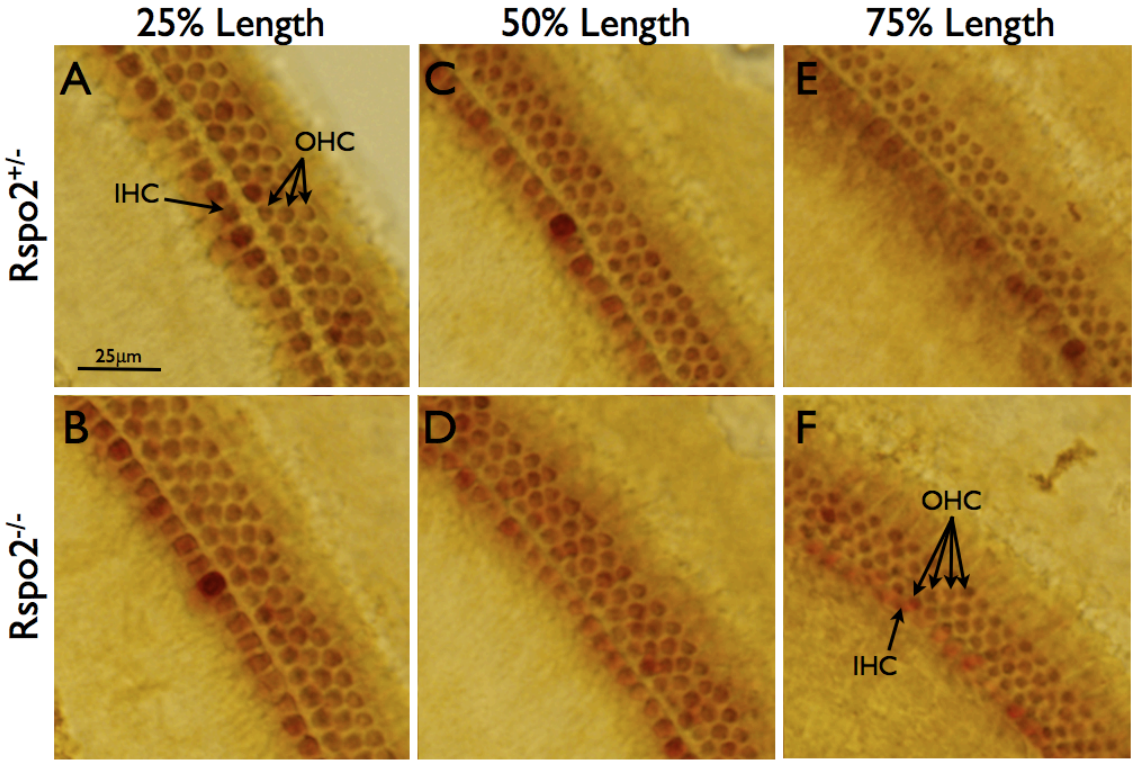

Figure 3: Four rows of OHCs are present in the apical half of Rspo2$^{-/-}$ cochleae. Myosin 6 staining on E18 mouse cochleae labels mechanosensory hair cells. Panels A, C and E show Rspo2$^{+/-}$ heterozygotes at 25%, 50% and 75% of total length, respectively. Panels B, D and F show Rspo2$^{-/-}$ homozygotes at the same proportions of length. Scale bar in A is 25µm and applies to A-F.

While *Rspo2* heterozygotes had normal hair cell patterning throughout the entire length of the cochlea (Figure 3A, C, E), the *Rspo2*$^{-/-}$ cochleae were found to have a fourth row of outer hair cells in portions of the cochlea. The extra row of OHCs was intermittently present over short lengths in the basal regions of the organ of Corti (Figure 3B, D), yet in the more apical regions it became consistently present (Figure 3F) so that at 75% length the extra row was observed throughout. After quantifying the amount of hair cells in both *Rspo2*$^{+/-}$ and *Rspo2*$^{-/-}$ cochleae and comparing the counts, it was found that there was a significant difference between the two groups in the number of outer hair

cells (Figure 4A), with the knock-out embryos showing supernumerary amounts of OHCs. *Rspo2*$^{+/-}$ cochleae (n=3) averaged 14 inner hair cells and 48 outer hair cells per segment, while *Rspo2*$^{-/-}$ cochleae (n=3) averaged 16 inner hair cells and 61 outer hair cells per segment. There was no significant difference in the number of inner hair cells between the control and *Rspo2*$^{-/-}$ cochleae (Figure 4B). The increased number of OHC's in *Rspo2*$^{-/-}$ cochleae resulting from the extra row was statistically significant ($P \leq 0.001$) in the region at 75% of the total length of the cochlea.

