ABSTRACT

Title of Document:	THE ROLE OF *SOX2* IN INNER EAR DEVELOPMENT
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The vertebrate inner ear is a structurally complex sensory organ responsible for detecting sound and maintaining balance. These functions are mediated by specialized sensory epithelia comprised of a mosaic of mechano-transducing hair cells and supporting cells. The sensory hair cells are innervated by neurons of the cochleo-vestibular ganglion (CVG, the VIIIth cranial nerve). Both neuronal and sensory lineages are thought to be specified early in the neural-sensory competent domain (NSD) of the ear rudiment. First, neuroblasts delaminate from the NSD to form neurons of the CVG. Then, cells remaining in the NSD adopt a sensory fate and develop into various sensory organs. The molecular mechanisms that specify neuronal and sensory cell fates are unclear. The aim of this dissertation is to provide a better understanding by examining the roles of the HMG (high mobility group)-box containing transcription factors Sox2 and Sox3 in developing chicken inner ears using gain and loss-of-function approaches. Over-expression of *Sox2 in ovo* readily induces *Neurogenin 1* (*Ngn1*) expression, an important gene required for the neurogenic fate.

Nevertheless, neurogenesis fails to proceed based on the lack of *Neurod1* up-regulation and consequently the size of the CVG is reduced. In contrast, over-expression of *Ngn1* is capable of up-regulating *Neurod1* and causes increased neuroblast formation, as well as Sox2 down-regulation. Similar increases in neurogenesis are obtained with over-expression of *Neurod1*. I provide evidence that Ngn1 and Neurod1 inhibit *Sox2* transcription via the *E-box* of the *nasal-otic placode specific enhancer 1* (*NOP-1*) within the *Sox2* promoter. On the other hand, loss of Sox2 function paradigms did not result in loss of *Ngn1* expression, suggesting that other factors may be required to induce *Ngn1* normally. Furthermore, while *Sox3* has been proposed to be up-stream of *Sox2*, it does not induce *Ngn1* in a similar manner as Sox2. Taken together, my results suggest that Sox2 and likely other factors are involved in initiating neurogenesis by up-regulating *Ngn1*. The up-regulated Ngn1, in turn, down-regulates *Sox2* expression and up-regulates *Neurod1* to mediate progression of neurogenesis. Finally, I show that Sox2 and the Notch signaling pathway interact to specify neuronal and sensory cell fate choices.

THE ROLE OF *SOX2* IN INNER EAR DEVELOPMENT

By

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Dedication

I dedicate this dissertation to Maria C. Carr,

to Rose Marie Ostrowski,

and to my parents Mesut and Ayşe Evsen.

Per aspera ad astra.

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I foremost would like to thank my mentor Dr. Doris K. Wu for her excellent guidance, and for training me into becoming an independently thinking researcher. I also would like to thank her for being caring and supportive when I needed her.

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En içten çok teşekkür ederim.

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Chapter 1: Introduction

The vertebrate inner ear develops in a precise spatio-temporal pattern. Specialized sensory epithelia within the inner ear, which are comprised of hair cells and their associated supporting cells, are responsible for mediating balance and hearing. These sensory patches are innervated by neurons of the cochlea-vestibular ganglion. Both sensory and neuronal lineages are thought to arise from a common neural-sensory competent domain (NSD) in the ear rudiment. Much work and efforts are focused on unraveling how sensory organs develop and how cells in the sensory and neuronal lineages adopt their fates. Despite these efforts a clear understanding of the molecular mechanisms is still lacking. The aim of this dissertation is to seek a better understanding of the molecular mechanisms involved in the regulation of neuronal and sensory cell fate choices. To this end, I have conducted gain and loss-of-function studies in chicken inner ears *in ovo* using an electroporation method that I have developed. This study focuses on the functions of *Sox2* and *Sox3*, because the roles of these genes in inner ear development are largely unknown. Yet, mutations in the *SOX2* gene lead to sensorineural hearing loss in humans and mice (Hagstrom et al., 2005; Kiernan et al., 2005).

Development of the inner ear

The development of the inner ear depends on many molecular processes including inductive signals emanating from the hindbrain, mesoderm, and ectoderm. This organ originates from a thickening of the ectodermal tissue adjacent to the hindbrain know as the otic placode (Fig.1.1). This placode then deepens to form a

cup and quickly closes to form the fluid-filled otocyst, which gives rise to the entire membranous labyrinth of the inner ear and the neurons that innervate it. Genetic fate-mapping data indicate that neuronal and sensory lineages arise from the NSD located in the antero-ventral region of the otic cup or otocyst (Raft et al., 2007). First, neuroblasts delaminate from the NSD to form the neurons of the cochleo-vestibular ganglion (CVG, the VIIIth cranial nerve), which eventually split into auditory and vestibular ganglia. These neurons innervate the sensory hair cells of the inner ear and nuclei in the brainstem. The cells that remain in the NSD are thought to give rise to various sensory patches consisting of the mechano-transducing sensory hair cells and their associated supporting cells.

Chicken inner ears have seven vestibular sensory organs for maintaining balance and one auditory organ for detecting sound. The vestibular organs consist of the three cristae, utricular macula, saccular macula, macula neglecta, and macula lagena. The auditory organ is the basilar papilla. The total number of sensory organs in chicken differs from mammalian inner ears, as mammals do not have a lagena. Furthermore, the auditory organ in mammals is the organ of Corti, which is more complex in structure than the basilar papilla. Despite these differences, the molecular pathways for patterning and morphogenesis between avian and mammalian inner ears are quite similar.

Figure 1.1

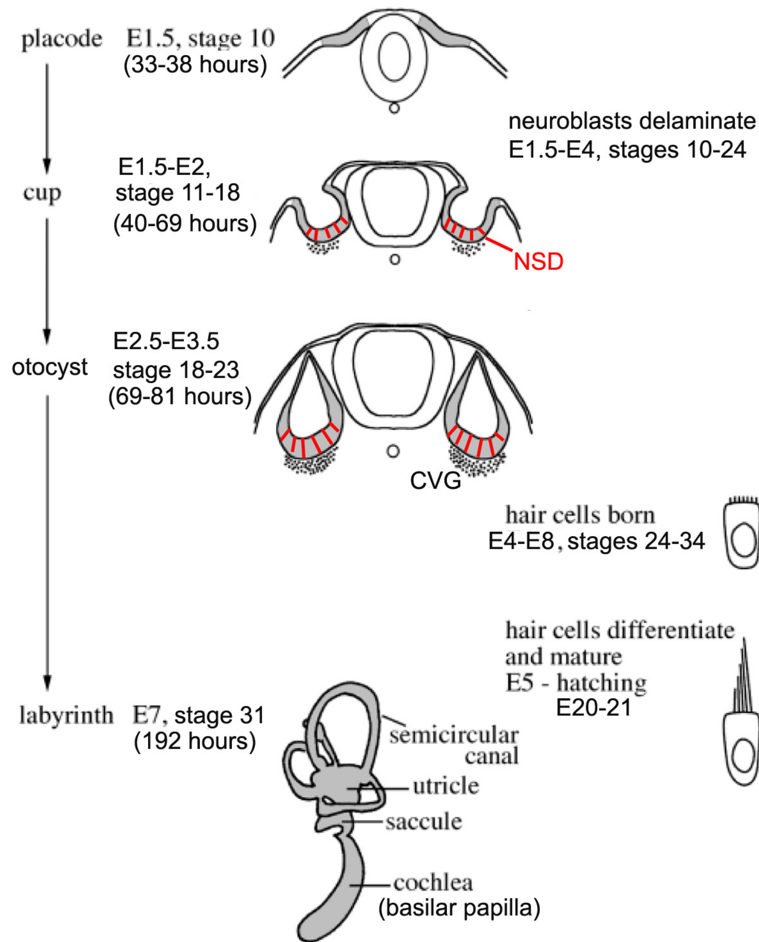


Figure 1.1. Timetable of chicken inner ear development. Stages are based on Hamburger & Hamilton 1951. Abbreviations: E, embryonic day; NSD, neural-sensory competent domain; CVG, cochleo-vestibular ganglion. Figure modified from (Adam et al., 1998).

A common NSD gives rise to the neuronal and sensory lineages

Several studies suggest that neuronal and sensory fates are related by lineage (Fig.1.2). For instance, fate-mapping studies using replication incompetent retroviruses in chicken inner ears show that sensory cells in the utricular macula and the CVG are clonally related (Satoh and Fekete, 2005). In addition, genetic fate-mapping studies of *Neurogenin1* (*Ngn1*)-positive cells in the NSD of the mouse shows that these cells give rise to neuroblasts of the CVG as well as to sensory cells of the utricular and saccular maculae (Raft et al., 2007). *Ngn1* encodes a pro-neural basic-Helix-Loop-Helix (bHLH) transcription factor associated with the neuronal fate and is expressed in a subset of cells within the NSD. In the absence of *Ngn1* CVG formation is severely affected (Liu et al., 2000; Ma et al., 2000; Matei et al., 2005). Besides *Ngn1*, the molecular mechanisms that lead up to the induction of *Ngn1* and subsequent neuroblast differentiation are unclear.

The NSD is marked by the expression domains of several genes in the inner ear

The NSD of the inner ear lies anterior-ventral-medially and is specified and marked by a temporal cascade of molecules, which are proposed to mediate neurogenesis: Fgf10>Ngn1/Delta1/Hes5>Neurod1/NeuroM (Alsina et al., 2004). Fgf10 belongs to the fibroblast growth factor family. Forced expression of *Fgf10* in the chicken inner ear leads to an increase in *Neurod1* (also known as *NeuroD1*) and *NeuroM*-positive neuroblasts, and inhibition of Fgf receptor signaling leads to a loss of *Ngn1*, *Neurod1*, *Delta1*, and *Hes5* expression (Alsina et al., 2004). Therefore, Fgf signaling is stipulated in playing a role in initial neuronal specification (Alsina et al.,

2004). Delta1 is a ligand for the Notch receptor involved in specification of the neuronal fate through Notch-signaling mediated lateral inhibition, and Hes5 belongs to the bHLH family of transcription factors that is a negative regulator of pro-neural bHLH transcription factors Ngn1, Neurod1, and NeuroM (Cau et al., 2000; Daudet et al., 2007). *Hes5* is known to be expressed in cells destined to become sensory and shown to be involved in the formation of hair cells (Zine et al., 2001). Expression of *Delta1*, *Hes5*, *Ngn1*, *Neurod1*, and *NeuroM* all have a salt-and-pepper pattern in the NSD, suggesting that cells within the NSD are selectively expressing these genes destined to become neuronal or sensory cells (Alsina et al., 2004). *Ngn1* is required for the neuronal fate and it is transiently expressed in neuroblasts (Ma et al., 2000; Matei et al., 2005; Raft et al., 2007). One of Ngn1's functions is thought to be up-regulation of *Neurod1*, which is required to mediate neuroblast differentiation (Liu et al., 2000; Ma et al., 1996).

***Sox2* and *Sox3* are expressed in the NSD**

Sry-related HMG-box 2 and 3 transcription factors (Sox2 and Sox3) are expressed in the NSD. Their expression patterns overlap with that of *Lunatic fringe* (*Lfng*) and *Ngn1*. *Lfng* encodes an extra-cellular protein, which modulates Notch signaling. Its expression domain in the otic cup encompasses *Ngn1*, *Sox2*, and *Sox3* positive regions. However, *Lfng* null mice mutants have no apparent inner ear phenotype (Zhang et al., 2000). For the purpose of this study *Lfng* serves as a marker for the NSD domain.

The expression patterns of *Sox2* and *Sox3* are dynamic within the neuronal and sensory lineages (Fig.1.2). While *Sox2* and *Sox3* are expressed in the NSD, both gene activities are down-regulated in the delaminated *Neurod1*-positive neuroblasts and only the expression of *Sox2* and not *Sox3* persists in the sensory lineage (this study and (Neves et al., 2007)). By the time various sensory patches start to differentiate, *Sox2* is further down-regulated in differentiating hair cells but its expression remains in supporting cells (Neves et al., 2007). These expression patterns suggest that *Sox2* and *Sox3* may be required to be down-regulated upon neuronal differentiation and *Sox2* expression in the sensory patches undergoes further down-regulation during hair cell differentiation. This is the hypothesis being addressed in this dissertation.

Figure 1.2

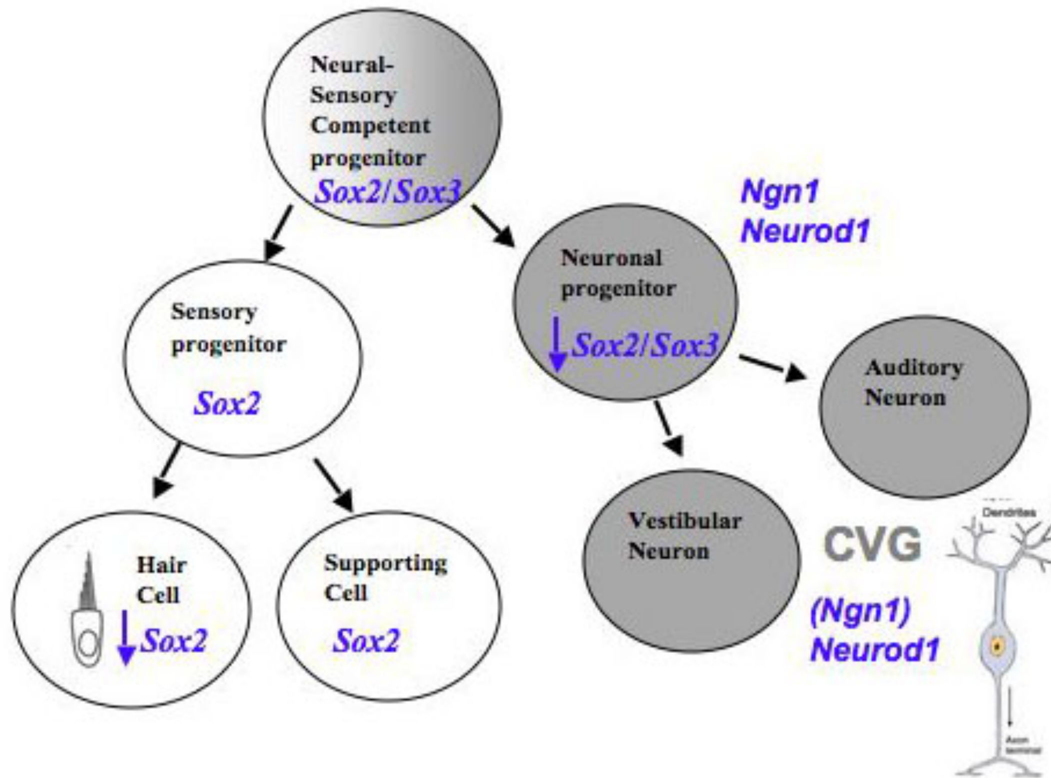


Figure 1.2. A common neural-sensory competent domain gives rise to the neuronal and the sensory lineages. *Sox2* and *Sox3* are both initially expressed in the NSD. They are soon down-regulated in delaminating neuroblasts (this study and (Neves et al., 2007)), whereas the expression of *Sox2* persists in the sensory lineage. These expression patterns suggest that there is a requirement for *Sox2* and *Sox3* to be down-regulated for neuronal differentiation and that there is a requirement for *Sox2* down-regulation for hair cell differentiation. *Ngn1* and *Neurod1* are both expressed in neuronal progenitors and required for the formation of the CVG. *Ngn1* is transiently expressed in delaminated neuroblasts, whereas expression of *Neurod1* persists for some time.

The requirement of *Sox2* and *Sox3* in the central nervous system and inner ear development

Sox1, Sox2, and *Sox3* belong to group B1 of the evolutionarily conserved family of *Sry*-related *High-Mobility-Group* (*HMG*) box genes, which encode transcription factors (Lefebvre et al., 2007). The HMG domain of these transcription factors mediates nuclear translocation and partners with other factors for binding to DNA (Kamachi et al., 2000; Lefebvre et al., 2007; Wilson and Koopman, 2002). The C-termini of Sox proteins are responsible for transcriptional activation or repression (Kamachi et al., 2000; Lefebvre et al., 2007; Wilson and Koopman, 2002). Sox proteins require the interaction of a binding co-partner that interacts with the HMG domain in a cell-context dependent manner (Kamachi et al., 2000; Wilson and Koopman, 2002). However, the C-terminal activation or repression domains are not cell-specific and not dependent on co-partners, but they do require stable DNA binding to the target through the HMG domain and the co-partner (Kamachi et al., 2000; Wilson and Koopman, 2002). Therefore, the specificity of the mode of action of Sox proteins is cell-context dependent, relying on co-partners (Kamachi et al., 2000; Wilson and Koopman, 2002).

There are about 20 *Sox* genes expressed in vertebrates and the overall role of these genes is to maintain stemness, i.e. a self-renewing progenitor cell state, and to regulate cell fate and differentiation (Lefebvre et al., 2007). Some regulatory elements of *Sox2* have been identified within the *Sox2* promoter, including the conserved *nasal-otic placode-specific enhancers 1* and *2* (*NOP-1* and *NOP-2*), which as the names imply, confer expression in the nasal and otic tissues (Uchikawa

et al., 2003). Sox2 and Sox3 appear to function as transcriptional activators in the central nervous system and *Sox2* is involved in the development of several other organs, such as the lung, tongue, and eye (Bani-Yaghoub et al., 2006; Bylund et al., 2003; Gontan et al., 2008; Okubo et al., 2006; Taranova et al., 2006). In the chicken neural tube, Sox2 and Sox3 block neurogenesis by maintaining a progenitor status, whereas Ngn2 counteracts *Sox2* and *Sox3* activity presumably to promote neurogenesis through a yet to be determined molecular mechanism (Bylund et al., 2003). In blastocysts, Sox2 mediates self-renewal and pluripotency in embryonic stem cells (Boyer et al., 2005; Chew et al., 2005; Loh et al., 2006). In the developing lung, *Sox2* expression has to be down-regulated for branching morphogenesis and is required for epithelial cell differentiation (Gontan et al., 2008). In the developing tongue, *Sox2* is required for differentiation of taste buds (Okubo et al., 2006). Therefore, Sox2's role of initially maintaining a proliferative progenitor pool, and its subsequent requirement for its expression to be down-regulated to mediate cellular differentiation seems to be a common theme underlying Sox2 function throughout development, including the inner ear. Nevertheless, the mechanisms that regulate the levels of Sox2 activities required for these various cellular processes are not clear.

In humans, mutations in the *SOX2* gene cause sensorineural hearing loss (Hagstrom et al., 2005). Furthermore, analyses of two mouse mutants, *light coat and circling* (*lcc*) and *yellow submarine* (*ysb*), show that Sox2 is required for sensory organ development (Kiernan et al., 2005). The *lcc* mutant has no *Sox2* expression, whereas the *ysb* mutant has reduced levels of *Sox2* transcripts. *Lcc* mutants fail to

develop sensory organs and *ysb* mutants have only some sensory patches. These findings indicate that *Sox2* function is essential for the development of the sensory organs and *Sox2* is commonly thought of as a pro-sensory gene. The role of *Sox2* or *Sox3* in the neurogenic fate of the inner ear is unclear. Given the dynamic expression patterns of *Sox2* and *Sox3* during early stages of inner ear development, these genes are good candidates for mediating neuronal as well as sensory fates. This dissertation addresses the roles of *Sox2* and *Sox3* in this context, in particular with regard to the neuronal fate (Chapter 2). Furthermore, because *Sox1* is not expressed in the NSD and it is only expressed in the anterior and lateral cristae starting at E3.5 (not shown), experiments with *Sox1* were not pursued in this study.

The neuronal fate in the inner ear is in part regulated through Notch signaling

The Notch signaling pathway plays important roles during embryogenesis. It generates cell type diversity through lateral inhibition, cell lineage decisions, and cell boundary formation (Weir et al., 2000). The process of lateral inhibition is in part responsible for regulating neurogenesis (Fig. 1.3). The working model for Notch signaling in vertebrates is similar to Notch signaling during the acquisition of the neuronal fate in *Drosophila* (Bertrand et al., 2002). Initially, Notch receptors such as Notch1 are expressed equivalently in neuroepithelial progenitors. As levels of pro-neural genes such as *Ngn1* increase in progenitors destined to become neuronal, levels of the Notch ligand such as Delta1 also increase in these cells, and this inhibits neighboring cells from adopting the same neuronal fate. Ligand-specific binding of the Notch receptor leads to proteolytic cleavage of the Notch

receptor by the enzyme γ-secretase and the release of the intracellular domain of Notch (Notch-ICD). The Notch-ICD in turn translocates to the nucleus to activate a set of genes including *Hes* and *Hey*, which inhibit the neuronal fate. For example, Hes5 has been shown to negatively regulate pro-neural *bHLH* genes including *Ngn1* during mouse olfactory placode neurogenesis (Cau et al., 2000). Another aspect of Notch signaling is lateral induction (see below). As opposed to lateral inhibition, this is a process of positive feedback and is involved in establishing the pro-sensory domain and the hair cell fate in the inner ear (Brooker et al., 2006; Daudet and Lewis, 2005; Hartman et al., 2010; Kiernan et al., 2001; Kiernan et al., 2005; Lanford et al., 1999; Pan et al., 2010; Tsai et al., 2001).

In the chicken inner ear, *Notch1* receptors and their ligands, *Serrate1* (*Ser1,* also known as *Jag1* in the mouse) and *Delta1* are specifically expressed in the NSD (Adam et al., 1998). When Notch signaling in the chicken otic cup is blocked with DAPT, a chemical compound that inhibits γ-secretase activity, neurogenesis is increased because lateral inhibition is alleviated and genes that are normally activated by Notch signaling such as *Hes5.1* (a chicken homolog of mammalian *Hes5*) are down-regulated (Daudet et al., 2007).

In addition, studies of mouse cochlear explant cultures suggest that Notch signaling functions up-stream of *Sox2*. Over-expression of Notch-ICD leads to up-regulation of Sox2*,* and when Notch signaling is blocked with DAPT, Sox2 expression is down-regulated (Dabdoub et al., 2008). In the chicken otic cup, it has been proposed that *Sox2* expression is induced through Ser1-mediated Notch signaling (Daudet et al., 2007). However, studies in retina suggest that Notch

signaling could also be down-stream of Sox2 since a direct Sox2 binding site in the promoter of *Notch1* has been identified (Taranova et al., 2006). Therefore, *Sox2* and the Notch signaling pathway may function in close association to mediate neuronal and sensory cell fate choices in the inner ear. This dissertation addresses the potential relationships between *Sox2* and the Notch signaling pathway within this context (Chapter 3).

Figure 1.3

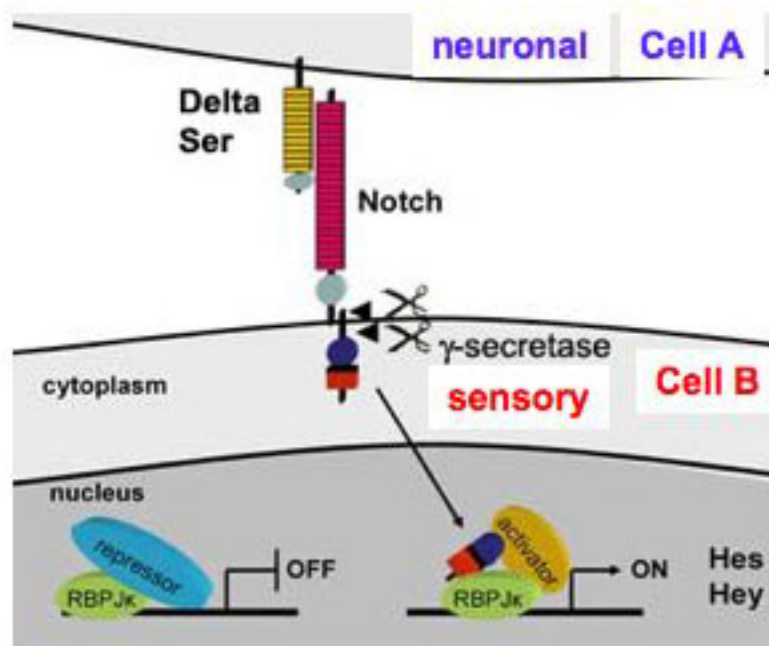


Figure 1.3. Notch signaling mediates cell fate choices. Cells that express Delta or Serrate ligands signal to neighboring cells not to adopt the same fate via the Notch receptor; a process known as lateral inhibition. Upon ligand-binding, the intracellular domain of Notch gets cleaved by γ-secretase and translocates into the nucleus to

activate genes such as *Hes* and *Hey* with the help of other binding proteins and co-factors. Figure modified from http://www.angiolab.de/research/fig1_Fischer.jpg

Notch signaling creates cell diversity through lateral induction and inhibition

The mammalian sensory epithelium of the cochlea, the organ of Corti, consists of one row of inner hair cells and three rows of outer hair cells. This patterning is absent in the chicken basilar papilla. Nevertheless, tall and short hair cells in the basilar papilla are analogous to inner and outer hair cells in mammals, respectively. Hair cells and their associated supporting cells are generated in a mosaic through lateral inhibition. However, the pro-sensory domain from which hair cells and supporting cells arise is established through lateral induction. Hair cells in the mouse and chicken cochlea differentiate several days after the initiation of neurogenesis. After neuroblasts delaminate from the NSD, cells remaining in this domain presumably adopt the sensory fate. It has been proposed that from this equipotential patch of sensory tissue, the hair cell fate is the default fate (Lanford et al., 1999). In this proposed model, initially all cells within the equipotential patch express Notch, then these cells developmentally progress and sensory progenitors express Ser/Jag ligands with decreased expression of Notch, whereas the surrounding cells have a high level of Notch expression (Daudet and Lewis, 2005; Lanford et al., 1999). Then, nascent hair cells emerge expressing Delta1 and Ser2/Jag2 ligands with surrounding cells expressing Ser1/Jag1 (Daudet and Lewis, 2005; Lanford et al., 1999; Tsai et al., 2001). The current dogma is that Ser1/Jag1-mediated Notch signaling induces the sensory fate, whereas Delta1/Ser2/Jag2-mediated Notch

signaling is responsible for generating the mosaic of hair and supporting cell types via lateral inhibition (Brooker et al., 2006; Daudet and Lewis, 2005; Hartman et al., 2010; Kiernan et al., 2001; Kiernan et al., 2005; Lanford et al., 1999; Pan et al., 2010; Tsai et al., 2001).

