Roles of *Sox3* and *Lmx1b* in early development of the inner ear

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DEDICATION

To memories of my parents

For all the times wanted to see me as a successful person

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SUMMARY

During the last years, a great progress has been made in understanding the mechanisms involved in otic induction but the mechanism behind otic patterning into neural and non-neural domains is still an open question and the major aim of this work was to address this question.

We have analyzed the molecular mechanisms underling the early regionalization of the otic placode, and explored the role of *Sox3* and *Lmx1b* in the establishment and maintenance of a neural competent domain in the otic placode by using the chick as a model system.

The results show that *Sox3* expression initially expressed in a broad domain gets regionalized in otic/epibranchial proneural domain. Overexpression of *Sox3* at preotic stages can induce ectopic neuronal precursor cells expressing *Sox2* and *Delta1* but does not allow the ectopically developed neuronal precursor cells for further differentiation. *Sox3,* besides providing neural competence to the proneural domain, regulates the posterior non-neural gene *Lmx1b* suggesting that its final expression pattern depends on the neural activity. Finally, I present evidence that BMP signaling has an early role in inducing *Lmx1b* expression in the otic field but that neither BMP activity nor *Lmx1b* expression influence neural commitment.

Taken together, our results provide new information and shed light on the molecular mechanisms that underlie the first steps of the neural competence and otic patterning in proneural and non-neural domain.

RESUM

En els darrers anys s'ha avançat substancialment en l'enteniment dels mecanismes moleculars implicats en el procés d'inducció de la placoda òtica. Tanmateix, encara existeix poca informació de com s'estableix un domini amb competència neural i un altre no-neural i aquest ha estat l'objectiu d'aquesta tesi doctoral.

Hem analitzat els mecanismes moleculars rellevants per la regionalització primarenca de la placoda òtica i hem explorat el paper de *Sox3* i *Lmx1b* en l'establiment i manteniment d'un territori competent neural, emprant l'embrió de pollet com organisme model.

Els resultats mostren que el gen *Sox3*, inicialment expressat en un territori extens, es regionalitza en un domini òtic i epibranquial proneural. La sobreexpressió de *Sox3* a estadis preòtics, indueix la generació de precursors neuronals que expressen *Sox2* i *Delta1*, malgrat aquests no progressen a estadis de major diferenciació. A la vegada, *Sox3* és capaç d'inhibir l'expressió de *Lmx1b*, un gen expressat en el domini no-neural, fet que suggereix que el seu patró final ve determinat per l'activitat neurogènica. Finalment, presento evidències que la senyalització mitjançada per BMP té un paper primerenc en l'establiment de l'expressió de *Lmx1b* en el territory òtic, però que ni l'activitat de BMP ni l'expressió de *Lmx1b* influencien el procés de determinació neural.

En conclusió, els nostres resultats posen de relleu nova informació dels mecanismes moleculars que governen els primers passos de la competencia neural i la regionalizació de la placoda òtica en un territori neural versus un no-neural.

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1. INTRODUCTION

1.1 Inner ear as sensory organ: Structure and histology

The ear is one of the main sensory organs responsible for the sensation of hearing, balance and acceleration. It is composed of three anatomical parts: (1) the external ear, that funnels sound waves to the middle ear; (2) the middle ear, that transforms sound waves through the auditory ossicles into mechanical vibration of the endolymphatic fluid contained in the inner ear; and, (3) the inner ear, which is responsible for the transduction of mechanical stimuli into electrical impulses and their propagation to the brain (Fig. 1A; Purves *et al.*, 2001).

The inner ear consists of the vestibular portion located in the dorsal portion of the organ, which contains the sensory organs responsible for the sense of motion and position, and the ventrally located auditory region, which contains the sensory organ responsible for the sense of hearing (Fig. 1A; Purves *et al.*, 2001). Three different cell types constitute the functional unit of all inner ear sensory organs: the hair-cells, the supporting cells (both two cell types are referred as sensory) and the otic neurons (neurogenic component) (Fig. 1C; Purves *et al.*, 2001). Hair-cells are specialized mechano-receptors that transduce the auditory and vestibular mechanical stimuli into electrical signals. Hair-cells are innervated by otic neurons which are bipolar primary afferent neurons that transmit the information to second order neurons in the vestibular and auditory nuclei in the brainstem. Their somas are intermingled with the glial Schwann cells forming the cochlear and vestibular ganglia (reviewed in Rubel and Fritzsch, 2002). Supporting cells are non-sensory cells that maintain the correct ionic environment for the function of hair-cells and they release factors that maintain the trophism and survival of the hair-cells (Haddon *et al.*, 1999). They are also capable of serving as

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progenitors to regenerate hair-cells after injury (reviewed Stone and Cotanche, 2007).

The number of sensory organs in the inner ear varies among animal species, but all have at least six differentiated sensory domains (Fig. 1B, depicted in red). The vestibular system contains three *cristae*, one per semicircular canal, and two *maculae*, one for the saccule and one for the utricle. The auditory system only contains one sensory organ (organ of Corti in mammals and *basilar papilla* in birds) lying along the cochlear duct. In birds, amphibians, and fish, several other small *maculae* of uncertain function are also present (Fritzsch, 1999). The *macula* of the utricle and saccule detect linear accelerations in the horizontal and vertical axis, respectively, and the gravitational pull (Purves *et al.*, 2001). The *cristae*, which are located in chambers called *ampullae* at the base of the semicircular canals, detect angular accelerations (Purves *et al.*, 2001). In the auditory sensory epithelium, sound-wave frequency discrimination is based on the position of the hair-cells along the longitudinal cochlear axis, which is correlated with the position of the sensory neurons in the cochlear ganglion.

B

Endolymphatic duct

Semicircular canals

Posterior crista ampullaris

Organ of Corti

Lateral crista ampullaris

Anterior crista ampullaris

Utricular macula

Saccular macula

Cochlear duct

C

Supporting cell

Hair-cell

Sensory neuron
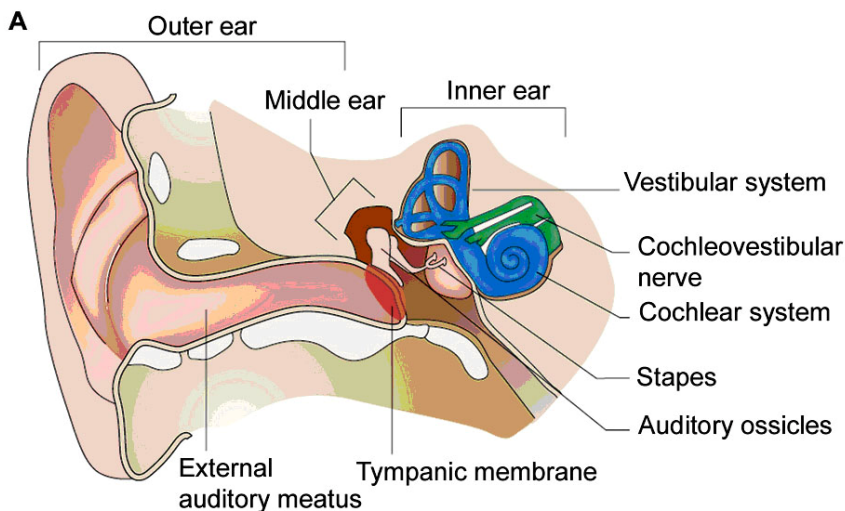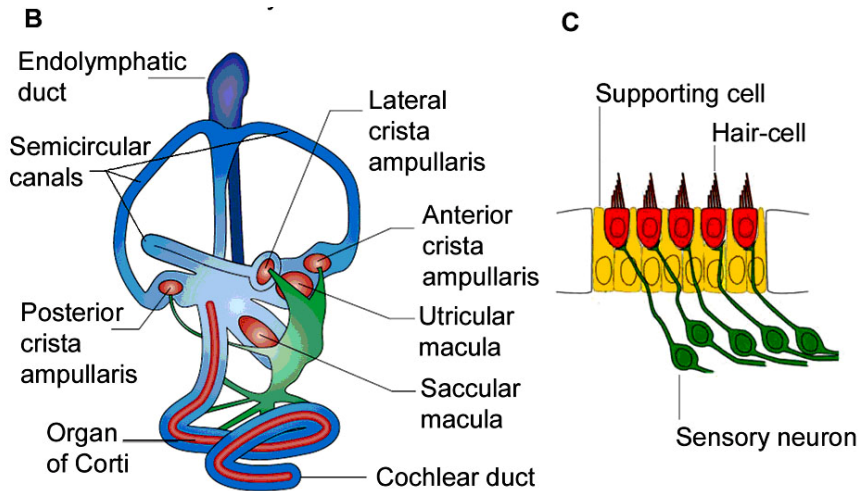
Figure 1: Anatomy of inner ear
A) Schematic representation of the ear showing its different anatomical parts: the outer, the middle and the inner ear.   **B)** Schematic drawing showing the mammalian adult inner ear and sensory patches depicted in red.   **C)** The sensory unit is build up by three different cell types: supporting cells, hair-cells and sensory neurons. From Kelley, 2006 and Adam *et al.*, 1998

This tonotopical order is conserved in the central auditory nuclei, where sensory neurons project, reproducing in the brain the hair-cell order of the cochlea (Torres and Giraldez, 1998; Purves *et al.*, 2001). In addition to the sensory structures, the inner ear includes the endolymphatic duct (ED), which extends dorsally to communicate with the Central Nervous System (CNS) and is involved in the turnover of the endolymph (Fig. 1B).

1.2 Cranial placodes and their origin

1.2.1 Cranial Placodes

Cranial placodes have been defined as transient embryonic thickenings of columnar epithelium that appear in the ectoderm adjacent to the neural crest and neuroectoderm (Bhattacharyya *et al.*, 2004). Common properties of all placodal cells include their origin at the border of the neural plate and ectoderm (Baker and Bonner-Fraser 2001), and their ability to

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invaginate and to produce cells that delaminate from the epithelium. Additionally, at a molecular level, these placodes are unified by their expression of at least a single member of each of the *Pax*, *Sox*, *Six*, *Eya*/*Dach* and *Fox* gene families (Baker and Bonner-Fraser 2001; Bhattacharyya *et al.*, 2004). From rostral to caudal are identified: the olfactory placode that gives rise to the regenerating olfactory sensory neurons; the lens placode that differentiate into lens fibers and cells of the eye; the trigeminal ganglion that relays touch, pain and temperature information from the face and jaw; the otic placode which forms the inner ear; and finally the epibranchial placodes (Figure 2A).
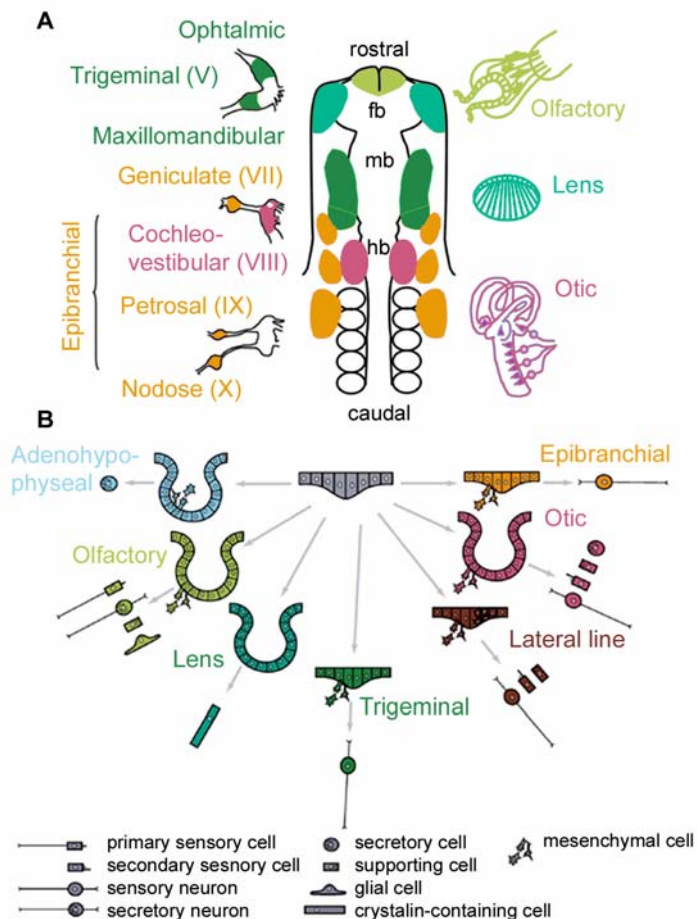
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A) Schematic representation of the sensory placodes and their deriatives at the 10 somite stage in the chick embryo (Streit, 2007). **B)** Schematic summary of morphogenesis and cellular derivatives of various cranial placodes. Invagination occurs in adenohypophyseal, olfactory, lens, and otic placodes. Moreover, in all placodes except the lens placode, some cells migrate away from the placodal epithelium as mesenchymal cells to form sensory neurons, secretory cells, or glial cells. In lateral line placodes, another subset of cells migrate along the basement membrane and forms the lateral line primordium. From Schlosser, 2006. Abbreviations: fb, forebrain; hb, hindbrain; mb, midbrain

The otic placode gives rise to visceral sensory neurons innervating taste buds and transmit vital information about heart rate, blood pressure and visceral dilation (Bhattacharyya *et al.*, 2004). Cranial placodes are divided into different groups: a) the pure neurogenic placodes such as the trigeminal and epibranchial placodes that only generate sensory neurons; b) the ones that produce sensory neurons and other cell-types such as the olfactory and otic placodes and; c) the lens placode that is non-neurogenic (Graham and Begbie, 2000; Schlosser, 2005; Schlosser, 2006). Ectodermal placodes form a wide variety of cell types, including ciliated sensory receptors, sensory neurons, endocrine cells, glia, and other supporting cells (Fig. 2B; reviewed in Streit, 2004 and Brugmann and Moody, 2005).

1.2.2 Development of cephalic sensory placodes

The inner ear derives from a domain that is shared with other cephalic sensory structures. Early in development, ectodermal placodes are formed in an area called the pre-placodal ectoderm (PPE). The PPE is located in the anterior region of the embryo, medial to the epidermis and lateral to the neural plate and the neural crest (Knouff, 1935).

Within the PPE, precursors for different placodes are initially interspersed, but then they separate to form the individual placodes at discrete positions along the neural tube (reviewed in Streit, 2004; Schlosser, 2006). Fate maps of embryos of different animal species indeed

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suggest that all cranial placodes originate from this horseshoe-shaped PPE (teleosts: Dutta *et al.*, 2005; amphibians: Streit, 2002; Bhattacharyya *et al.*, 2004). Preplacodal state can first be identified at neurula stages and is defined by the expression of unique set of molecular markers as well as by common properties of all cells contained in it (Jacobson, 1963; Streit, 2002; Bhattacharyya, *et al.*, 2004; Schlosser and Ahrens, 2004; for review: Streit, 2004; Bailey and Streit, 2006; Schlosser, 2006). At molecular level the PPE can be visualized by the expression of a number of genes belonging to the *Dlx*, *Six*, *Eya*, *Iro*, *BMP*, *Foxi* and *Msx* families (Ohyama and Groves, 2004a; Streit, 2004; Ohyama *et al*, 2007). Among the many transcription factors expressed in the PPE, only members of the *Six* and *Eya* families match precisely the location of all placode precursors and are subsequently maintained in all placodes, but lost from nonplacodal ectoderm (Figure 3A and B). Members of the *Six* and *Eya* families of nuclear factors begin to be expressed in a horseshoe-shaped domain surrounding the rostral neural plate from fore- to hindbrain levels. They have been implicated in multiple processes during placode formation and are therefore likely candidates to be involved in defining the placode territory at early developmental stages (Schlosser and Ahrens, 2004; Litsiou, *et al.*, 2005; Streit A, 2007).

As development proceeds, the PPE becomes regionalized in smaller sub-domains and when placodes are morphologically identifiable, each one exhibits a unique transcription factor code (Torres and Giraldez, 1998; Bailey and Streit, 2006; Schlosser, 2006). The current view is that localized signals from surrounding tissues drive the PPE to differentiate into distinct developmental fates (reviewed by Baker and Bronner-Fraser, 2001). For instance, FGFs play a key role in inducing olfactory, adenohypophysis, otic and epibrancial placodes (Ladher *et al.*, 2000; Maroon *et al.*, 2002; Wright and Mansour, 2003; Nikaido *et al.*, 2007; Sun *et al.*, 2007). Wnt signaling is also involved in placode development as

shown by the zebrafish mutants masterblind and headless, in which Wnt signaling is overactivated. This mutants exhibit a loss of anterior placodes (lens, olfactory), but an expansion of trigeminal neurons around the anterior neural plate, suggesting that the differential activation of the Wnt pathway along the rostral-caudal axis influences patterning of the PPE (Heisenberg *et al*., 1996; Kim *et al*., 2000).
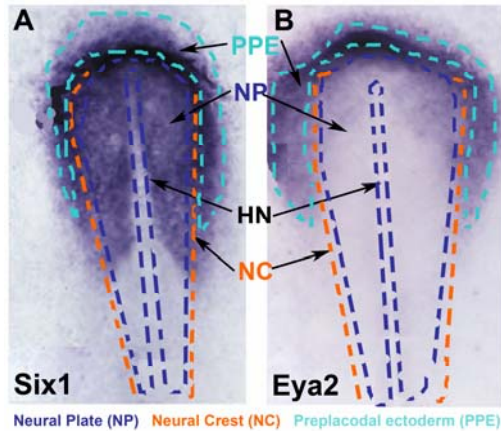
Figure 3: Segregation of placode region and neural crest markers
Segregation of placode ectoderm (PPE) and neural crest markers between head-fold. At stage 5-6 *Six1* **A)**, and *Eya2* **B)** are expressed in the ectoderm encircling the anterior neural plate. Modified from Litsiou, *et al*., 2005.

Within the PPE the precursors for different placodes are intermingled. However, there is some segregation of individual placode populations along the anterior-posterior axis. Precursors for the anterior placodes (adenohypophysis, olfactory, lens) are located in the rostral PPE, while precursors for posterior placodes (trigeminal, epibranchial, otic, lateral line) are restricted more caudally (D'Amico- Martel and Noden, 1983; Streit, 2002; Bhattacharyya *et al*., 2004; Litsiou *et al*., 2005).