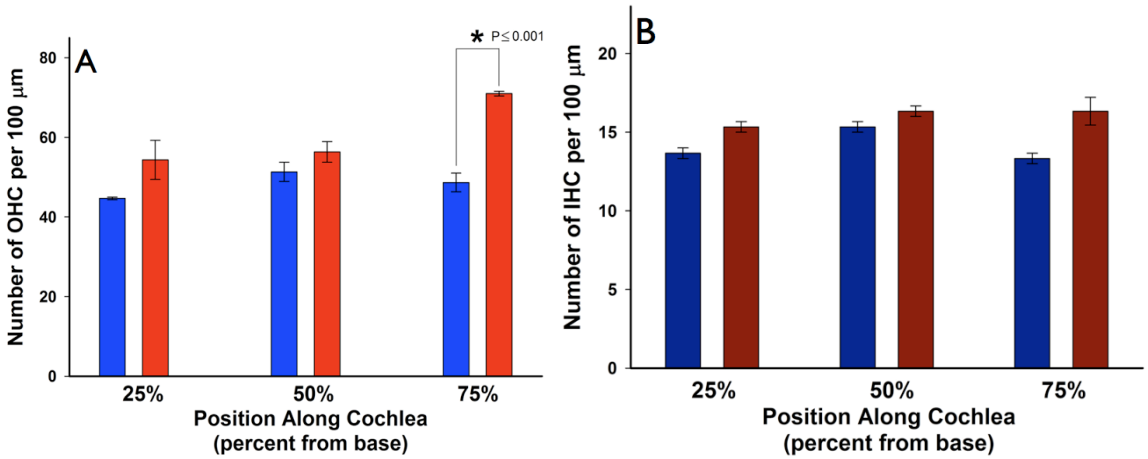

Figure 4: *Rspo2*$^{-/-}$ cochleae have significantly more outer hair cells than *Rspo2*$^{+/-}$ cochleae in the apex. Blue bars represent *Rspo2*$^{+/-}$ cochleae; red bars represent *Rspo2*$^{-/-}$ cochleae. Panel A contains the counts of outer hair cells; panel B contains the counts of inner hair cells. Error bars are SEM. Significance was determined using a t-test, $P \leq 0.001$.

Because each hair cell has an associated support cell, it was hypothesized that there would be an increase in support cells that accompanied the increased number of hair cells in *Rspo2*$^{-/-}$ cochleae. Also of interest were the pillar cells, as *FGFR3*$^{-/-}$ cochleae had

disrupted pillar cell development that accompanied the similar phenotype of hair cells. To examine support cells, immunolabeling of Prox1, a transcription factor, was used to mark pillar cells and Deiters' cell nuclei. In examining the phenotype of *Rspo2*$^{-/-}$ support cells, supernumerary Deiters' cells were observed. Normally, there are three rows of Deiters' cells, one for each OHC, in the organ of Corti as was the case in *Rspo2*$^{+/+}$ and *Rspo2*$^{+/-}$ cochleae (Figure 5A). In examining the Deiters' cells of *Rspo2*$^{-/-}$ cochlea in which an extra row of outer hair cells was present, the cochleae contained an extra row of Deiter cells (Figure 5B). Supernumerary Deiters' cells were also found in the *FGFR3*$^{-/-}$ cochleae in regions where extra rows of outer hair cells were present. However, in the FGFR3 mutant cochlea, the inner pillar cells were absent and the pillar head did not develop due to the lack of outer pillar cell differentiation (Hayashi et al., 2007). The Prox1 staining revealed the presence of two rows of pillar cells - one inner, one outer - that ran along the entire length of the cochlea of *Rspo2*$^{-/-}$ mice. This stands in contrast to the *FGFR3*$^{-/-}$ phenotype, as those cochleae lose one row of pillar cells in regions with supernumerary outer hair cells.