Several mouse models and studies in the chick support the above models of lateral induction and inhibition. *Ser1/Jag1* is expressed early in the sensory domain before hair cells differentiate, but later *Ser1/Jag1* is expressed in the supporting cells (Adam et al., 1998; Brooker et al., 2006; Cole et al., 2000; Neves et al., 2011). This expression pattern is consistent with an early role for Ser1/Jag1 function in establishing the pro-sensory fate and a late role in mediating lateral inhibition of the hair cell fate (Brooker et al., 2006; Kiernan et al., 2001; Tsai et al., 2001). Forced expression of *Notch-ICD* in the chicken inner ear induces ectopic sensory patches with the induction of *Ser1* expression (Daudet and Lewis, 2005). Similar phenotypes were observed in mouse mutants over-expressing *Notch-ICD* (Hartman et al., 2010; Pan et al., 2010). These results are consistent with the notion of Jag1's early role. The *Jag1* conditional knockout mouse (cko) exhibits a loss of outer hair cells and an increase in inner hair cell number together with the loss of the cell cycle cyclin-dependent kinase inhibitor p27Kip1 (Brooker et al., 2006). These hair cell phenotypes are also evident in heterozygous *Jag1* mutants, although less severe (Kiernan et al., 2001; Tsai et al., 2001). The more severe loss in outer than inner hair cells is attributed to reduced cell proliferation preferentially affecting outer hair cell formation (Brooker et al., 2006).

More supporting evidence for the lateral inhibition model in mediating hair cell fate comes from analyses of *Delta1* and *Jag2* knockout mutants, as well as mutants lacking downstream effectors of Notch signaling such as *Hes1* and *Hes5*. The *Delta1* cko mutant exhibits premature production and excess number of hair cells consistent with the role for Delta1 in mediating lateral inhibition (Brooker et al., 2006). In the *Jag2* null mutant, both inner and outer hair cells numbers are increased and the effect is more profound in *Delta1/Jag2* double mutants (Kiernan et al., 2005; Lanford et al., 1999). These results are consistent with the lateral inhibitory role for these ligands and suggest that Delta1 and Jag2 cooperate with each other and are both required for this function (Kiernan et al., 2005). Hes1 and Hes5 are effectors of the Notch signaling pathway downstream of Notch-ICD. They belong to the *bHLH* gene family and are repressors of pro-neural *bHLH* genes during lateral inhibition of the neuronal fate. In sensory epithelia of the inner ear, Hes1 and Hes5 negatively regulate the expression of the pro-sensory *bHLH* gene *Math1* (also known as *Atoh1*) in supporting cells. Atoh1 is necessary and sufficient for generating hair cells (Woods et al., 2004). *Hes1* null mutants show an increase in inner hair cell number and *Hes5* nulls have an increase in outer hair cells (Zine et al., 2001). These results are in agreement with the expression pattern of *Hes1* and *Hes5* in the greater and lesser epithelial ridges adjacent to the organ of Corti, respectively (Zine et al., 2001).

Sox2's role in sensory formation

There is evidence of Sox2's role in regulation of the sensory fate based on results of the two mouse mutants *lcc* and *ysb,* which have no or reduced level of *Sox2*

expression, respectively (Kiernan et al., 2005). Sensory epithelia are either missing in the *lcc* mutant, or expression of the sensory markers is reduced in the *ysb* mutant (Kiernan et al., 2005). In wildtype, after the initial down-regulation of *Sox2* in delaminating neuroblasts, *Sox2* expression is maintained in the sensory lineage until it is again down-regulated during hair cell differentiation but its expression is maintained in supporting cells (Dabdoub et al., 2008; Neves et al., 2007). Furthermore, *Sox2* and *Atoh1* are co-expressed in the sensory lineage transiently until hair cells start to differentiate (Dabdoub et al., 2008). There is evidence that Sox2 and Atoh1 negatively regulate each other. Over-expression of *Sox2* in mouse cochlear explants leads to an inhibition of hair cell formation while having no effect on supporting cells (Dabdoub et al., 2008). Over-expression of *Atoh1* in P-19 cell culture leads to down-regulation of endogenous *Sox2* expression, whereas forced expression of *Atoh1* in cochlear explants resulted in the induction of hair cell markers and hair cells (Dabdoub et al., 2008). Forced expression of *Sox2* alone or together with *Atoh1* also in cochlear explants also causes an absence or reduced formation of hair cells (Dabdoub et al., 2008). These results suggest an antagonistic relationship between Sox2 and Atoh1 in regulating the hair cell fate (Dabdoub et al., 2008). Although this dissertation does not address directly Sox2's role in the sensory lineage and development of hair cells, I do provide evidence that Sox2 and the Notch signaling pathway potentially interact in mediating cell fate choices between neurons and sensory cells (Chapter 3).

Sox2 is a stem cell factor

The dual functions of Sox2 in maintaining a proliferative progenitor status and mediating differentiation are most crucial during early development. Sox2 is expressed in the morula, embryonic stem cells of the inner cell mass of the blastocyst, in the epiblast, and in germ cells where it mediates self-renewal and pluripotency (Avilion et al., 2003; Chew et al., 2005). In neural stem cells, Sox2 mediates maintenance of progenitor status, proliferation, and differentiation (Pevny and Nicolis, 2010). In the embryonic stem cells of the blastocyst, *Sox2*, *Oct4* (also known as *Oct3*), and *Nanog* form a close regulatory network through auto-regulation, cross-regulation, and regulation of down-stream targets, thereby being the core regulatory elements (Boyer et al., 2005; Chew et al., 2005; Loh et al., 2006). Recently, another core set of transcription factors Oct3/4, cMyc, Klf4, together with Sox2 have been found to induce pluripotent stem cells from already differentiated adult mouse and human fibroblasts (skin cells) (Takahashi et al., 2007; Takahashi and Yamanaka, 2006). Embryonic stem cells have the unique ability to adopt any cell type, to differentiate into all three germ layers, and to grow and maintain pluripotency seemingly forever (Boyer et al., 2005; Chew et al., 2005; Loh et al., 2006; Takahashi et al., 2007; Takahashi and Yamanaka, 2006). A variety of diseases could be potentially treated through the technology of induced patient-specific somatic cells, and *Sox2* is at the forefront of active stem cell and regenerative medical research in this context (Takahashi et al., 2007; Takahashi and Yamanaka, 2006).

Sox and Sex

The roles of *Sox* genes and their homolog *Sry* in mediating cellular differentiation are perhaps best highlighted during sex determination. Mammalian sex determination is a complex interplay between intrinsic (e.g. transcription factors) and extrinsic factors (e.g. growth factors and signaling molecules) (Bowles and Koopman, 2010). During embryonic development, primordial, bipotential germ cells, which eventually will give rise to reproductive cells of an egg or sperm, migrate from the posterior primitive streak into the developing hindgut (Bowles and Koopman, 2010). There, they populate the urogenital ridge, which gives rise to the impartial gonads (Bowles and Koopman, 2010). The gonad develops either into a testis or an ovary depending on a master switch and on environmental factors (Bowles and Koopman, 2010; Collignon et al., 1996; Nagai, 2001; Waters et al., 2007; Wegner, 1999; Weiss et al., 2003). It has been proposed that the ovary is the default pathway, and only in the presence of the master switch together with an ovary repressing environmental signal will the gonad develop into a testis (Bowles and Koopman, 2010). The master switch has been found to be the *Sox* gene homolog *Sry* (sex determining region of Y chromosome), which encodes a transcription factor, and the environmental signaling molecule is thought to be retinoic acid, or rather the degradation of retinoic acid (Bowles and Koopman, 2010; Collignon et al., 1996; Nagai, 2001; Waters et al., 2007; Wegner, 1999; Weiss et al., 2003).

Sry is expressed in Sertoli cells, which direct the male-specific pathway of the testes (Bowles and Koopman, 2010; Collignon et al., 1996; Wegner, 1999).

Without *Sry*, XX genital ridges develop as ovaries and in the presence of *Sry*, XY ridges as testes (Bowles and Koopman, 2010). Germ cells in the ovary undergo meiosis, whereas in the testes they do not and instead arrest in the cell cycle until after birth (Bowles and Koopman, 2010). Whether germ cells undergo meiosis or not secures the female or the male fate, respectively (Bowles and Koopman, 2010). The decisive factor in this case is the degradation of retinoic acid through the retinoic acid degradation enzyme Cyp26b1 (Bowles and Koopman, 2010). Retinoic acid is thought to induce meiosis and *Cyp26b1* is expressed initially in both male and female gonads, but then it is up-regulated in the testis and down-regulated in the ovary (Bowles and Koopman, 2010). Therefore, the presence of *Cyp26b1* prevents meiosis and seals the male fate (Bowles and Koopman, 2010). Although *Sry* is thought to be up-stream of *Cyp26b1,* these two genes are thought to act in different pathways (MacLean et al., 2007).

The *Sox* gene family is grouped together based on sequence homology to the *Sry HMG* domain (Collignon et al., 1996; Lefebvre et al., 2007; Nagai, 2001; Wegner, 1999). Group B1 *Sox* genes, *Sox1*, *Sox2*, and *Sox3*, are closest in homology to *Sry* with *Sox3* sharing the highest homology (Collignon et al., 1996; Lefebvre et al., 2007; Nagai, 2001; Wegner, 1999). *Sox3* is located on the X chromosome, thereby being the only *Sox* gene so far located on a sex chromosome (Nagai, 2001; Waters et al., 2007). *Sox3* is expressed in the urogenital ridge like *Sry*, and then in both male and female genital ridges (Collignon et al., 1996; Waters et al., 2007; Wegner, 1999). *Sox2* is expressed in primordial germ cells where it mediates

pluripotency, and its expression has been found in oocytes, suggesting it plays a role there as well (Bowles and Koopman, 2010; Collignon et al., 1996; Wegner, 1999).

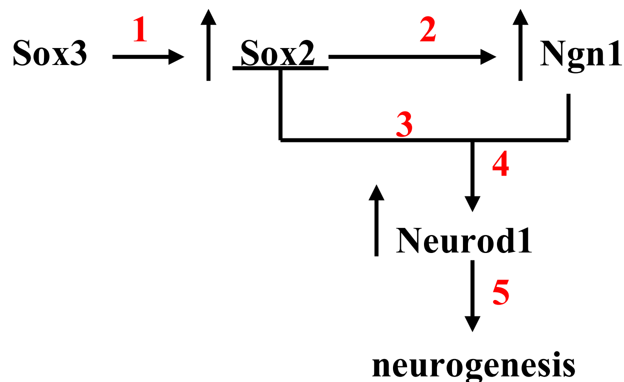
It is thought that *Sry* evolved from an ancestral *Sox3* gene because of the high *HMG* sequence homology and because *Sox3* is located on the X chromosome and *Sry* is located on the Y chromosome (Nagai, 2001; Waters et al., 2007). The X and Y-chromosomes are thought to have evolved from an ancestral autosomal pair where the Y chromosome progressively differentiated and lost most of its encoding genes and *Sry* evolved only in mammals (Waters et al., 2007; Wegner, 1999). Despite the link between *Sry* and *Sox3*, Sox3 is not necessary for sex determination but has been found to be important for the normal development of the central nervous system, and ovarian and testis development in mice (Weiss et al., 2003). X-linked mental retardation in humans is associated with mutations in *SOX3* (Weiss et al., 2003). In mice, *Sry* is sufficient to induce testis formation in XX animals (Weiss et al., 2003). Another Sox gene, *Sox9* is also expressed in the male and female genital ridges until the onset of *Sry* expression, and further in the male gonad and development of the testes (Wegner, 1999). In humans, mutations in *SRY* and in *SOX9* lead to sex reversal in affected XY males, and mutations in *SOX9* also lead to skeletal deformations (Nagai, 2001; Wegner, 1999; Weiss et al., 2003). All these studies illustrate that *Sry* and *Sox* genes play crucial roles in mediating differentiation throughout normal development including sex determination.

Aims and Hypotheses

The aims of this project are to understand the roles of *Sox2* and *Sox3* in mediating neurogenesis in the inner ear and to understand the potential relationship between *Sox2* and the Notch signaling pathway in mediating cell fate choices between neurons and sensory cells.

Hypothesis 1: Sox3 up-regulates *Sox2*. Sox2 up-regulates *Ngn1* to initiate neurogenesis, and Ngn1 inhibits *Sox2* expression for neurogenesis to proceed and promotes neurogenesis by up-regulating *Neurod1* (Fig.1.4).

Figure 1.4



Hypothesis 2: Notch signaling is up-stream of *Sox2* and Sox2 mediates parts of its functions via Notch signaling (Fig.1.5).

Figure 1.5

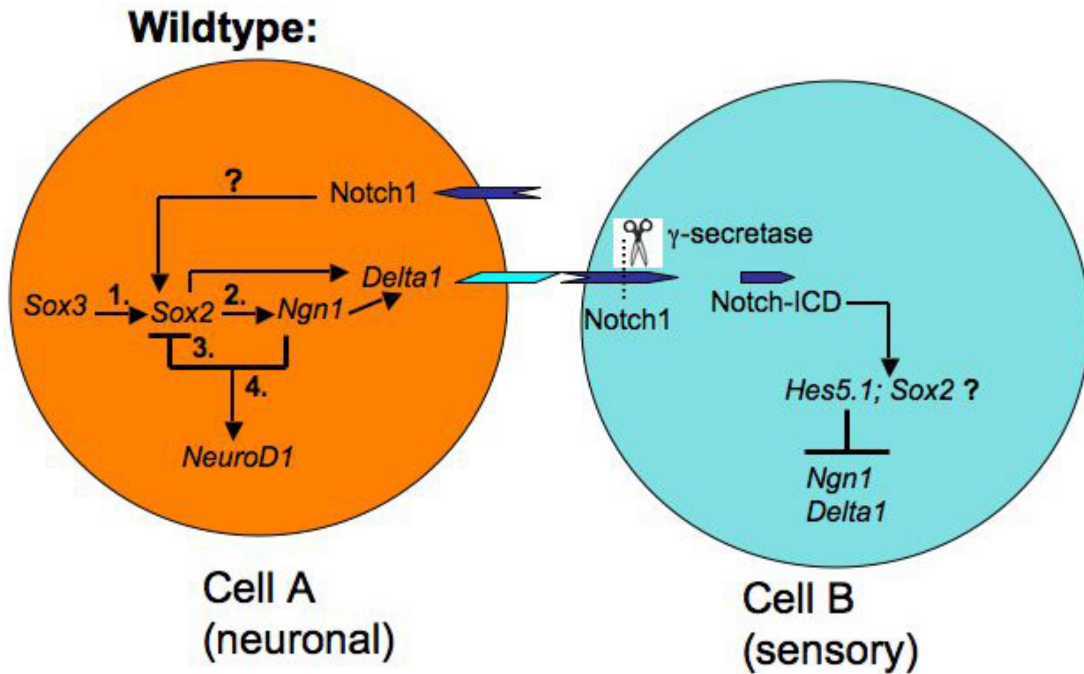


Figure 1.5. Hypothesis 2 Model

Sox2 up-regulates *Ngn1* and *Delta1* cell-autonomously. The up-regulation of *Delta1* by Sox2 may be direct or indirect through the up-regulation of *Ngn1*. In order for neurogenesis to proceed, the up-regulated Ngn1 inhibits *Sox2* activity to activate *Neurod1,* and cell A eventually develops into a neuron. Delta1 binds to the Notch1 receptor in neighboring cells (cell B) to inhibit the neuronal fate. Upon ligand-binding, the Notch intracellular domain (Notch-ICD) gets cleaved by γ-secretase. Notch-ICD translocates into the nucleus to up-regulate *Hes5.1* and presumably *Sox2*. Hes5.1 in turn inhibits *Ngn1* and thereby *Delta1*. Thus, cell B goes on to adopt a sensory fate. Missing from the illustration are some temporal elements.

Experimental Strategy and the Electroporation Method

All experiments were conducted in chicken inner ears *in ovo*. For both gain and loss-of-function studies, genes of interest were over-expressed using the electroporation method (Fig.1.6). The principle of the electroporation method is based on the fact that DNA is negatively charged due to the PO_4^- (phosphate) groups in the backbone of the DNA molecule. When an electrical current is placed across the DNA, the DNA flows toward the anode (positively charged electrode), driving the DNA toward a specific area of the tissue of interest. In this case the right otic cup is the tissue targeted. The left ear was used as an internal control. This method, though widely used, has not been demonstrated to work with high efficiency in the developing inner ear. I have devised an electroporation method which yields approximately ~80-90% of efficiency in targeting and survival.

The genes used in this study were sub-cloned into expression vectors that express either Green Fluorescent Protein (GFP) or Red Fluorescent Protein (RFP) as a marker. The chicken embryos were staged according to developmental stages established by Hamburger & Hamilton (1951). The genes were over-expressed at stages 10-12 with 10-17 somites at embryonic day 1.5. The ears were analyzed for expression of the fluorescent marker within 6-48 hours after electroporation. The ears were scored on a scale of 1-3 with a score of 1 for ears with the best expression of the fluorescent protein covering the entire ear (see Fig. 1.7 below). Ears with expression of the fluorescent marker that included the anterior-ventral NSD such as expression in the anterior or ventral half of the ear received a score of 2. Robust expression of the marker outside the NSD domain received a score of 3. Embryos with scores 1-3 were

harvested. Embryos with scores beyond 3, with poor GFP or RFP expression, were not harvested and were discarded. Harvested embryos were processed and analyzed for gene or protein expression by RNA *in situ* hybridization or by immunohistochemistry, respectively. Data were statistically quantified where appropriate.

Figure 1.6

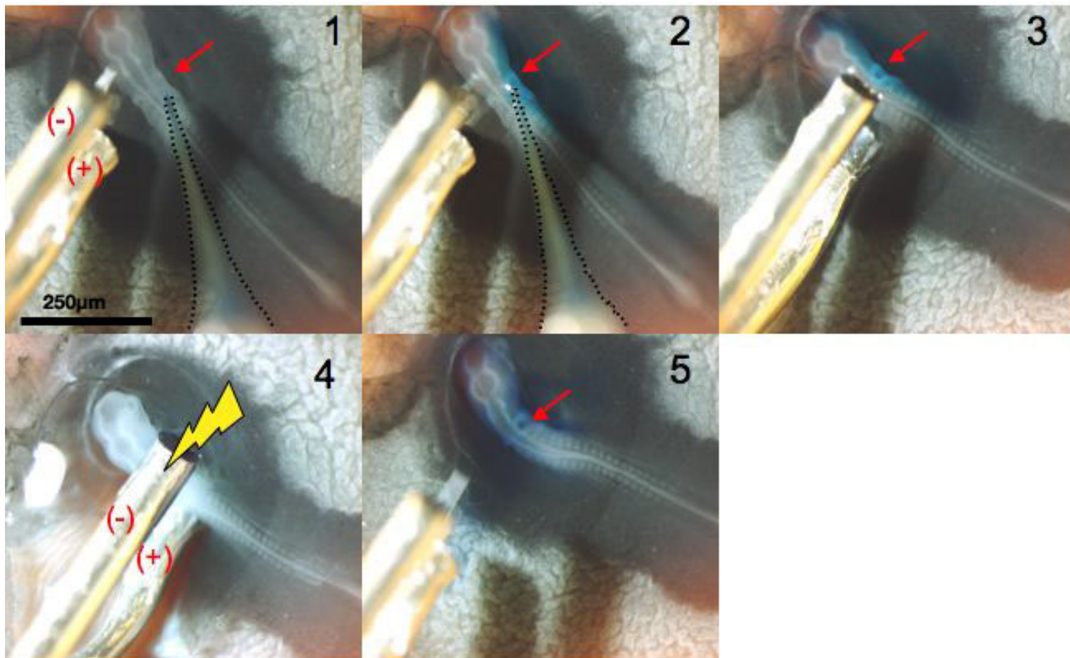


Figure 1.6. The Electroporation Method

The electrodes were made out of two platinum needles, cut, and bent with ~1 mm distance between them. The cathode (-) is placed on top of the anode (+). Panel 1 shows a chicken embryo at approximately ~17 somite stage *in ovo*. The right otic cup (arrow) and left otic cup are visible. The glass capillary needle that holds the DNA solution is also visible (outlined with black dashes). The electrodes are initially placed by the left side of the embryo. The embryo is bathed in albumin overlaid with

Phosphate Buffered Saline (PBS) to allow easy access with the electrodes. The right otic cup is filled with the DNA solution containing 0.1% Fast Green for visualization (Panel 2). Then, the electrodes are quickly inserted on the left side sandwiching the embryo (Panels 3 and 4). The placement of the electrodes turns the embryo slightly toward the left and helps to position the cathode right above the right otic cup (Panel 4). Then, two pulses at 7 volts with 100 msec duration and spacing are applied (Panel 4). The electric current opens transient pores in the ear epithelium allowing the DNA to be taken up. After the electrodes are removed, one drop of PBS is applied to cool down the embryo. The egg is then sealed and returned to the incubator for analysis and harvesting between 6-48 hrs.

Figure 1.7

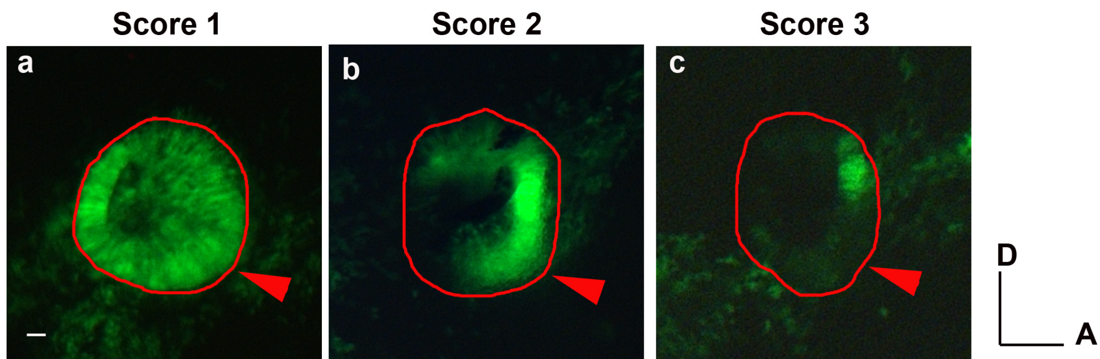


Figure 1.7. Scoring Strategy. (a-c) Whole-mount ears 24 hrs after electroporation with GFP. (a) Score 1 has fluorescent signal in the entire ear including the NSD located anterior-ventrally (a, red arrowhead). (b) Score 2 has signal that includes the NSD (red arrowhead). (c) Score 3 has signal outside the NSD. The ears are outlined in red (a-c).

Chapter 2: Inhibition of *Sox2* transcription by Neurogenin 1 is required for neurogenesis

Abstract

In the vertebrate inner ear sensory patches and neurons that innervate them are thought to derive from a common neural-sensory competent domain (NSD) at the ear rudiment. *Sry*-related *HMG-box 2* and *3* (*Sox2, Sox3*) are expressed in the NSD but their expression is down-regulated in neuroblasts. These expression patterns suggest a requirement for *Sox2* and *Sox3* to be down-regulated for neurogenesis. I addressed the roles of Sox proteins in neurogenesis by conducting gain and loss-of-function experiments in developing chicken inner ears using an electroporation method. While over-expressing *Sox2* readily induces *Neurogenin 1* (*Ngn1*) expression, an important gene required for specifying the neurogenic fate, the size of the cochleo-vestibular ganglion (CVG) is reduced. Over-expression of *Ngn1* causes inhibition of *Sox2* transcription, *Neurod1* up-regulation, and increased neuroblast formation. Over-expression of *Neurod1* causes similar increases in neuroblast formation. The lack of neurogenesis progression is attributed to the inability of Ngn1 to down-regulate an exogenous promoter driving *Sox2*. Furthermore, I provide evidence that Ngn1 and Neurod1 inhibit *Sox2* transcription via the *E-box* containing *nasal-otic placode-specific enhancer 1* (*NOP1*) of the endogenous *Sox2* promoter. Despite the induction of *Ngn1* by Sox2, attempts of loss-of-function approaches suggest that a factor(s) in addition to Sox2 is likely required for the induction of *Ngn1 in vivo*. While *Sox3* has

been proposed to be up-stream of *Sox2*, it does not induce *Ngn1* in a similar manner as Sox2.