1.3 Development of the chick Inner ear

1.3.1 Inner ear induction

The development of the inner ear begins when inducing signals from surrounding tissues instruct to the ectoderm from the PPE adjacent to the posterior hindbrain to follow an otic developmental pathway. It is now accepted that embryonic induction rarely occurs in a single step, but rather as a series of changes in the responding tissue, leading ultimately to the complete differentiation of the tissue or organ (Gurdon, 1987; Ginsburg, 1995; Grainger, 1996). The inner ear is an attractive system for the study of embryonic induction; this is why many studies over the last 80 years have examined the interactions between presumptive placodal ectoderm and candidate-inducing tissues leading to the formation of the inner ear. In this regard members of the Fibroblast Growth Factors (FGF) have been shown to play a critical role in inner ear induction in several species. Their expression in the developing hindbrain adjacent to presumptive otic tissue or in the underlying mesoderm suggested a role for FGF family members as possible ear inducers. FGF signaling has now been demonstrated to be necessary for otic placode induction in fish, chick and mouse. In zebrafish, treatment with the FGF receptor inhibitor SU5402 resulted in the complete loss of otic vesicles as well as the loss of expression of some ear markers, *Pax2a* and *Dlx3b* (Leger and Brand, 2002, Maroon *et al.,* 2002). In chick, future ear ectoderm failed to express *Pax2* and *EphA4* when cultured in presence of SU5402 (Martin and Groves, 2006). In fish, *Fgf3* and *Fgf8* have been implicated as being the main candidates for FGF-dependent ear inductive signaling. Both are expressed in the vicinity of the pre-otic domain at the time of placode induction (Reifers *et al.,* 1998, Furthauer *et al.,* 2001, Phillips *et al.,* 2001, Maroon *et al.,* 2002). When both *Fgf3* and *Fgf8* were targeted, no otic vesicles was formed (Phillips *et al.,* 2001, Leger and Brand, 2002, Maroon *et al.,* 2002, Liu *et*

al., 2003). In mouse, *Fgf3* and *Fgf10* are considered as potential inducers, with *Fgf3* being expressed in rhombomere 5 and 6 of the hindbrain and *Fgf10* in the mesoderm underlying the presumptive placode (Mahmood *et al.,* 1995, McKay *et al.,* 1996, Wright and Mansour, 2003). *Fgf10* mutant mice develop smaller otic vesicles (Ohuchi *et al.,* 2000), whereas *Fgf3* mutants develop an otic placode, although later ear differentiation is highly abnormal (Mansour *et al.,* 1993). Mutants of both *Fgf3* and *Fgf10* failed to form otic vesicles or form microvesicles and expression of *Pax2* was absent in the ear tissue, while *Pax8* and *Gbx2* were reduced (Alvarez *et al.,* 2003, Wright and Mansour, 2003). *Fgf8* is also involved in placode induction in chick and mouse, although its role appears to be indirect. It is expressed in cranial endoderm and is necessary for the mesodermal expression of *Fgf19* in chick and *Fgf10* in mouse (Ladher *et al.,* 2005, Ohyama et al, 2007).

Several genes have been found to be specifically expressed in the region that will eventually thicken to form the otic placode. The earliest ear marker reported so far is *Foxi1* in zebrafish (Solomon *et al.,* 2003). It has been proposed by Westerfield and colleagues that *Foxi1* and *Dlx3b* may provide competence to form the inner ear because loss of both *Foxi1* and *Dlx3b* results in ablation of all otic tissue even in the presence of a fully functional Fgf signaling pathway (Hans *et al.,* 2004). However, none of the mouse or chick *Foxi* class genes are expressed early in the otic placode (Ohyama and Groves, 2004a), although mouse *Foxi3* marks the entire pre-placodal domain at slightly earlier stages. The second earliest molecular marker is *Pax8* which is expressed in the presumptive otic ectoderm in fish, frogs and mice (Pfeffer *et al.,* 1998; Heller and Brandli, 1999; Ohyama and Groves, 2004b). *Pax2* expression follows shortly after, although its domain is initially larger than the otic placode in chick and mouse encompassing the epibranchial placodes and the lateral epidermis (Nornes *et al.,* 1990; Krauss *et al.,* 1991; Groves and Bronner-Fraser,

2000). A number of signaling molecules (FGFs, BMP7) and transcription factors (Eya1, GATA3, Nkx5.1, Gbx2, Sox3, Sox9) are also expressed in the otic ectoderm prior to invagination (Rinkwitz-Brandt *et al.,* 1995; Wright *et al.,* 1995; Penzel *et al.,* 1997; Shamim and Mason, 1998; Sheng and Stern, 1999; Groves and Bronner-Fraser, 2000; Liu and Joyner, 2001, Ohyama *et al*., 2007).

1.3.2 Otic development

After inner ear induction the ectoderm gets thickened and the otic placode becomes morphologically visible. In chick, this happens by 9-10 somite stage (ss) embryos, although probably otic specification starts before as shown by explant experiments (Groves and Bronner-Fraser, 2000). Then the placode undergoes a process of invagination to form otic cup and later pinches off from the ectoderm to form the otic vesicle (Figure 4).
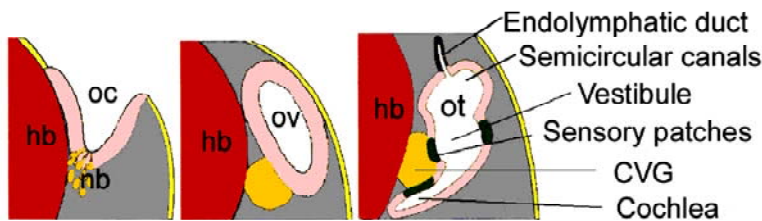
Figure 4: Otic development scheme
Schematic drawing of transverse sections showing the transition from otic cup to the otocyst. At otic cup stage, neuroblast shown in yellow delaminate from the otic epithelium. From Torres and Giraldez, 1998. Abbreviations: CVG, cochleovestibular ganglion; hb, hindbrain; nb, neuroblasts; oc, otic cup; op, otic placode; ov, otic vesicle; ot, otocyst.

The otic vesicle is a transient structure that undergoes multiple developmental changes associated with cell proliferation, differentiation, morphogenesis and cell death. If the otic vesicle is explanted outside embryo, it shows developmental autonomy which means that it can exhibit patterning, morphogenesis and cell diversification events,

including the genesis of mechanosensory hair-cells and sensory neurons
(Swanson *et al*., 1990; Alsina *et al*., 2003).

Once the placode starts to invaginate, some epithelial cells are identified
as neuroblasts, loose their connectivity and delaminate into the
mesenchymal space where they remain as a group, forming the cochleo-
vestibular ganglion (CVG) (Figure 4, neuroblasts depicted in yellow;
D´Amico-Martel, 1982; Hamond and Morest, 1991; Adam *et al*., 1998).
In the CVG, neuroblasts undergo a series of amplifying rounds of cell
divisions (Camarero *et al*., 2003) to enter into differentiation, extend their
axons and innervate the epithelial region from where they have been
generated (Fritzsch *et al*., 2002). Sensory precursors that will develop
hair-cells and supporting cells emerge two days later in chick (Pujades *et
al*., 2006).

1.3.3 Molecular steps of neurosensory development

The early steps of cell fate specification in the different sense organs are
controlled by proneural genes, which were first identified in *Drosophila*
mutants lacking the ability to develop external sense organs and bristles
(Ghysen and Richelle, 1979). Proneural genes encode for transcription
factors of the *b*asic *H*elix-*L*oop-*H*elix (bHLH) class that bind to a
common DNA sequence called the E-box sequence (Bertrand *et al.*,
2002). In *Drosophila*, two major proneural gene families control neuronal
development of the PNS, the *achaete-scute* (*asc*) and the *atonal* (*ato*) gene
family (Simpson, 1990). Interestingly, vertebrates, in addition to the
achaete-scute homologues (*ash*) and the *atonal homologues* (*atoh*), other
related proneural families are encoded in the genome: the E proteins, *Olig*,
NeuroD, *Neurogenin* and *Nscl* protein families (Bertrand *et al.*, 2002). In
Drosophila, the mechanotransduction and electrical propagation to the
brain for sensory information is performed by a single cell, while in

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vertebrates the two functions are performed by two distinct cells: the hair-cells specified by *Atoh1* and the sensory neurons specified by the *Neurogenin* proneural gene. Thus, it has been postulated that the appearance of new proneural genes in vertebrates allowed the molecular segregation of two specification events (see Fritzsch and Beisel, 2001). Furthermore, the two processes are also segregated temporally and few scattered cells expressing the proneural *Atoh1* are detected in the prosensory patches in chick E3.5 otic vesicles (Pujades *et al.*, 2006), while the neuronal precursors labeled by *Neurogenin1* or *Delta1* are found by E1.5 in chick.

How neurogenesis commences in the otic placode? In vertebrates otic neurogenesis does not differ in a great measure to the events of CNS neurogenesis (Bertrand *et al.*, 2002). In brief, in the chick inner ear low expression of *Neurogenin1* is expressed in the anterior subdomain of the otic placode. In this domain, scattered cells with increased levels of *Neurogenin1* and *Delta1* appear in the otic cup in mouse and chick (Adam *et al.*, 1998, Ma *et al.*, 2000, Alsina *et al.*, 2004). Following *Neurogenin1* expression, neuronal precursors emerge by a process of lateral inhibition mediated by the Delta-Notch transmembrane proteins. Notch-ligand, *Delta1* is expressed in those cells that are committed to become neurons and inhibit neuronal fate in neighboring cells. This is carried out by activation of Notch receptor and Notch effectors like *HES* genes (homologous to the *Drosophila <u>Hairy</u>* or *<u>Enhancer of Split</u>*), which in turn repress neuronal fate. By double fluorescent ISH, it was shown in our laboratory that indeed *Hes5* expressing cells are in adjacent *Delta1* expressing cells (Figure 5D; Abello *et al.*, 2007). Thus, the Delta-Notch pathway contributes to establish cell assymetry and diversification within an equally competent domain.
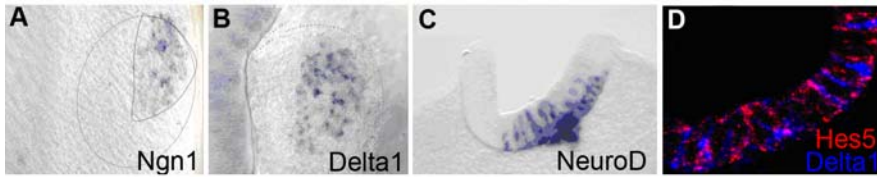
Figure 5: Neurosensory development
A) and B) Dorsal view of whole mounts embryos. C) and D) Sagittal sections. From Abello *et al.*, 2007.

Once the neuronal precursors are expressed, other genes of the proneural bHLH family (i.e. *NeuroD* and *NeuroM*) are switched on whose expression is detected in epithelial and delaminated neuroblasts (Figure 5C). Functional analysis of *Neurogenin1* and *NeuroD* suggests that they are necessary and sufficient for neurogenesis. Overexpression of *Neurogenin1* drives formation of ectopic neurons (Perron *et al.*, 1999, Kim *et al.*, 2001), while targeted inactivation of *Neurogenin1* results in loss of proximal cranial sensory ganglia (Ma *et al.*, 2000). Disruption of *NeuroD* function also results in a severe loss of sensory neurons associated with the inner ear (Kim *et al.*, 2001). The sequence of activation of *Neurogenin1*, followed by *NeuroD*/*NeuroM* expression plus the phenotypes observed supports the notion that *Neurogenin* genes have conserved the neuronal determination functions of the *atonal Drosophila* counterparts, whereas the *NeuroD/NeuroM* bHLH transcription factors exhibit neuronal differentiation and survival functions.

1.3.4 Signaling pathways regulating neurogenesis and sensory cell specification

Numerous studies have provided evidence that cell specification is regulated by a cascade of transcription factors as well as by signaling molecules that influence positively or negatively the sequential steps of cell commitment. During the process of inner ear neurogenesis, *Fgf10* is expressed in the neurogenic domain in chick and mouse and positively

regulates the determination of otic neuroblasts (Alsina *et al.*, 2004). Moreover, the mouse KO of *Fgf10* presents a reduction in the number of otic neurons and defects in the sensory epithelium (Pauley *et al.*, 2001). Knocking down *Fgf3* that is also found in the neurogenic territory in mice but not chick (Wilkinson *et al.*, 1989), showed impaired otic neurogenesis. Later on, otic neuroblasts that have populated the mesenchymal space are influenced by IGF-1 which is crucial for regulating neuroblast cell transit amplification (Camarero *et al.*, 2003). IGF-I is currently accepted as a neurotrophic factor (Savage *et al.*, 2001; Varela-Nieto *et al.*, 2003)., and it is being considered for treatment of neurodegenerative diseases (Dore *et al.*, 1997).

On the other hand, Bmp4 is expressed during multiple stages of development in the chick inner ear in association with presumptive sensory organs and semicircular cannal formation (Wu and Oh, 1996; Cole *et al.*, 2000; Mowbray *et al.*, 2001). Blockade of BMP-signal by Noggin produce anomalies in semicircular canal formation and structural patterning of the sensory organs (Chang *et al.*, 1999). Furthermore, a defined balance between Noggin and BMP4 has been shown to be required for proper specification of hair-cells (Li *et al*, 2005; Pujades *et al.*, 2006). The role BMP signaling in CNS development is well defined. In the development of ear, the focus of BMP signaling has primarily been in sensory development with little attention to a putative role in regulating neural specification.

1.3.5 Otic patterning and axis formation

An important step during early inner ear development is the acquisition of axial identities from the surrounding tissues, which in turn influence the positional information and development of all inner ear components (Fekete and Wu, 2002). It is not clear when otic tissue acquires its axial identity, and the timing of this specification appears to vary across

different species. Nevertheless, it is evident from different studies that the AP axis is specified before the DV axis, suggesting that axial specification occurs in multiple stages (Harrison, 1936; Wu *et al.*, 1998). The mechanisms involved in acquiring axial identity, however, remain elusive. The role of hindbrain in inner ear development has been well established (Kiernan *et al.*, 2002). Hindbrain mutants with defects in the region of rhombomere 5 (R5) are often associated with inner ear malformations. Therefore, it is possible that the hindbrain confers AP axial identity to the inner ear. Nevertheless, it is not clear from the typical hindbrain mutants such as $Hoxa1^{-/-}$ and kreisler whether inner ear defects reflect a failure in AP patterning (Ruben, 1973; Gavalas *et al.*, 1998; Vazquez-Echevarria *et al.*, 2008). Furthermore, as the border between rhombomere 5 (R5) and rhombomere 6 (R6) corresponds to the AP midline of the otocyst, it has been postulated that unique signals from each rhombomere may provide AP axial information required for inner ear patterning (Brigande *et al.*, 2000). In chick, when the relative positions of R5 and R6 were switched *in ovo* before the AP axis of the chicken inner ear was fixed, AP axial pattern of the inner ear was not affected (Bok *et al.*, 2005), suggesting that AP orientation of R5 and R6 does not play a major role in conferring AP axial identity to the inner ear (Bok J *et al.*, 2007). In zebrafish the *valentino* and the vHNF1 mutant in which hindbrain patterning is perturbed, display an expansion of the anterior otic domain and overproduction of hair-cells, suggesting that hindbrain influence in AP patterning may vary among vertebrate species (Kwak *et al.*, 2002).

Previous work has shown that otic patterning in neurogenic and non-neurogenic domain occurs as soon as the otic placode is morphologically visible. We have demonstrated that the anterior proneural region is characterized by the expression of *Sox3*, *Fgf10* and *Ngn1*, whereas *Iroquois1* (*Irx1*) and *Lmx1b* are restricted to the posterior domain in complementary fashion (Abello *et al.*, 2007). In addition, some

components of Notch pathway are also differentially expressed in proneural and non-neural domain (*Hes5*, *LFng*, *Delta1* in anterior, *Serrate1* and *Hes1* in posterior). Furthermore, deregulation of the Notch pathway leaded to expansion of posterior otic markers such as *Lmx1b* and *Irx1* into the neurogenic territory (Abello *et al.*, 2007). Although, AP patterning is visible in the otic ectoderm at HH10 by molecular markers, this event is probably not fixed until later stages and the specification process occurs gradually over approximately a 12 hour-period until the otic cup is half-closed as shown by otic rotation studies in chicken embryos (Wu *et al.*, 1998, Bok *et al.*, 2005).
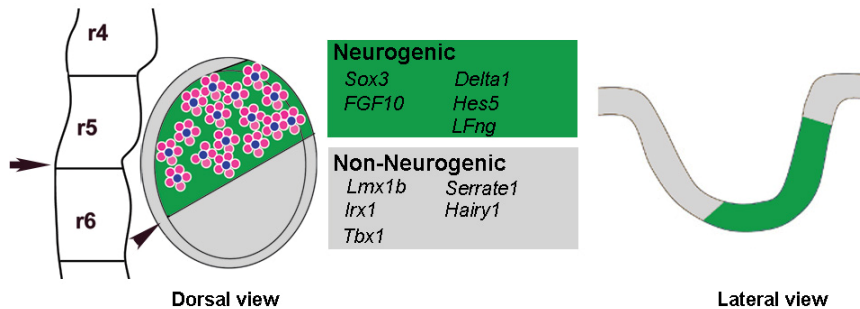
Figure 6: Otic patterning and axis formation
Dorsal and ventral views of the otic cup in which the neurogenic domain is depicted in green and the non-neurgenic in gray. Rossettes of *Delta1* (blue) and *Hes5.2* (pink) are detected in the neurogenic domain.

Recent studies suggest that *Tbx1* could be a key downstream target of extrinsic AP signaling (Vitelli *et al.*, 2003; Raft *et al.*, 2004, Arnold *et al.*, 2006). In the mouse, *Tbx1* expression is found exclusively in the posterior half of the otic cup (Vitelli *et al.*, 2003, Raft *et al.*, 2004, Arnold *et al.*, 2006). Interestingly, in *Tbx1* null mutants, the expression domains of genes such as *Ngn1*, *NeuroD1*, *Lfng* and *Fgf3* lose their normal anterior restriction in the otocyst and expand into more posterior regions. In contrast, the expression domains of posterior-expressed genes such as

Otx1, Otx2 and Goosecoid are abolished (Vitelli *et al.*, 2003, Raft *et al.*, 2004, Arnold *et al.*, 2006). Moreover, the expression domains of *Ngn1* and *NeuroD1* are significantly reduced in a transgenic mouse line carrying multiple copies of human *TBX1* (Raft *et al.*, 2004). Together, these results suggest that *Tbx1* may functions to suppress or restrict the anterior, neurosensory fate and that *Tbx1* could be an important determinant of AP axial identity within the inner ear (Bok *et al.*, 2007).