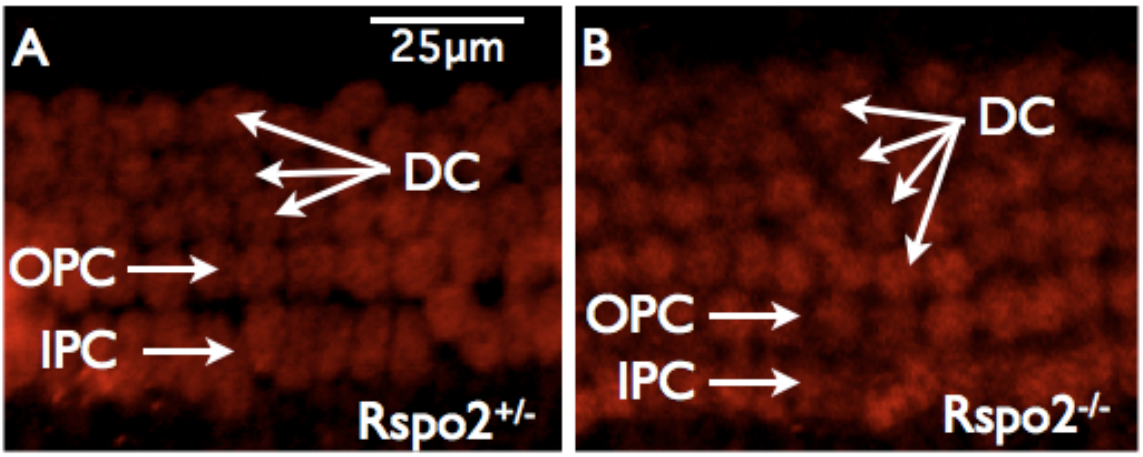

Figure 5: An extra row of Deiters' cells is present in *Rspo2*$^{-/-}$ cochleae. A. Prox1 fluorescent immunostaining on E18 mouse cochleae showing the normal single row of inner pillar cells (IPC), a row of outer pillar cells (OPC) and Deiter cells (DC) in the *Rspo2*$^{+/-}$ cochlea. B. In *Rspo2*$^{-/-}$ cochleae there were two rows of pillar celsl and four rows of Dieters cells instead of the normal three in an area of the cochlea where an extra row of outer hair cells was present.

To further examine the pillar cells of the *Rspo2*$^{-/-}$ cochleae, staining of the p75 neutrophin receptor (ntr) was used to mark the pillar heads and their development, which protrude upwards in between the inner hair cells and the first row of outer hair cells.

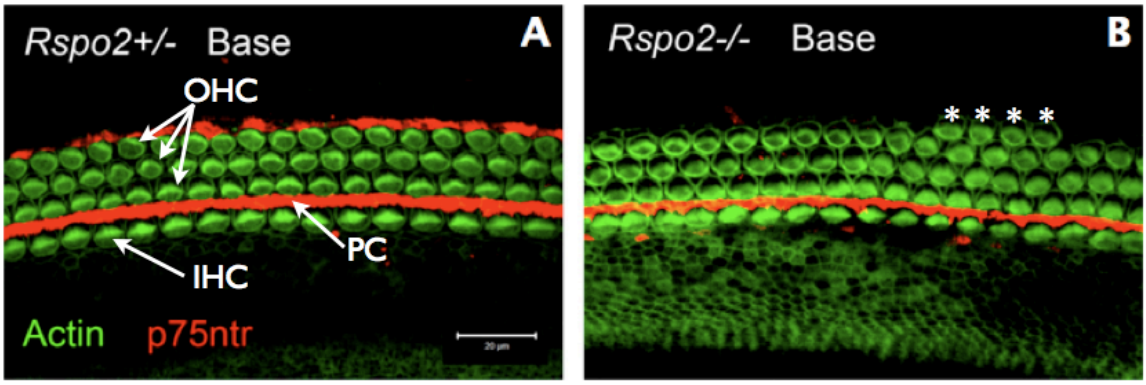

Figure 6: Normal pillar cell phenotype in E18 Rspo2$^{-/-}$ cochleae. Actin staining in green marks inner and outer hair cells, p75ntr staining in red marks the pillar heads which protrude upwards to the level of hair cells and separate the inner hair cells from the first row of outer hair cells. IHCs, OHCs and PCs are indicated with arrows in panel A, extra OHCs are indicated with asterisks in panel B. Hensen's cells may also pick up p75ntr staining, as seen on the strial edge in panel A.

Pillar cells were present in the knock-out embryos. Normal patterning of pillar cells in the organ of Corti places one inner pillar cell and one outer pillar cell between the IHCs and first row of OHCs. Both inner and outer pillar cells were present throughout the entire length of the organ of Corti in *Rspo2*$^{+/-}$ and *Rspo2*$^{-/-}$ cochleae. P75 staining revealed that the pillar heads were present in the pillar cell space between the IHCs and first row of OHCs throughout the entire length of the cochlea (Figure 6). It can also be seen that in basal regions - where the extra row of OHCs is present in only short spans - that when the extra row of OHCs appears, the pillar heads are uninterrupted (Figure 6B). This again is in contrast to the *FGFR3*$^{-/-}$ phenotype in which pillar heads are disrupted in regions where an extra row of OHCs was present.