Introduction

The vertebrate inner ear develops in a precise spatio-temporal manner into a highly intricate labyrinth of specialized sensory epithelia of sensory and non-sensory components. In chicken, seven sensory epithelia, the utricular macula, saccular macula, three cristae, macula neglecta, and the macula lagena, are responsible for mediating balance, and one auditory organ, the basilar papilla detects sound. These sensory epithelia are arranged in a mosaic of specialized mechanosensory hair cells and their associated supporting cells. Cell fate decisions between the sensory and neuronal lineages are determined early during inner ear development. First, neuroblasts delaminate from the otic neural-sensory competent domain (NSD) to give rise to the neuronal subtypes of the cochleo-vestibular ganglion (CVG, VIIIth cranial nerve), which extend dendrites to innervate the hair cells in various patches. After neuroblast delamination, cells that remain in the NSD are thought to split into various sensory patches and develop into hair cells and supporting cells. Fate-mapping studies indicate that the neuronal and sensory fates in the inner ear are lineage related (Raft et al., 2007; Satoh and Fekete, 2005), even though the molecular mechanisms regulating the fate choices are not well understood.

Pro-neural bHLH genes regulate neurogenesis

Vertebrate neurogenesis is regulated by a molecular cascade of evolutionary conserved *basic-Helix-Loop-Helix* (*bHLH*) genes, which encode transcription factors. These genes are grouped together into distinct families based on the sequence similarity of the *bHLH* DNA binding domain (Bertrand et al., 2002). This includes the families of pro-neural *Neurogenin* and *Neurod* genes. The Neurogenin family specifies neuronal subtypes whereas the Neurod family promotes differentiation (Bertrand et al., 2002; Chae et al., 2004; Ma et al., 1998; Ma et al., 1996). Ectopic over-expression of *Ngn1* has been shown to lead to ectopic up-regulation of *Neurod1* in *Xenopus* (Ma et al., 1996). Therefore, *Ngn1* is thought to be up-stream of *Neurod1*. Consistently, the *Ngn1* null mouse has no *Neurod1* expression and the CVG is absent, whereas in the *Neurod1* null mouse the CVG is much reduced (Liu et al., 2000; Ma et al., 2000; Matei et al., 2005).

The basic region of the bHLH domain mediates DNA binding to target genes and the HLH region mediates dimerization with other members of the bHLH family (Markus et al., 2002). The *bHLH* family of genes are grouped into three classes (Markus et al., 2002). Class I makes up the ubiquitously expressed E-proteins, which are required within a tissue-specific and cellular-specific context as partners for transcriptional activity of the class II members (Markus et al., 2002). Class II comprises the pro-neural genes such as *Ngn1* and *Neurod1*, and class III makes up the dominant negative repressors, the inhibitors of differentiation and DNA binding (Id) proteins (Markus et al., 2002). The pro-neural transcription factors together with E-proteins bind at *E-box* motifs within the target genes for transcriptional regulation.

The specificity of the interaction of the E-protein and the pro-neural protein at the target gene is determined by the specificity of the two central nucleotides within the consensus *CANNTG E-box* motif and by nucleotides flanking this motif (Bertrand et al., 2002; Seo et al., 2007). The Id proteins also interact with E-proteins but because the Id proteins lack the basic region they cannot bind to the target genes and hence act as negative transcriptional regulators (Markus et al., 2002). Although much effort is underway in understanding the events of neurogenesis, molecular mechanisms initiating neurogenesis and how pro-neural bHLH genes are regulated are unclear (Kintner, 2002; Ming and Song, 2005).

Role of Sox2 in organogenesis

Sox2 is part of a large family of evolutionary conserved transcription factors that contain a high-mobility-growth (HMG) DNA binding domain. *Sox* genes share high homology of the *HMG* domain with the *Sry* (Sex determining region of Y chromosome) gene, which encodes a testis determination factor (Collignon et al., 1996; Lefebvre et al., 2007; Nagai, 2001; Wegner, 1999). There are about 20 *Sox* genes in vertebrates that are divided into eight groups based on sequence homology (Lefebvre et al., 2007). *Sox1, Sox2*, and *Sox3* all belong to group B1 (Lefebvre et al., 2007). The HMG domain of Sox proteins mediates translocation to the nucleus and it partners with other factors for DNA binding to promoter regions of target genes (Kamachi et al., 2000; Lefebvre et al., 2007; Wilson and Koopman, 2002). Sox family of transcription factors could function as transcription activators or repressors depending on their C-terminal domain (Kamachi et al., 2000; Lefebvre et

al., 2007; Wilson and Koopman, 2002). Sox2 is postulated to function as an activator in the development of several organs including the central nervous system, taste bud, eye, and lung (Bani-Yaghoub et al., 2006; Bylund et al., 2003; Gontan et al., 2008; Okubo et al., 2006; Taranova et al., 2006). In blastocysts, Sox2 mediates self-renewal and pluripotency in embryonic stem cells (Chew et al., 2005). Sox2 also functions to maintain a proliferative progenitor pool during vertebrate neurogenesis, similar to its role as an embryonic stem cell factor (Pevny and Nicolis, 2010). However, the expression of *Sox2* is down-regulated in the chicken neural tube and the mouse neocortex upon neuronal differentiation (Bani-Yaghoub et al., 2006; Bylund et al., 2003). There is evidence that suggests the down-regulation of *Sox2* in the chicken neural tube is mediated by bHLH transcription factors such as Neurogenin 2 (Ngn2) (Bylund et al., 2003). In the mouse neocortex, Sox2 is thought to be degraded specifically in the neuronal lineage by serine protease(s) to alleviate Sox2 repression on neurogenesis (Bani-Yaghoub et al., 2006). Whether this post-translational regulation of Sox2 is a universal mechanism for regulating progression of neurogenesis in all neural tissues is not clear.

The tissue-specific expression of *Sox2* is mediated by several identified regulatory elements, including the conserved *nasal-otic placode-specific enhancers 1* and *2* (*NOP-1* and *NOP-2*) (Uchikawa et al., 2003). *NOP-1* and *NOP-2* have been shown to be active in the developing chick inner ear (Uchikawa et al., 2003). The *NOP-1* enhancer has been identified to contain an *E-box* sequence, whereas *NOP-2* does not contain an *E-box* (personal communication with Hisato Kondoh). *E-boxes* are short stretches of DNA with a consensus *CANNTG* motif (Bertrand et al., 2002;

Blackwell et al., 1993; Seo et al., 2007). *E-boxes* are binding sites for bHLH transcription factors of E-proteins together with pro-neural bHLH factors such as Ngn1 and Neurod1 (Markus et al., 2002). Pro-neural bHLH transcription factors require the interaction of E-proteins to regulate the expression of target genes though the *E-box* motif (Markus et al., 2002). In this study, I investigated whether there is an interaction of Ngn1 and Neurod1 with the *NOP-1* enhancer. Since the *NOP-2* enhancer is not conserved between chicken and mouse (personal communication with Hisato Kondoh) and the fact that *NOP-2* does not contain an *E-box*, making it an unlikely target binding site for bHLH proteins, experiments with *NOP-2* were not pursued further in this study.

Mutations in the *SOX2* gene lead to sensorineural hearing loss in humans and loss of *Sox2* function leads to deafness in mice (Hagstrom et al., 2005; Kiernan et al., 2005). Here, I sought to address the role of Sox2 and Sox3 in regulating neuronal fate specification using gain and loss-of-function experiments in developing chicken inner ears. I show that while over-expressing *Sox2* readily induces *Ngn1* expression, the size of the CVG is reduced. Over-expression of *Ngn1* inhibits *Sox2* transcription, induces *Neurod1* expression, and leads to increased neuroblast formation. Over-expression of *Neurod1* is sufficient for neurogenesis. I provide evidence that Ngn1 and Neurod1 inhibit *Sox2* at the transcriptional level via the *E-box* containing *NOP-1* enhancer of the *Sox2* promoter. Furthermore, my loss-of-function results suggest that Sox2 may potentially be required in the NSD for neurogenesis. I propose that Sox2 and another unknown factor(s) initiate neurogenesis by up-regulating *Ngn1*, and that

one of the important roles of pro-neural bHLH proteins is to inhibit *Sox2* transcription in order for neurogenesis to proceed. While *Sox3* has been proposed to be up-stream of *Sox2*, it does not induce *Ngn1* in a similar manner as Sox2.

Materials and Methods

Eggs, *in ovo* Electroporation, and Expression Constructs

Fertilized chicken eggs (B&E York Springs, PA) were incubated at 38° C and staged according to Hamburger and Hamilton (Hamburger and Hamilton, 1992). Full-length cDNA of chicken *Sox2* and *Sox3,* as well as the region encoding the *HMG* domain of *Sox2* and *Sox3* fused to the *Drosophila Engrailed* repressor domain (*HMG-EnR*), and the *HMG* domain of *Sox2* fused to the *Herpes simplex viral protein 16* activator domain (*HMG-VP16*) were subcloned into the *pMES-IRES-GFP* expression vector, in which various cDNA is driven by a chicken β-actin promoter. The *internal ribosomal entry site* (*IRES*) mediates bicistronic expression of the Green Fluorescent protein. Mouse *Ngn1* cDNA was subcloned into *pMES-IRES-GFP* and *pCMV-DsRed-Express* (Clontech) vectors. Chicken *Neurod1* was sub-cloned into the *pCI-IRES-H2B-RFP* vector. The *NOP-1,* and *NOP1-Ebox Mutant* (*E-box* sequence *CAGGTG* mutated to *AGCTAA*) enhancers of *Sox2* were subcloned into an Enhanced Green Fluorescent Protein vector, *ptkEGFPv2*. Various plasmids were delivered to the right otic cup *in ovo* between 10-17 somite stages (E1.5) by electroporation. This was conducted by filling the right otic cup with plasmids at a concentration of 3-5 μg/μl in 0.1% Fast Green. Then, a negative platinum electrode was placed above the right otic cup and the positive electrode

inserted underneath the embryo at the location of the left otic cup. Two pulses at 7 volts with 100 msec duration and spacing were applied using a CUY21 electroporator. Then, the eggs were sealed and returned to the incubator between 6 to 48 hrs before harvesting. For co-electroporations, the respective plasmid constructs were used at approximately 3 μg/μl concentrations each.

For consistency throughout the study the constructs are named as follows (also see Table 2.1 below): *GFP*, *Sox2-GFP, Sox3-GFP, Ngn1-GFP, EnR(Sox2)-GFP, EnR(Sox3)-GFP, VP16(Sox2)-GFP, DsRed, Ngn1-DsRed, RFP, Neurod1-RFP, NOP1-GFP,* and *NOP1-EboxMut-GFP*.

Table 2.1 List of constructs and proteins they encode

pMES-IRES-GFP	Dicistronic expression of Green Fluorescent Protein driven by a chicken β-actin promoter
pMES-Sox2-IRES-GFP	Full-length chicken Sox2 driven by a chicken β-actin promoter
pMES-Sox3-IRES-GFP	Full-length chicken Sox3 driven by a chicken β-actin promoter
pMES-Ngn1-IRES-GFP	Full-length mouse Ngn1 driven by a chicken β-actin promoter
pMES-HMGEnR(Sox2)-IRES-GFP	HMG domain of Sox2 fused to Drosophila Engrailed repressor domain driven by a chicken β-actin promoter
pMES-HMGEnR(Sox3)-IRES-GFP	HMG domain of Sox3 fused to Drosophila Engrailed repressor domain driven by a chicken β-actin promoter

pMES-HMGVP16(Sox2)-IRES-GFP	HMG domain of Sox2 fused to Herpes simplex viral protein 16 activator domain driven by a chicken β-actin promoter
pCMV-DsRed-Express-Ngn1	Mouse Ngn1 fused to Red Fluorescent Protein driven by a cytomegalovirus promoter
pCI-IRES-H2B-RFP	Dicistronic expression of Red Fluorescent Protein driven by a chicken β-actin/rabbit β-globin hybrid promoter and a cytomegalovirus enhancer, as well as histone 2B for nuclear localization
pCI-Neurod1-IRES-H2B-RFP	Full-length chicken Neurod1 driven by a chicken β-actin/rabbit β-globin hybrid promoter and a cytomegalovirus enhancer, as well as histone 2B for nuclear localization
ptk-Nop1-EGFPv2	NOP1 enhancer fused with Enhanced Green Fluorescent Protein and thymidine kinase cassette
ptk-Nop1EboxMut-EGFPv2	E-box within NOP1 enhancer mutated fused with Enhanced Green Fluorescent Protein and thymidine kinase cassette

***In situ* hybridization**

Whole mount and section *in situ* hybridization were carried out as previously described (Raft et al., 2007; Wu and Oh, 1996). Chicken Dig-labeled anti-sense RNA probes were generated for *Sox2*, *Sox3*, *GFP, Ngn1, Neurod1,* and *Lfng.*

Double labeling of tissue sections with *in situ* and Immunohistochemistry

To analyze gene expression at a cellular level, ear sections were first probed for RNA transcripts (e.g. *Ngn1*), followed by labeling with rabbit polyclonal-anti-GFP antibody (1:500 Invitrogen) overnight at 4° C. The time for Proteinase K treatment during the *in situ* procedure was reduced to 1 min and the time for colorimetric development was also monitored and terminated when the color precipitates first became evident to avoid hindering subsequent immunostaining signals. The secondary antibody was goat anti-rabbit Alexa Fluor 488 (1:250 Invitrogen). Antibody labeling was performed according to standard protocol (Raft et al., 2007).

Immunohistochemistry

Cryo-sections were prepared as described above for *in situ* hybridization. The primary antibodies used were rabbit polyclonal anti-Sox2 (1:4000 Chemicon), goat polyclonal anti-GFP-FITC-(conjugated) (1:400 GeneTex), rabbit polyclonal anti-DsRed-Express (1:100 Clontech), and mouse monoclonal anti-Neuron-specific β-III Tubulin-Nothern Lights 557-(conjugated) (TuJ-1-NL557, 1:25 R&D Systems). The secondary antibodies were goat anti-rabbit Alexa Fluor 568 (1:250 Invitrogen), goat anti-rabbit Alexa Fluor 680 (1:250 Invitrogen), and goat anti-rabbit Alexa Fluor 488 (1:250 Invitrogen). Antibody labeling was performed according to standard protocol (Raft et al., 2007), except the sections were subjected to antigen retrieval by citrate boiling for 5 min before immunostaining for anti-Sox2.

Statistical Analyses

For analyzing the size of ganglia after electroporation, serial ear sections were subjected to *Neurod1 in situ* hybridization and photographed with a Zeiss microscope. The outlines of the *Neurod1*-positive ganglionic regions and the otocyst were traced, and the areas were summed and computed using NIH image J software. Two-tailed Student's *t*-tests were performed between control and treated samples.

For analyzing the enhancer activity of *Sox2* in the presence of *Ngn1*, *NOP1-GFP,* or *NOP1-EboxMut-GFP* was co-electroporated with *Ngn1-DsRed* or *DsRed* control. Similar experiments were conducted with *Neurod1-RFP*. Ear sections were subjected to double antibody labeling against anti-GFP-FITC and anti-DsRed-Express, counterstained with DAPI, and photographed with a Zeiss microscope. The images were merged in Adobe Photoshop and the total number of cells that co-expressed anti-GFP-FITC and anti-DsRed-Express per ear were counted. Two-tailed Student's *t*-tests were performed on the total number of co-expressing cells. For analysis of NOP-1 activities within the NSD, sections adjacent to the ones processed for double antibody labeling of anti-GFP and anti-DsRed were probed for *Ngn1* transcripts by *in situ* to identify the NSD. Double-labeled cells within the NSD were scored and a two-tailed Chi-Square test was used for quantification.

Results

Sox2* is expressed in the NSD and overlaps in expression with *Sox3* and *Lfng

I sought to address the expression patterns of *Sox2* and *Sox3* at early stages of inner ear development and neuroblast delamination by performing RNA *in situ*

hybridization. Figure 2.1 illustrates the overlapping expression patterns of *Sox2*, *Sox3* (Fig. 2.1a, b, g, and h) and *Lfng* (Fig. 2.1d) in the presumed NSD located in the anterior ventral region of the chicken otic cup and otocyst. While *Ngn1* is also expressed in the NSD (Fig. 2.1c), its expression is only localized in a sub-set of cells within the domain (Fig.2.1d). Notably, both *Sox2* and *Sox3* are only weakly expressed in the delaminated neuroblasts of the CVG (Fig. 2.1e and f). Based on the over-lapping expression pattern of *Sox2* and *Sox3* in the NSD and their subsequent down-regulation in the CVG, I hypothesized that both *Sox2* and *Sox3* play a role in regulating the neuronal fate.

Figure 2.1

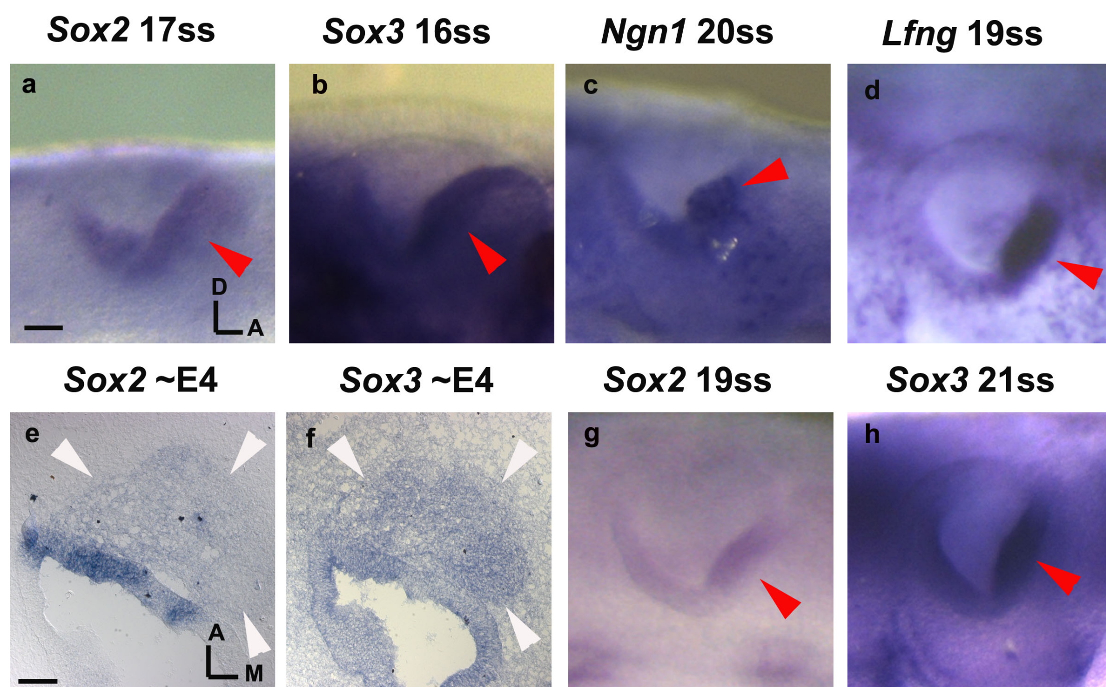


Figure 2.1. Expression patterns of *Sox2*, *Sox3*, *Ngn1*, and *Lfng* in the otic cup and otocyst. In the otic cup (a and b) or otocyst (c, d, g, h) *Sox2*, *Sox3* and *Ngn1* are

expressed within the *Lfng*-positive NSD (a-d, g and h; red arrowheads). At the otocyst stage, *Sox2* expression persists in the sensory patches (e), whereas *Sox3* is down-regulated in sensory epithelia (f). Both *Sox2* and *Sox3* are only weakly expressed in the delaminated neurons of the CVG (e and f; white arrowheads). ss, somite stages; E, embryonic day.

Ectopic *Sox2* causes a decrease in the size of CVG but an up-regulation of *Ngn1*

I investigated the role of *Sox2* in neurogenesis by over expressing *Sox2* in the developing inner ear. Inner ears electroporated with *Sox2-IRES-GFP* (*Sox2-GFP*) or control (*GFP*) plasmid at otic cup stages were analyzed for GFP signal within 24 and 48 hrs after electroporation. *Sox2-GFP* treated ears appear to have a smaller CVG (Fig. 2.2b, b'). Some of the 48 hrs specimens were subsequently sectioned, processed for *in situ* hybridization of *Neurod1* transcripts, and used to quantify the size of the otocyst and ganglion (see Materials and Methods). While there is no difference in the size of the otocyst between the right electroporated and the left non-electroporated ears and there is no significant difference in the size of CVG between non-electroporated and GFP control ears, there is an approximately 50% reduction in the size of *Sox2-GFP* treated CVGs compared to GFP controls and untreated controls (Fig. 2.2c).

The decrease in CVG size suggested an inhibition of neurogenesis by over-expressing *Sox2*. This led me to analyze the expression patterns of *Ngn1* and *Neurod1 in Sox2-GFP* specimens. Unexpectedly, *Sox2-GFP* treated ears show ectopic expression of *Ngn1* in GFP positive regions within (Fig. 2.2f, f') as well as outside (Fig. 2.2e, e') of the NSD. Most of the *Ngn1* expression is well correlated

with the GFP-positive domains, suggesting the up-regulation is cell-autonomous (Fig.2.2e, f; arrowheads). This up-regulation is also rapidly detectable within 6-7 hrs after electroporation (Fig. 2.3), suggesting a possible direct effect of Sox2 on *Ngn1*. In contrast, while GFP positive cells are present throughout the otic epithelium in *GFP* controls, no up-regulation of *Ngn1* is observed (Fig. 2.2d, d'). Notably, despite the up-regulation of *Ngn1,* there is no concomitant up-regulation of *Neurod1* in the *Sox2*-treated ears 48 hrs after electroporation (not shown), which suggests there is no progression of neurogenesis beyond *Ngn1* expression and this result is consistent with the finding of reduced CVG sizes.

Figure 2.2

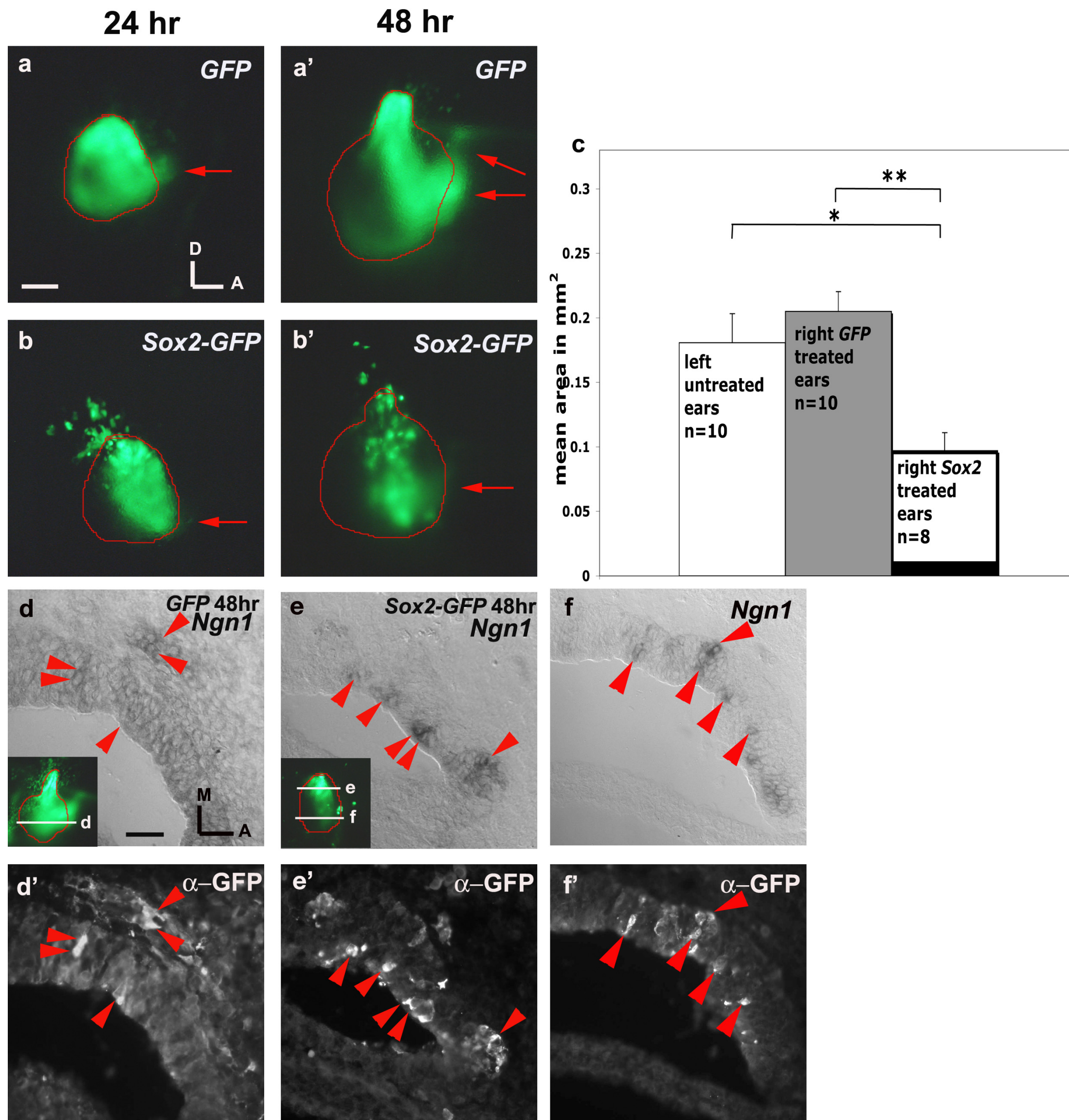


Figure 2.2. Over-expression of *Sox2* leads to a decrease in the size of CVG but an ectopic cell-autonomous up-regulation of *Ngn1*. (a-b') Otocysts electroporated with *GFP* or *Sox2-GFP* at 24 hrs and 48 hrs. The otocyst is outlined in red. Red arrows point to the presumably delaminated neuroblasts. *GFP* control ears show more neuroblast delamination than *Sox2-GFP* ears at both 24 and 48 hrs. (c) Quantitative analysis of CVG sizes. CVGs of *GFP*-treated ears are not significantly different than

non-electroporated control ears, whereas CVGs of *Sox2*-treated ears are approximately 50% smaller than controls and *GFP*-treated ears. Student's *t*-test * p=0.0068; ** p=0.000084; error bars represent s.e.m.. (d-f') Sectioned *GFP* control and *Sox2-GFP* treated ears double-labeled for *Ngn1* transcripts and anti-GFP antibody 48 hrs after electroporation. *GFP* control ears (n=2) show *Ngn1* expression in the NSD and in delaminating neuroblasts that are double-labeled with anti-GFP (d, d'; red arrowheads). In *Sox2-GFP* ears (n=4/5) double-labeled cells are present in dorsal (e, e') as well as ventral (NSD, f, f') otic regions (red arrowheads).