Unlike the AP axis, the DV axial specification of the inner ear is mainly dependent on signals emanating from the hindbrain (Bok *et al.*, 2005). When a segment of hindbrain adjacent to the inner ear is rotated along its DV axis *in ovo*, genes normally expressed in the ventral otocyst such as *Lfng*, *NeuroD* and *Six1* are shifted dorsally. On the other hand, the expression of a dorsal otic gene, *Gbx2*, is abolished. These results indicate that by rotating the DV axis of the hindbrain, ventral hindbrain tissues are sufficient to confer ventral fates to dorsal otic tissue (Bok *et al.*, 2005). Thus, the ability of the ventral hindbrain to override other potential dorsalizing signals from neighboring tissues further suggests that the hindbrain provides the major DV axial information to the inner ear (Bok *et al.*, 2007). Secreted molecules from the hindbrain such as Wnts from the dorsal neural tube and Sonic hedgehog (Shh) from the floor plate and notochord play important roles in establishing the DV axis of the inner ear (Liu *et al.*, 2002, Riccomagno *et al.*, 2002, Bok *et al.*, 2005, Riccomagno *et al.*, 2005). Analyses of the inner ears from *Wnt1/Wnt3a* double knockouts show that Wnt signaling is responsible for the expression of a subset of dorsal otic genes such as *Dlx5, Dlx6 and Gbx2* and for the development of vestibular structures (Riccomagno *et al.*, 2005; Bok J *et al.*, 2007). Moreover, tissue ablation experiments carried out in otic explant cultures clearly demonstrated that the Wnts responsible for the expression of these dorsal otic genes are emanating from the dorsal hindbrain (Riccomagno *et al.*, 2005). On the other hand, Shh is required

for the ventral patterning of the inner ear in both chicken and mice (Liu *et al.*, 2002, Riccomagno *et al.*, 2002, Bok *et al.*, 2005). In *Shh* $^{-/-}$ mouse embryos, the expression levels of genes in the ventral portions of the otocyst such as *Otx1*, *Otx2*, *Lfng*, *Fgf3*, *Ngn1* and *NeuroD1* are reduced, resulting in the complete absence of ventral inner ear structures (Riccomagno *et al.*, 2002). In chicken, injecting hybridoma cells that secrete antibodies blocking Shh bioactivity into the ventral midline at the otic cup stage also resulted in inner ears devoid of ventral structures (Bok *et al.*, 2005).

Finally, the last patterning event is the specification of sensory patches within the otic vesicle. The otocyst consists of both sensory and non-sensory regions and the morphogenesis of these regions are most likely coordinated during development. One major morphogenetic event is the subdivision of the putative neurosensory competent region into various sensory patches after neuroblast delamination (Figure 1). The molecular mechanisms underlying the specification of the neurosensory domain are thought to involve *Sox2*, *Six1* and the Notch signaling pathway (Kelley, 2006). The lack of *Sox2, Six1*, or genes within the Notch signaling pathway such as *Jagged1* and *Delta1,* affects ganglion formation as well as sensory organ development (Zheng *et al.*, 2003, Ozaki *et al.*, 2004, Kiernan *et al.*, 2005, Brooker *et al.*, 2006, Kiernan *et al.*, 2006).

1.4 Hypothesis of neural specification

Previous work in the laboratory has focused in the understanding of the early events of otic neural/non-neural patterning. However, when and how otic neural specification takes place is still an open question in all vertebrate species. When trying to understand how a proneural field is established several questions arise. Does the process of placodal induction by default involve proneural induction or, alternatively successive induction events lead first to placodal and subsequently to neural fate?

Secondly, is neural competence initially acquired in a subdomain of the otic fated cells or repression of neural fate in the non-neural competent region is required? Three models have been proposed to understand how the proneural domain is established (Figure 7, Abelló and Alsina, 2007). Model A, *single induction and repression of neural fate,* suggests that the competence of neural fate is conditioned by the acquisition of a placodal identity and once the placode is specified proneural fate is inhibited in the posterior otic territory (Figure 7A). The fact that FGF signaling is required both for otic/epibranchial induction, as well as for *Sox1-3* gene expression in the otic/epibranchial field in zebrafish (Sun *et al.*, 2007) suggest that both events could, in fact, be part of the same process.
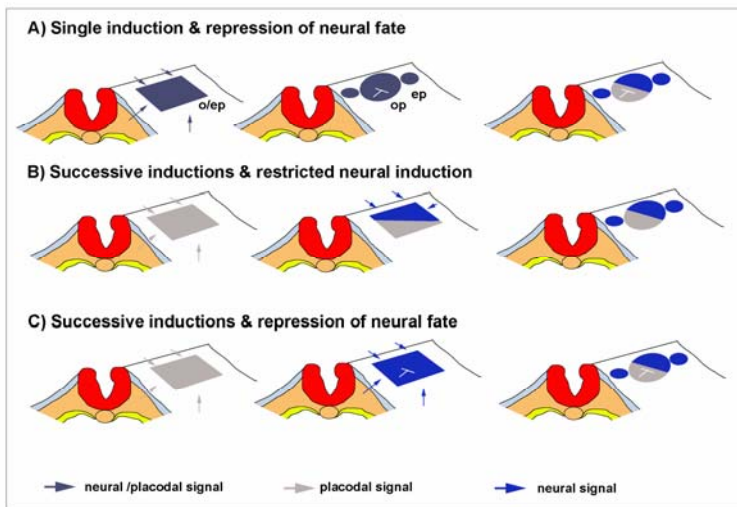
Figure 7: Proposed models for neural induction in the otic/epibranchial fiels
Dorsal views of the cranial ectoderm at the level of the hindbrain. o/ep, otic-epibranchial placode; ep, epibranchial placode; op, otic placode. From Abelló and Alsina 2007.

Model B, *successive inductions and restricted neural induction*, postulates that placodal and neural induction are successive events (Figure 7B). Graft experiments at different axial levels performed by Noden and van-de-Water (1986) revealed that presumptive otic placode ectoderm could

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ectopically generate otic vesicles without the ability to form neurons. Again, Groves and Bronner-Fraser (Groves and Bronner-Fraser, 2000) observed that quail anterior epiblast grafted in the presumptive otic region of host of 3-10ss could start to express *Pax2* and *Sox3*, while grafts performed at 11-21 ss, only expressed *Sox3* but not *Pax2*, suggesting that *Pax2*-inducing signals are lost before *Sox3*-inducing signals.

This model hypothesizes for a restricted source of neural inducers in the anterior otic field. Model C, *successive inductions and repression of neural fate*, predicts on successive inductions, as in model B, but unrestricted neural inducing signals activate *Sox* genes in a broad otic/epibranchial field. Broad proneural competence is subsequently restricted to the anterior otic region by an inhibitory signal from the posterior region (Figure 7C).

Recent studies from our laboratory (Abello *et al.*, ms in preparation and results shown here) suggest that in chick *Sox3* expression initially distributes in a broad preotic domain at 6 ss to progressively restrict to the anterior otic territory that will later undergo neurogenesis. On the other hand, restricted ectodermal expression of *Fgf8* after otic induction in the anterior field, its ability of inducing *Sox3* and disassociation of otic placode induction and *Sox3* induction by explanting embryos at different stages (Figure 8, Abello *et al.*, ms in preparation), favors models **B** and **C** over model **A** in which successive waves or sustained FGF signaling is required for neural induction in the otic territory.
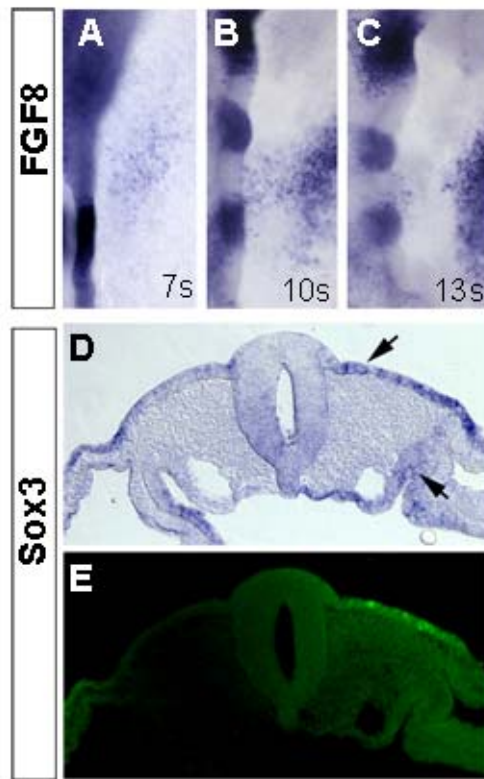
Figure 8: Role of *Fgf8* in inducing *Sox3*
A-C). Expression of *Fgf8* appears trqansiently in the anterior ectoderm
from 7ss to 13ss. **D-E)** Overexpression of *Fgf8* in the ectoderm induces
Sox3 expression (arrows).

In parallel to restriction of *Sox3* in the anterior otic field, *Lmx1b* is also
expressed in the otic territory. *Lmx1b* is initially found in a medial band to
progressively concentrate to the caudal otic territory. As neurogenesis
proceeds from otic placode to otic vesicle stage, *Lmx1b* expression is
down-regulated in the neurogenic domain, suggesting that posterior non-
neural genes might be regulated by genes in the proneural territory. In the
neural tube, *Lmx1b* is expressed in the roof and floor plate where no
neurogenesis occurs. This segregated spatial distribution suggests that
either both genes regulate each other or use different signaling
mechanisms that specifically maintain them in distinct pools of cells.

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Notch signaling has proven to be necessary for the down-regulation of *Lmx1b* in the neurogenic domain (Abelló *et al.*, 2007), while *Fgf8* was found to positively regulate *Sox3* expression in the anterior otic territory. In this work the functional role of *Sox3* and *Lmx1b* during otic neural development, their possible relationship and the signals that regulate them have been explored in order to better understand otic patterning in a neurogenic and non-neurogenic territory. In the following section I will describe what is known about *Sox3* and *Lmx1b* genes in the field of development to better comprehend the results presented here with respect to their putative role in inner ear development.

1.5 Role of Sox3 in ear development and neurogenesis

1.5.1 Sox3 Structure and function

The Sox proteins comprise a group of transcription factors characterized by the presence of a Sry-related box initially found in the Sry gene, a mammalian testis determining factor box. Sry-related box is a 79 amino acid motif that encodes HMG-type (high mobility group) DNA binding domain. The *S*ox genes encode proteins that are highly conserved throughout evolution and are identified through homology. The Sox family falls into a subclass of HMG box proteins, the members of which show greatly restricted tissue distribution and bind to specific DNA sequences. Most strikingly, upon binding Sox proteins cause DNA to bend at an acute angle (Pevny and placzek, 2005). Cumulative evidence indicates that Sox proteins by themselves do not exert regulatory functions to activate or repress gene transcription but do so through specific interactions with a partner factor (Kamachi *et al.*, 2000). In most cases, the C-terminal region of the Sox protein carries a cryptic transactivating domain, but the potential for activation becomes explicit only when a

highly specific interaction with the partner factor is achieved (Pevny and placzek, 2005).

To date, 30 vertebrate *Sox* genes have been identified and are classified into 10 subgroups (A–J) based on sequence identity (Bowles *et al.*, 2000; Pevny, Lovell-Badge, 1997; Wegner, 1999). At least 12 members of the *Sox* gene family are expressed in the nervous system. The role of *SoxB1* and *SoxB2* subgroups is well defined in the specification and maintenance of neural progenitor identity in the CNS and the *SoxE* subgroup in the PNS. The first class, comprising *Sox1*, *Sox2* and *Sox3*, share greater than 90% amino acid residue identity in the HMG-DNA binding domain (Figure 9) and are classified as subgroup B1 (Pevny and placzek, 2005). Within one group of Sox proteins the amino acid sequences of the HMG domains are more than 90% identical, but between the groups the identity is significantly lower. Subsequent cloning of wider regions of coding sequences generally verified the HMG domain-based classification of the groups: within a group amino acid sequences are fairly conserved throughout the polypeptide length, implying related regulatory functions, but between the groups no significant similarities exist except for the HMG domain (Uchikawa *et al.*, 1999). All Sox proteins bear activation domains and are involved in activation of the genes (Pevny and Lovell-Badge, 1997).

Transient transfection studies have shown that subgroup B1 factors are transcriptional activators and that the activation domains are located in the COO-terminal half (Kamachi *et al.*, 2000). All three factors are co-expressed in proliferating neural progenitors of the embryonic and adult CNS. The SoxB2 subgroup of Sox factors, including Sox14 and Sox21, are very similar to SoxB1 in their HMG-DNA binding domain and can bind to identified Sox2 target sites.
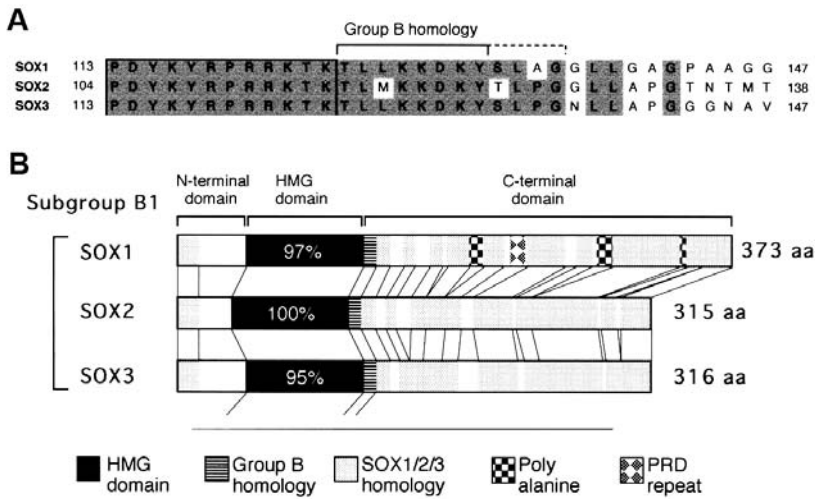
Figure 9: Comparasion of organization of Sox1, -2, -3
A) Comparison of amino acid sequences at the junction of the HMG domain (left of the vertical bar) and its immediate C-proximal region showing `Group B homology' among SOX1, -2, -3.   **B)** Comparison of the domain organizations among the SOX1, -2, -3 proteins, with indication of the conserved subdomains. Percent identity of the amino acid sequence of the HMG is shown in comparison with the SOX2 HMG domain. The regions and the domains conserved among SOX1/2/3, are indicated as respective `homologies'. Amino acid sequences with polyalanine stretch and PRD repeat are also indicated. Modified from Uchikawa et al., 1999.

However, the COO-terminal portions of Sox14 and Sox21 act as transrepression domains. Sox21 is widely expressed in the developing CNS, whereas Sox14 expression is limited to small subset of interneurons in the developing CNS (Rex et al., 1997; Uchikawa et al., 1999). By contrast, Sox9 and Sox10 belong to the SoxE subgroup, and define newly-induced neural crest cells and proliferating crest progenitors during embryogenesis (Wegner, 1999; Buescher et al., 2002; Kim et al., 2003). A key common feature of SoxB1, SoxB2 and SoxE, however, is their ability to maintain neural progenitor or stem cell identity (Pevny and placzek, 2005).

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1.5.2 Role of SOXB1 genes in neural competence and neurogenesis

1.5.2.1 Defining neural potential in embryonic ectoderm

Throughout evolution, the expression of the *SoxB*1 genes, *Sox1*, *Sox2* and *Sox3*, directly correlates first, with ectodermal cells that are competent to acquire a neural fate, and second, with the commitment of cells to a neural fate (Pevny and placzek, 2005). The *Drosophila*, *Amphioxus*, *Xenopus*, zebrafish and avian orthologs of SoxB1 all show expression throughout cells that are competent to form the neural primordium, and then become restricted to cells that are committed to a neural identity (Rex *et al*., 1997; Cremazy *et al*., 2000; Hardcastle and Papalopulu, 2000; Holland *et al*., 2000; Mizuseki *et al*., 1998; Nambu and Nambu, 1996; Penzel *et al*., 1997; Russell *et al*., 1996; Streit *et al*., 1997; Uwanogho *et al*., 1995). In the mouse the initial phase of *Sox2* and *Sox3* expression is pan-ectodermal along the entire proximal–distal axis of the egg cylinder (Collignon *et al*., 1996; Wood *et al*., 1999). *Sox1* expression then appears as one of the earliest transcription factors to be expressed in ectodermal cells committed to the neural fate: the onset of expression of Sox1 appears to coincide with the induction of neural ectoderm (Pevny *et al*., 1998). Concomitantly, expression of *Sox2* and *Sox3* becomes confined to cells that are committed to a neural fate. In chick embryos, *Sox3* is initially expressed throughout ectoderm that is competent to form nervous tissue before neural induction. *Sox2* expression then increases dramatically in neural ectoderm cells (Linker and Stern, 2004; Uchikawa *et al*., 2003). In *Xenopus* embryos, *Sox2* and *Sox3* similarly define the newly induced neural plate (Hardcastle and Papalopulu, 2000; Mizuseki *et al*., 1998). In *Drosophila*, the ortholog of *Sox2*, designated Dichaete, is first expressed in the syncytial blastoderm but by early gastrulation is restricted to neuroblasts of the newly formed nerve cord (Nambu and Nambu, 1996; Russell, 1996). Likewise, the *Drosophila* group B1 *Sox* gene, *SoxNeuro* is

dynamically expressed, first marking the presumptive ventro–lateral neuroectoderm, then confined to cephalic and ventral neurogenic regions (Cremazy, 2000). Taken together, SoxB1 factors have an evolutionarily conserved role (Pevny and placzek, 2005).