DISCUSSION

Our phenotype characterization of *R-spondin2* null mutant mice strongly suggests that *Rspo2* is a novel genetic factor for cochlear development. The results of this study are supportive of a role for *Rspo2*$^{-/-}$ in the development of the cochlea, specifically in regulating the number of outer hair cells in the organ of Corti. The somewhat similar hair cell phenotypes between *FGFR3*$^{-/-}$ and *Rspo2*$^{-/-}$ cochleae suggest that *Rspo2* might modulate the FGF signaling pathway during hair cell development. However, the differences in support cell phenotype imply a more complex interaction, possibly involving Wnt signaling or other pathways. Studies have shown that the FGF and Wnt pathways influence one another at different stages in development through multiple feedback loops, with Wnt acting upstream of FGF in some instances and conversely in other cases (Nam et al., 2007; Urness et al., 2010). R-spondins have been shown to interact with both of these pathways during development, appearing mostly to have a potentiating effect on the signaling. It is unclear at this point the mechanism through which *Rspo2* regulates hair cell differentiation, or exactly which components of the Wnt or FGF pathway it interacts with during this process.

After quantifying the number of hair cells, it was apparent that the loss of R-spondin2 led to a significant increase in the number of outer hair cells. The supernumerary hair cells appeared as an extra row of outer hair cells that was intermittently present throughout the basal portions of the organ of Corti and consistently present in the apical portions. This phenotype appears similar to that observed in *FGFR3*$^{-/-}$ cochlea, in which supernumerary hair cells were observed in the more apical

portions of the organ of Corti suggesting that *Rspo2* might affect cell differentiation through some mechanism involving the FGF signaling pathway. However, a notable difference between the phenotypes of *FGFR3*$^{-/-}$ and *Rspo2*$^{-/-}$ cochleae lies in the support cells. While the *FGFR3*$^{-/-}$ cochleae showed a decrease in the number of pillar cells - with only one PC present in the areas with extra hair cells, and a loss of the characteristic pillar head morphology in that singular PC - the *Rspo2*$^{-/-}$ cochleae have both inner and outer pillar cells present throughout the organ of Corti, both with normal morphology. As in the *FGFR3*$^{-/-}$ phenotype, in regions where extra outer hair cells are present, there is also a supporting Deiters' cell associated with each extra OHC. Because hair cells induce a neighboring cell into differentiating as a support cell, it is reasonable to assume that the loss of *Rspo2*$^{-/-}$ did not directly influence the differentiation of extra Deiters' cells, but rather that these support cells were formed as a result of induction by the extra outer hair cells.

In examining the cochleae of *Rspo2*$^{-/-}$ embryos, we found that there was no difference in the total length of the cochleae between mutants and wild type littermates. Furthermore, the orientation of the stereociliary bundles on the surface of the mechanosensory hair cells was not affected. These data indicate that the loss of R-spondin2 did not affect non-canonical Wnt/PCP signaling in the cochlea, as that pathway is involved in convergent extension (cochlear length) (Wang et al 2006) and planar cell polarity (uniform orientation of stereociliary bundles) (Dabdoub et al., 2003). We conclude that *Rspo2* alone does not influence the Wnt/PCP pathway in the cochlea. To examine whether canonical Wnt signaling in the cochlea is affected by the absence of

Rspo2, the Wnt signaling reporter *TOPGAL* transgenic mice (DasGupta and Fuchs 1999) can be utilized. The *TOPGAL* transgene consists of a β-galactosidase gene driven by a Tcf/Lef β-catenin-responsive promoter, which enables canonical Wnt/β-catenin signaling to be measured by β-galactosidase activity. Therefore, the β-galactosidase activity in *Rspo2*$^{-/-}$ cochlea containing the *TOPGAL* transgene can be compared to *Rspo2*$^{+/+}$, *TOPGAL* cochlea to indicate a possible role for Rspo2 in modulating canonical Wnt signaling in the developing cochlea.

The current state of knowledge on the R-spondin family of proteins is largely centered on their interactions with the Wnt and FGF signaling pathways. *Rspo2* has been shown to affect the maintenance of *FGF8* expression in the apical ectodermal ridge of the developing hindlimb (Nam et al., 2007) where a reduction in the expression of *FGF8* - a likely ligand of FGFR3 - was observed in embryos with targeted disruption of *Rspo2*. These effects of Rspo2 were thought to be mediated through canonical Wnt signaling providing some evidence to support a model in which Rspo2 is a mediator in both FGF and Wnt/β-catenin signaling. To investigate whether FGF8 is affected by Rspo2, real time qPCR could be utilized to compare the levels of FGF8 in wild type versus mutant *Rspo2* cochleae. More generally, the effects of *Rspo2* can be further investigated using a growth factor PCR array profiling the expression of several genes related to FGF-mediated signaling. Thus additional analysis of the cellular and molecular defects in *Rspo2* mutant mice as well as elucidation of the regulatory roles and signaling mechanisms will advance our knowledge of *Rspo2* signaling in cochlear development.

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