Figure 2.3

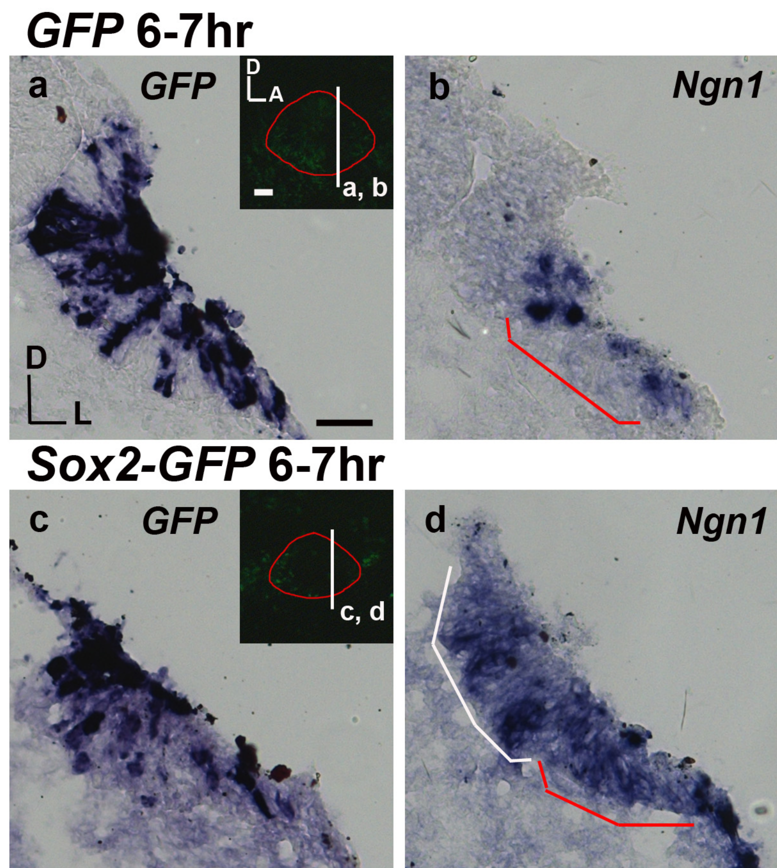


Figure 2.3. Over-expressing *Sox2-GFP* leads to *Ngn1* up-regulation by 6-7 hrs. Otic cups electroporated with *GFP* control or *Sox2-GFP* at 6-7 hr harvest (a, c; inset). Ears are outlined in red and the level of section is indicated in white lines (a, c; insets). Adjacent sections probed for *GFP* and *Ngn1* (a-d). *Ngn1* expression is up-regulated in the NSD (d, red bracket) as well as other regions outside the NSD (d, white bracket) in *Sox2*-treated ears (n=7/7) compared to *GFP* control (b, NSD red bracket; n=7).

Ngn1 is sufficient to promote neurogenesis and up-regulate *Neurod1*

Despite the observed up-regulation of *Ngn1* in Sox2 treated specimens, neurogenesis fails to proceed. This led me to investigate the consequence of over-expression of *Ngn1*. Similar electroporated experiments conducted with *Ngn1-IRES-GFP* (*Ngn1-GFP*) show broad ectopic cell delamination beyond the NSD from all over the otocyst within 24 hrs (Fig. 2.4a-b). Delamination is more evident by 48 hrs, to the extent that the otocysts are malformed and smaller in size (Fig. 2.4b', m). Section analyses indicate that the delaminated cells are indeed neuroblasts based on *Neurod1* expression (Fig. 2.4n, q). The ectopic expression of *Neurod1* and the subsequent delamination of these cells could be a result of an expansion of the NSD. However, there is no obvious expansion of the NSD based on *Lfng* expression (Fig. 3i, l, o, r; n=15). Since a majority of the GFP-positive cells appear to have delaminated from the otic epithelium already in the *Ngn1-GFP* specimens by 48 hrs, I harvested some specimens at 15 hrs after electroporation to confirm the ectopic expression of *Neurod1* within as well as beyond the NSD (Fig. 2.4c-f), which was

not observed in the *Sox2-GFP* specimens. The induction of *Neurod1* by Ngn1 is consistent with previous reports that *Ngn1* is up-stream of *Neurod1* (Ma et al., 1996). Together, these results show that over-expressing *Ngn1* is sufficient to up-regulate *Neurod1* and induce neurogenesis in the inner ear.

Figure 2.4

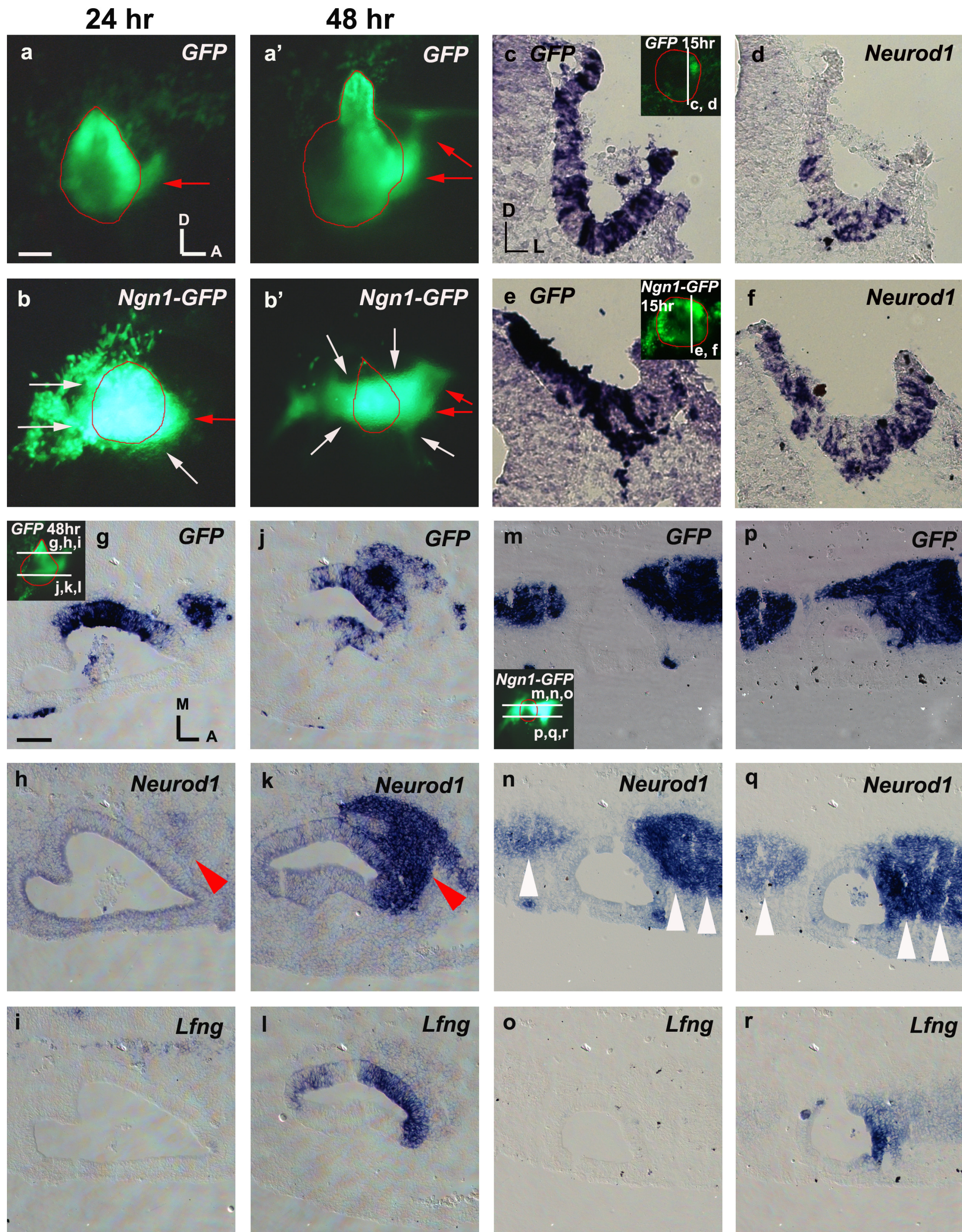


Figure 2.4. Ngn1 is sufficient to promote neurogenesis and up-regulate *Neurod1*.

(a-b') Otocysts electroporated with *GFP* (a, a') or *Ngn1-GFP* (b, b') at 24 hrs (a, b)

and 48 hrs (a', b'). The outline of the otocyst is indicated in red. Red arrows point to the presumably delaminated neuroblasts. *Ngn1-GFP* ears show neuroblast delamination from the NSD (b' red arrows) as well as ectopically from other regions of the otocyst (b, b'; white arrows) compared to *GFP* ears. Adjacent tissue sections probed for *GFP* and *Neurod1* transcripts in *GFP* control (c, d) and *Ngn1-GFP* (e, f) treated ears 15 hrs after electroporation. *Neurod1* expression is up-regulated in the ear epithelium in *Ngn1-GFP* treated ears (f, n= 8/10) but not in the *GFP* control (d, n=7). Adjacent tissue sections probed for *GFP, Neurod1*, and *Lfng* transcripts in *GFP* control (g-l) and *Ngn1-GFP* (m-r) treated ears 48 hrs after electroporation. In the *GFP* control ears *Neurod1* is expressed only in the CVG (h, k; red arrowheads; n=10) and the *Lfng*-positive NSD (k, l), but not outside of the NSD (h) that is *Lfng*-negative (i). In *Ngn1-GFP* ears, *Neurod1*-positive neuroblasts are detected in both dorsal and ventral sections anterior and posterior to the otic epithelium (n, q; white arrowheads; n=4/4). *Neurod1* is expressed in the NSD (q) that overlaps with the *Lfng* domain (r). Despite the ectopic, delaminated *Neurod1*-positive cells dorsally, there is no expansion of the *Lfng* domain (m, n, o).

Over-expressing *Ngn1* leads to down-regulation of Sox2

In the chicken neural tube, *Ngn2* inhibits Sox2 expression (Bylund et al., 2003). To test if a similar relationship occurs in the inner ear, I have analyzed *Ngn1-GFP* treated ears for Sox2 expression at 15 hrs after electroporation. Cells double-labeled for GFP and Sox2 immunoreactivity are present in the NSD of *GFP* electroporated control ears, whereas GFP-positive cells in *Ngn1-GFP* electroporated

ears do not show Sox2 immunostaining (Fig. 2.5). These results suggest that over-expression of *Ngn1* leads to a down-regulation of Sox2 similar to findings in the chicken neural tube and further suggests that there may be a requirement for *Sox2* to be down-regulated by Ngn1 for neurogenesis to proceed in the developing inner ear as well.

Figure 2.5

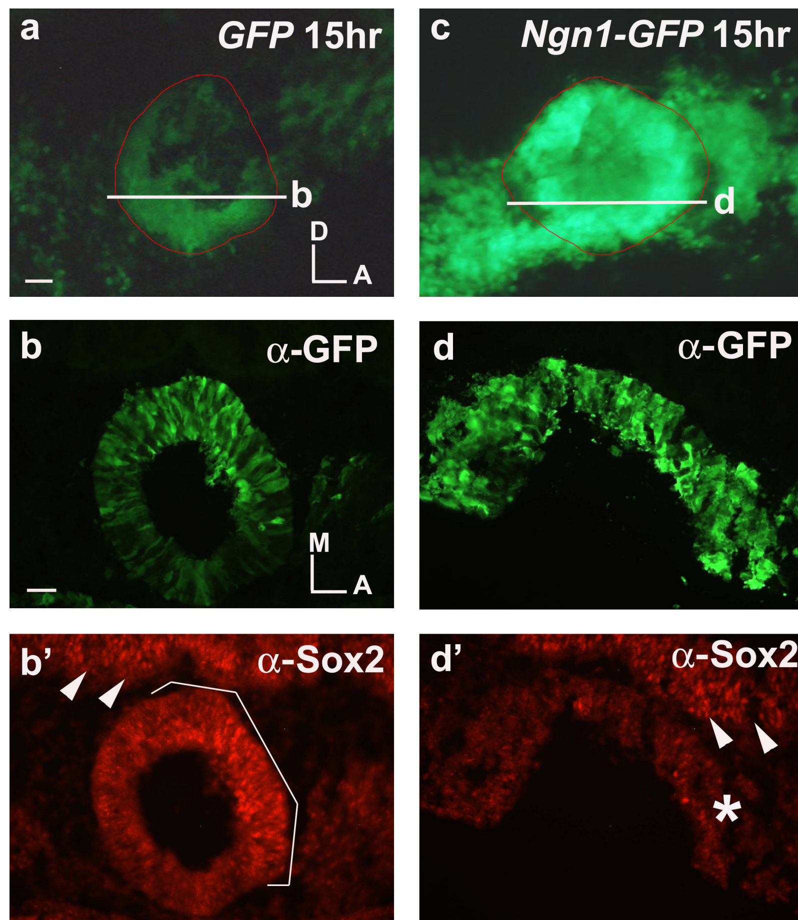


Figure 2.5. Over-expressing *Ngn1* leads to down-regulation of Sox2. Wholemounts (a, c) and sections (b, d) of *GFP* control (a-b') and *Ngn1-GFP* (c-d') treated ears. Sections of *GFP* control and *Ngn1-GFP* treated ears double-labeled with anti-Sox2 and anti-GFP antibodies 15 hrs after electroporation (b-b'; d-d').

GFP control ears show Sox2 immunostaining in the NSD (b' white brackets; n=4), in which some of the cells are GFP positive. (d) The GFP-positive region in *Ngn1-GFP* ears show down-regulated Sox2 immunostaining (d' asterisk; n=4/5), but staining in the neural tube is similar to controls (b', d'; white arrowheads). *Ngn1-GFP* electroporated ears often show a delay in otic cup closure (c, d, d'). The ears are outlined in red (a, c), and the levels of sectioning are indicated in white lines (a, c).

***NOP-1* enhancer of *Sox2* is inhibited by exogenous Ngn1**

The tissue-specific expression of *Sox2* is mediated by multiple regulatory elements within the Sox2 promoter, which includes the conserved *nasal-otic placode-specific enhancer 1* (*NOP-1*), which confers expression in the developing chicken inner ear (Uchikawa et al., 2003). To test if Ngn1 might inhibit *Sox2* transcription at the *NOP-1* site, *NOP-1* tagged with *EGFP* (*NOP1-GFP*), or *NOP-1* with a mutation in *E-box* (*NOP1-EboxMut-GFP*) were co-electroporated to the otic cup in the presence or absence of *Ngn1-DsRed* and samples were analyzed 15 hrs after electroporation and the total number of anti-GFP and anti-DsRed co-expressing cells per ear were quantified (Fig. 2.6). The GFP activity driven by the *NOP-1* enhancer activity is significantly reduced in the presence of *Ngn1-DsRed* compared to control (Fig. 2.6c). However, GFP activity driven by *NOP-1* with a mutated *E-box*, *NOP1-EboxMut-GFP*, is not significantly reduced (Fig. 2.6f). These findings suggest that Ngn1 could inhibit *Sox2* transcription by binding to *NOP-1*.

Figure 2.6

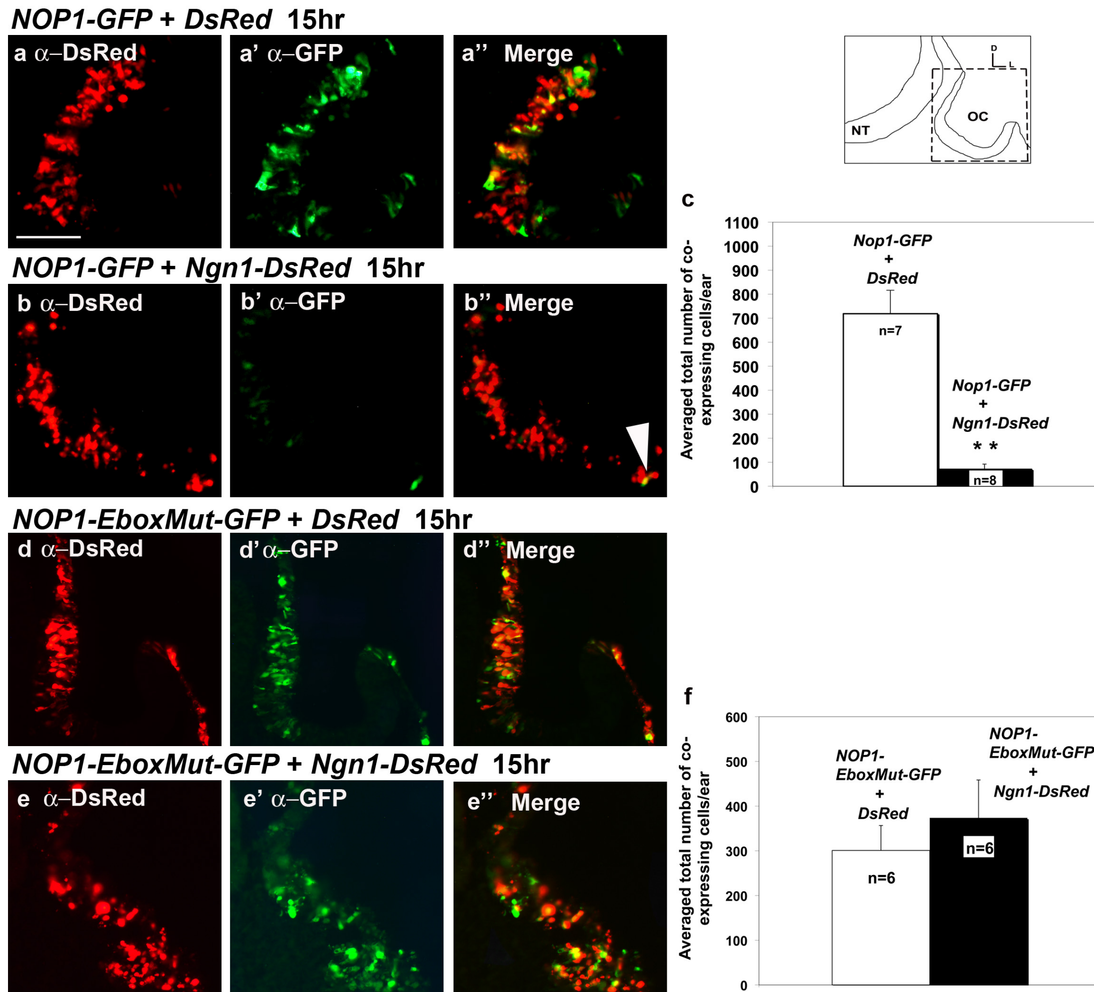


Figure 2.6. Ngn1 inhibits *NOP-1* activity. Ears co-electroporated with *NOP1-GFP* (a-b'') or *NOP1-EboxMut-GFP* (d-e'') with *DsRed* control (a-a''; d-d'') or *Ngn1-DsRed* (b-b''; e-e'') 15 hrs after electroporation. Ear sections were double-labeled with anti-GFP and anti-DsRed, and counterstained with DAPI (not shown). Images were merged in Adobe Photoshop (a'', b'', d'', e''). Total number of GFP and DsRed co-labeled cells were quantified (c, f). The activity of *NOP1-GFP* is significantly reduced in the presence of *Ngn1-DsRed* compared to controls ** p=0.00036; error bars represent s.e.m. (c). The activity of *NOP1-EboxMut-GFP* is not significantly

affected by *Ngn1-DsRed* compared to controls, p=0.49; error bars represent s.e.m. (f). The level of sections is indicated in the schematic. NT, neural tube; OC, otic cup.

Neurod1 is sufficient to promote neurogenesis and inhibits *NOP-1* activity

Neurod1, which is thought to be activated by Ngn1 (Ma et al., 1996), has been shown to be required for the formation of the CVG (Liu et al., 2000). To test if *Neurod1* is able to induce neurogenesis similar to *Ngn1*, *Neurod1-IRES-RFP* (*Neurod1-RFP*) or *IRES-RFP* (*RFP*) control were electroporated at the otic cup stage and harvested 48 hrs later (Fig. 2.7). *Neurod1-RFP* ears show double-labeled DsRed and TuJ1-positive cells delaminating from within and ectopically outside the NSD (Fig. 2.7e, e', f, f') compared to RFP control (Fig.2.7b-c"). Therefore, expression of *Neurod1* is also sufficient to mediate neurogenesis in the developing inner ear.

In addition, I tested if Neurod1 is also capable of inhibiting *NOP-1* activity. Similar *NOP1-GFP* or *NOP1-EboxMut-GFP* experiments were conducted with *Neurod1-RFP* (Fig. 2.7). The *NOP1-GFP* enhancer activity is significantly reduced in the presence of *Neurod1-RFP* compared to control (Fig. 2.7i), whereas the *NOP1-EboxMut-GFP* activity is not (Fig. 2.7l). These findings suggest that Neurod1 is capable of inhibiting *Sox2* transcription in a similar manner to Ngn1.

Figure 2.7

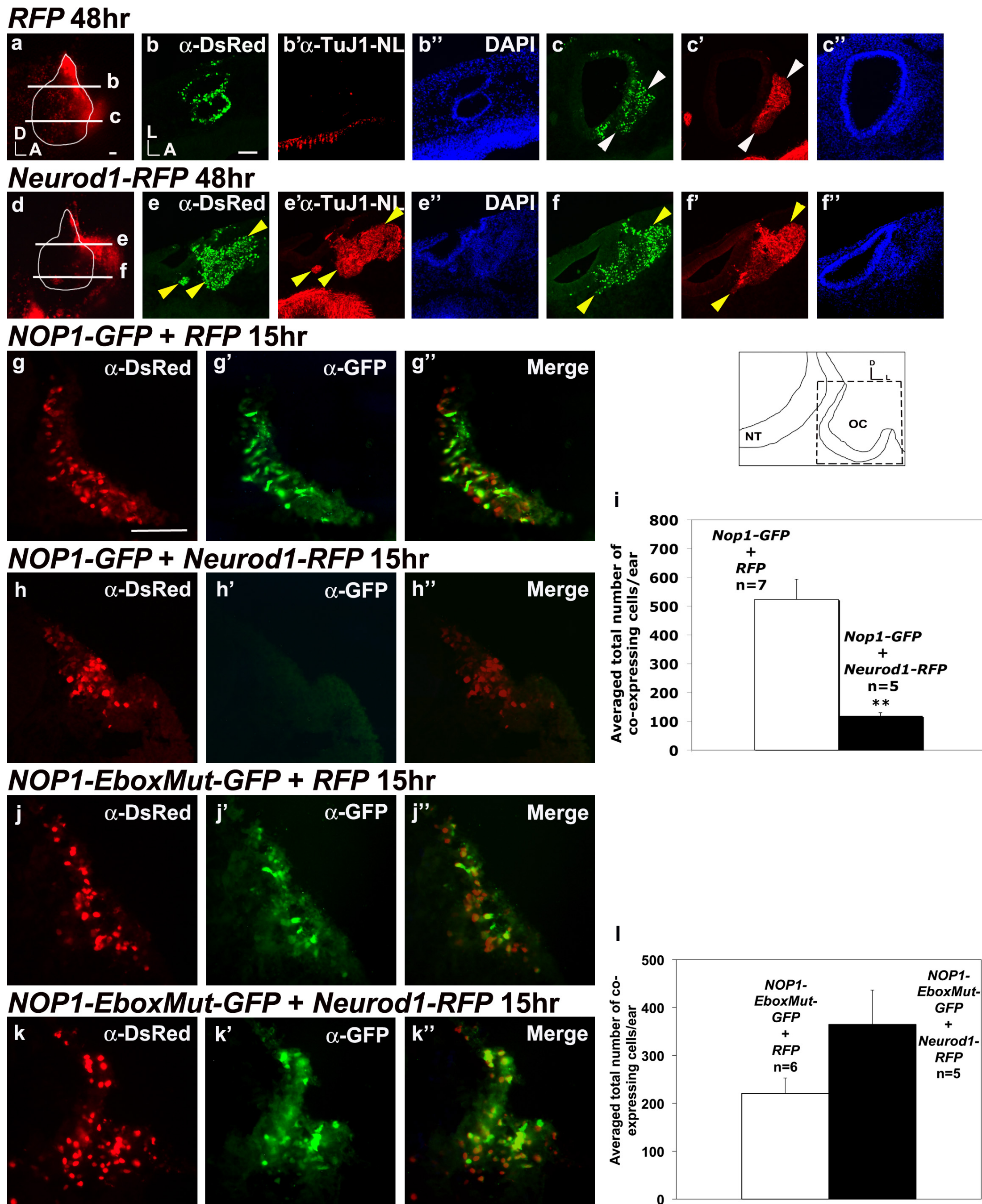


Figure 2.7. Neurod1 is sufficient to promote neurogenesis and inhibits *NOP-1* activity. Otocysts electroporated with *RFP* (a) or *Neurod1-RFP* (d) at 48 hrs after electroporation. Ears are outlined and levels of sections are indicated in white. Ear sections were double-labeled with anti-DsRed and anti-TuJ1-NL(conjugated), and

counterstained with DAPI (b-b'', c-c'', e-e'', f-f''). *Neurod1-RFP* treated ears show double-labeled ectopic DsRed and TuJ1-NL-positive neuroblast delamination (e, e'; yellow arrowheads) as well as increased delamination form the NSD (f, f'; yellow arrowheads) compared to control (c, c'; white arrowheads). Ears co-electroporated with *NOP1-GFP* (g-h'') or *NOP1-EboxMut-GFP* (j-k'') with *RFP* control (g-g''; j-j'') or *Neurod1-RFP* (h-h''; k-k'') 15 hrs after electroporation. Ear sections were double-labeled with anti-GFP and anti-DsRed, and counterstained with DAPI (not shown). Images were merged in Adobe Photoshop (g'', h'', j'', k''). Total number of GFP and DsRed co-expressing cells were quantified (i, l). The activity of *NOP1-GFP* is significantly reduced in the presence of *Neurod1-RFP* compared to controls ** p=0.0013; error bars represent s.e.m. (i). The activity of *NOP1-EboxMut-GFP* is not significantly affected by *Neurod1-RFP* compared to controls, p=0.12; error bars represent s.e.m. (l). The level of sections is indicated in the schematic. NT, neural tube; OC, otic cup.