1.5.2.2 Regulation of SoxB1 by neural inducing signals

Much progress has been made in recent years in identifying the signaling molecules that induce neural tissue (Stern, 2006). In *Xenopus* embryos, a large body of evidence suggests that the antagonism of Bone Morphogenetic Protein (BMP) signaling by molecules such as Chordin and Noggin is a requisite step in the acquisition of neural fate. In the chick, the attenuation of BMP activity and acquisition of neural fate appears to require signaling by fibroblast growth factors (FGFs) and concomitant abrogation of Wnt signals (Wilson *et al*., 2000). However, despite these advances in understanding how neural tissue is induced, little is known of the molecular mechanisms that interpret the inducing signal in responding cells. In particular, the nature of transcriptional regulators that carry out the differentiation program, or whether or not distinct neural inducing signals regulate common transcriptional mediators of neural fate. Consistent with SoxB1 factors providing ectodermal cells with neural potential initially and committing ectodermal cells to a neural fate subsequently, their expression is modified by signaling molecules involved in neural induction (Pevny and placzek, 2005). Elegant studies in the chick embryo have provided evidence that neural inducing signals like FGFs directly regulate an enhancer of Sox2 that is conserved across diverse species (Uchikawa *et al*., 2003).

Furthermore, studies in *Xenopus* embryos have demonstrated that *Sox2* is upregulated by overexpression of Chordin and suppressed by BMP (Mizuseki *et al*., 1998). In *Drosophila*, Dpp and Sog, the counterparts of vertebrate BMP4 and Chordin respectively, similarly regulate expression

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of the *SoxB1* gene, SoxNeuro (Buescher *et al*., 2002; Cremazy *et al*., 2000). Thus, in both *Drosophila* and *Xenopus*, the antagonistic actions of BMP signals, and their inhibitors, govern *SoxB1* gene expression in the neuroectoderm (Pevny and placzek, 2005). In contrast, Storey and colleagues have precluded that *Sox3* expression is mainly influenced by FGF signaling but not BMP signaling pathway (Stravidis *et al*. 2007).

1.5.2.3 Neural determination and SoxB1

The early onset of expression of *SoxB*1 genes, together with the observation that the expression of the *SoxB*1 gene subfamily has been evolutionarily conserved in neural primordial cells and is regulated by signaling molecules involved in specification of neural fate suggests a role for *SoxB*1 genes in establishing neural fate. Compelling evidence for a requirement of SoxB1 factors in the acquisition of neural fate by naive ectodermal cells is derived from studies in *Xenopus* embryos. In *Xenopus*, ectopic expression of *Sox2*, in combination with FGF, initiates neural differentiation of ectoderm (Mizuseki *et al.*, 1998). Conversely, injection of dominant interfering forms of *Sox2* (dnSox-2) into *Xenopus* inhibits neural differentiation, both in whole embryos and in animal caps in which neural tissue is induced by the attenuation of BMP signals (Mizuseki *et al.*, 1998). Intriguingly, dnSox2 injected cells that fail to differentiate into neural tissues are also unable to adopt epidermal cell fates, indicating that inhibition of *Sox2* signaling in naive ectodermal cells might generally restrict their differentiation potential (Pevny and placzek, 2005). Recently, similar results have been obtained by overexpressing *Sox3* in *Xenopus* and zebrafish (Dee et al., 2008; Rogers *et al.*, 2009). Thus, SoxB1 signaling is required for the consolidation of neural identity.

Is there complementary evidence that demonstrates a requirement for SoxB1 transcription factors in neural determination? To date, loss-of-function mutations in the mouse have been unable to determine the role of

SoxB1 genes in neural development. Targeted inactivation of *Sox2* results in peri-implantation lethality of homozygous embryos (Avilion *et al.*, 2001). *Sox1* and *Sox3* homozygous mutant mice are viable and gross morphological defects are only apparent at sites where the gene is uniquely expressed, the developing lens and pituitary (respectively) (Nishiguchi *et al.*, 1998; Rizzoti *et al.*, 2004). The requirement for *Sox2* in early mammalian embryogenesis and the possibly redundant functions of Sox1 and *Sox3* have therefore precluded an analysis of the role of these factors during neural induction. Genetic demonstration that *SoxB1* genes are required to specify neural progenitor fate in mice awaits the generation and characterization of conditional *SoxB1* alleles. Genetic evidence for a role of SoxB1 factors, however, has been provided through the study of Dichaete and SoxN fly mutant embryos (Buescher *et al.*, 2002; Cremazy *et al.*, 2000; Overton *et al.*, 2002). Specifically, SoxNeuro–Dichaete double mutant embryos show a severe neural hypoplasia throughout the CNS (Pevny and placzek, 2005).

1.5.2.4 Maintenance of neural progenitor and differentiation potential

After neural induction, *Sox1*, *Sox2* and *Sox3* together with *Sox21* are co-expressed in proliferating neural precursors along the entire AP axis of the developing embryo, and are detected in neurogenic regions in the postnatal and adult CNS. There is increasing evidence that SoxB factors are also required for the maintenance of neural progenitor identity. Studies in chick have shown that SoxB1 factors have a role in maintaining the undifferentiated state of embryonic chick neural progenitors (Pevny and Placzek, 2005). Specifically, overexpression of *Sox2* and/or *Sox3* inhibits neuronal differentiation of chick neural progenitors and causes them to retain their undifferentiated properties, with the ability to proliferate and express progenitor markers (Graham *et al.*, 2003; Bylund *et al.*, 2003). Conversely, expression of a dominant interfering form of *Sox2* and/or

Sox3 in neural progenitors results in their premature exit from cell cycle and the onset of neuronal differentiation. The phenotype elicited by this inhibition can be rescued by coexpression of *Sox1*, providing support for redundant SoxB1 function in neural progenitor cells, at least as assayed by these experimental conditions. Second, Kondo and Raff (Kondo and Raff, 2004) investigated the molecular mechanisms regulating the conversion of rat oligodendrocyte precursors (OPCs) into multipotent neural stem-like cells (NSCLs) and identified *Sox2* as a key player in this process. The conversion of rat OPCs into NSCLs is dependent upon the re-initiation of *Sox2* expression, and inhibition of *Sox2* signaling results in premature exit from the cell cycle and neuronal differentiation of OPCs (Kondo and Raff, 2004). Interestingly, it has also recently been demonstrated that *Sox2* expression is maintained in neurogenic regions of the adult rodent nervous system (Ellis *et al.*, 2004) and that regulatory mutations of mouse *Sox2* cause neurodegeneration and impaired adult neurogenesis (Ferri *et al.*, 2004). These findings suggest that a *Sox2* regulates pathway that initially defines neural progenitor identity during embryogenesis which is maintained throughout ontogeny. Finally, *Sox21*, a member of the SoxB2 subgroup, is coexpressed with SoxB1 members in proliferative neural progenitors in both embryonic and adult mouse CNS and represses nerve growth factor (NGF)-induced neuronal differentiation (Ohba *et al.*, 2004). Taken together, SoxB factors are both necessary and sufficient for maintaining pan-neural properties of neural progenitor cells (Pevny and placzek, 2005).

The precise mechanism by which SoxB1 factors repress neuronal differentiation and maintain progenitor identity remains unclear. The temporal progression of differentiation from a proliferating neural progenitor to a postmitotic neuron is accompanied by the ordered regulated expression of transcription factors marking progressive steps of differentiation. Members of the basic-helix-loop-helix (bHLH) family of

transcription factors can direct the exit of neural progenitors from cell cycle and promote terminal neuronal differentiation (Fode *et al*., 1998; Farah *et al*., 2000; Bellefroid *et al*., 1996; Arman *et al*., 1998; Casarosa *et al*., 1999). To date, two lines of evidence have raised the possibility that proneural genes are directly counteracted by SoxB1 activity. First, the capacity of proneural bHLH proteins to direct neuronal differentiation crucially depends on their ability to suppress *Sox1–3* expression in chick CNS progenitors (Kondo and Raff, 2004). And second, *SoxNeuro* in *Drosophila* acts upstream and in parallel to the proneural genes of the *achaete–scute* gene complex (Buescher *et al*., 2002; Overton *et al*., 2002). These experiments provide a possible mechanism by which SoxB1 factors maintain progenitor identity by direct inhibition of neuronal differentiation (Pevny and placzek, 2005). An alternative possibility is that *SoxB1* genes play an important role in maintaining neural progenitors in cell cycle and thus regulate the timing of their exit from cell cycle. The finding that the inhibition of *Sox2* in chick neural progenitor cells (Graham *et al*., 2003; Bylund *et al*., 2003) and rat OPCs (Kondo and Raff, 2004) resulted premature exit from mitosis supports this idea.

To date, however, neither expression nor genetic experiments have led to the identification of a direct transcriptional target of SoxB1 factors in neural progenitors. To begin addressing this issue, Tanaka *et al.* (Tanaka *et al*., 2004) have taken a candidate approach to identify direct targets of SoxB1 signaling. The intermediate filament protein Nestin is expressed by the majority of neural progenitors in the CNS from embryonic to adult stages. This study demonstrates that SoxB1 proteins interact with POU domain factors to activate a Nestin neural enhancer directly. Recombinant Sox2 and Brn2 proteins bind to sites within the enhancer, and mutational abolition of the Sox binding site eliminates the activity of the Nestin enhancer in electroporated chicken spinal cord and transfected neural stem cell lines (Tanaka *et al*., 2004). Using a chromatin immunoprecipitation

assay, Kan *et al.* confirmed that Sox1 binds to a putative consensus Sox binding sequence in the promoter of mouse basic helix-loop-helix transcription factor Hes1 (Kan *et al., 2004*).

In short, three main conclusions can be drawn. First, subsets of Sox transcription factors appear to prefigure, and predict, the ability of cells to adopt a neural fate. Second, expression of that same subset of Sox transcription factors sustains neural cells in a progenitor or stem cell mode, maintaining their ability to either proliferate or differentiate. Third, subsets of Sox transcription factors appear to have evolved in parallel to control the identity of neural progenitor or stem cells in the CNS and PNS (Pevny and Placzek, 2005).

1.5.2.5 Role of SoxB1 in placodes

SoxB1 genes are also expressed in placodes where they play different roles. *Sox2* and *Sox3* are expressed in the lens, otic and epibranchial placodes in chick (Abu-Elmagd *et al*., 2001; Ishii *et al*., 2001; Uchikawa *et al*., 2003) and zebrafish (Okuda *et al*., 2006), but only *Sox3* has been reported to be expressed in epibranchial placodes in frogs (Mizuseki *et al*., 1998) and *Medaka* (Köster *et al.*, 2000). It has been shown that *Sox2* together with *Pax6* is able to specify the ectoderm to lens fate (Kondoh *et al.*, 2004). In medaka, ectopic *Sox3* is sufficient to induce supernumerary otic vesicles (Köster *et al.*, 2000), suggesting that SoxB1 genes can induce placodal fate. In agreement with this role, ectopic *Sox3* in epibranchial territory induced ectopic ectodermal thickenings similar to placodal tissue (Abu-elmagd *et al., 2001*). On the other side, the latter experiments have shown the regulation of the number of epibranchial neuroblast by *Sox3*. In the ear, the role of Sox3 has not been addressed yet. In mouse, *Sox2*-deficient mice like *light coat and circling* (*Lcc*), and *yellow submarine* (*Ysb*), show hearing and balance impairment (Kiernan *et al.*, 2005a). This

correlates with the fact that mutations of *Sox2* in humans cause sensoryneural hearing loss (Hagstrom *et al.*, 2005).

1.6 Role of LMX1B in inner ear development

1.6.1 Structure of Lmx1b gene

Lmx1 is a member of the LIM-homeodomain (LIMHD) family of transcription factors that play a variety of roles during development to determine body pattern in vertebrates and invertebrates (Dawid *et al*., 1995; Curtiss *et al*., 1998). LIM proteins are found in invertebrate and vertebrate and have diverse biochemical functions. Originally named for the LIM domain-containing transcription factors Lin11, Isl1, and Mec3, the LIM protein super family includes cytoskeleton-associated proteins that contribute to cellular architecture, intracellular signaling proteins such as protein kinases and GTPase-activating factors, transcription factors, and transcriptional coactivators. At least one hundred LIM proteins are presently annotated as gene products of the human genome (NCBI, http://www.ncbi.nlm.nih.gov/). The LIM domain is a multi functional protein/protein interaction domain of approximately 50–60 amino acids. Each cysteine-rich LIM domain binds two zinc ions to form a finger-like structure. LIM proteins can contain single or multiple LIM domains that are sometimes found in association with other domains, including homeodomains, PDZ domains, kinase domains, SH3 domains, and GAP domains (Hunter and Rhodes, 2005). The homeodomains of LIM-HD proteins contain a characteristic signature that has been conserved through evolution; this signature is the amino acid sequence TGL at positions 38–40 in the homeodomain (Banerjee-Basu and Baxevanis, 2001).

Two paralogue genes, *Lmx1b* and *Lmx1a* are expressed in the inner ear in vertebrates, *Lmx1a* expressed in mice and *Lmx1b* in chick. Mice homozygotic mutant for *Lmx1a*, the Dreher mice, exhibit defects of three

classes of anatomic structures: the hindbrain, roof plate and neural crest derived tissues, and the axial skeleton (Millonig *et al.* 2000). Deafness may be the consequence of serious morphogenetic defects of the otic vesicle due to the dorsal neural tube abnormalities or to the direct effects of *Lmx1a* loss of expression in the inner ear. No direct human syndromes have been associated with *Lmx1a* dysfunction. On the other hand, humans Nail-patella syndrome (NPS) is characterized by developmental defects of dorsal limb structures, nephropathy, glaucoma and sensorineural hearing loss caused by heterozygous mutations in the LIM homeodomain transcription factor Lmx1b (Bongers *et al.*, 2005). However, the specific role of *Lmx1* in inner ear development and its relevance to hearing loss is still unknown. Understanding the molecular mechanisms of *Lmx1b* in inner ear development is part of this thesis.

Chick Lmx1b gene is located on chromosome 17 and contains eight exons spliced to give a ~1.4-kb mRNA. Alternative splicing results predicted protein of 377 amino acids with the molecular weight of ~42.6KDa. Chick *Lmx1b* has also 2 LIM-Zn domains and a Homeodomain (http://www.ensembl.org/Gallus_gallus/Gene/Summary?g=ENSGALG00 000000936).

1.6.2 Function of *Lmx1b* in development

Briefly, the role of *Lmx1b* in limb and midbrain/hindbrain development will be discussed as basis for understanding *Lmx1b* regulation during inner ear development.

1.6.2.1 Role of *Lmx1b* in Limb development

The vertebrate limb is a model system for studying pattern formation during development. The proper elaboration of this structure requires that cells of the developing limb integrate and respond to patterning signals

from each of the three limb axes: proximal-distal (P-D), anterior-posterior (A-P) and dorsal-ventral (D-V) (Johnson *et al*., 1994; Tickle, 1995).

Lmx1b expression begins at the onset of limb bud outgrowth and marks a sharp DV boundary that is maintained throughout embryonic development (Riddle *et al*., 1995; Vogel *et al*., 1995; Schweizer *et al*., 2004). Fate mapping studies suggest that this DV boundary is functional: cells that are specified as dorsal (*Lmx1b*-positive) or ventral (*Lmx1b*-negative) do not cross this boundary throughout the development of the limb (Arques *et al*., 2007; Pearse *et al*., 2007). *Lmx1b* is sufficient to induce dorsal fates when overexpressed in chick limbs, demonstrating that *Lmx1b* is a downstream determinant of dorsal cell fate (Riddle *et al*., 1995; Vogel *et al*., 1995). *Lmx1b* is also required for DV limb patterning in mice and humans. *Lmx1b*$^{-/-}$ limbs lack dorsal structures (Kania *et al*., 2008) and have duplications of ventral ectodermal and mesodermal derivatives, including the foot pads, ventral muscle masses, tendons, and ventral sesamoid bones (Chen *et al*., 1998).

The onset of *Lmx1b* expression is concurrent with the onset of *Wnt7a* expression in the overlying ectoderm (Riddle *et al*., 1995). *Lmx1b* expression in the distal limb appears to be dependent on signals from the dorsal ectoderm, since removal of the dorsal ectoderm results in a loss of *Lmx1b* expression in distal limb mesenchyme (Riddle *et al*., 1995; Vogel *et al*., 1995). Cells expressing *Wnt7a* are sufficient to maintain *Lmx1* expression in the distal limb mesenchyme in the absence of limb ectoderm, indicating that Wnt7a is sufficient to activate *Lmx1* (Riddle *et al*., 1995). *Lmx1b* expression can also be induced in ventral mesenchyme in response to ectopic *Wnt7a* in the ventral ectoderm (Riddle *et al*., 1995; Vogel *et al*., 1995). It has been suggested that *Lmx1b* mediates the dorsalizing Wnt7a signal, since ectopic expression of *Lmx1b* in ventral mesenchyme of the chick limb is sufficient to dorsalize the distal ventral limb in the absence of *Wnt7a* (Riddle *et al*., 1995; Vogel *et al*., 1995).

1.6.2.2 Role of *Lmx1b* in Midbrain-Hindbrain boundary establishment and Roof Plate Development

The development of the midbrain and cerebellum are controlled by the isthmic organizer (IsO), a constriction at the midbrain-hindbrain boundary (MHB). The IsO serves as one of the best models to study the molecular mechanism of the regionalization of the central nervous system (CNS) along the AP axis. The IsO is thought to secrete planar signals for regulating the development of the mid/hindbrain (Liu and Joyner, 2001b; Nakamura *et al.*, 2005; Wurst and Bally-Cuif, 2001).

In mouse, *Lmx1b* is expressed in the anterior embryo as early as E7.5, and becomes progressively restricted to the isthmus at E9.0. Among many genes that are expressed in the early embryo, *Lmx1b* is the earliest gene, besides *Otx2*, to be essential for mid/hindbrain development (Guo *et al.*, 2007).

The growth factor Fgf8 has been shown to be responsible for the inducting activity of the IsO. In mice the expression of *Lmx1b* precedes that of *Fgf8* and *Fgf8* expression is absent in the MHB in *Lmx1b*$^{-/-}$ embryos (Guo *et al.*, 2007). Importantly, misexpression of *Lmx1b* in chick embryos induces ectopic expression of *Fgf8* and causes expansion of the tectum and cerebellum (Matsunaga *et al.*, 2002), suggesting that *Lmx1b* is sufficient for the initiation of *Fgf8* expression. Conversely, knockdown of *Lmx1b* in zebrafish results in the loss of *Fgf8* expression (O'Hara *et al.*, 2005). These studies suggests that *Lmx1b* is required for the initiation of *Fgf8* expression in the IsO of mouse embryos and that *Lmx1b* is necessary and sufficient for the initiation of *Fgf8* expression in the MHB during IsO development, although the intrinsic molecular machinery may be different among the different species (Guo *et al.*, 2007).

Furthermore, it is interesting to note that misexpression of *Fgf8* in the chick embryo (Matsunaga *et al.*, 2002), or addition of Fgf8-coated beads

to mouse midbrain slice culture (Liu and Joyner, 2001a), is able to induce ectopic *Lmx1b* expression. Thus, *Lmx1b* and *Fgf8* appear to cross-regulate each other during the development of the IsO.

The secreted factor *Wnt1* is necessary for the proper development of the IsO and mice lacking *Wnt1* show abnormal development of the midbrain and hindbrain (McMahon and Bradley, 1990; McMahon *et al.*, 1992). *Lmx1b$^{-/-}$* embryos show downregulation of *Wnt1* in the MHB and complete lost by E9.5 suggesting that *Lmx1b* is required for the maintenance of *Wnt1* expression in the MHB. Misexpression of *Lmx1b* in chick embryos induces ectopic *Wnt1* expression (Adams *et al.*, 2000; Matsunaga *et al.*, 2002), and knockdown of *lmx1b* in zebrafish leads to the loss of *Wnt1* expression (O'Hara *et al.*, 2005). Whether *Wnt1* is directly regulated by *Lmx1b* is unclear. Because *Fgf8* is required for maintaining *Wnt1* expression in the MHB (Chi *et al.*, 2003; Reifers *et al.*, 1998), and *Fgf8* is absent in the MHB of the *Lmx1b$^{-/-}$* embryo, it is possible that the *Fgf8* activity mediates the maintenance of *Wnt expression* by *Lmx1b* in the MHB.
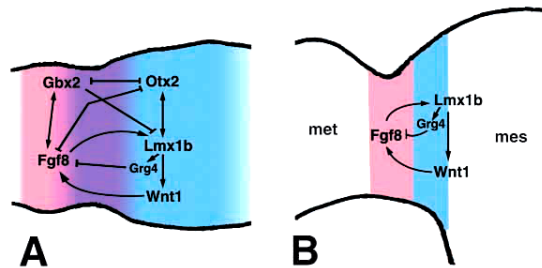
Figure 10: Role of Lmx1B and Wnt1 in isthmic organizing activity

At 10-somite stage, *Lmx1b* and *Wnt1* (blue) are expressed in the mesencephalon and metencephalon **A)**. Their expression overlaps with *Fgf8* (red) expression around the isthmic region. Gbx2 and Fgf8 induces each other's expression, and Gbx2 repressed *Lmx1b* expression. Otx2 and Gbx2 repress each other's expression. As a result of this complicated gene expression cascade, *Fgf8* expression may be set and kept in the isthmic region just rostral to the Lmx1b and Wnt1 expression ring by E2.5 **B)**. From Matsunaga *et al.*, 2002.

The IsO-related genes encoding transcription factors Pax2, En1 and En2 are expressed in the MHB (Liu and Joyner, 2001b; Nakamura *et al*., 2005; Wurst and Bally-Cuif, 2001; Ye *et al*., 2001). In mice expression of *Lmx1b* precedes that of *Pax2* and *En1*, and *Lmx1b* deletion downregulates their expression (Guo *et al*., 2007). Because the downregulation of these transcription factors occurred at a time prior to the initiation of *Fgf8* expression in the normal embryo, it is unlikely that this downregulation is caused by the loss of *Fgf8* activity in the mutant embryo (Guo *et al*., 2007). This does not exclude the possibility that *Fgf8* normally may contribute to the expression of these transcription factors at later stages, because *Fgf8* is required for their maintenance (Chi *et al*., 2003). A positive maintenance loop involving *Fgf8*, *Wnt1*, *En* and *Pax* genes during development of the IsO has been proposed (Joyner *et al*., 2000; Nakamura *et al*., 2005; Wurst and Bally-Cuif, 2001). This is based on the evidence that the loss of *En1*, *Pax2*, *Fgf8* or *Wnt1* function affects the maintenance but not the initiation of expression of these genes, with the exception of *Pax2* that is required for the initiation of *Fgf8* expression (Chi *et al*., 2003; Joyner *et al*., 2000; McMahon *et al*., 1992; Meyers *et al*., 1998; Wurst *et al*., 1994; Ye *et al*., 2001). This indicates that *Lmx1b* is a crucial component of the positive maintenance loop for IsO development.

In summary, there are two steps in the regionalization of the developing mid/hindbrain (Adams *et al*., 2000; Beddington and Robertson, 1998). The first step is the positioning of the future MHB in the early embryo, for which *Otx2* and *Gbx2* are two major players; this process is *Lmx1b*-independent because in the *Lmx1b$^{-/-}$* embryo do not show caudal shift of the *Otx2*+ domain, which marks the MHB. The second step of the regionalization begins when the IsO is established in the MHB. As a key component of the genetic pathway underlying the isthmic organizer activity, *Lmx1b* is responsible for the initiation of *Fgf8* expression and the maintenance of other key IsO genes (Guo *et al*., 2007).

Lmx1b is also expressed in the roof plate and floor plate of the neural tube preceding *Lmx1a* (yuan and Schoenwolf, 1999, Muller *et al.*, 2003). By using chick in ovo electroporation in conjuction with mutant mouse analysis, Chizhikiv and Millen (Chizhikiv and Millen, 2004) determined that in chick *Lmx1b* functions upstream of *Lmx1a* in the process of roof plate induction. Upregulation of BMP signaling by expressing constitutively active BMP receptors (Bmpr1a and Bmpr1b) resulted in expansion of dI1 progenitors and differentiated dI1 interneurons at the expense of other interneuronal cell types (Chizhikov and Millen, 2004). The same phenotype was observed after overexpression of *Lmx1b*. Interestingly, although in mice *Lmx1b* is not expressed in the caudal roof plate, *Lmx1b* overexpression in mice can partially rescue mice Dreher roof plate defects, again reinforcing the idea that both genes can have partially overlapping functions.

1.6.3 Dreher Mutants

Dreher (dr) is an autosomal recessive mutation in the mouse LIM homeobox gene, *Lmx1a* (Costa *et al.*, 2001). Adult homozygotes mice display a variety of behavioral phenotypes including ataxia, hyperactivity, and running in circles. CNS structures including the cerebellum, the hippocampus, and the cortex are hypoplastic (Millonig *et al.*, 2000; Sekiguchi *et al.*, 1994, 1996).

Major expression domains of *Lmx1a* include the dorsal midline (roof plate) of the neural tube, the cortical hem, the otic vesicles, the developing cerebellum and the notochord. The *Lmx1a* expression domains are probably responsible for the short tail (tail bud expression) and the deaf (otic vesicle expression) phenotype observed in homozygous dreher mouse mutants (Manzanare *et al.*, 2000; Failli *et al.*, 2002). Interestingly, *Lmx1a* is not expressed in developing limbs or gonads (Failli *et al.*, 2002). Only *Lmx1b* is expressed in the limb, IsO and the ear of the chick whereas

Lmx1a is not expressed in chick suggesting that both genes can play similar functions.

The early inner ear defects of dreher mice are preceded by defects in the shape and closure of the neural tube in the region of the hindbrain (Deol, 1964b). Complete examination of dreher mouse revealed that the overall process of segmentation and regional anterior-posterior specification is normal in the neural tube. However, in the otic region of the hindbrain the most dorsal population comprising the roof plate or dorsal roof is abnormal. Abnormal sensory and motor projections were also detected in the R4 to R6 territory which could be associated with deafness in dreher mice. However, they are unlikely to be the sole or major cause of loss of hearing as there are serious morphogenetic defects within the otic vesicle that arise due to the dorsal neural tube abnormalities. These are likely to have a major impact on hearing and so far analysis does not distinguish between primary and secondary phenotypes (Manzanares *et al.*, 2000).

A recent study of ear phenotypes of dreher mice indicate that major developmental defects in sensory epithelium are found although *Lmx1a* is expressed in non-sensory epithelia. It has been proposed that sensory and non-sensory regions reciprocally interact as loss of *Lmx1a* expands *Sox2* sensory expression (Nichols *et al.*, 2008).

1.6.4 Role of chick *Lmx1b* in ear development

Studies conducted by Giraldez (Giraldez 1998) analyzed the early expression of chick *Lmx1b* during inner ear development and its regulation by neural tube. The expression of *Lmx1b* in the preotic ectoderm starts before otic placode formation (Torres and Giraldez, 1998; Yuan and Schoenwolf, 1999; Abello *et al.*, 2007). *Lmx1b* is expressed in the otic field in 7 to 8 ss embryo. This is probably a landmark for otic field specification by which placode competence is restricted to the prospective ear region (Torres and Giraldez, 1998). In this connection, it

is interesting to note that before *Lmx1b* is detectable in the otic placode, it is expressed in a paraaxial band of dorsal ectoderm in the 4 to 5 ss embryo. Later in development, this expression stripe disappears and *Lmx1b* is not detected in other placode derivatives.
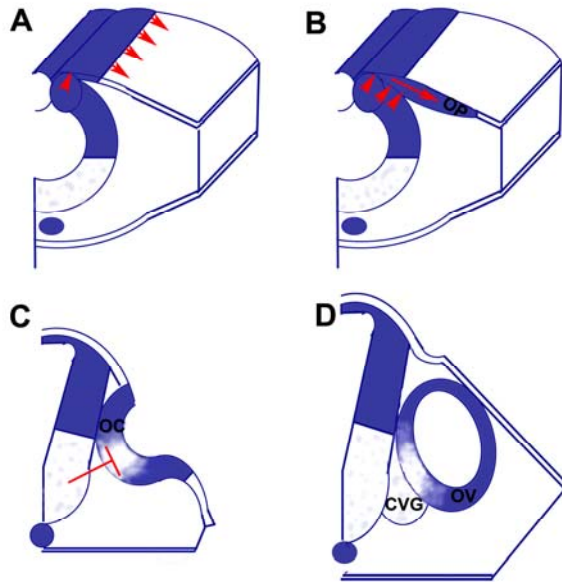
Figure 11: Model for patterning the otic vesicle by the neuroectoderm
Lmx1b is expressed in the neural tube and the paraxial stripe of ectoderm as a consequence of early dorsoventral/ mediolateral patterning. From there, dorsal neural activity induces the expression of *Lmx1b* in the otic placode. *Lmx1b* expression remains high in the otic vesicle but is downregulated by in the ventral neural tube, facing the medial and ventral aspects of the otic cup and otic vesicle. Assuming that *Lmx1b* specifies the dorsal character in the otic vesicle, the dorsalizing and ventralizing activities of the neuroectoderm would be spatially segregated to the dorsal and ventral neuroectoderms, respectively (Modified from Giraldez, 1998).

As the otic vesicle closes, *Lmx1b* expression is down regulated in the medial and ventral epithelium (the neurogenic domain) and high expression levels are maintained in the dorsal and lateral aspects. The expression pattern of *Lmx1b*, however, may be better described as dorsal–

posterior–lateral rather than simply dorsal. With this added complexity in mind, the asymmetry of *Lmx1b* expression suggests that, as in the limb, *Lmx1b* may also be involved in regional specification and the establishment of dorsoventral polarity of the otic vesicle (Fekete, 1996).

The requirement of the neural tube for the formation of the otic vesicle has been extensively documented *in vivo* (Yntema, 1933, 1950; Waddington, 1937; Jacobson, 1963; Gallagher *et al.,* 1996) and *in vitro* (Noden and Van de Water, 1986; Represa *et al.,* 1991). Neural tube removal experiments suggested that the formation of the otic vesicle is strictly dependent on the presence of the neural tube until the 6 ss (Giraldez, 1998). Beyond 7 to 9 ss the inner ear develops in the absence of the neural influence (stage 9 of Hamburger and Hamilton, 1951). The removal of either the dorsal and ventral neural tube had opposite effects on *Lmx1b* expression: the dorsal neural tube was able to induce and maintain the expression of *Lmx1b*, whereas the ventral neural tube down regulated *Lmx1b*. These two activities may contribute to the normal patterning of *Lmx1b* in the otic vesicle as illustrated in the model of Figure 11. This pattern would originate from two types of inductive interactions between the otic presumptive ectoderm and the neural tube.

The expression of *Lmx1b* in the otic placode may originate from the paraaxial ectoderm stripe expressing *Lmx1b*. This would require the neural ridge in the stages that precede the formation of the otic placode, 5 to 7 ss (Figure 11A). Thereafter, *Lmx1b* may be partially autonomous or maintained in part by the neural ridge, until stage 10 (Figure 11B). During invagination of the otic placode and the formation of the otic cup, the ventral aspect of the neural tube contributes to the down regulation of Lmx1b (Figure 11C). The final result is the normal expression pattern exhibited by the otic vesicle (Figure 11D). Since ventral ablations removed the notochord but dorsal ablations left the notochord in place, the possibility that the notochord is necessary for the ventralizing effect either

directly or indirectly by patterning the neural tube cannot be excluded (Giraldez, 1998).

One interesting question is the mechanism that triggers the expression of *Lmx1b* in the otic ectoderm. It may have at least two possible origins, which are not necessarily mutually exclusive. First, it may be set by cell to cell signaling by neural cells, probably the neural crest (Giraldez, 1998). This type of inductive mechanism has been recently proposed for the induction of the trigeminal placode (Stark *et al.,* 1997). Alternatively, it may be due to the migration of neuroectodermal cells and colonization of the otic placode (Giraldez, 1998). Cell tracing with intracellular dyes indicates that very few cells from the neural ridge of rhombomeres 4 to 6, which at some stage may be in spatial relation with the otic primordium, do incorporate into the otic vesicle (Sechrist *et al.,* 1993, 1994). Neural crest cells do migrate toward the otic vesicle, but they exclude the epithelium and only a small cell population incorporates to the VIII ganglion (D'Amico- Martel and Noden, 1983). It is more likely; therefore, that otic *Lmx1b* is induced by short-range signals coming from the dorsal neuroectoderm rather than the expansion of clonal population (Giraldez, 1998).

The other major question relates the nature of both positive and negative signals that may regulate *Lmx1b* (Giraldez, 1998). Lim genes are regulated by a variety of factors, and some of these factors or related genes are expressed in the neural tube. *Wnt7a* and *BMP4* have been shown to induce *Lmx1b* in the limb (Johnson and Tabin, 1997) and roof plate respectively (Chizhikiv and Millen, 2004).

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OBJECTIVES

2 OBJECTIVES

The first regionalization event detected in the otic placode is the specification of a neurogenic territory in the anterior otic field. We have hypothesized that anterior otic neural specification is mediated by restriction of *Sox3* in this domain. On the other hand, *Lmx1b* is complementary to the non-neurogenic territory. However, there are no functional studies on the role of both genes in the neurogenic versus non-neurogenic patterning event. The present thesis work was aimed at studying the role of both genes in early otic development by using chick as a model system.

Specific aimes proposed were:

1. To analyze the role of *Sox3* in the acquisition of neural fate

2. To study the role of *Sox3* in regulating patterning genes

3. To study the role of *Lmx1b* in early otic patterning

4. To study the regulation of *Lmx1b* by BMP signaling and the influence of BMP signaling in otic neural development

5. To study the role of *Sox3* and *Lmx1b* in promoting otic fate

MATERIAL AND METHODS

3 MATERIALS AND METHODS

3.1 Embryos

Chick embryos were used in all the experiments. Fertilized hen's eggs (Granja Gibert, Tarragona, Spain) were incubated at 38°C in an humidified atmosphere for appropriate times (Covatutto incubators). Embryos were staged according to the Hamburger and Hamilton staging table (Hamburger and Hamilton, 1992). Embryos were dissected from the yolk and fixed by immersion in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS; pH 7.4) at 4°C for 24-h.

3.2 Whole embryo explants in BD Matrigel Matrix

� Matrigel (Invitrogen, 354234) was defrost on ice at least 2-h before use.

� 10μl of Matrigel per well in a 24-well plate (NUNC, Roskilde, Denmark) was added to make a round-shaped dome and allowed it to solidify at room temperature (RT) for at least 10-minutes.

� Embryos were dissected out from egg around area pellucida and were gathered in a 4-well plate containing Medium 199 (Gibco, 22350) according to their developmental stage.

� Dissected embryos (one embryo/well of 24-well plate) were transferred with dorsal up on top of the Matrigel dome containing medium 199.

� Medium 199 was removed and embryos were left for 5-minutes (min) at RT to get attached to the Matrigel.

� Final solution of control or experimental culture media was added in each well containing embryo. Make sure that the embryos are covered with the medium (250-300μl/well). If embryos do not have enough medium the development of the DV axis will be affected.

� Incubation is carried out at 37.5ºC in a water-saturated atmosphere containing 5% CO_2.

Culture media

CONTROL	EXPERIMENTAL
♣ 2% FBS	♣ 2% FBS
♣ 1X Antibiotic Antimycotic	♣ 1X Antibiotic Antimycotic
♣ 4mM L-Glutamine	♣ 4mM L-Glutamine
♣ 1X DMEM	♣ 1X DMEM
	♣ 1µg/ml Noggin (in 0,1%BSA)

Noggin and DorsomorphinTreatment

In order to assess the role of BMP in early ear development, BMP pathway was blocked by recombinant mouse Noggin/ FC chimera (R&D systems, 719-NG). Noggin has been shown to be a high-affinity BMP (bone morphogenic protein) binding protein that antagonizes BMP bioactivities. Dorsomorphin inhibitor ((6-[4-(2-Piperidin-1-yl-ethoxy)phenyl]-3-pyridin-4-yl-pyrazolo[1,5-a]pyrimidine)) is a small compound recently found to specifically block the BMP type I receptors (Yu *et al.,* 2008). The inhibitor was used at 10µM in culture explants of chick embryos.