The activity of *NOP-1* is inhibited within the endogenous *Ngn1*-positive domain

Next, I asked if the inhibition of *NOP-1* by Ngn1 or Neurod1 is biologically relevant and could be inhibited by the level of endogenous pro-neural bHLH factors. Therefore, I focused my analyses of *NOP1-GFP* or *NOP1-EboxMut-GFP* electroporated specimens only in the NSD, where *Ngn1 and Neurod1*-positive cells are normally expressed (Fig. 2.8). The total numbers of anti-GFP and anti-DsRed co-expressing cells in the NSD were scored for quantification (see Materials and Methods). The *NOP-1* enhancer activity is significantly reduced in the NSD

compared to the control *NOP-1* with a mutated *E-box* (Fig. 2.8e). These findings indicate that endogenous levels of pro-neural bHLH factors are capable of inhibiting *NOP-1* activity.

Figure 2.8

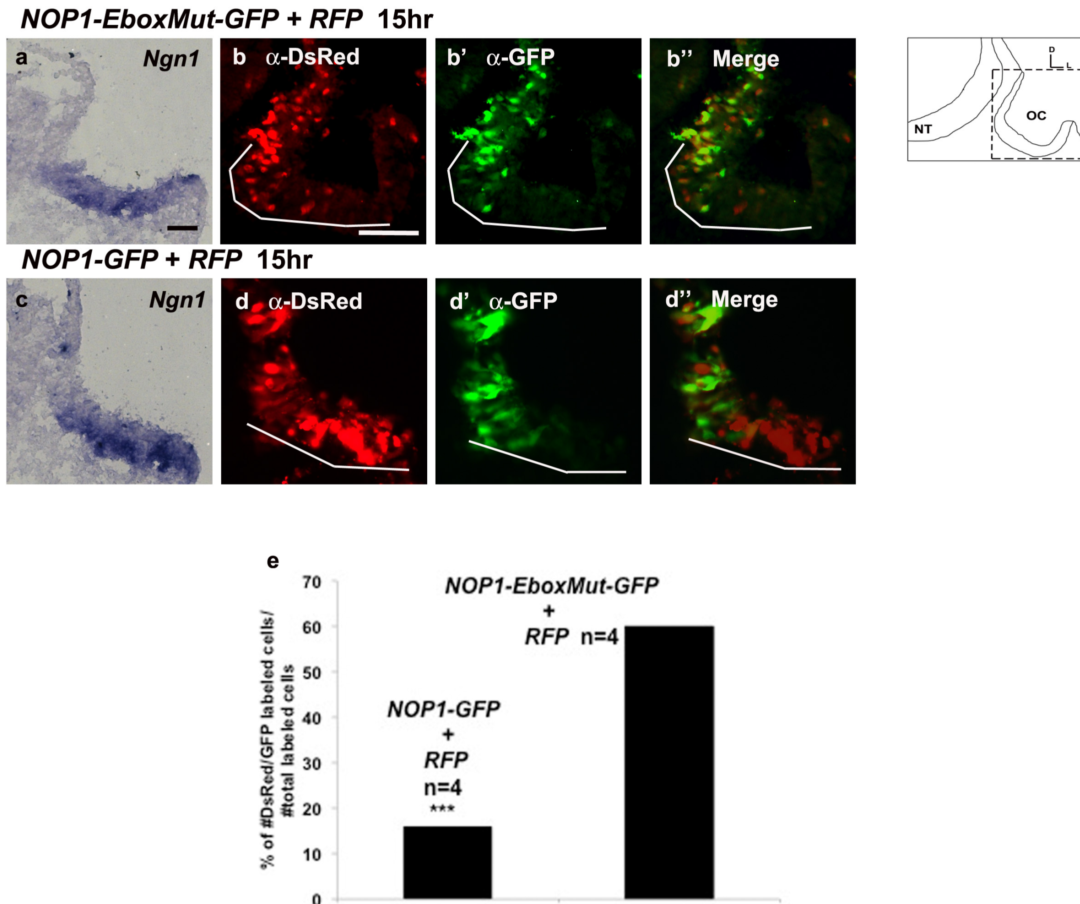


Figure 2.8. The activity of *NOP-1* is inhibited within the NSD. Ears co-electroporated with *NOP1-EboxMut-GFP* and *RFP* as control (a-b'') or *NOP1-GFP* and *RFP* (c-d'') 15 hrs after electroporation. Adjacent sections probed either for *Ngn1* transcript or double-labeled against anti-DsRed and anti-GFP (a, b and c, d) and counterstained with DAPI (not shown). Images were merged in Adobe Photoshop (b'', d''). Total number of GFP and DsRed co-expressing cells in the endogenous

Ngn1-postive area were quantified (e). The activity of *NOP1-GFP* is significantly reduced in the *Ngn1*-positive domain compared to the control *** $p<0.0001$; $\chi^2=591$ (e). The level of sections is indicated in the schematic. NT, neural tube; OC, otic cup.

Over-expression of *Sox3* has no effect on *Ngn1* expression or neurogenesis

Similar to *Sox2*, *Sox3* is also expressed in the NSD (Fig. 2.1). Sox3 has been shown to up-regulate *Sox2* in the developing chicken inner ear (Abello et al., 2010). To address whether Sox3 has a similar function in regulating neurogenesis as Sox2, similar experiments were conducted using a *Sox3-GFP* construct. Over-expression of *Sox3* does not induce *Ngn1* expression inside or outside of the NSD (Fig.2.9). It also has no apparent effect on *Neurod1* expression (not shown, n=6), or the size of the CVG (not shown, n=8). Therefore, despite the induction of *Sox2* by ectopic Sox3, Sox3 does not have a similar effect on neurogenesis as Sox2 in these experimental paradigms.

Figure 2.9

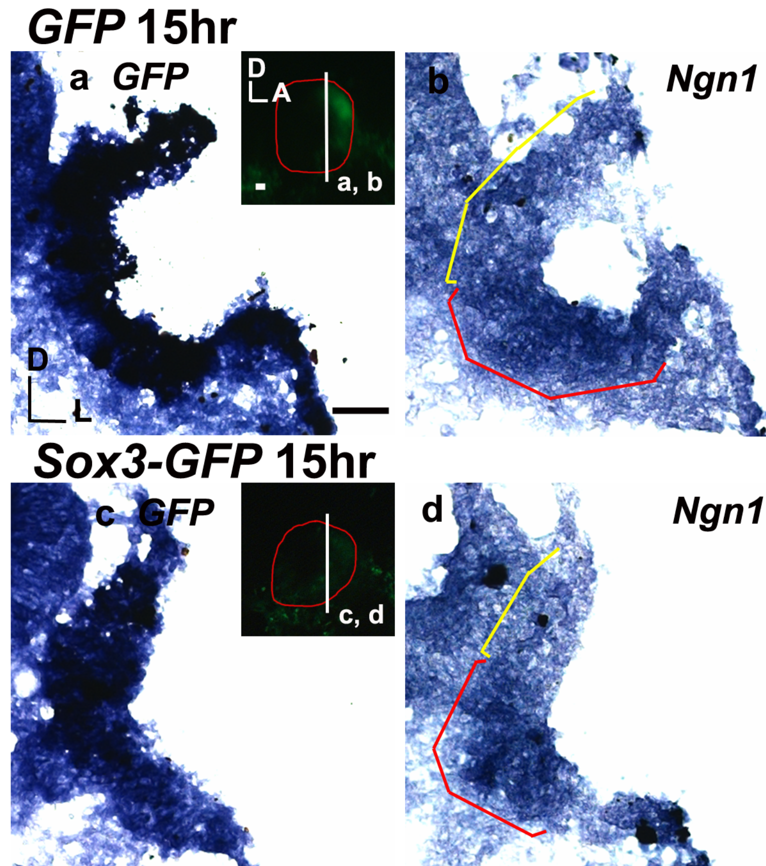


Figure 2.9 Over-expressing *Sox3* is insufficient to up-regulate *Ngn1*. Otic cups electroporated with *GFP* control or *Sox3-GFP* at 15 hr harvest (a, c; insets). The ears are outlined in red and the level of sectioning is indicated in white lines (a, c; insets). Adjacent sections probed for *GFP* and *Ngn1* transcripts (a-d). Similar *Ngn1* expression within the endogenous NSD (b, d; red brackets) in both control (b; n=3) and *Sox3*-treated ears (d; n=5). No *Ngn1* up-regulation outside the NSD is observed in *Sox3*-treated ears or controls (b, d; yellow brackets).

Sox2 appears to act as a repressor

It is well established that Sox proteins can function as a transcription activator or repressor (Kamachi et al., 2000; Lefebvre et al., 2007; Wilson and Koopman, 2002). Two previously published constructs used successfully to demonstrate Sox2 and Sox3 proteins functioning as activators in the neural tube were employed (see (Bylund et al., 2003)): an obligatory repressor version of Sox2, *EnR(Sox2)-GFP,* in which the *HMG* domain of *Sox2* is fused to the *Drosophila Engrailed* repressor domain and *IRES-GFP*, and an activator version of Sox2, *VP16(Sox2)-GFP*, in which the *HMG* domain of *Sox2* is fused to the activator domain of *Herpes simplex viral protein 16* and *IRES-GFP.* Over-expression of *EnR(Sox2)-GFP* shows that there is an approximately 32-50 % reduction in the size of the CVGs compared to controls (Fig.2.10), similar to ears over-expressing *Sox2*. In addition to the reduction in CVG sizes, section analyses indicate that there is an ectopic up-regulation of *Ngn1* dorsally outside the NSD and an up-regulation within the NSD in some specimens analyzed at different time points of harvests (shown at 20 hr harvest in Fig.2.11). This increase in *Ngn1* expression is very robust in specimens co-electroporated with *EnR(Sox2)-GFP* and *EnR(Sox3)-GFP* (not shown, n=4/5). Although *Sox3-GFP* alone (Fig.2.9) or *EnR(Sox3)-GFP* alone-treatments (not shown) have no obvious effect on *Ngn1* expression or the sizes of the CVG (not shown), the combined effects of EnR(Sox2)-GFP and EnR(Sox3)-GFP on *Ngn1* up-regulation suggest that these proteins function co-operatively with each other.

So far, my results suggest that Sox2 is functioning as a repressor in the inner ear, in contrast to its activator function in the neural tube. However, specimens with

over-expression of *VP16(Sox2)* also showed ectopic, dorsal up-regulation of *Ngn1* but for the most part no change in expression within the NSD (Fig.2.12). It is not clear why both EnR(Sox2) and VP16(Sox2) induce *Ngn1* expression ectopically outside the NSD (see Discussion). Nevertheless, my results are more consistent with Sox2 functioning as a repressor within the NSD because of the similarity in phenotypes obtained between specimens with over-expression of *Sox2* and *EnR(Sox2).* The lack of down-regulation of *Ngn1* in *VP16(Sox2)* specimens could be attributable to other factors regulating *Ngn1* expression in addition to Sox2.

Figure 2.10

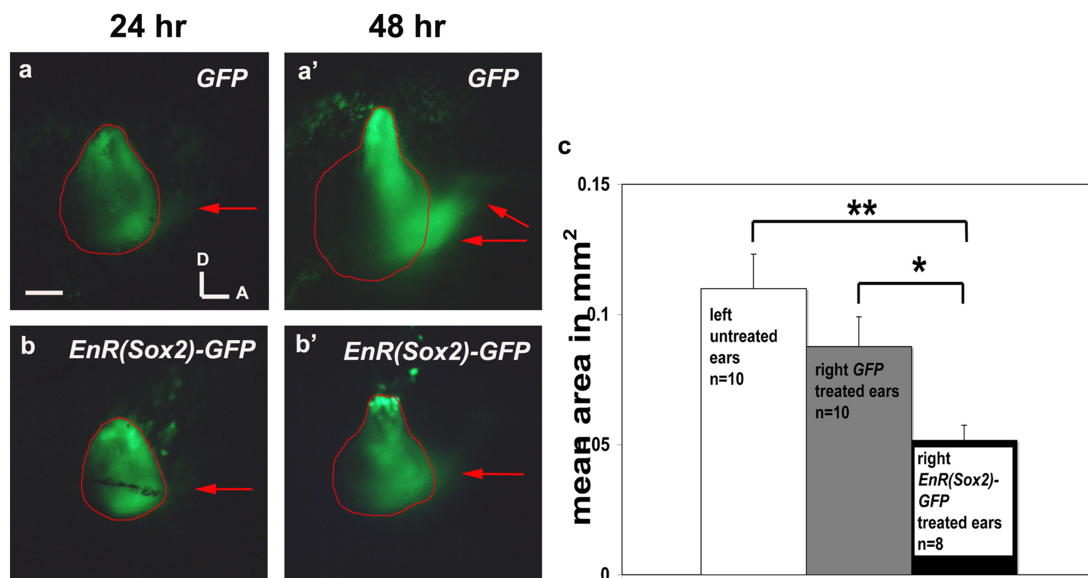


Figure 2.10. EnR(Sox2) causes a reduction in the size of the CVG. Otocysts electroporated with *GFP* (a, a') or *EnR*(*Sox2)-GFP* (b, b') at 24 hrs (a, b) and 48 hrs (a', b'). The outline of the otocyst is indicated in red. Red arrows point to the presumably delaminated neuroblasts. *GFP* control ears show more neuroblast delamination than *EnR*(*Sox2)-GFP* ears at both 24 and 48hrs. (c) Quantitative

analysis of CVG sizes. CVGs of *GFP* treated ears are not significantly different than non-electroporated ears, whereas CVGs of *EnR*(*Sox2)*-treated ears are approximately 32-50% smaller than controls. Student's *t*-test * p=0.015 ** p=0.0017; error bars represent s.e.m..

Figure 2.11

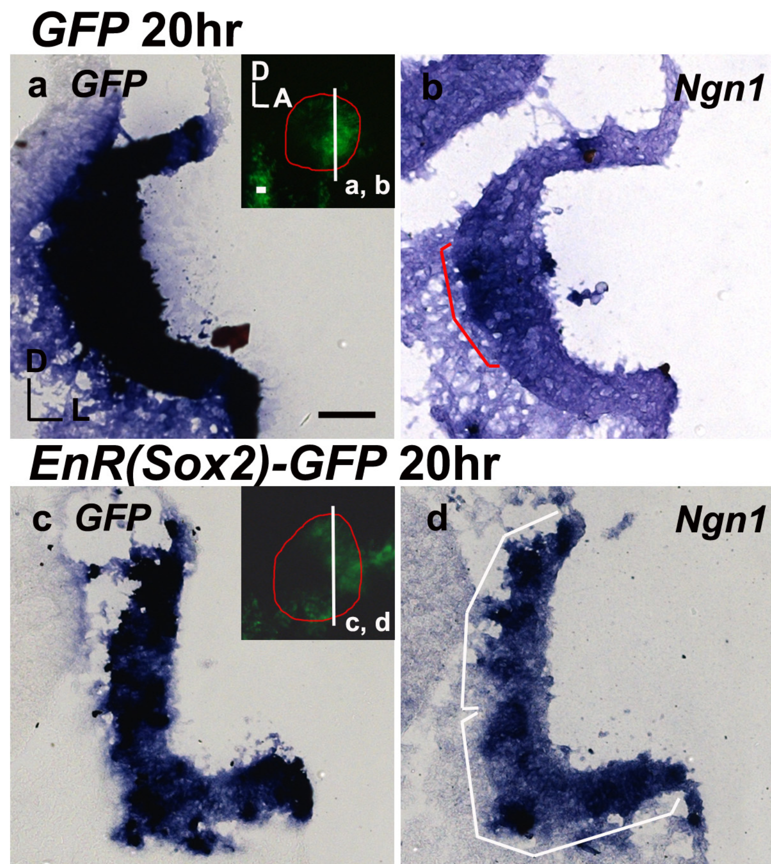


Figure 2.11 EnR(Sox2) causes *Ngn1* up-regulation within and outside the NSD. Otic cups over-expressed with *GFP* (a, b) or *EnR(Sox2)-GFP* (c, d) at 20 hr harvest. The ears are outlined in red and levels of sectioning are indicated in white lines (a, c; insets). Adjacent sections probed for *GFP* and *Ngn1* transcripts (a-d). *Ngn1* expression appears to be up-regulated within and outside the NSD in *EnR(Sox2)-*

treated ears (d, white brackets; n=3/4) compared to *GFP* controls (b, red bracket; n=2).

Figure 2.12

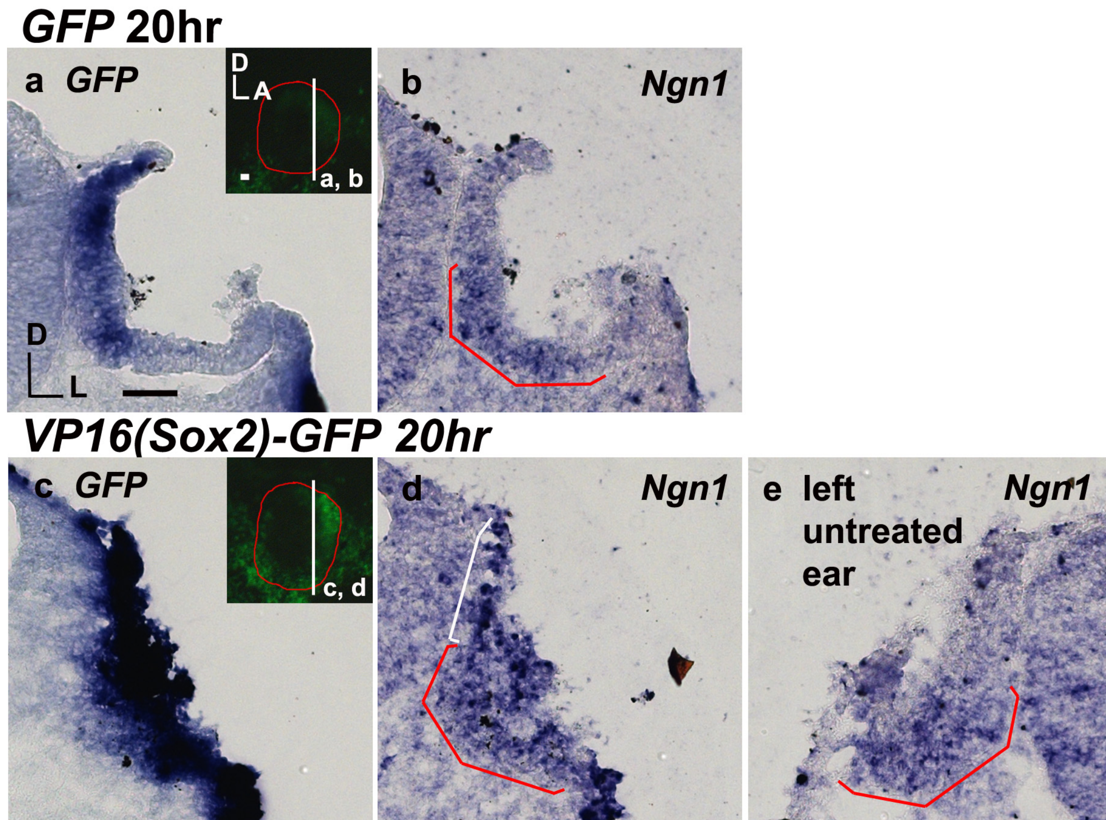


Figure 2.12 VP16(Sox2) causes only up-regulation of *Ngn1* outside the NSD. Otic cups over-expressed with *GFP* (a, b) or *VP16(Sox2)-GFP* (c, d) at 20 hr harvest. The ears are outlined in red and levels of sectioning are indicated in white lines (a, c; insets). Adjacent sections probed for *GFP* and *Ngn1* transcripts (a-d). (e) *Ngn1* expression in left untreated ear of the specimen in (c). *Ngn1* expression appears unchanged within the NSD (d, red bracket), but up-regulated outside the NSD (d, white bracket) in *VP16(Sox2)*-treated ears (n=4/5) compared to left untreated control (e, red bracket) and *GFP* controls (b, red bracket; n=2).

Figure 2.13

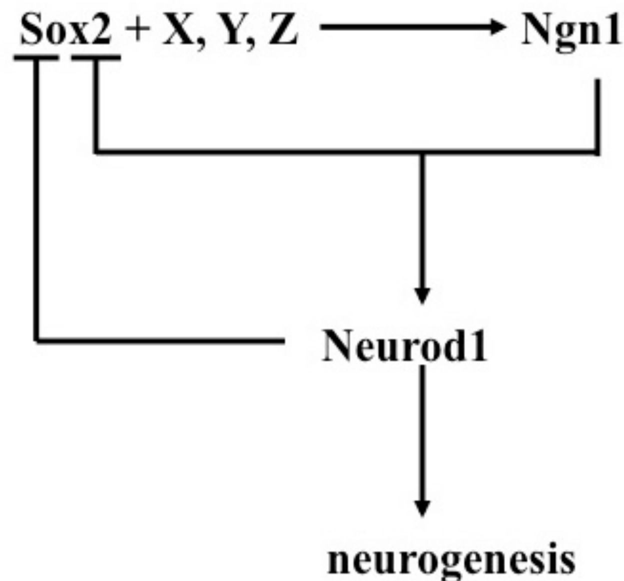


Figure 2.13 Summary Model

Sox2 activity together with other potential unknown factors (X, Y, and Z) up-regulate *Ngn1* within the NSD. Ngn1 initiates neurogenesis by inhibiting *Sox2* transcription and thus up-regulates *Neurod1,* which is an obligatory step for neurogenesis. Neurod1 further inhibits *Sox2* transcription.

Discussion

Sox2's role in neurogenesis

Vertebrate neurogenesis is regulated by a molecular cascade of pro-neural bHLH transcription factors including Ngn1 and Neurod1 (Kintner, 2002). The current dogma of neurogenesis in the vertebrate CNS is that neural stem cells proliferate and undergo self-renewal. Over time these stem cells develop into progenitors, which subsequently become specified and lineage restricted into forming neurons or glia cells (Kintner, 2002; Ming and Song, 2005). Ngn1 specifies

the neuronal fate lineage and Neurod1 mediates the differentiation of neuroblasts into neuronal subtypes (Bertrand et al., 2002; Chae et al., 2004; Ma et al., 1998; Ma et al., 1996). It is unclear how neural stem cells progress into becoming neuronal progenitors and then become lineage restricted into neurons and glia. However, growth factors and mitogens such as fibroblast growth factors (Fgf's) and Sonic hedgehog (Shh) are thought to play a role in the proliferation of the neural stem cells, and Bone morhpogenetic proteins (Bmp's) and Shh are thought to play a role in the progression from neural stem cell to neuronal progenitor pool (Kintner, 2002; Ming and Song, 2005). It is also unclear how pro-neural *bHLH* genes themselves are regulated (Kintner, 2002). Sox2's role is to maintain a self-renewing, proliferating neural stem cell pool (Pevny and Nicolis, 2010). Accumulating evidence suggests that *Sox2* expression is down-regulated in the neuronal lineage upon differentiation, but maintained in expression in the glial lineage, i.e. down-regulation of *Sox2* appears to be a pre-requisite for the occurrence of neurogenesis, while Sox2 function is required for the generation of glia (Bani-Yaghoub et al., 2006; Cavallaro et al., 2008). Compared to previous reports, the up-regulation of *Ngn1* by Sox2 in the developing inner ear is an unexpected finding. For instance, over-expression of *Sox2* in the chicken neural tube has led to no detectable changes in *Ngn1* or *Ngn2* expression (Bylund et al., 2003). However, a binding site for Sox2 has been identified within the *Ngn1* promoter using chromatin immunoprecipitation analyses (Okuda et al., 2010). This binding site has been proposed to be involved in the up-regulation of *Ngn1* during early embryogenesis in zebrafish, since knock-down of *Sox* group B1 genes leads to down-regulation of *Ngn1* expression in that

system (Okuda et al., 2010). Furthermore, in the developing *Xenopus* retina, a specific Frizzled trans-membrane receptor, Fz5, which is involved in Wnt/β-catenin signaling, has been demonstrated to be up-stream of *Sox2* (Van Raay et al., 2005). In this case, knock-down of Fz5 leads to loss of *Sox2* and expression of neuronal genes such as *Ngn1* (Van Raay et al., 2005). These results suggest that the Wnt/β-catenin signaling pathway regulates retinal neuronal fate specification indirectly by regulating *Sox2* expression (Van Raay et al., 2005). Here, the activation of *Ngn1* expression by Sox2 in the developing inner ear suggests that Sox2 is involved in initiating the molecular cascade of pro-neural *bHLH* genes required for neurogenesis. Both Ngn1 and Neurod1 engage in an inhibitory feed-back loop in inhibiting *Sox2,* which is required for the progression of neurogenesis. It will be interesting to see if Sox2 functions in a similar context in other placode-derived sensory organs such as the olfactory placode and the trigeminal ganglion (Streit, 2008).