3.3. Assay of cell proliferation by BrdU incorporation

  ✴ Electroporated embryos were incubated with 10 µg/µl of 5-Bromo-2′-deoxyuridine (BrdU, Roche) prepared in Phosphate Buffer Saline (PBS) for 30-min before fixation.

  ✴ After in situ hybridization procedure, embryos underwent the process of DNA denaturation by incubating in 2N HCl for 30-min.

  ✴ Embryos were neutralized by washing in Sodium Borate pH 8.9 three times and processed for immunohistochemistry.

  ✴ BrdU mAb (BMC9318 antibody -Roche) was used in whole-mount at 1:200 dilution for analyzing replicating cells.

3.4. Assay of cell death by TUNEL

  ✴ Well electroporated embryos were sectioned and kept at -20°C

✰ Slides were taken out from -20°C and were left at 37°C for 2hrs to let sections adhere properly on the slides whenever TUNEL assay was made.

✰ Sections were hydrated with PBT (0.1%) and permeabilized with Proteinase K (sigma-P2308) with 1:1000 dilutions in PBT (0.1%) for 2 min at RT

✰ Slides were washed with PBT (0.1%) once for 5 min and fixed with 0.2% Gluteraldehyde and 4% PFA (1:100) for 20 min at RT.

✰ After washing with PBT (0.1%) for 15 min, slides were incubated in 0.3% hydrogen peroxide (H_2O_2)/ PBS for 5 min and were washed again once for 15 min with PBT (0.1%).

TUNEL reaction

Sections were incubated in TUNEL mix (Roche, 3333566) (5ul of TdT enzyme + 45ul of Reaction mix) for 60 min at 37°C in humidified chamber in dark and were washed twice for 10 min with PBS.

3.5 Immunohistochemistry

3.5.1 Immunohistochemistry on whole mounts

✰ Embryos were fixed with 4% PFA in PBS at 4ºC (O/N after dissection or 30 min after ISH).

✰ Embryos were washed 3 x 10 min in 1xPBS and then 3 x 10 min in 1% PBT (PBS plus 1%Tween-20)

✰ Blocking was done at room temperature with Blocking Solution (heat inactivated and filtered 10% Goat Serum (GS) and filtered 3% Bovine Serum Albumin (BSA) in 1%PBT) for 60 min

✰ After incubating O/N at 4ºC with the primary antibody diluted in blocking solution with a concentration of 1:50 to 1:800 (see TABLE-1 for each antibody dilutions), embryos were washed with 1%PBT for several times (preferably whole day) at RT on rocker.

✰ Embryos were incubated with secondary antibody in blocking solution O/N at 4ºC in dark (see TABLE-1 for antibody dilutions).

✱ After rinsing several times in 1%PBT in dark, embryos were preserved in 100% glycerol and kept in dark until taken images and/or sectioned.

3.5.2. Immunohistochemistry on frozen sections

✱ Slides were taken out from -20°C and left at 37°C for 2hrs to let sections adhere properly on the slides.

✱ Slides were washed 3 x 10 min in 0.1% PBS, then 3 x 10 min in 0.1% PBT

✱ Blocking of the sections was done in 10% GS, 3% BSA prepared in 0.1%PBT for 1hr by adding 100μl on top of the slide and covered with paraffin.

✱ Paraffin was removed carefully and 100μl of primary antibodies prepared in blocking solution with appropriate dilution was added.

✱ Incubation in primary antibodies was carried overnight at 4°C in humidified chamber.

✱ Slides were washed several times in 0.1% PBT (preferable whole day) and slides were incubated with secondary antibodies prepared in blocking solution with appropriate dilution for 4hrs ON at 4°C covered with paraffin and kept in humidified chamber.

✱ After washing sections in PBT and PBS, slides were mounted in Mowiol

Protein	Polyclonal/ Monoclonal	Company	Dilution
PRIMARY ANTIBODIES			
GFP	Rabbit polyclonal	Molecular probes	1:400
BrdU	Monoclonal	Roche	1:200
Beta-tubulin III	Rabbit polyclonal	Covence	1:400
Sox2	Goat polyclonal	Abcam	1:400
Sox3	Monoclonal	Abcam	1:400
Islet	Monoclonal	Developmental Studies Hybridoma Bank; DSHB	1:400

SECONDARY ANTIBODIES			
Mouse IgG-alexa 488	Goat polyclonal	Molecular probes	1:400
Mouse IgG-alexa 594	Goat polyclonal	Molecular probes	1:400
Rabbit IgG-alexa 488	Goat polyclonal	Molecular probes	1:400
Rabbit IgG-alexa 594	Goat polyclonal	Molecular probes	1:400

Table 1: Antibodies used for the experiments described in this thesis

3.6. Antisense RNA probe synthesis for in situ hybridization

This process takes place in two steps:

1) DNA linearization and 2) DIG labelled RNA transcription.

DNA linearization

* DNA sequence to be transcribed was cloned into a vector and flanked by T7, T3 or SP6 RNA-polymerases sequence. Detail of the constructs to be transcribed is shown in TABLE-2.

* Circular DNA was linearized using appropriate restriction enzyme with a final volume of 100μl in 1.5ml eppendorf tube as followed

DNA to be linearized	10-15μg
Digestion buffer	1X
Restriction enzyme	15U
Water	up to 100μl

* The above reaction mix was kept at 37°C for 2hrs.

* Protein degradation was done at 37°C for 30 minutes in the presence of 5μl of Proteinase-K [from the stock of 10 mg/ml to make final concentration of 500μg/ml] + 5μl of SDS [from the of stock 10 % to make final concentration of 0.5%].

* DNA was purified by adding equal volume of phenol (100μl) and the mix was centrifuged for 5 minutes at 4°C at13 rpm.

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✳ Aqueous solution (upper layer) was transferred in another eppendorf tube and the lower layer was discarded.

✳ 0.5X volume of phenol (50μl) and 0.5X volume of chloroform (50μl) was added in the upper layer containing DNA and centrifuged for 5 minutes at 4°C at 13 rpm.

✳ Upper layer was transferred in another eppendorf tube and the lower layer was discarded.

✳ DNA was precipitated by adding 2.5X volumes of 100% ethanol [250μl] and the eppendorf tube was left for 30 minutes at –20°C.

✳ Precipitated DNA was recovered by centrifuging for 15 minutes at 4°C at 13 rpm.

✳ Supernatant was thrown away

✳ DNA was further cleaned up by adding 1ml of 70% ethanol followed by centrifugation for 15 minutes at 4°C at 13 rpm

✳ Pellet containing DNA was kept for 10 minutes at 37°C to evaporate rest of the ethanol and was resuspended in 10μl of water.

✳ 1% agarose gel was run with 1μl of linearized and non-linearized DNA in 6X loading dye.

DIG Labeled transcription

✳ DIG mix was prepared in a final volume of 20 l in 1.5ml eppendorf tube as followed

✳ The Dig Mix was mixed well by vortexing and spinning down and then transcription mixture was prepared in 1.5ml eppendorf tube as followed

NOTE: T7, T3 or SP6 polymerases (Roche) are used for RNA transcription

DIG Mix	
↓ Water	7 μl
↓ UTP [1:10diluted]	6.5 μl
↓ Digoxigenin-11-UTP (Roche)	3.5 μl
↓ ATP (Roche)	1 μl
↓ CTP (Roche)	1 μl
↓ GTP (Roche)	1 μl

- ✶ The Transcription Mix was kept at 37°C for 2hrs in order to transcribe DNA.

- ✶ DNA was degraded by adding 2μl of DNase-1 RNase free in the mix at 37°C for 15 min.

- ✶ DNA was precipitated in a total volume of 100μl by adding 10μl of 4M LiCl and 3 volumes of ethanol followed by vortex and centrifugation at 4°C for 10 minutes at 13rpm.

- ✶ Pellet of RNA was recovered and purified by adding 70% ethanol (500μl) along with centrifugation at 4°C for 10 minutes at 13 rpm.

- ✶ Purified RNA pellet was left at 37°C for not more than 10 minutes in order to evaporate ethanol.

- ✶ RNA pellet was resuspended in 10μl of H_2O.

- ✶ RNA transcription was confirmed by running 1% agarose gel.

Transcription Mix	
Buffer	2 ul
DIG mix (1X)	4 ul
Linearized DNA	1μg
RNase inhibitor (40U)	1 ul
Polymerase (40U)	2 ul
Water	up to 20 μl

3.7. Gene expression analysis by In Situ Hybridization (ISH)

3.7.1. In Situ Hybridization (ISH) on whole embryo

- ✶ Dissected embryos were fixed in 4% PFA for O/N.

- ✶ A cut in the head of the embryos was made to facilitate the free entry of the probe inside the embryo.

- ✶ Whole-mount in situ hybridization was carried out according to Wilkinson and Nieto (1993). Briefly the following protocol was followed.

Pretreatment

- ✶ Embryos were washed 2x5 min with PBS and passed through the series of 10 min dehydration steps [methanol-25%, 50% 75% and 100%] followed by rehydration series.

- ✶ After 2x10 min washes with 0.1%PBT, embryos were permeabilized by incubating in 10ug/ml Proteinase K (in winter) or 5μg/ml (in summer) diluted in 0.1%PBT at RT for 5-60 min without shaking depending on the stage of the embryos and laboratory temperature.

Proteinase K solution

- ♣ 50mM TRIS pH7.4
- ♣ 5mM EDTA pH8
- ♣ Proteinase K (10mg/ml; 1/1000)
- ♣ ddH$_2$O final volume

Time (min)	Stage
5	0-4 ss
7	5-10 ss
10-15	HH11-14
30-40	HH15-18

- ✶ Embryos were carefully washed 2X5 min with 0.1%PBT at RT and were refixed immediately for 20 minutes in 0.2% Gluteraldehyde-4%PFA at room temperature.

- ✶ After 2X5 min washes with 0.1%PBT at RT, embryos were ready for hybridization process.

Hybridization

- ✶ Embryos were prehybridized in pre-hot hybridization buffer for 1hr at 70°C.

⚹ Probes were taken out from –20°C and a heated at 80°C for 2 minutes.

⚹ Probes were diluted in prewarmed hybridization buffer in 1:100 to 1:500 dilution and embryos were incubated in hybridization buffer ON at 70°C.

⚹ Following day probes were kept in an eppendorf tube to recycle. Probes can be recycled for 4-5 times.

> **Hybridization buffer:**
> - 50% Formamide (FAD)
> - 5x SSC pH 4.5
> - 1% Sodium dodecyl sulphate (SDS)
> - 50 μl/ml yeast RNA
> - 0.05 mg/ml heparin
> - ddH$_2$O final volume

Washing

⚹ Embryos were washed 3x30 min with wash solution 1 at 70°C, followed by [3x30 min washes in solution 2 at 65°C.

> **Wash1:**
> - 50% FAD
> - 5x SSC pH 4.5
> - 1% SDS
> - ddH$_2$O final volume

> **Wash2:**
> - 50% FAD
> - 2x SSC pH 4.5
> - ddH$_2$O final volume

⚹ Final 2X5 min washes were done with TBST

Immunohistochemistry

⚹ Blocking was done for 60 min at RT in blocking solution [10 % GS in 0.1% TBST]. Then embryos were incubated with anti-dig antibody diluted in blocking 1:4000 for 2 hrs at RT.

⚹ After 3X5 min washes with 0.1% TBST embryos were left O/N in 0.1% TBST.

Color development

✱ Next day 2x5 min wahes of 0.1% TBST were performed followed by 3X10 min washes with NTMT at RT.

> **NTMT:**
> - 100mM NaCl
> - 100mM TRIS pH 9.5
> - 50mM $MgCl_2$
> - 0.1% Tween-20
> - ddH_2O final volume

✱ Colour reaction was developed with NBT/ BCIP mix ((3,5µl NBT and 4,5µl BCIP for 1ml of TBST) (Roche Diagnostics) in dark at RT and embryos were observed after every 15 minutes.

✱ Once appropriate colour was achieved, embryos were rinsed with 0.1% PBT [4x15 minutes or O/N if required] and fixed in 4% PFA at RT for 20 min on rocking.

✱ Final rinsing was done in 0.1% PBT.

3.7.2. In Situ Hybridization (ISH) on sections:

✱ All the slides that are needed to be preceded for ISH were taken out of -20°C and were kept at 37°C for 2hrs to avoid any detachment or flip of the sections during ISH procedure.

✱ Probe was denatured at 80°C for 2 minutes and was spin down.

✱ Denatured probe was diluted in pre-warmed hybridization buffer with appropriate concentration or pre-warmed recycled probe was used.

✱ 100µl of diluted probe was put on top of each slide and slide was covered with paraffin (Note: paraffin should be bigger than the slides to avoid uncovering of any section after paraffin shrinkage due to overnight incubation at 70°C in a humidified chamber).

✱ Next day slides were washed with wash solution 1 (see above) for 3x30 min followed by wash solution 2 (see above) for 3x30 min.

✴ After wash 3 X 30 min with 0.1% TBST, sections are ready for blocking for 1hr (see above) and immunohistochemistry with anti-dig diluted 1/4000 in 10%GS in TBST (see above).

✴ After 3X5 min washes with 0.1%TBST embryos were left O/N in 0.1%TBST.

Color development

✴ Next day wash once again with 0.1% TBST followed by 3x 10min washes with NTMT at RT.

✴ Colour reaction was developed in dark at RT by putting 100μl of NBT/ BCIP mix (as above) (Roche Diagnostics) mix on top of each slide and covered with paraffin.

✴ Sections were observed after every 15 minutes for the colour development.

✴ Once appropriate colour is developed, slides were rinse with 0.1% PBT [4x5 minutes] and fixed in 4% PFA for 20 min at RT on rocking.

✴ Final rinsing was done in 2x5 min 0.1% PBT.

3.7.3. In Situ Hybridization (ISH)-DNase treatment to avoid cross-hybridization in Electroporated Embryos

In order to avoid cross hybridization of embryos that are electroporated, whole mount in Situ hybridization was done according to Arede N and Tavares A T (2008) with slight modifications.

Only differences with the standard protocol are illustrated here

Pretreatment

✴ Embryos were hydratated and rehydrated in methanol series (see standard ISH protocol)

✴ Embryos were washed with 0.1% PBT [2x10 minutes]

✴ Electroporated embryos were treated with RNase-free DNase I (Ambion- 50U/ml in DNase I buffer) for 1hr at 37°C in order to remove electroporated vector DNA.

✲ After bleaching embryos with 6% hydrogen peroxide in 0.1% PBT for 1h, embryos were rinsed in 0.1% PBT [3 X 5 min].

✲ Embryos were permeabilized by incubating in Proteinase K (see above).

✲ Embryos were carefully washed with 0.1% PBT [2X5 minutes] at RT followed by fixing once in 2mg/ml glycine-0.1% PBT for 20 min.

✲ Post fixing was done with 0.2% Gluteraldehyde-4%PFA [1:100] dilution at RT [1X20 minutes] followed by washing with 0.1% PBT at RT [2X5 minutes].

Hybridization

✲ Embryos were prehybridized in pre-heated hybridization buffer for 1hr at 70°C.

✲ Probes were taken out from -20°C and given a heat shock at 80°C for 2 min.

✲ Probes were diluted in hybridization buffer [hot] in appropriate dilution.

✲ Embryos were incubated in diluted probes for O/N at 70°C.

Hybridization buffer:

⬥ 50% Formamide (FAD)

⬥ 5x SSC pH 4.5

⬥ 0.1% Tween®20

⬥ 50 μl/ml yeast RNA

⬥ 50 μg/ml heparin

⬥ 50 μg/ml salmon sperm DNA

⬥ ddH$_2$O final volume

Washing

✴ Embryos were washed with wash solution 1 [3X30 minutes] at 70°C followed by wash solution 2 [3X30 minutes] at 65°C.

Wash1:
+ 50% FAD
+ 4x SSC pH 4.5
+ 1% SDS
+ H$_2$O final volume

Wash2:
+ 50% FAD
+ 2x SSC pH 4.5
+ H$_2$O final volume

MABT
+ 100 mM maleic acid
+ 150 mM NaCl (pH 7.5)
+ 0.1%Tween®20

Immunohistochemistry and colour development

Blocking and incubation with anti-Dig antibody was performed as described in standard ISH procedure. MABT instead of TBST was used for diluting blocking solution. Colour development was performed as standard ISH procedure.

Gene	Vector	Origin	Reference	RE site/ RNA pol
BMP4		A. Graham		BamHI/T3
Serrate1	Blue Skript-KSII (-)	D. Henrique		HindIII/ T7
Delta1	Blue Skript-KSII (+)	D. Henrique	Henrique al., 1995	EcoRI/T3
fgf10	pGEM-Teasy	T. Shimmang	Ohuchi *et al.*, 1997	NcoI/ SP6
FGF-8	Blue Skript-KSII (-)	G. Martin		EcoRI/T7
Lunatic Fringe		Cliff Tabin	Luafer et al, 1997	ClaI/T3
Hairy1	Blue Skript-KSII (+)	D. Henrique	Palmeirim *et al.*, 1997	HindIII/ T7

Hes5-2	EST	D. Henrique	Fior and Henrique 2005	Not/T3
Irx1	EST	Ruth Diez del Corral		Not/T3
Lmx1		M. Maden	Giraldez, 1998	EcoRI/T3
MafB	Blue Skript-KSII (-)	M. Nishisawa	Eichmann 1997	NotI/T3
Msx1		Paola Bovolenta		ApaI/T3
NeuroD	Blue Skript-KSII (+)	D. Henrique	Luafer et al, 1997	EcoRI/T3
Ngn1		A.Graham		SacI/T7
Pax2		T. Schimmang		XbaI/T3
cSerrate1	Blue Skript-KSII (-)	D. Henrique	Henrique *et al.*, 1997	HindIII/ T7
Sox2	Blue Skript-SK	P. Scotting		SmaI/ T7 (-HMG box)
Sox2	Blue Skript-SK	P. Scotting	Rex *et al.*, 1997	BamHI/ T7
Sox3	Blue Skript-SK (+)	P. Scotting?	Rex *et al.*, 1997	SalI/T7
Tbx1	EST	MRC Geneservice		Not1/T3
Wnt1	Blue Skript-SK	O. Pourquie		ClaI/T7
Krox20	Blue Skript-KSII (-)	P Charnay		Stu1/T7

Table 2: List of probes used in the experiments

3.8. In Ovo electroporation

✯ Eggs were incubated horizontally until required stage is obtained.