Although previous studies have tried to address Sox2's role during neurogenesis and assigned its function as inhibitory to neurogenesis, the molecular mechanism has remained unclear (Bani-Yaghoub et al., 2006; Bylund et al., 2003). Contrary to this assigned role, recent studies suggested that *Sox2* is a pro-neural gene in the inner ear based on the positive effects of Sox2 on neuronal formation in mouse cochlear cultures, specifically in the Kolliker's organ (Puligilla et al., 2010). However, my results suggest that while Sox2 may be required for neurogenesis it needs to be inhibited in order for neurogenesis to proceed. Based on my results, Sox2 should not be able to induce neurons in the Kolliker's organ. It has been

demonstrated previously that cells in the Kolliker's organ are derived from the *Ngn1*-positive cells in the NSD. This lineage relationship may have biased the cells in the Kolliker's organ to have a pre-disposition toward the neuronal fate particularly in the presence of exogenous Sox2 activity (see Puligilla et al., 2010 and Raft et al., 2007).

Sox2 has to be down-regulated for neurogenesis to proceed

My results indicate that while Ngn1 is sufficient to induce the neurogenic fate, commitment to the neuronal fate is somehow blocked in cells over-expressing *Sox2,* despite their high levels of *Ngn1* expression. It is possible that the neuronal fate cannot progress in *Sox2* over-expressed cells because of the high exogenous *Sox2* levels. Ngn1 fails to repress *Sox2* in *Sox2*-over-expressing cells, in which *Sox2* is driven by an exogenous promoter (*β-actin* promoter) instead of its endogenous promoter.

Inhibition of *Sox2* transcription is an important molecular step before neurogenesis can proceed (Summary Model Fig. 2.13). Both Ngn1 and Neurod1 are capable of inhibiting *NOP-1* activity and thus presumably down-regulate *Sox2* transcription. The fact that *Sox2*-overexpressing cells show up-regulation of *Ngn1* but no up-regulation of *Neurod1* whereas *Ngn1*-overexpressing cells do show up-regulation of *Neurod1,* suggests that *Sox2* needs to be down-regulated before *Neurod1* expression can be induced. Neurod1 function is an obligatory step for the occurrence of neurogenesis. In the mouse hippocampus, the removal of Sox2 from the *Neurod1* promoter is required, before Wnt-signaling can mediate activation of

Neurod1 (Kuwabara et al., 2009). It is possible that Sox2 negatively regulates *Neurod1* expression through a similar mechanism in the inner ear, and this negative regulation needs to be alleviated by Ngn1 before neurogenesis can proceed. This may be the reason for the requirement of down-regulation of *Sox2*. Once Neurod1 is induced, this bHLH protein could further inhibit *Sox2* transcription via *NOP-1*.

Sox2, Ngn1, and Neurod1 form an intrinsic regulatory network within the NSD

My results suggest that Sox2, Ngn1, and Neurod1 form an intrinsic regulatory network in regulating the NSD. Previous studies have demonstrated that the NSD is regulated through extrinsic signaling pathways (Bok et al., 2011; Riccomagno et al., 2005). Based on my results obtained here it appears that over-expressing *Ngn1* or *Neurod1* can over-ride extrinsic Wnt, Bmp, and retinoic acid activity to promote neurogenesis at these early stages of development. Normally, rostro-caudal retinoic acid signaling directly regulates the expression of the T-box gene, *Tbx1,* and the anterior/posterior axial specification of the inner ear in a dose-dependent manner (Bok et al., 2011). *Tbx1* expression is thought to restrict the *Ngn1*-positive neurogenic domain to the anterior otic region (Raft et al., 2004). Signals from the hindbrain pattern the dorsal/ventral axis of the otocyst (Bok et al., 2007). Secreted Wnt molecules from the dorsal hindbrain induce dorsal-specific otic genes, whereas Shh emanating from the ventral hindbrain confers the otocyst its ventral identity (Bok et al., 2007). It has been proposed that Wnt together with Bmp signaling from the dorsal hindbrain and ectoderm antagonize ventralizing signals such as Shh (Riccomagno et al., 2005). Forced activation of Wnt/β-catenin signaling causes

ventral expansion of dorsal otic genes *Dlx5/6* and *Gbx2*, and removal of the dorsal hindbrain leads to dorsal expansion of Shh-dependent ventral otic genes *Pax2* and *Ngn1* (Riccomagno et al., 2005). My findings of Ngn1 and Neurod1's ability to induce neurogenesis from all around the ear epithelium suggest that these proteins can over-ride the extrinsic inhibitory signaling for neurogenesis outside the NSD, and that the entire inner ear is initially competent to be neurogenic.

Sox3 does not play a direct role in regulating neurogenesis

A previous publication has demonstrated that *Sox3* over-expression leads to *Sox2* up-regulation in the developing chicken inner ear (Abello et al., 2010). Therefore, *Sox3* is postulated to be up-stream of *Sox2* (Abello et al., 2010). Under my experimental conditions, loss of Sox3 function approaches using the *EnR(Sox3)* construct has no apparent effect on neurogenesis as analyzed for *Ngn1* expression and quantification of CVG sizes. In the Abello et al 2010 study, over-expressing *Sox3* causes *Delta1* to be up-regulated, but there is no ectopic neurogenesis. In these experiments, *Delta1* is postulated to be up-regulated by Sox3, indirectly through an up-regulation of *Sox2*. This hypothesis is consistent with my findings in chapter 3 where I show over-expression of *Sox2* leads to *Delta1* up-regulation. Similar to the Abello et al study, I found that over-expressing *Sox3* has no apparent change in *Ngn1* or *Neurod1* expression, or the size of the CVG. Taken together, the previous publication and my results, I propose that Sox3, though expressed in the NSD, its role in neurogenesis may be indirect through regulating *Sox2*. Neurogenesis is an

event further down-stream from activation of *Sox3* but closely related to activation of *Sox2*.

Sox2 appears to function as a repressor

The similarity in the phenotypes elicited by the *EnR(Sox2)-GFP* construct compared to that of *Sox2-GFP* suggests that Sox2 is functioning as a repressor to promote neurogenesis within the inner ear. If Sox2 were acting as an activator, then over-expressing *VP16(Sox2)-GFP* should have yielded the same phenotype for *Ngn1* expression similar to the Sox2 gain-of-function results. This was not the case. In addition, no apparent change in *Ngn1* expression within the NSD was obtained with the Sox2 activator construct *VP16(Sox2)-GFP*. This suggests that another factor(s) is involved in regulating the NSD. Alternatively, it is possible that there is not enough cellular resolution to detect a possible change in *Ngn1* expression using the *in situ* hybridization method as this technique is not quantitative.

The two constructs, *EnR* and *VP16*, are designed to operate with different mechanisms. Herpes simplex viral protein 16 (VP16) recruits the transcriptional complex including RNA polymerase II and other factors for transcription of the target gene, whereas the Engrailed repressor binds to the target gene promoter and thereby blocking the transcription machinery from transcribing the target gene. However, in this study both *VP16* activator or *EnR* repressor are fused to the *HMG* domain of *Sox2*. Therefore, the phenotype obtained with either construct should be relevant to the function of Sox2. The phenotype obtained with *EnR(Sox2)* is closer to the phenotype obtained to Sox2 gain-of-function than with the phenotype

obtained with the *VP16(Sox2)* construct, indicating that Sox2 normally acts as a repressor and not an activator with respect toward *Ngn1*.

Both *VP16(Sox2)* and *EnR(Sox2)* specimens yielded up-regulation of *Ngn1* expression dorsally, outside the NSD. The reason for this phenotype is unclear, especially since neither *Sox2* nor *Sox3* appear to be normally expressed outside the NSD (Uchikawa et al., 1999; Uchikawa et al., 2011). The region dorsal to the NSD may be regulated by another intrinsic factor(s) and/or Sox protein(s). The *EnR(Sox2)* and *VP16(Sox2)* constructs, though appear to be specific for Sox2 in the neural tube (see Bylund et al., 2003), may be cross-reacting with binding sites of other Sox proteins. Notably, *Sox21,* which functions as a transcriptional repressor for *Sox* group B1 genes (*Sox1-3*) in the vertebrate CNS, is expressed broadly in the otic cup beyond the NSD (Sandberg et al., 2005; Uchikawa et al., 1999; Uchikawa et al., 2011). The function of Sox21, or whether it acts as a repressor or activator in the inner ear is not known. One possibility is that Sox21 normally represses *Sox2* expression dorsally and restricts the NSD to the ventral region. As a result, there could be a low level of *Sox2* or even *Sox3*, below the level of detection by *in situ* hybridization, in the dorsal otic region. The ectopic up-regulation of *Ngn1* in Sox2 gain-of-function specimens may be due to Sox2 over-riding Sox21's inhibitory effects. Under this scenario, EnR(Sox2) would be mimicking Sox2's function both dorsal and ventrally to up-regulate *Ngn1*. This up-regulation could be somewhat dampened by a weak cross-reactivity of EnR(Sox2) to Sox21 binding sites, which could have resulted in inhibition of Sox2 activities. Nevertheless, a net increase of Sox2 activities as indicated by the up-regulation of *Ngn1* in *EnR(Sox2)* specimens

was obtained. Furthermore, the cross-reactivity of VP16(Sox2) to Sox21 binding sites in the dorsal otic region could alleviate the inhibitory action of Sox21 on *Sox2* and cause an up-regulation of *Sox2*, thereby up-regulating *Ngn1* as well.

Sox proteins require the interaction of a binding co-partner that interacts with the HMG domain in a cell-context dependent manner (Kamachi et al., 2000; Wilson and Koopman, 2002). The binding partner(s) for Sox2 in the inner ear is largely unknown. Although, *Eya1*, which encodes a transcription co-activator has been proposed to be a binding partner of Sox2 in the formation of hair cells (Zou et al., 2008). It is possible that Sox2 selectively partners with a co-activator or co-repressor in a cell-context dependent manner within the inner ear, and the two constructs EnR(Sox2) and VP16(Sox2) could be behaving differently in ventral versus dorsal otic regions. Based on my Sox2 gain and loss-of-function approaches, my working hypothesis is that Sox2 normally acts as a repressor in the NSD and its activity leads to inhibition of an inhibitor of *Ngn1*, thereby leading to the induction of neurogenesis by up-regulation of *Ngn1*.

Sox2's role in the sensory fate

Sox2 is expressed in the NSD, but its expression is down-regulated in delaminating neuroblasts. The expression of *Sox2* persists in the sensory lineage until hair cells start to differentiate (Dabdoub et al., 2008; Neves et al., 2007). In humans, mutations in *SOX2* lead to senorineural hearing loss, and mice without *Sox2* are deaf (Hagstrom et al., 2005; Kiernan et al., 2005). In wildtype mice, the expression of Sox2 is down-regulated in nascent hair cells, which express the bHLH

transcription factor Atoh1 (Dabdoub et al., 2008). Forced expression of *Atoh1* in a P-19 cell line leads to down-regulation of endogenous *Sox2* expression, suggesting that Atoh1 counteracts Sox2 activity, whereas over-expression of *Sox2* in mouse cochlear explants leads to loss of hair cell formation and up-regulation of a pro-sensory gene, *Prox1* (Dabdoub et al., 2008). These findings are in agreement with the notion that there is a requirement for Sox2 to be down-regulated for hair cell differentiation similar to the earlier requirement for Sox2 to be down-regulated for neuronal differentiation as demonstrated here. Forced expression of *Sox2* in the NSD leads to a decrease in neurogenesis. It will be interesting to determine the consequence of these heightened Sox2 levels on the sensory lineage. My prediction is that forced *Sox2* expression will lead to an expansion or ectopic formation of sensory domains and possibly an increase in hair cell formation. Alternatively, if *Sox2* levels remain high in the sensory domains, it could inhibit subsequent hair cell formation. In chapter 3 of this dissertation, I demonstrate the potential interaction of Sox2 and the Notch signaling pathway in mediating neuronal and sensory cell fate choices, and my results indicate that Sox2 plays a non-cell-autonomous role in regulating neurogenesis and specifying the sensory fate, in addition to its cell-autonomous role in neurogenesis described here.

Conclusion

I have found an important molecular mechanism of regulation of the NSD by Sox2. I have demonstrated that Sox2 and Ngn1 play pivotal roles in regulating neurogenesis, and that Sox2, Ngn1, and Neurod1 form a close, intricate regulatory

network within the NSD. A tight temporal regulation of the levels of Sox2 is critical for neurogenesis to proceed normally. While Sox2 is capable of inducing *Ngn1* in the developing inner ear, it is unlikely to be the only factor involved in this process. The proposed repressor role of Sox2 in inducing *Ngn1* also requires further investigation. Nevertheless, it will be interesting to investigate whether Sox2 utilizes similar molecular mechanisms in the formation of other cranial placodes and sensory tissues.

Chapter 3: Sox2 and the Notch signaling pathway interact to regulate neuronal and sensory cell fate choices in the developing inner ear

Introduction

Notch signaling regulates neurogenesis via lateral inhibition

Notch is a trans-membrane receptor and its known ligands in chicken are Delta1, Serrate1 (Ser1), and Serrate2 (Ser2) (equivalent to Jagged1 and Jagged2 in mouse, respectively). There are four known vertebrate Notch receptors and so far only *Notch1* has been shown to be expressed in the inner ear (Lewis et al., 1999). In vertebrate inner ears Notch signaling mediates cell fate choices between neuronal and sensory cells during neurogenesis and between sensory hair cells and supporting cells within the sensory lineage. The mechanisms for these cell fate choices are similar to mechanosensory bristles formation in *Drosophila* (Adam et al., 1998). *Notch1*, *Ser1,* and *Delta1* are expressed in the NSD of the developing chicken inner ear (Adam et al., 1998; Cole et al., 2000). The expression domain of *Notch1* is broad in the ear rudiment and encompasses more than the presumed NSD (Adam et al., 1998; Shailam et al., 1999). *Ser1* is involved in formation of the pro-sensory patch in the chicken inner ear, and Delta1-Notch signaling mediates cell fate choices between neuronal and sensory cells through lateral inhibition (Daudet et al., 2007). Delta1 is expressed in cells destined to become neuronal and it induces a sensory fate in neighboring cells via the Notch receptor (Bertrand et al., 2002; Daudet et al., 2007). Ligand-binding to the trans-membrane Notch receptor leads to proteolytic

cleavage of the intracellular domain of Notch by the enzyme γ-secretase. The intracellular domain (Notch-ICD) then translocates into the nucleus to activate *Hes* and *Hey* genes, which are negative regulators of pro-neural *bHLH* genes such as *Ngn1* (Bertrand et al., 2002; Cau et al., 2000). When Notch signaling is blocked with a γ-secretase inhibitor, DAPT, expression of *Delta1* and neurogenesis initially increase because lateral inhibition is alleviated, and *Hes5.1* expression (chicken homolog of mammalian *Hes5*) is down-regulated presumably consequently in adjacent cells (Daudet et al., 2007). The similar lateral inhibition process is thought to mediate hair cell and supporting cell fates within the sensory patch via ligands such as Delta1, Jag1, and Jag2 (Brooker et al., 2006; Hartman et al., 2010; Kiernan et al., 2001; Kiernan et al., 2005; Lanford et al., 1999; Pan et al., 2010; Tsai et al., 2001; Zhang et al., 2000).

Known interactions between *Sox2* and the Notch signaling pathway

Notch signaling has been proposed to act up-stream as well as down-stream of *Sox2*. Jag1/Ser1 mediated Notch signaling has been proposed to induce *Sox2* expression in mouse cochlear explant cultures and chicken otic cup (Dabdoub et al., 2008; Daudet et al., 2007). Consistently, *Jag1* conditional knockout mouse mutants show down-regulated *Sox2* expression, whereas in *lcc* mutants, which has no *Sox2* expression in the inner ear, *Jag1* expression is not affected (Dabdoub et al., 2008; Pan et al., 2010). These results suggest that the Notch signaling pathway is up-stream of *Sox2*. Nevertheless, studies in the mouse retina suggest that Notch could also be down-stream of *Sox2*, since a direct binding site for Sox2 within the *Notch1*

promoter was found *in vitro*, even though it is not clear whether this binding is biologically relevant (Taranova et al., 2006). In mouse neocortical cultures, over-expression of *Sox2* leads to up-regulation of *Notch1* and *Hes5* transcripts, and to decreased neurogenesis (Bani-Yaghoub et al., 2006). Additionally, over-expressed *Notch-ICD* mouse mutants show ectopic sensory patches with the up-regulation of both *Jag1* and *Sox2* (Hartman et al., 2010; Pan et al., 2010). More recent studies in the chicken inner ear suggest that Jag1 cannot induce *Sox2*, but is required to maintain its expression (Neves et al., 2011). Taken together, these studies suggest that Sox2 and Notch signaling are interacting closely to mediate cell fate choices.

In chapter 2 of this dissertation, I have demonstrated that Sox2 plays a cell-autonomous role in regulating neurogenesis. In this chapter, I address the potential interactions of Sox2 and the Notch signaling pathway in mediating neuronal and sensory cell fate choices. I show that over-expressing *Ngn1* leads to a subsequent expansion of the Sox2 domain after *Ngn1*-postive cells have left the ear epithelium. Over-expressing the intracellular domain of *Notch* (*NICD*) leads to an expansion of the *Sox2* domain. Over-expressing *Ngn1* is sufficient to up-regulate *Delta1*, and over-expressing *Sox2* leads to up-regulation of *Delta1* and *Hes5.1*. These findings suggest that Notch signaling is up-stream of *Sox2*, and that Sox2 plays a cell-autonomous and non-cell-autonomous role in regulating the NSD.

Materials and Methods

Eggs, *in ovo* Electroporation, and Expression Constructs

Fertilized chicken eggs (B&E York Springs, PA) were incubated at 37° C and staged according to Hamburger and Hamilton (Hamburger and Hamilton, 1992). Full-length cDNA of mouse *Ngn1*were subcloned into the *pMES-IRES-GFP* expression vector (*GFP*), in which various cDNA is driven by a chicken β-actin promoter (*Ngn1-GFP*). The Notch intracellular domain *NICD* was subcloned into the *pCAB-IRES-GFP* vector (*NICD-GFP*). The *internal ribosomal entry site* (*IRES*) mediates bicistronic expression of the Green Fluorescent protein. The plasmids were delivered to the right otic cup between 10-17 somite stages (E1.5) by electroporation. This was conducted by filling the right otic cup with plasmids at a concentration of 3-4 μg/μl in 0.1% Fast Green. Then, a negative platinum electrode was placed above the right otic cup and the positive electrode inserted underneath the embryo at the location of the left otic cup. Two pulses at 7 volts with 100 msec duration and spacing were applied using a CUY21 electroporator. After electroporation, the eggs were sealed and returned to the incubator and harvested between 20 to 48 hrs.

In situ hybridization

Tissue-section *in situ* hybridization was carried out as previously described (Raft et al., 2007; Wu and Oh, 1996). Chicken Dig-labeled anti-sense RNA probes were generated for *GFP, Delta1, Hes5.1, Notch1,* and *Sox2.*

Immunohistochemistry

Cryo-sections were prepared as described above for *in situ* hybridization. The primary antibodies used were rabbit polyclonal anti-Sox2 (1:4000 Chemicon)**,** and goat polyclonal anti-GFP-FITC-conjugated (1:400 GeneTex). The secondary antibody was goat anti-rabbit Alexa Fluor 568 (1:250 Invitrogen). Antibody labeling was performed according to standard protocol (Raft et al., 2007), except the sections for labeling with anti-Sox2 were first subjected to citrate boiling for 5 min for antigen retrieval prior to immunostaining.

Results

Over-expressing *Ngn1* leads to a subsequent expansion of the Sox2 domain

In chapter 2, I have demonstrated the initial down-regulation of Sox2 in *Ngn1* treated ears within 15 hrs of electroporation (see Fig.2.4). Despite this down-regulation, the Sox2-positive domain was expanded by 45 hrs (Fig.3.1). Double-labeling with anti-Sox2 and anti-GFP antibodies indicate that while Sox2 is expressed in the NSD of *GFP* control ears (Fig.3.1 c'), the Sox2-positive domain in *Ngn1-GFP* ears is expanded anterior-dorsally beyond the NSD (Fig.3.1 e'). These Sox2-positive cells are GFP-negative, and most GFP-positive cells are outside of the otic epithelium where GFP-positive neuroblasts have delaminated from an earlier time (Fig.3.1 f, red asterisk). Furthermore, in control as well as the *Ngn1-GFP* treated ear, the delaminated neuroblasts show down-regulated Sox2 expression (Fig.3.1 white asterisks). This is consistent with findings in the central nervous system (CNS) (Bani-Yaghoub et al., 2006; Bylund et al., 2003), as well as with my

observation in the inner ear (see Fig.2.1) that *Sox2* expression is down-regulated in differentiating neuroblasts. The finding that there is a secondary up-regulation of Sox2 expression in the otic epithelium after neuroblast delamination suggests that over-expressing *Ngn1* has a non-cell-autonomous effect on Sox2 expression, which could be mediated by the Notch signaling pathway.

Figure 3.1

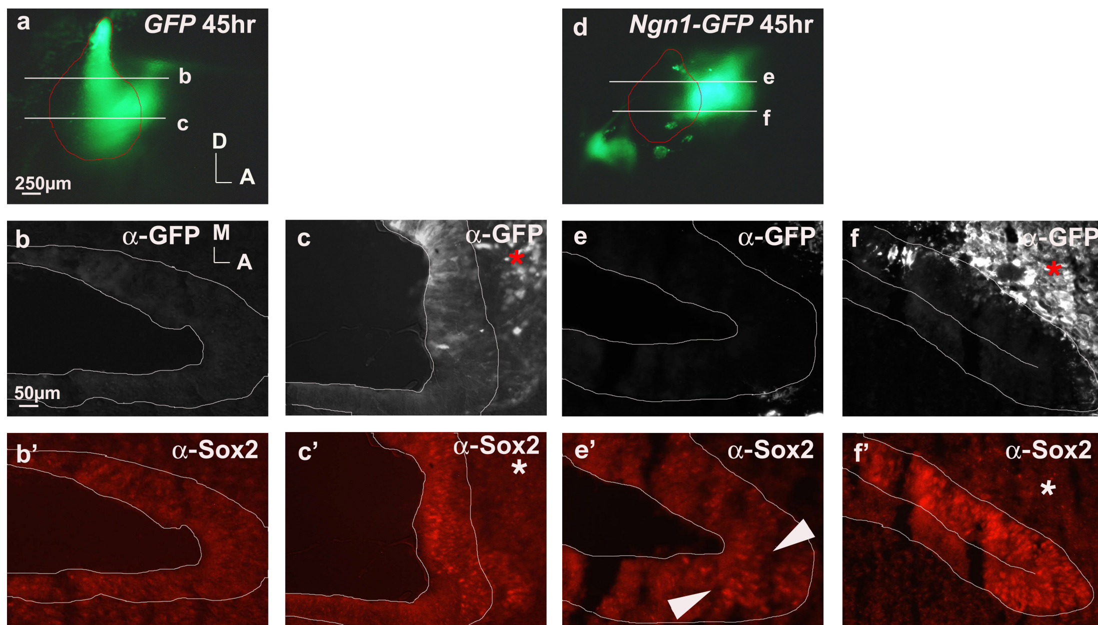


Figure 3.1. Over-expressing *Ngn1* leads to an expansion of the Sox2 domain. Sections of *GFP* control and *Ngn1-GFP* treated ears double-labeled with anti-Sox2 and anti-GFP antibodies 45 hrs after electroporation. Sox2 expression in the *GFP* control ear is in the NSD (c') and not in regions outside the NSD (b') or the delaminated neuroblasts (c', asterisk; n=3). GFP-positive cells can be found within the NSD domain and the CVG (c, asterisk). *Ngn1-GFP*-treated specimens show expanded Sox2 staining (e' between white arrowheads) dorsally beyond the NSD (f' n=4/6). A majority of the Sox2-positive regions (f') in *Ngn1-GFP* ears are GFP

negative (f), whereas large numbers of GFP-positive cells are in the ganglion, which are Sox2 negative (f' asterisk). Levels of sections are indicated in the wholemounts (a, d; white lines). The outline of the otocyst is indicated in red (a, d). Ear epithelia are outlined in white (b-c', e-f').