✯ An oval window was made to open up the eggs and diluted Fast green was poured on top of the embryo to visualize embryos.

✯ A small hole was made into the vitelline membrane to expose the otic placode.

✯ Electroporation was done in two different ways depending on the stage of the embryos.

✯ Briefly for younger embryos the cathode platinium electrode was placed next to the otic territory and anode electrode underneath the embryo. For older embryos both cathode and anode platinum electrodes were kept parallel on top of the embryo beside the otic cups

✯ The desired vector mixed (final concentration 1-2ug/ul) with 0,5-1ul of fast green (3mg/ml) was electroporated by injection onto the otic placode/ otic cup by gentle air pressure through a fine micropipette.

✯ Square pulses (4-6 pulses of 8-10 V) were generated by an electroporator Square CUY-21 (BEX Co., LTd, Tokiwasaiensu, Japan).

✯ Medium-199 was added immediately after each electroporation.

✯ Eggs were sealed by squash tap and incubated for 6-20h.

✯ Embryos were collected based on GFP and fixed overnight in 4% PFA at 4ºC for further analysis.

1. Open oval window

2. Set electrodes for electroporation and inject vector

3. Sealed back the eggs

4. Select GFP+ embryos
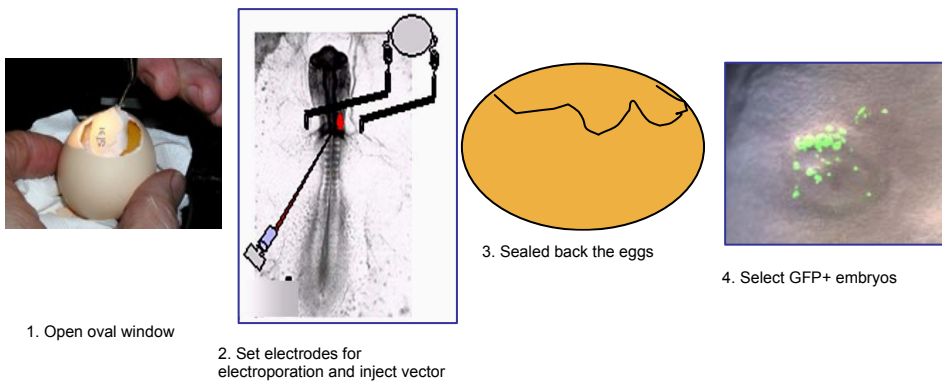
Figure: Illustrated method showing electroporation

3.9. In Ovo gene silencing by electroporation of specific morpholinos

Morpholinos specific for the starting translation site of chick *Lmx1b gene* were injected by electroporation. As a control random 5 mismatch sequences of the Morpholinos specific for *Lmx1b* were used.

- ✶ Briefly, antisense morpholino oligonucleotides (MOs) designed to target the 5' region of *Lmx1b* were obtained from Genetools (Philomath, OR) tagged with Carboxyfluorescein.

- ✶ The MO sequences were:

- ✶ *Lmx1b*-MO 5'- CTTGATGCCGTCCAGCATCTTGGCG-3'

- ✶ Embryos were injected with a mixture of *Lmx1b*-MO (stock concentration of 1uM) and were further diluted with Medium 199 to achieve final concentration of (750 nM).

- ✶ *Lmx1b*-MO were injected through electroporation in the same way as described before

- ✶ Embryos were selected for Carboxyfluorescein fluorescence.

3.10. Quantification of gene expression by quantitative real time Polymerase chain reaction (QRT-PCR)

3.10.1. Embryos dissection

- ✶ Electroporated embryos after 6hrs and 20hrs of post electroporation were dissected out

- ✶ Embryos were first taken out from the eggs and collected in PBS.

- ✶ Embryos showing GFP in right otic side of the embryos were selected for RNA extraction.

- ✶ For RNA extraction, the embryo was first cut from the middle of the NT and head and tail was removed

- ✶ Very carefully the two otic sides were separated and GFP positive right side was directly added in an eppendorf containing TRIZOL (invitrogen) placed in ice. The same procedure was followed for the non electroporated left side used as a control.

- ✶ The whole dissection procedure was done rapidly and placed immediately on the ice in order to avoid any RNA degradation.

✯ 5-6 well-electroporated embryos were dissected in 70μl of TRIZOL reagent and proceded for RNA extraction as described by the manufacturer.

3.10.2. RNA extraction

3.10.2.1 Phase Separation

✯ Homogenized samples were incubated for 5 minutes at 15 to 30°C to permit the complete dissociation.

✯ 0.2 ml of chloroform per 1 ml of TRIZOL® Reagent was added and sample tubes were caped securely.

✯ Tubes were shaken vigorously by hand for 15 seconds and incubated at 15 to 30°C for 2 to 3 minutes.

✯ Samples were centrifuged at no more than $12,000 \times g$ for 15 minutes at 2 to 8°C.

✯ Following centrifugation, the mixture separates into a lower red, phenol-chloroform phase, an interphase, and a colorless upper aqueous phase.

✯ RNA remains exclusively in the aqueous phase. The volume of the aqueous phase is about 60% of the volume of TRIZOL® Reagent used for homogenization.

3.10.2.2. RNA Precipitation

✯ The aqueous phase was transferred to a fresh tube.

✯ RNA was precipitated from the aqueous phase by mixing with isopropyl alcohol. 0.5 ml of isopropyl alcohol per 1 ml of TRIZOL® Reagent was used for the initial homogenization.

✯ Samples were incubated at 15 to 30°C for 10 minutes and centrifuged at no more than $12,000 \times g$ for 10 minutes at 2 to 8°C.

✯ The RNA precipitate, often invisible before centrifugation, forms a gel-like pellet on the side and bottom of the tube.

3.10.2.3. RNA Wash

✯ Supernatant was removed.

✴ RNA pellet was washed once with 75% ethanol, adding at least 1 ml of 75% ethanol per 1 ml of TRIZOL® Reagent used for the initial homogenization.

✴ The sample was mixed by vortexing and centrifuged at no more than $7,500 \times g$ for 5 minutes at 2 to 8°C.

3.10.2.4. Redissolving the RNA

✴ At the end of the procedure, the RNA pellet was briefly dried (air-dry for 5-10 minutes). Note not to over-dry RNA pellet as decreases its solubility.

✴ Dissolve RNA in RNase-free water by passing the solution a few times through a pipette tip, and incubating for 10 minutes at 55 to 60°C.

✴ Check the RNA purity by calculating A260/280 ratio and RNA quantity by (Nanodrop). Partially dissolved RNA gives a A260/280 ratio < 1.6.

3.10.3. First-Strand cDNA Synthesis

First strand cDNA synthesis was done according to the manufacturer (Invitrogen) supplied with SuperScriptIII reverse transcriptase. The following procedure is designed to convert total RNA into first-strand cDNA (and works in a range of initial concentration of RNA of 10pg to 5μg):

✴ Each component was mixed and briefly centrifuged before use.

✴ The following components were combined in a 0.2- or 0.5-ml microcentrifuge tube:

> **RNA/primer mixture** (Vf=20 μl)
>
> ◈ up to 10pg-5 μg total RNA
> ◈ Primer
> - 50 μM oligo(dT)$_{20}$, *or*
> - 50-250 ng/μl random hexamers
> ◈ 10 mM dNTP mix (10mM of each dATP, dCTP, dGTP, dTTP at neutral pH)
> ◈ Sterile distilled water 13μl

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✶ The tubes were incubated at 65°C for 5 min, and placed on ice for at least 1 min.

✶ The content of the tube was collected by brief centrifugation and the following cDNA Synthesis Mix was prepared, adding each component in the indicated order.

> **cDNA Synthesis Mix**
> ♣ 4μl 5X First strand buffer
> ♣ 1μl 0.1 M DTT
> ♣ 1 μl RNaseOUT (40 U/μl)
> ♣ 1 μl SuperScript. III RT (200 U/μl)

✶ 10μl of cDNA Synthesis Mix was added to each RNA/primer mixture

✶ After mixing gently, the content was collected by brief centrifugation.

✶ The mix was incubated as followed.

✶ Oligo(dT)20: 50 min at 50°C

✶ Random hexamer primed: 10 min at 25°C, followed by 50 min at 50°C

✶ The reactions were terminated at 70°C for 15 min. Chill on ice.

✶ The reactions were collected by brief centrifugation.

✶ cDNA synthesis reaction was either stored at -20°C or used for PCR immediately.

3.10.4. Gene Expression Analysis by qRT-PCR

✶ Gene expression was assessed by real-time PCR using the Roche LightCycler 480.

✶ PCR reactions were run in triplicate using 5-20ng cDNA per reaction

✷ 400 M forward and reverse primers (Table-3) with 2X SYBR Green PCR master mix (Applied Biosystem) and 50 cycles of amplification (95°C 10 s; 60°C 10 s; 72°C 10 s).

✷ Specificity of primers was determined by melt curve analysis, and standard curves were generated to control for primer efficiency.

✷ Gene expression was determined by either relative or absolute quantification

Gene	Primer sequences (F)	Primer sequences (R)
GADPH	TTGGCATTGTGGAGGGTCTT	GTGGACGCTGGGATGATGTT
PAX2	ATGTTCGCTTGGGAGATTCG	CTGGTGTTGGGTGGAAAGGTT
NGN1	AGCGGAACCGCATGCA	AGGGCCCAGATGTAGTTGTAAGC
NEUROD	GCTACTCCAAGACGCAGAAACTC	CACAGCGTCTGCACGAATG
FGF8	TCAGTCCCCACCTAATTTTACACA	GTCCAAGATCTGCACGTGCTT
FGF10	CAAGGAGACGTGCGCAAGA	CTACAGATGTTATCTCCAATATGCTGAA
HAIRY1	GGCGCGCATCAACGA	TGGAGGTTCCTCAGGTGCTT
LMX1B	AGCGACTGTTTTCTTGCCTCTT	GCCGGCTGTTTGTTTTCGT
SERRATE1	GGTGCCCAGTGCTTCAATCT	CTGTACAGCTGTCGATTACTTCACAA
TBX1	CAGGACAGCCTATGCCAACA	CGTGTCACGATCATCTGGTTACA
HES5.2	GGATGCCTGGCTTCCCTTCTT	AGTCGGCGTTCCTCTCTGAA
SOX3	GATTGACACTTACGCCCATATGAAC	CATGTCGTAGCGGTGCATCT
SOX2	ACAACGCGGCGCAGAT	GTCCCTTGCTGGGAGTACGA

Table 3: List of primers used for the RNA quantification by qRT-PCR

3.10.4.1. Data analysis and interpretation in qRT-PCR

The RNA concentration in the sample was quantified by comparing the Ct obtained from the sample with the Ct obtained from a dilution series of a

standard, (we used RNA from control non electroporated side of the embryo for this purpose), in the same run. The Ct values of the standard were plotted against RNA copy number to generate a standard curve.

The accuracy of quantification by real-time PCR depends on the quality of the standard curve. Major sources of error in quantitative real-time PCR are the measurement threshold, variations during sample preparation and pipetting errors. If the sample contains inhibitors, or the probe has lost fluorescence, absence of amplification, erroneously high Ct values or low signal strength may occur. The accuracy of quantitative real-time PCR is also dependent on variations in PCR efficiency. The efficiency of PCR reactions can be estimated from the slope of the crossing threshold against copy number line. The expected slope at 100% PCR efficiency is 3.3 cycles per tenfold concentration difference. Real-time PCR results are dependent on the optimal master mix reagents, since the amount of the fluorescent signal can be affected by the correct amount of polymerase, the optimal concentration of dNTPs, MgCl2, primer and probe. The purity of the probe is also important. Mistakes during synthesis or mishandling (e.g. exposure to light, possibly also freezing and thawing) can adversely affect probe function

RESULTS

4. RESULTS

CHAPTER I. ROLE OF SOX3 IN PRONEURAL SPECIFICATION

4.1. Sox3 is regionally expressed in the proneural domain

Otic neurogenesis in chick starts at the otic placode/otic cup transition stage (HH11) in the anterior otic epithelium as revealed by the expression of *Ngn1* and *Delta1* at the level of rhombomere 5 of the hindbrain (Henrique *et al.*, 1995; Adam *et al.*, 1998; Alsina *et al.*, 2004). Figure 12A show the expression of *Sox3* in an ectodermal band encompassing the anterior otic placode/cup and the epibranchial placodes. In the same domain otic neurogenesis starts with the appearance of scattered neuronal precursors expressing *Delta1* (Figure 12B). At higher magnification the expression of the above mentioned genes is confined to the proneural territory where neurogenesis takes place during inner ear development (Figure 12C and D). The proneural territory labeled with *FGF10* becomes ventral with the invagination of the otic pit (Figure 12E and F) where otic neuroblasts (in red) immunostained for the transcription factor Islet1 delaminate from the anterior neurogenic epithelium and populate the ventral mesenchymal space surrounding the otic cup (Figure 12G and H). Complementary to the proneural epithelium, *Lmx1b* is expressed in the non-neural otic subdomain (Figure 12I and J).

One of the main interests of our laboratory is to understand when and how neural competence is acquired in the otic territory. As expression of the *SoxB1* genes directly correlates with ectodermal cells that are competent and committed to a neural fate (Rex *et al.*, 1997; Pevny and placzek, 2005), we hypothesized that the expression of *SoxB1* genes could foreshadow the establishment of a proneural domain in the otic field. It has already been shown that *Sox3* appears in the otic ectoderm at 6 somites in chick, after the expression of the otic marker *Pax2* (Groves and Bronner-Fraser, 2000). However, as Sox3 is also expressed in the

underlying endoderm, a detailed analysis of the ectodermal expression in the otic territory before the otic placode is morphologically visible (also referred as preotic stages) was missing. Previous studies have demonstrated that two members of SoxB1 family of transcription factors, the chick *Sox2* and *Sox3* genes were expressed in the CNS in similar but not with identical patterns. *Sox3* is expressed throughout the epiblast before primitive streak formation and becomes progressively restricted to the forming CNS, while *Sox2* appears in the presumptive neural ectoderm at the time of neural induction before the CNS becomes morphologically apparent, and its expression is then restricted to the CNS as it develops. Careful examination of *Sox2* and *Sox3* expressions at preotic placode stages was performed after dissecting out the underlying mesoderm and endoderm tissue. At 5-6 somites Sox2 and *Sox3* was diffusely expressed in a broad area of the ectoderm from rhombomere 3 (R3) to rhombomere 6 (R6).
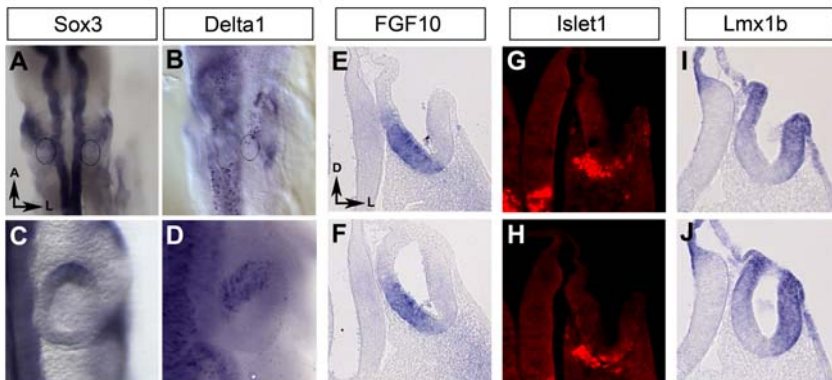
Figure 12: Patterning of the otic cup into neurogenic and non-neurogenic epithelium
A and C) Whole mount ISH of *Sox3*. B and D) Whole mount ISH of *Delta1*. E and F) Transversal sections of *Fgf10*. G and H) Transversal sections of Islet1. I and J) Transversal sections of *Lmx1b*. A: Anterior, L: Lateral, D: Dorsal, Circles mark otic placodes

Sox2 expression was very weak in comparison to *Sox3* and *Sox2* expression was artificially enhanced in these pictures (Figure 13A-C). The

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relative broad expression of *Sox3* progressively became refined and enhanced to the more rostral part of the preotic territory from 7-8 to 9-10 somites. The highest levels of *Sox3* draw a lateral to medial band encompassing the geniculate placode and part of the otic field (Figure 13D-F) foreshadowing a putative neural competent territory shared between both placodes. On the other hand *Sox2* was broadly expressed in ectoderm from rhombomere 3 (R3) to rhombomere 6 (R6) mapping the whole otic ectoderm (Figure 13A-C). *Sox2* at later stages is restricted in the sensory parches from where hair-cells emerge, while *Sox3* expression is confined to the neurogenic epithelium but is not detected in sensory epithelium (Neves *et al.*, 2007). The broader expression of *Sox2* could be due to this additional role of *Sox2* in sensory specification.
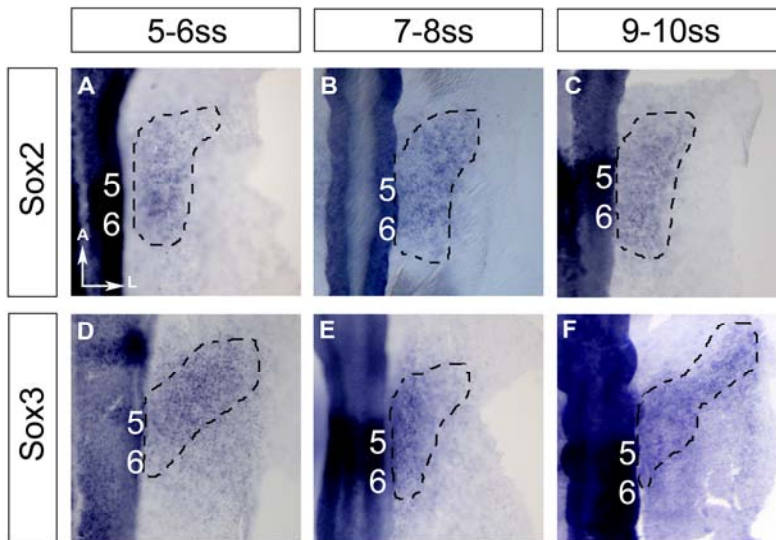
Figure 13: *Sox2* and *Sox3* expression profile
A-C) *Sox2* expression in presumptive otic placode broadly expressed from R3-R6. D-F) *Sox3* expression in the presumptive otic region. A: Anterior, L: Lateral, Numbers 5 and 6 denote rhombomere 5 and 6.