Over-expressing the intracellular domain of Notch (*NICD*) leads to up-regulation of *Sox2*

To investigate whether Notch signaling could be involved in the secondary up-regulation of Sox2 in *Ngn1* over-expressed specimens, I first over-expressed *NICD-IRES-GFP* at otic cup stages by electroporating and analyzed *Sox2* expression at 20 hrs later. Normally, *Notch1* is expressed broadly in the inner ear dorsally and ventrally (data not shown, see (Adam et al., 1998; Lewis et al., 1999; and Shailam et al., 1999)). In the *NICD*-treated ear, where Notch signaling is constitutively active, the *Sox2* expression domain is expanded dorsally in addition to its normal expression in the NSD compared to control (Fig.3.2). These results show that constitutively activated Notch signaling is sufficient to up-regulate *Sox2*.

Figure 3.2

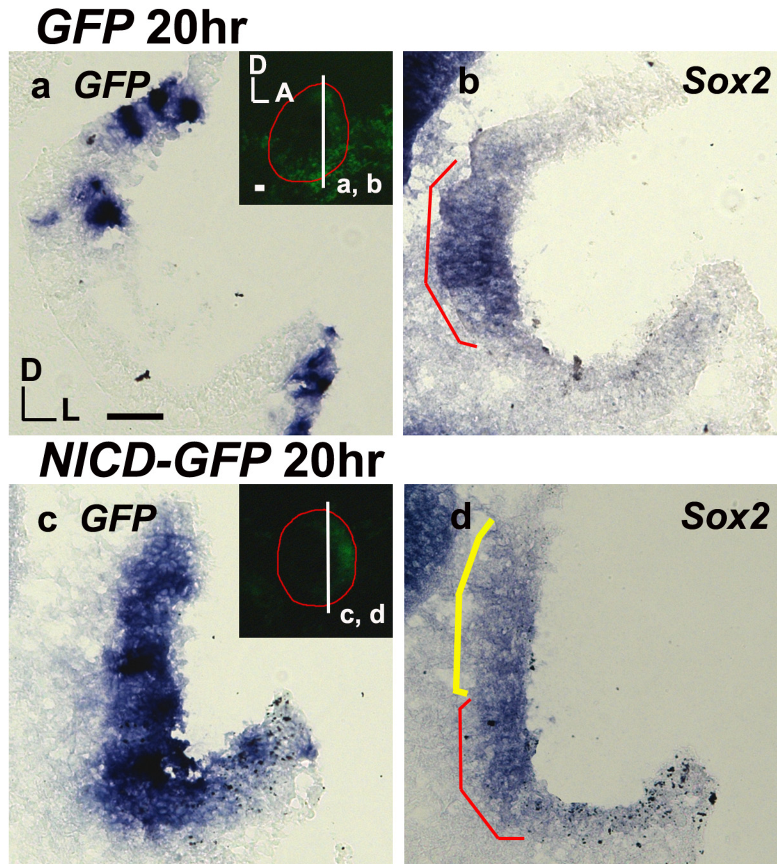


Figure 3.2 Over-expressing *NICD* is sufficient to up-regulate *Sox2*. Otic cups electroporated with *GFP* control or *Sox2-GFP* at 20 hr harvest (a, c; inset). Ears are outlined in red and the level of sectioning is indicated with white lines (a, c; insets). Adjacent sections probed for *GFP* and *Sox2* transcripts (a-d). *Sox2* expression is restricted to the NSD of controls (b red bracket, n=2), whereas its expression in *NICD*-treated ears is within (d, red bracket) as well as beyond (d, yellow bracket; n=2/4) the NSD.

Over-expressing *Ngn1* leads to ectopic *Delta1* up-regulation

Taken together the above results suggest that the secondary, non-cell-autonomous up-regulation of Sox2 in *Ngn1* over-expressed specimens could be mediated through the Notch signaling pathway. If so, what might be the Notch ligand(s) responsible for activating the Notch receptors? Delta1 is a likely candidate since *Delta1* is expressed in neuroblasts within the NSD (Adam et al., 1998), and this ligand has been proposed to mediate lateral inhibition of the neuronal fate (Daudet et al., 2007). Additionally, forced expression of *Ngn1* in *Xenopus* leads to ectopic up-regulation of *Delta1* (Ma et al., 1996). To analyze if a similar relationship occurs in the inner ear, *Ngn1*-treated ears were probed for *Delta1* transcripts 20 hrs after electroporation (Fig. 3.3). Normally, *Delta1* expression is restricted to the NSD (Fig.3.3b-e), whereas in the *Ngn1*-treated ear, *Delta1* expression is ectopically expressed outside of the NSD (Fig.3.3g-j). These results show that Ngn1 is sufficient to up-regulate *Delta1* expression in my experimental conditions and suggests that Delta1 is the main ligand for activating Notch signaling in neighboring cells, which leads to up-regulation of Sox2 expression.

Figure 3.3

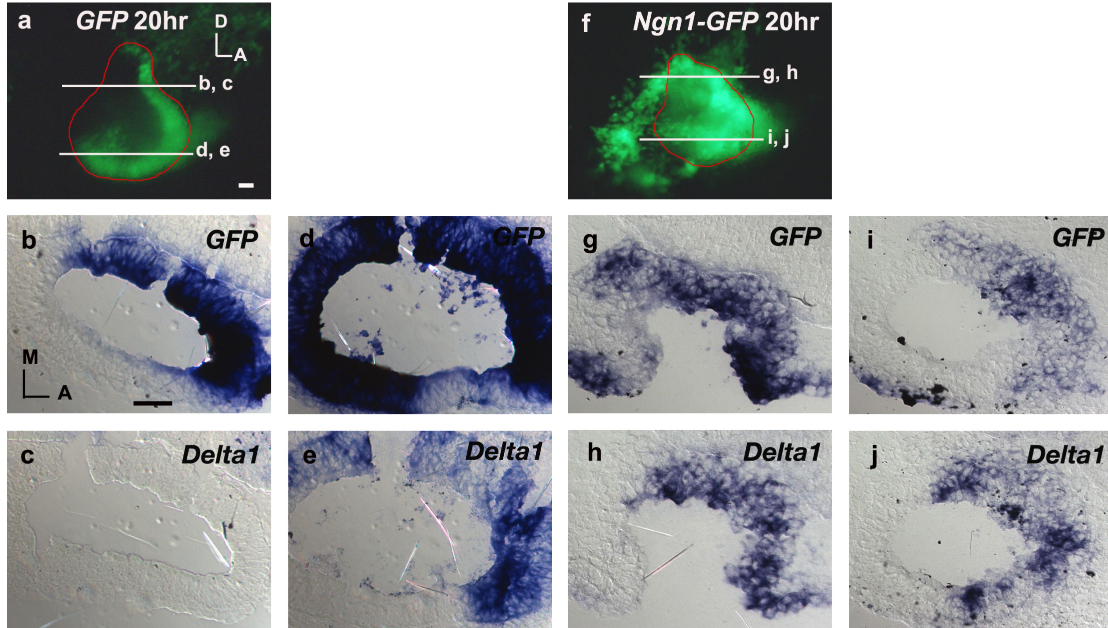


Figure 3.3 Over-expressing *Ngn1* leads to ectopic up-regulation of *Delta1*. Otic cups over-expressed with *GFP* (a) or *Ngn1-GFP* (f) at 20 hrs after electroporation. The ears are outlined in red and levels of sectioning are indicated in white lines. Adjacent sections probed for *GFP* and *Delta1* transcripts (b-e, g-j). *GFP* control shows *Delta1* expression in the NSD domain only (e, n=2), and not in dorsal *GFP*-positive regions (c). *Ngn1-GFP* specimens show ectopic *Delta1* expression dorsally (h) and ventrally (j), which overlaps with the *GFP* domain (g, i; n=3/3).

Over-expressing *Sox2* leads to ectopic *Delta1* and *Hes5.1* up-regulation

In chapter 2, I have demonstrated that over-expression of *Sox2* leads to cell-autonomous *Ngn1* up-regulation (see Fig.2.2 and Fig.2.3). Yet, this up-regulated Ngn1 is insufficient to drive neurogenesis. Here, *Ngn1* over-expression leads to *Delta1* up-regulation (Fig.3.3). These results suggest that increased lateral inhibition

could be occurring in *Sox2*-over-expressed specimens. This prompted me to analyze expression patterns of *Delta1* in *Sox2* over-expressed ears. Figure 3.4 illustrates that *Delta1* expression is up-regulated ectopically in *Sox2*-treated ears (Fig.3.4a-d). To investigate whether this Delta1 up-regulation actually resulted in activating the Notch signaling pathway, I investigated the expression pattern of *Hes5.1*, which is a gene down-stream of Notch signaling. Indeed, *Hes5.1* is ectopically up-regulated outside the NSD in *Sox2*-treated ears compared to control (Fig.3.4e-h). These results suggest that there is heightened Notch signaling in *Sox2*-over-expressed specimens and a possible non-cell-autonomous inhibition of the neuronal fate.

Figure 3.4

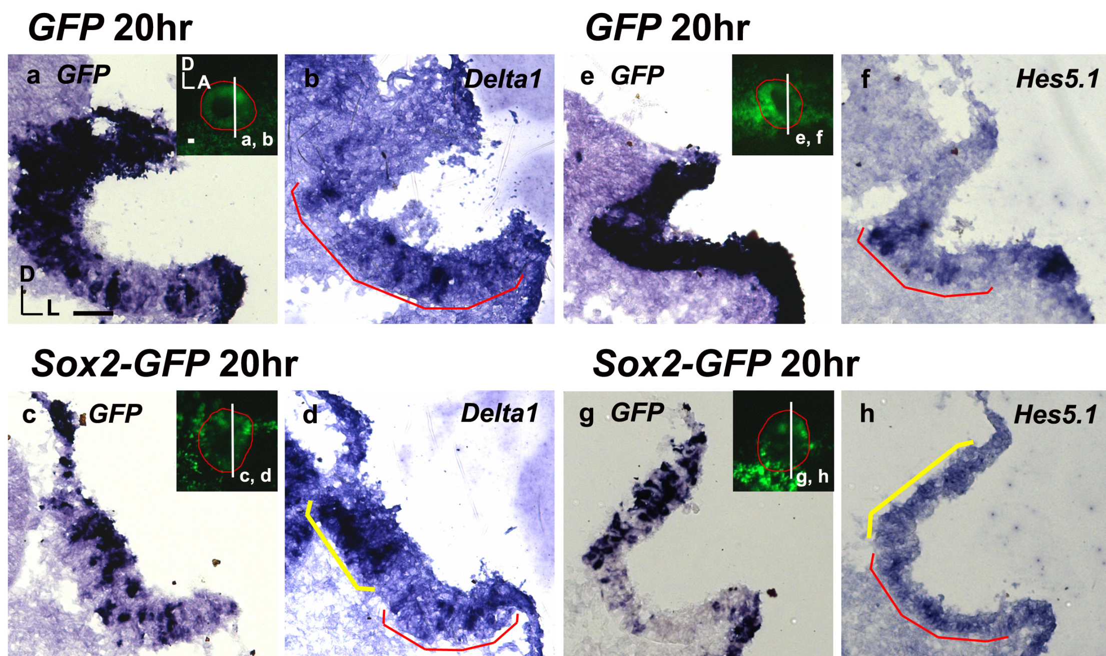


Figure 3.4 Over-expressing *Sox2* leads to ectopic up-regulation of *Delta1* and *Hes5.1*. Otic cups electroporated with *GFP* and *Sox2-GFP* at 20 hr harvests (a, c, e, and g; insets). The ears are outlined in red and levels of sectioning are indicated in

white lines (a, e, c, g; insets). Adjacent sections probed for *GFP*, *Delta1*, and *Hes5.1* transcripts (a-d and e-h). *Delta1* (d, n=4/4) and *Hes5.1* (h, n=4/4) are expressed within the NSD (d and h; red brackets) but are also up-regulated outside the NSD (d and h; yellow brackets) of *Sox2*-treated ears compared to controls (b, n=3; and f, n=4), which have expression only in the NSD (b and f; red brackets).

Discussion and Future Studies

Over-expressing *Sox2* causes non-cell-autonomous inhibition of the neuronal fate via Notch-activated lateral inhibition

The reason for the phenotype of ectopic *Ngn1* up-regulation but no ectopic neurogenesis obtained in chapter 2 when *Sox2* is over-expressed is two-fold. One, Ngn1 can't inhibit *Sox2* transcription of the exogenous construct. Two, *Delta1* is up-regulated in *Sox2* over-expressed cells through the up-regulation of Ngn1, and directly by Sox2, leading to activation of the Notch signaling pathway and thereby lateral inhibition of the neuronal fate in adjacent cells. Up-regulation of *Delta1* in *Sox2* over-expressed cells further suggests that these cells are going down the neuronal lineage. Furthermore, activated NICD leading to up-regulation of *Sox2* seems to be one cause of the expanded Sox2 domain in *Ngn1* over-expressed specimens, suggesting that cells adjacent to once *Ngn1*-positive cells are presumably going down the sensory lineage. Therefore, over-expressing *Sox2* causes heightened Notch activity and lateral inhibition of the neuronal fate.

The neural-sensory competent domain appears to be broader than commonly thought

The fact that Sox2, Ngn1, and NICD all seem to induce the neuronal/neural-sensory competent fate dorsally suggests that the NSD is broader than commonly thought and includes the dorsal region. *Delta* and *Serrate* ligands are normally not expressed in the dorsal region, but *Notch1* is (Adam et al., 1998; Cole et al., 2000; Lewis et al., 1999; Shailam et al., 1999). Some extrinsic factors like Wnt signaling could be inhibiting the expression of these ligands in the dorsal region. Wnt signaling is known to restrict the *Ngn1*-positive NSD ventrally (Riccomagno et al., 2005). Taking all the results into consideration, it appears that Sox2, Ngn1, and NICD are capable of over-riding Wnt activity in the dorsal domain.

Sox2 has a cell-autonomous and non-cell-autonomous role in regulating neuronal and sensory fates

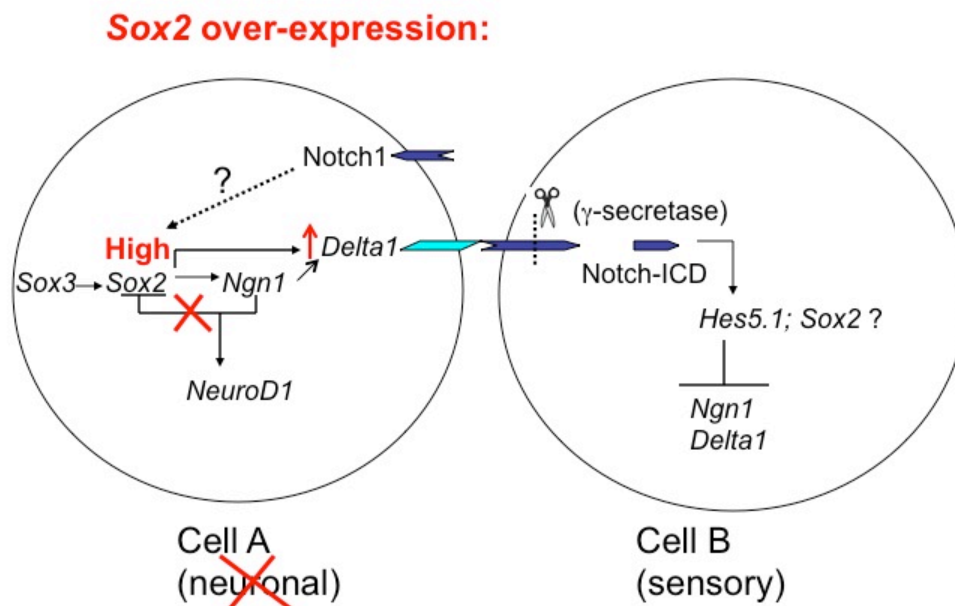
In addition to the cell-autonomous up-regulation of *Ngn1* in *Sox2* over-expressed ears shown in chapter 2, the up-regulation of *Delta1* by Ngn1 and the up-regulation of *Delta1* and *Hes5.1* by Sox2 suggest possible cell-autonomous and non-cell-autonomous relationships between these genes and the Notch signaling pathway. Previous studies indicate that *Delta1* is expressed in pro-neural progenitors similar to *Ngn1*, and *Hes5.1*, a negative regulator of *Ngn1*, is expressed in non-neuronal progenitors (Cau et al., 2000; Daudet et al., 2007). It remains to be demonstrated that the up-regulations of *Delta1* by Ngn1 and Sox2 is cell-autonomous, whereas the up-regulation of *Hes5.1* by Sox2 is non-cell-autonomous. This could be accomplished

using immunohistochemical techniques. Dr. Ram Mishra in our laboratory has generated rabbit polyclonal antibodies against Delta1 and Hes5.1 and these antibodies are currently being characterized. I would expect the double-antibody labeling experiments to confirm that Delta1 is up-regulated cell-autonomously by Ngn1 and Sox2, and that Hes5.1 is up-regulated in adjacent cells that over-express Sox2 or Ngn1.

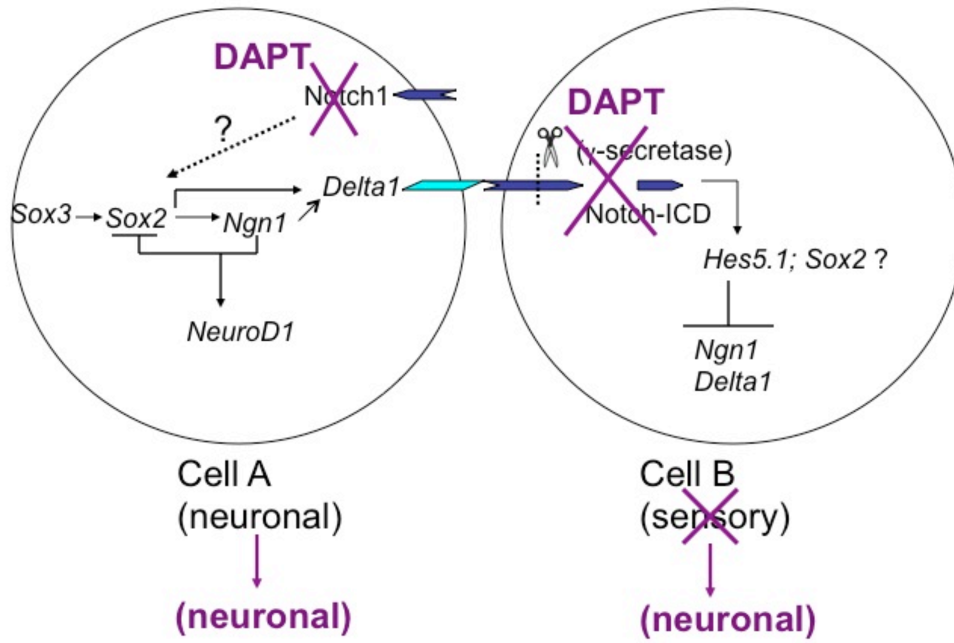
To demonstrate that Sox2 interacts non-cell-autonomously with the Notch signaling pathway in regulating neurogenesis, I recommend that further studies are performed by inhibiting the Notch signaling pathway concomitantly with over-expressing *Sox2* (see Working Models Fig.3.5 below). This could be accomplished by using a γ-secretase inhibitor DAPT (*N*-[(3,5-Difluorophenyl)acetyl]-L-al anyl-2-phenyl]glycine-1,1-dimethylethyl ester), which blocks the Notch intracellular domain NICD from being cleaved and translocated into the nucleus to up-regulate a set of genes including *Hes5.1*. The use of DAPT should be followed immediately by electroporating with *Sox2-GFP*. In my pilot studies, I have begun to optimize the method and narrowed the concentration of optimal DAPT use *in vivo* to 5mM in 100% DMSO, as some specimens show down-regulation of *Hes5.1* at this concentration (n=2/3, data not shown). I would predict that *Sox2* expression will be down-regulated by DAPT alone treatments because Notch signaling is postulated to be up-stream of *Sox2* and neurogenesis will be increased because lateral inhibition mediated by Notch signaling will be impaired. Alternatively, no changes in *Sox2* expression may be obtained in DAPT alone treatments because *Sox3* is postulated to be up-stream of *Sox2,* thereby possibly compensating for the loss of *Sox2* expression

when Notch is blocked by DAPT. Over-expressing *Sox2-GFP* in the presence of DAPT will test if exogenous Sox2 mediates parts of its effects on neurogenesis via Notch signaling. I would expect an improvement in neurogenesis compared to over-expressing *Sox2-GFP* without DAPT treatment. This prediction is based on the assumption that blocking Notch signaling will alleviate the non-cell-autonomous effects resulting from over-expression of *Sox2*.

Figure 3.5



DAPT treatment:



***Sox2* over-expression + DAPT:**

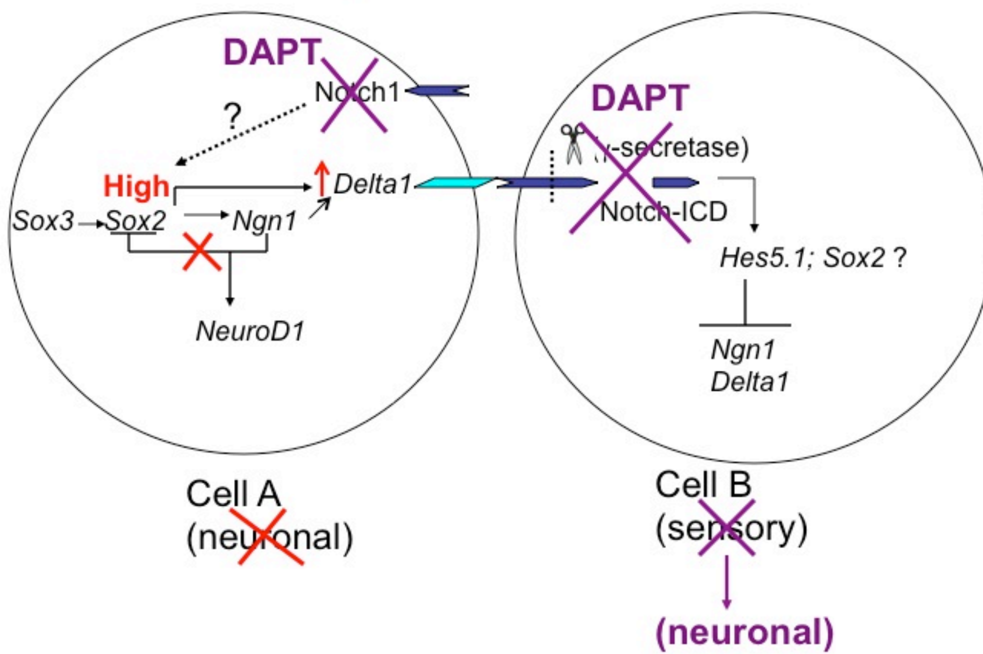


Figure 3.5 Working models of Sox2 function in *Sox2* over-expressed cells, DAPT treatment, and *Sox2* over-expression + DAPT treatment. *Sox2* over-expression has cell-autonomous and non-cell-autonomous effects between cells A and B in regulating neuronal and sensory cell fates.

When ***Sox2-GFP* is over-expressed in cell A**, Sox2 up-regulates *Ngn1* in cell A, but Ngn1 in turn can't block *Sox2* promoter activity in order for *Neurod1* to be up-regulated and neurogenesis to proceed. Therefore cell A cannot be committed to the neuronal fate because exogenous *Sox2* cannot be down-regulated. This is the cell-autonomous effect of *Sox2* over-expression in inhibiting neurogenesis. At the same time *Ngn1* is up-regulated, *Delta1* is also up-regulated in cell A. Delta1 activates Notch receptors in cell B. As a result, Notch is cleaved by γ-secretase, and the activated Notch (Notch-ICD) translocates to the nucleus to activate target genes such as *Hes5.1* and *Sox2* in cell B. Hes5.1 in turn blocks *Ngn1* and therefore *Delta1* expression in cell B. Consequently, cell B goes on to become a sensory cell. In contrast, if ***Sox2-GFP* is over-expressed in cell B (not illustrated),** the fate of cell B may not necessarily change, but the expected up-regulation of *Delta1* in cell B will block the neuronal fate in cell A. This is the non-cell-autonomous effect of *Sox2* over-expression in inhibiting neurogenesis.

Application of the chemical compound **DAPT** blocks the Notch signaling pathway by blocking γ-secretase. This will test the involvement of the Notch signaling pathway in mediating neurogenesis and whether Notch signaling normally functions up-stream of *Sox2*. *Sox2* expression should be down-regulated by DAPT treatments because Notch signaling is postulated to be up-stream of *Sox2,* and

neurogenesis will be increased because lateral inhibition mediated by Notch will be impaired. Alternatively, no changes in *Sox2* expression may be obtained since *Sox3* is also postulated to be up-stream of *Sox2,* thereby Sox3 could be compensating for the loss of *Sox2* expression when Notch is blocked by DAPT treatment.

Over-expressing *Sox2-GFP* in the presence of DAPT will test if Sox2 mediates parts of its functions via Notch signaling. An improvement in neurogenesis should be obtained because blocking Notch signaling would block non-cell-autonomous effects of over-expressing *Sox2*.

Chapter 4: Summary and Conclusions

Summary and Conclusions

This dissertation investigates the roles of Sox2 and Sox3 in inner ear development using gain and loss-of-function approaches in the developing chicken inner ear. My results show that Sox2 plays a major role during neurogenesis, whereas Sox3's role in neurogenesis seems to be indirect, mediated through regulation of Sox2. In chapter 2, I demonstrate that Sox2 and Neurogenin 1 regulate the neurogenic fate and in chapter 3, I address the potential interactions between Sox2 and the Notch signaling pathway in mediating neuronal and sensory cell fate choices. My results from chapter 2 indicate that over-expressing *Sox2* in the otic cup stage leads to up-regulation of *Ngn1* but no induction of *Neurod1* or subsequent increase in neurogenesis. In contrast, up-regulation of *Ngn1* alone is sufficient to induce *Neurod1* and increase neurogenesis. These results indicate that while Ngn1 is sufficient to induce the neurogenic fate, yet commitment to the neuronal fate is somehow blocked in cells over-expressing *Sox2,* despite their high levels of Ngn1 expression. Normally, *Sox2* is down-regulated in the neuronal lineage. In *Ngn1*-over-expressing cells, Sox2 expression is down-regulated within 15 hrs. Therefore, it is possible that the neural fate cannot progress in *Sox2*-over-expressed cells because of the high exogenous *Sox2* levels. Given the role of Ngn1 in inhibiting *Sox2* and *Sox3* in the neural tube, I interpret my results to indicate a requirement for a transcriptional repression of *Sox2* activity in order for neurogenesis to proceed. This repression could be normally mediated by Ngn1, which fails to repress exogenous *Sox2* activity, driven by a *β-actin* promoter. Thus, I propose that while

Sox2 and perhaps some other unknown factor(s) normally initiate neurogenesis by up-regulating *Ngn1*, *Sox2* needs to be down-regulated by Ngn1 in order for neurogenesis to progress. The fact that Ngn1 counteracts *NOP-1* activity suggests that Ngn1 counteracts Sox2 activity directly possibly by interacting with an E-protein at the *E-box* motif within the *NOP-1* enhancer of the *Sox2* promoter. Inhibition of *Sox2* transcription appears to be an important step before *Neurod1* can be activated and neurogenesis to proceed, since *Neurod1* is up-regulated in *Ngn1*- but not *Sox2*-over-expressed cells. Interestingly, Neurod1 is also able to inhibit the enhancer activities of *NOP-1*. Therefore, it is likely that once *Neurod1* expression is activated normally, *Sox2* expression in the neuronal lineage is further reduced.

In chapter 3, I have demonstrated a potential interaction of Sox2 and the Notch signaling pathway in mediating neuronal and sensory cell fate choices. I show that there is a secondary effect on Sox2 expression after *Ngn1*-positive cells have delaminated from the ear epithelium. Although Sox2 is initially down-regulated in *Ngn1*-treated ears, it is subsequently up-regulated presumably in adjacent cells to once *Ngn1*-positive cells; i.e. the Sox2 domain is expanded dorsally. This suggests a non-cell-autonomous effect on *Sox2* expression possibly through activation of the intracellular domain of the Notch receptor (NICD). In my hands, over-expressing *NICD* leads to an expansion of the *Sox2* domain. Members of the Notch signaling pathway, the expression of the ligand *Delta1* and the transcriptional repressor *Hes5.1* are up-regulated in *Sox2*-over-expressed ears, and *Delta1* is up-regulated in *Ngn1*-over-expressed ears. I take my findings to indicate that Sox2 and the Notch signaling pathway interact cell-autonomously and non-cell-

autonomously in regulating the NSD. I propose that over-expression of *Sox2* leads to up-regulation of *Delta1*, which activates Notch receptors in adjacent cells to inhibit neurogenic fate possibly by up-regulating *Hes5.1* and *Sox2.* Both Sox2 and Ngn1 are able to up-regulate *Delta1*. *Hes5.1* is up-regulated by Sox2 and predicted to be up-regulated by Ngn1 via Delta1 up-regulation in presumably adjacent cells. *Delta1*-expressing cells adopt a neuronal fate, whereas *Hes5.1* and *Sox2* expression in adjacent cells presumably leads them to adopt a sensory fate. Future studies will have to be performed to demonstrate the cell-autonomous and non-cell-autonomous relationships, possibly by using a double-antibody labeling approach and by over-expressing *Sox2* via electroporation concomitantly with inhibiting the Notch signaling pathway through DAPT treatment.

The fact that Sox2, Ngn1, NICD all induce the neuronal/neural-sensory competent fate dorsally suggests that the NSD is broader than commonly thought and includes the dorsal region. The limiting factor is that Notch ligands such as *Delta* and *Serrate* are normally not expressed in the dorsal area, whereas *Notch1* is. It is very likely that extrinsic signaling pathways such as Wnt signaling regulate this dorsal area and prevent the expression of these ligands, as Wnt signaling is known to restrict the *Ngn1*-positive NSD ventrally.

Figure 4.1

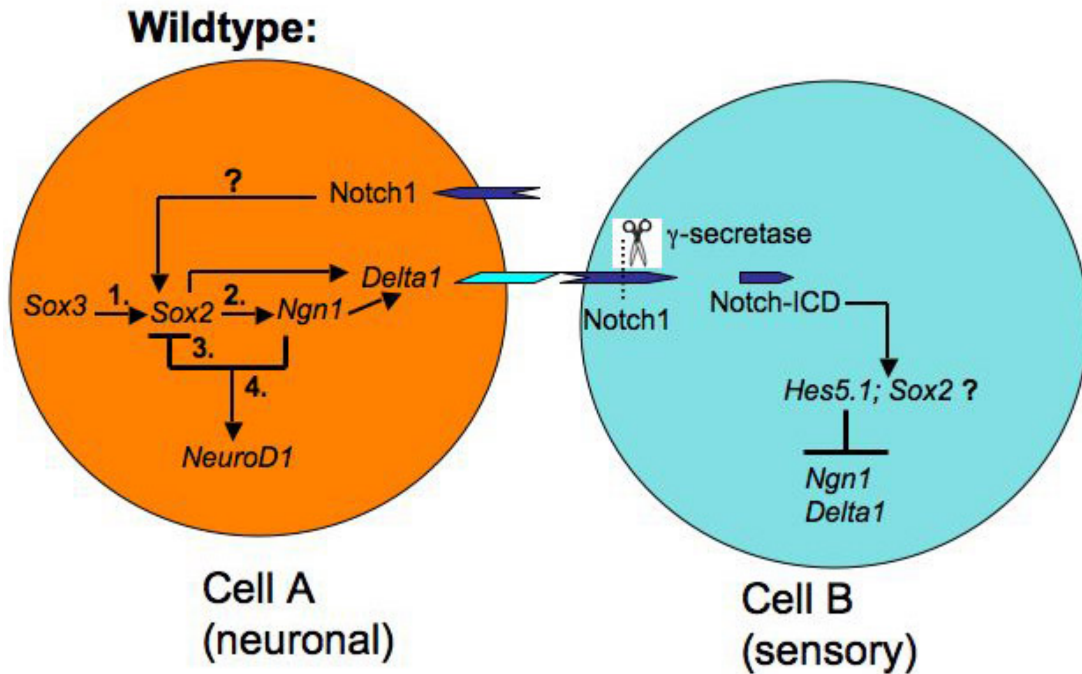


Figure 4.1. Summary Model.

Sox3 and Notch are up-stream of *Sox2,* and Sox2 and the Notch signaling pathway regulate neuronal and sensory cell fate choices cell-autonomously and non-cell-autonomously. In cell A Sox2 up-regulates *Ngn1*. Ngn1 in turn blocks *Sox2* promoter activity in order for *Neurod1* to be up-regulated and neurogenesis to proceed. Therefore, cell A becomes neuronal. At the same time, *Delta1* is up-regulated in cell A. Delta1 activates Notch receptors in cell B. As a result, Notch is cleaved by γ-secretase, and the activated Notch (Notch-ICD) translocates to the nucleus to activate target genes such as *Hes5.1* and *Sox2* in cell B. Hes5.1 in turn blocks *Ngn1* and therefore *Delta1* expression in cell B. Consequently, cell B goes on to become a sensory cell.

Future Studies

Loss of Sox2 function

Although I have extensively tried to test Sox2 loss-of-function in this study, the experiments yielded a mixed phenotype for the expression of *Ngn1* (see chapter 2). I recommend that future studies are performed with alternative methods to using the dominant negative repressor *Engrailed* construct. For instance, use of morpholinos or inhibitory RNA's (RNA interference (RNAi)) could proof more conclusive. These methods knock down gene function itself as opposed to interfering with down-stream target genes as in the case of the dominant negative repressor, and the oligos can be easily obtained and electroporated with the method I have developed. However, in my hands experiments with and analyses of two independent translation-blocking morpholinos to Sox2 has so far not yielded successful knock-down of Sox2 (data not shown).

Sox2 and the Notch signaling pathway

The cell-autonomous and non-cell-autonomous relationships between Sox2 and the Notch signaling pathway remain yet to be demonstrated (see chapter 3 discussion). I recommend the use of antibodies against Delta1 and Hes5.1 as well as blocking Notch signaling concomitantly with over-expressing *Sox2* to be performed in the future to accomplish this.

Sox2's role in the sensory lineage

Although I demonstrate that Sox2 and the Notch signaling pathway potentially interact in mediating neuronal and sensory cell fate choices, this dissertation investigates the role of Sox2 during neurogenesis and not the hair cell fate *per se*. However, my findings in chapter 3 suggest that Sox2 plays a role in sensory fate specification after *Ngn1*-postive cells have left the NSD epithelium. It would be interesting to see what happens to the hair cell fate if *Sox2* over-expressed ears are incubated past 48 hrs and analyzed for sensory and hair cell markers at 72 hr harvests. My hypothesis is that forced *Sox2* expression will lead to an expansion or ectopic formation of sensory domains and possibly to increased formation of hair cells. Alternatively, forced expression of *Sox2* in the NSD may lead to a decrease in hair cell numbers as high Sox2 activity will inhibit the hair cell fate (see chapter 2 discussion).

Research Implications

Why is Sox2 in inner ear development important?

The website of the National Institute on Deafness and Other Communication Disorders states that approximately 36 million Americans suffer from some form of hearing loss (http://www.nidcd.nih.gov/). The majority of the cases is due to sensorineural hearing loss caused by mutations, aging, certain tumors, infections, ototoxic drugs, or loud sound exposures. Depending on the situation, damages can be occurring at the level of sensory hair cells or neurons of the spiral ganglion, or both. Thus far, therapeutic interventions are in the forms of hearing aids and

cochlear implants. My study is not only important from the point of view of development but also for therapeutic approaches in combating hearing disorders. The findings in this study will provide a greater understanding of the molecular mechanisms involved in regulating neuronal and sensory fates and may provide better strategies for gene therapy in replacing damaged neurons or sensory hair cells.

Adult mammalian cochlear hair cells are not able to regenerate, although hair cells in the vestibular system are able to regenerate to some extent, whereas avian, fish, and amphibian hair cells are able to regenerate throughout life (Cotanche and Kaiser, 2010; Staecker and Van De Water, 1998). The mammalian cochlea consists of a mosaic and precise pattern of hair and supporting cells. There are three known possible mechanisms of hair cell renewal: regenerative proliferation of supporting cells (e.g. re-entering the cell cycle), trans-differentiation of supporting cells into hair cells, and repair of damaged hair cells (e.g. migration of macrophages to location of damaged hair cell) (Staecker and Van De Water, 1998). Miss-expression of the *Atoh1* gene in the guinea pig and mouse ear leads to trans-differentiation of supporting cells into hair cells, and pharmacological inhibition of the Notch signaling pathway in the organ of Corti leads to supernumary hair cells from supporting cells (Brigande and Heller, 2009). Furthermore, inhibiting cell cycle inhibitors such as p27Kip1 allows supporting cells to re-enter the cell cycle and trans-differentiate into hair cells (Brigande and Heller, 2009; White et al., 2006).

Manipulating stem cells in vitro by providing appropriate guidance signals and re-introducing them in vivo allows for the differentiation of a selective cell lineage such as hair cells or neurons. Supporting cells have been identified to be one source of potential stem cells/progenitor cells for hair cell replacement, the other sources being embryonic and induced pluripotent stem cells (Oshima et al., 2010; Smeti et al., 2011; White et al., 2006). Sox2 and the Notch ligand Jag1 are expressed in supporting cells of the post-natal organ of Corti, but their expression gradually declines in the adult (Smeti et al., 2011). *Sox2* and *Jag1* are considered pro-sensory genes and Jag1 is later involved during lateral inhibition of the hair cell fate (see chapters 1 and 3). Sox2 and Atoh1 have been demonstrated to interact in regulation of the hair cell fate (Dabdoub et al., 2008). Given the nature of Sox2 and Jag1 function in hair and supporting cell development, the temporal expression patterns of these genes may provide windows of opportunities for therapeutic interventions in hair cell regeneration from supporting cells. Furthermore, given Sox2's role as a key player in embryonic and induced pluripotent stem cells, it is actively being used for studies in regeneration of hair cells from stem cells ((Oshima et al., 2010) also see chapter 1). Current approaches for regenerating neurons of the inner ear also use stem cell methodology with the involvement of *Sox2* and the Notch signaling pathway. For instance, blocking Notch signaling in neurospheres generated from mouse utricular stem cells increases hair cell production at the expense of supporting cells and neurons, whereas forced expression of the Notch intracellular domain *NICD* leads to up-regulation of *Ngn1* indirectly through the up-regulation of *Sox2* (Jeon et al., 2011). Furthermore, *Ngn1* is proposed to be up-

regulated by a target of *NICD*, *RBP-J*, since a binding site for RBP-J has been identified in vitro within the *Ngn1* promoter (Jeon et al., 2011). This dissertation identifies an important molecular mechanism of Sox2 function in the regulation of the neuronal fate and makes strong implications for an interaction of Sox2 with the Notch signaling pathway in mediating neuronal and sensory cell fate choices. Although it does not deal with the hair cell fate *per se*, findings in this study could prove instrumental for future studies in combating hearing disorders and for both hair cell and neuronal cell regeneration.

Appendix

Generation of *Lunatic fringe Cre-ERT2* mice for fate-mapping sensory domains of the inner ear

Introduction

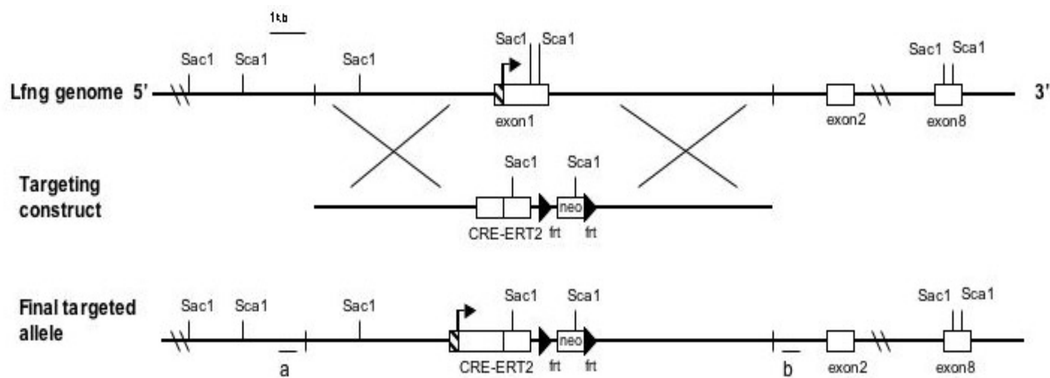
Lunatic fringe **is expressed in the neural-sensory competent region of the otic vesicle and in all subsequently derived sensory organs**

The vertebrate inner ear, which is made up of a labyrinth of sensory and non-sensory components, develops from a simple tear-drop shaped vesicle. The antero-ventral region of the otic vesicle is the presumed neural-sensory competent region. In mammals, this region gives rise to neurons of the auditory and vestibular ganglia, and to six sensory organs including three cristae, the utricle and saccule, and the organ of Corti. The precise region of the neural-sensory competent region within the ear rudiment is not clear and is thought to be marked by the expression of a number of genes such as *Lunatic fringe (Lfng)* and *Neurogenin 1* (*Ngn1)*. Lfng is an extracellular modulator of the Notch signaling pathway (Irvine, 1999), whereas Ngn1 is a bHLH (basic-Helix-Loop-Helix) transcription factor. Ngn1 specifies the neuronal fate and is expressed in neuronal progenitors. Genetic fate mapping studies of the neurogenic domain using *Ngn1-CreER* mice show that *Ngn1*-positive cells give rise to the neurons of the auditory and vestibular ganglia as well as sensory cells in the utricular and saccular maculae, and some non-sensory cells (Raft et al., 2007). Thus far, it is

not clear which areas of the neural-sensory competent domain give rise to the three cristae and the organ of Corti. Since *Lfng* is expressed in all sensory organs and its expression domain at the otic vesicle stage encompasses and is broader than that of *Ngn1* domain, I sought to fate map the neural-sensory competent region of the inner ear by generating *Lfng-creERT2* mice, in which a tamoxifen-inducible *cre* recombinase (*Cre-ERT2*) is knocked into the *Lfng* genomic locus. Fate mapping the progenies of *Lfng*-positive cells could provide a more complete picture of the neural-sensory competent region and insights into how individual sensory organs arise from a common domain.

Materials and Method

Generation of *Lfng-CreERT2* targeting construct

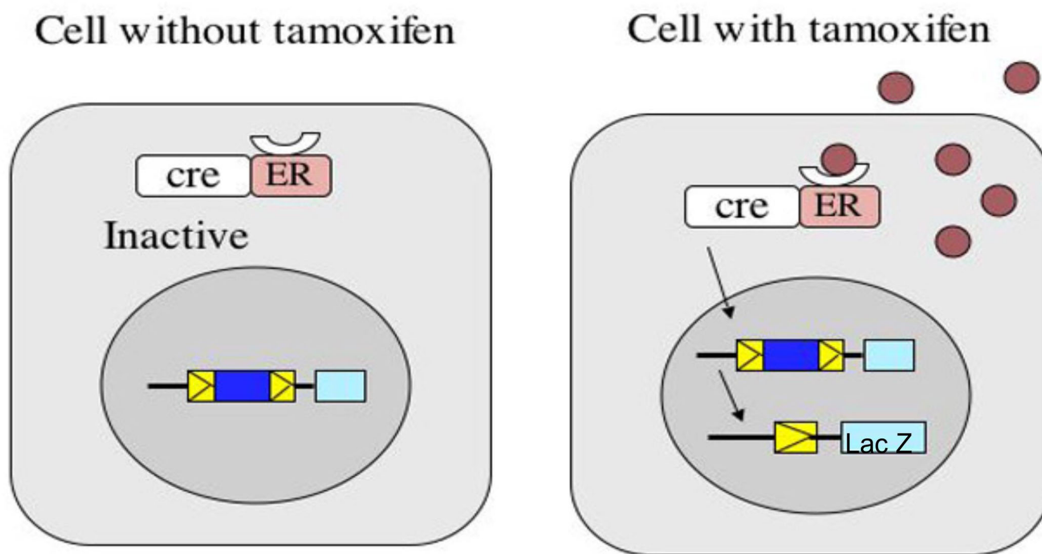


a, b: Southern probes

	Size of expected bands	
	Sca1 Probe a	Sac1 Probe b
Mut	10.4 kb	9.8 kb
WT	7.7 kb	7.3 kb

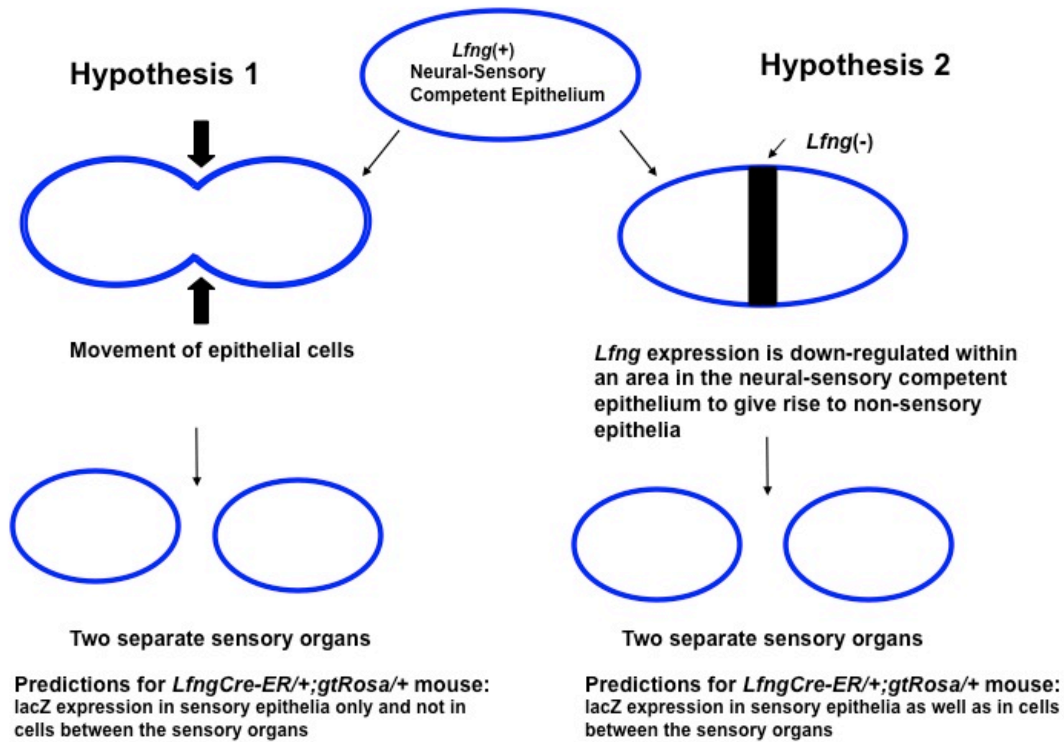
I replaced Exon1 of the genomic *Lfng* locus with a *Cre-ERT2-neo* cassette using homologous recombination via the bacterial artificial chromosome (BAC) recombineering technique. The construct was electroporated into embryonic stem cells (ES) strain 129 by the company Xenogen. I identified positive ES clones through Long Range PCR and confirmed by Southern blot. The mice were generated by Xenogen. I confirmed the genotypes of the mice using Southern blots.

Induction of Cre activity requires tamoxifen



The *cre* recombinase is fused to a synthetically modified estrogen receptor (*ERT2*). Thus, this cre fusion protein can only be translocated to the nucleus and mediate recombination in the presence of tamoxifen. Cells with successful recombination will be expressing *lacZ*.

Hypotheses



Hypothesis 1: Two separate sensory organs form from one *Lfng(+)* sensory patch by movement of non-sensory epithelial cells into the sensory patch and dividing it into two. This would lead to lacZ expression in the sensory organs only and not in non-sensory cells in between the sensory organs.

Alternative Hypothesis: *Lfng* expression is down-regulated within the *Lfng(+)* sensory patch to give rise to two separate sensory organs. Thus, lacZ expression should be in the sensory organs as well as in non-sensory cells in between the sensory organs.

Glossary

Cell-autonomous

Regulation of cellular changes within the same cell

Cochleo-vestibular ganglion (CVG)

The VIII cranial nerve; Population of neurons comprised of auditory and vestibular ganglia

High Mobility Growth domain (HMG)

DNA binding domain of Sox proteins

Ligand

In this study the term ligand is used for cell-surface proteins, which bind to and interact with a cell-surface receptor protein in adjacent cells

Neuronal fate

Neuroblasts that are both *Ngn1* and *Neurod1* positive, which delaminate from the otic epithelium and form neurons of the cochleo-vestibular ganglion (CVG)

Neural-sensory competent domain (NSD)

An area located in the antero-ventral region of the otic cup or otocyst; Fate-mapping data indicate that cells in this region can give rise to both neuroblasts and sensory cells

Neurosphere

Free-floating cluster of neural stem cells *in vitro*

Non-cell-autonomous

Signaling from one cell which affects cellular changes in a neighboring cell

Nucleotide

Molecule that is a building block of RNA or DNA

Oligo

Oligonucleotide; Part of a short sequence of RNA or DNA nucleotides

Plasmid

A circular piece of double-stranded DNA that will replicate episomally in bacteria

Pluripotency

Ability of stem cells to develop into many different cell types

Sensory fate

Sensory progenitors that develop into either hair cells or supporting cells

Subcloning

A term used to describe cutting and pasting fragments of DNA using restriction enzymes

Transcript

Messenger RNA that will allow translation into a protein

Transcription factor

A protein that activates transcription of a gene by binding directly or in a complex with other proteins to the promoter region of the gene

Table of Genes

Delta1, Serrate1 (Ser1), Serrate2 (Ser2)	ligands for Notch receptors
Hes5.1	basic-Helix-Loop-Helix (bHLH) transcription factor of the Hairy and Enhancer-of-Split family activated by Notch signaling pathway; negative regulator of pro-neural bHLH genes
Lunatic fringe (Lfng)	extracellular modulator of Notch signaling
Neurod1	pro-neural bHLH transcription factor activated by Ngn1
Neurogenin 1 (Ngn1)	pro-neural bHLH transcription factor
Notch1	trans-membrane receptor
Sox2, Sox3	Sry-related HMG (High-Mobility-Growth) box transcription factors

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