The progressive restriction of *Sox3* in the anterior otic field at the time of otic placode formation suggests that only the anterior otic territory is able to commit to a neural fate.

4.2. Analysis of the role of Sox3 in neural specification in the inner ear

4.2.1. Overexpression of Sox3 and analysis of its functionality

In order to study the role of *Sox3* in proneural specification in the inner ear, full-length *Sox3* cDNA cloned in-frame with GFP protein in the N-terminal end (kind gift from P. Scotting) was overexpressed from 5 to 9 ss embryos by in ovo electroporation.
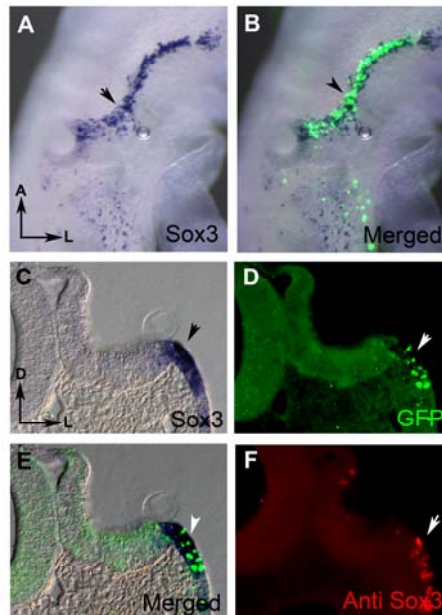
Figure 14: Checking *Sox3* vector functionality
A) Lateral view of *Sox3* electroporated embryo showing overexpressed *Sox3* mRNA by ISH. B) Lateral view of the same electroporated embryo showing GFP at the site where overexpressed Sox3 mRNA was detected. C-E) Transversal cryostat sections of the *Sox3* electroporated site of the otic cup showing overexpressed *Sox3* mRNA and GFP at the same site. F) Detection of Sox3 protein by immunohistochemistry with an antibody against Sox3 protein.

Figure 14 shows electroporated embryos that were in situ hybridized (ISH) with *Sox3* probe. When *Sox3* was overexpressed, strong mRNA transcription of *Sox3* was seen in all GFP positive cells as expected by in situ hybridization (ISH) (Figure 14A-E). To confirm if the mRNA

produced was translating into protein, we analyzed the electroporated embryos with *Sox3* antibodies (Figure 14F) and found that higher levels of *Sox3* protein correlated with the electroporated tissue, indicating that the construct was correctly translated.
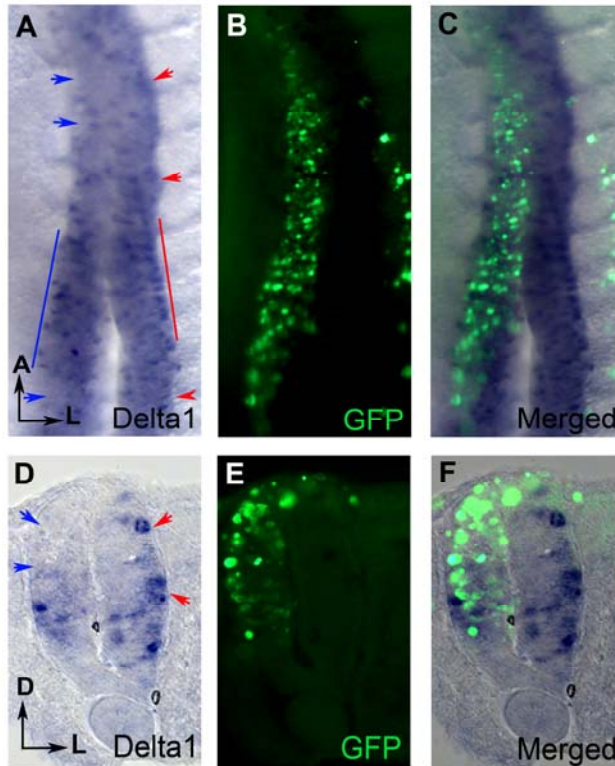
Figure 15: Analysis of EGFP-*Sox3* electroporation
A) Whole mount ISH after EGFP-*Sox3* electroporation. B) GFP after immunostaining C) merged image showing clearly that one side is electroporated and other side is not. D-F) Transversal sections of embryos showing down-regulation of *Delta1* expression on the electroporated side. Red arrows indicate normal expression and well aligned *Delta*1 expressing cells at non-electroporated side whereas blue arrows indicate inhibition of *Delta1* expression in cell autonomous fashion only on the electroporated side. D: dorsal, A: anterior, L: lateral, GFP: green fluorescent protein.

Bylund *et al*. (Bylund *et al., 2003*) have shown that when *SoxB1* genes were overexpressed in the neural tube, neuronal differentiation was

inhibited and a decrease in the number of neuronal committed cells was observed. From these results it was concluded that *Sox*B1 transcription factors keep the cells at progenitor state and allows differentiation of proneural genes only when they are down regulated. A similar experiment was conducted by electroporating full-length *Sox3* cDNA in the neural tube at HH10 stage embryos to verify the functionality of the construct and embryos were harvested after 24 hrs. After *Sox3* overexpression experiments, a clear down-regulation of *Delta1* in the neural tube was observed (Figure 15A-F). Furthermore, arrangement of the *Delta1* positive cells properly aligned along the control side of the neural tube, was disturbed in the electroporated side of the neural tube (compare red and blue lines in Figure 4A). *Delta1* inhibition was more obvious in the transversal section of electroporated neural tubes (Figure 15D-F). From these results we concluded that the vector is functioning as previously reported.

4.2.2. Sox3 overexpression can induce Sox2 ectopically

Gain- and loss-of-function experiments in zebrafish and *Xenopus* have explored the role of *Sox3* in determining neural fate. When *Sox3* was overexpressed ectopically, a substantial increase in the population of cells adopting a neural fate was observed by the induction of *Sox2* and other neural markers like N-tubulin (Dee *et al.*, 2008). In order to see whether *Sox3* has an evolutionary conserved role in regulating the expression of another *SoxB1* gene, *Sox2*, in the otic placode, we overexpressed EGFP-Sox3 in the presumptive otic placode at HH8-9 stage embryos and observed its effect on *Sox2* expression.

Embryos electroporated with EGFP-Sox3 showed a dramatic increase in the number of *Sox2* expressing cells (n=6/6) just within 6hrs of post-electroporation (Figure 16C and D). In sagittal cryostat sections cell

autonomous induction of *Sox2* can be appreciated by detection of increased *Sox2* transcripts in GFP positive cells (Figure 16G-I) as compared with low levels of *Sox2* expression in the non-electroporated side (Figure 16F). Embryos electroporated with vector expressing EGFP alone in the presumptive otic placode, did not show induced *Sox2* expression (0/7) (Figure 16A and B). To further confirm our results, full length *Sox3* (cloned in pCDNA) was electroporated in the presumptive otic ectoderm and expression of *Sox2* was analyzed after 6hrs. The same results were observed (6/6). It is reported that *Sox2* and *Sox3* coding sequences share a 95% percent amino acid sequence identity in the HMG domain (Uchikawa *et al.*, 1999), therefore a *Sox2* probe devoid of HMG box was also used for the analysis. When we checked the response of *Sox2* after induced *Sox3* overexpression, the same *Sox2* induction was seen in ectopic domains (4/4).
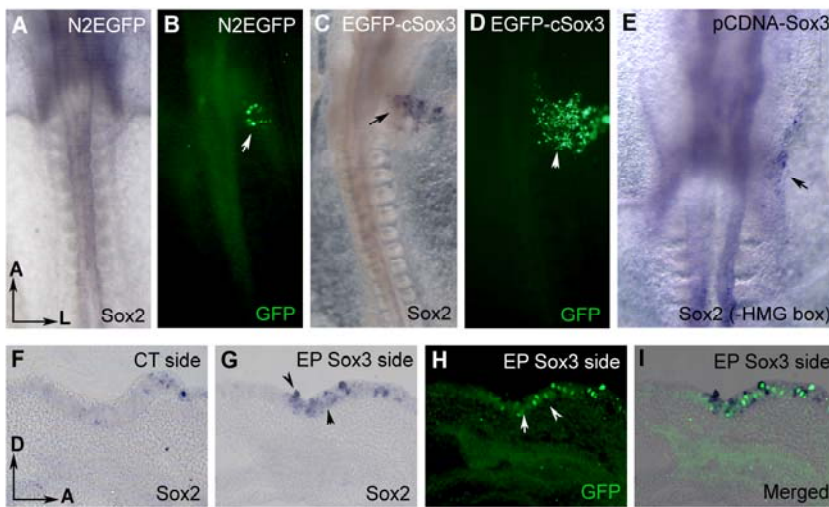
Figure 16: *Sox2* induction in response to *Sox3* over expression
A-E) Dorsal view of chick embryos electroporated at HH8-9 and dissected after 6 hrs of post-EP. A-B) Electroporation of empty N2EGFP vector. C-D) Electroporation of EGFP-Sox3. E) Electroporation of pCDNA-Sox3. F-I) Sagittal sections of the embryo electroporated with EGFP-Sox3 and dissected after 6hrs. F) Non-electroporated side. G) EGFP-Sox3 electroporated side. H) GFP seen in the electroporated side. I) Merged

image of ISH Sox2 and GFP. D: dorsal, A: anterior, L: lateral, GFP: green
fluorescence protein, EP: electroporated, CT: control
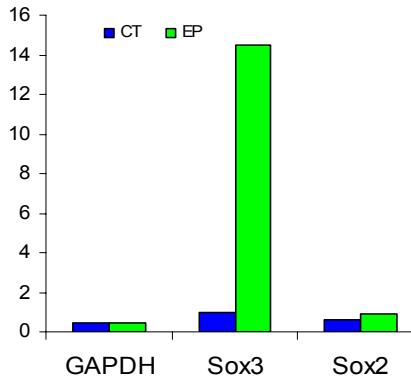
**Graph 1: qRT-PCR showing *Sox2* induction in response to
Sox3 over expression**

We verified the ability of *Sox3* to induce *Sox2* in the otic ectoderm by
quantitative real-time PCR (qRT-PCR) experiments. Embryos from three
different rounds of independent electroporation were used for qRT-PCR
analysis. Electroporated embryos presenting strong GFP in the
presumptive otic region were selected for RNA extraction and proceeded
for RNA quantification by qRT-PCR. Dissected otic ectoderms with part
of the mesoderm and neural tube of each side were processed for
extraction of total mRNA. The initial total RNA concentration of the two
sides was kept equal and GAPDH was used as an internal control to
equilibrate the amount of mRNA for each side. A slight increased level of
Sox2 mRNA was detected in the electroporated side as compared to the
non-electroporated side of the embryo by absolute qRT-PCR (Graph 1 for
GAPDH, CT=0.49 and EP=0.49; *Sox3*, CT=1 and EP=14; *Sox2*, CT=0.57
and EP=0.87). The fact that the increase in mRNA detected by this
technique is not high might be due to several reasons: i) only a fraction of
the ectodermal cells were electroporated; ii) at this stage removal of the
underlying mesoderm was impossible. The induction of *Sox2* by *Sox3* fits

with a model in which *Sox3* is responsible for initiating the cascade of events that lead to neural commitment in the otic placode.

4.2.3. Ectopic Sox3 expression induces Delta1 expression

Regionalization of *Sox3* in the anterior otic field suggests a role of ectodermal *Sox3* in providing competence to respond to the neural inducing signal only in the anterior otic domain.
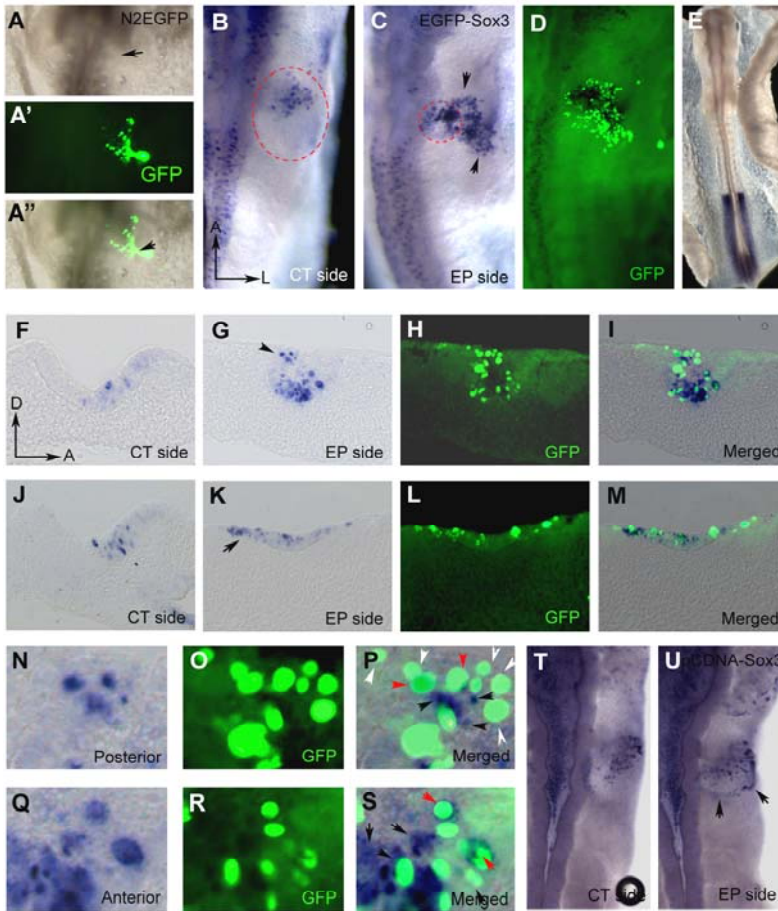
Figure 17: *Delta1* induction in response to *Sox3* overexpression after 6-hrs of post electroporation
A-A'') Whole mount ISH of *Delta1* after electroporation with an N2EGFP vector. B and C) Non-electroporated and electroporated side with EGFP-Sox3. Red circles indicate the otic cup and black arrow indicate *Delta1* induction outside otic domain. D) Electroporated side of chick embryo immunostained with GFP. E) Chick embryo electroporated with EGFP-Sox3 and incubated only for

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3hrs. F-M) Sagittal sections of embryos electroporated with EGFP-Sox3 and incubated for 6hrs post-EP. F and J) Control side. G and K) electroporated side. Black arrows indicate *Delta1* induction in the posterior non-neurogenic domain. N-S) Higher magnification images of a cryostat section showing *Delta1* induction after EGFP-Sox3 electroporation. N-P) posterior region and Q-R) Anterior region. Black arrows indicate non cell-autonomous *Delta1* induction; red arrows indicate cell-autonomous *Delta1* induction and white arrow indicate no induction. T and U) Electroporation with pCDNA-Sox3 vector and post EP incubation for 6hrs showing non electroporated side (T) and electroporated side (U). D: dorsal, A: anterior, L: lateral, GFP: green fluorescence protein, EP: electroporated, CT: control.

Moreover, the ability of *Sox3* to induce *Sox2* ectopically in the otic field led us to investigate if overexpression of *Sox3* was able to induce neurogenesis ectopically.

Gain-of-function strategy by over-expressing full-length chick in ovo electroporation was carried out. After 6 hours of overexpressing *Sox3* by in ovo electroporation, experimental embryos were analyzed for the expression of neuronal markers such as *Delta1*. Embryos electroporated with vectors expressing N2EGFP alone (0/6; Figure 17A) or the non electroporated side of the embryo electroporated with Sox3-GFP (Figure 17B) presented expression of *Delta1* restricted in the anterior proneural domain in a salt and pepper fashion at otic placode and otic cup stages. In contrast, when embryos were electroporated with EGFP-Sox3 a dramatic increase in the number of *Delta1* expressing cells was observed (29/29) just within 6hrs after electroporation in the anterior domain but interestingly also in the non-neurogenic territory (Figure 17F-M).

When *Delta1* expression in the otic cup stage in response to *Sox3* overexpression was compared with the control side of the same embryo, higher number of *Delta1* positive cells was found in the anterior domain (Figure 17F-M). In the control side, *Delta1* was expressed in isolated cells because of the lateral inhibition mechanism (Figure 17F and J). In contrast in areas of overexpression of *Sox3*, *Delta1* was not found in a salt and pepper pattern. Close analysis comparing GFP staining and *Delta1*

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activation, showed that most of the cells were showing overlapping expression of *Sox3* and *Delta1* (indicated by red arrows in Figure 17Q-S), indicating that *Sox3* was able to activate *Delta1* in cell autonomous manner. Besides GFP/*Delta1* overlapping cells, we have seen some *Delta1* positive cells that were in close vicinity of *Sox3* positive cells but not overlapping with GFP (indicated by black arrows in Figure 17N-S). Those could be normal neurogenic cells not induced by overexpression of *Sox3*.

In order to assess if overexpression of *Sox3* could induce *Delta1* expression in a cell non-autonomous manner, we calculated the total number of GFP and *Delta1* expressing cells in the posterior region. For this, we selected 3 random sections of 3 electroporated embryos (9 sections total) and found that on average from 30 cells ectopically expressing *Delta1*, 14 cells also showed GFP. Three possibilities can explain this observation: 1) *Sox3* can induce *Delta1* in the neighboring cells hence induction behaves in a non-cell autonomous manner; 2) the *Sox3* (GPF) was down-regulated as neurogenesis proceeded hence that cell had been exposed to high doses of Sox3, and 3) not all the cells were initially electroporated with same efficiency and low GFP is not observed due to strong mRNA signal after ISH (for detail please see discussion).

Several important conclusions were drawn from these experiments: the ability of *Sox3* to commit non-neurogenic ectoderm to a neural fate, to increase the number of *Delta1* expressing cells in the anterior domain; and to allow premature induction of *Delta1* expressing cell at early otic placode stage.

To further confirm our results, we electroporated full length *Sox3* cloned in pCDNA in the presumptive otic ectoderm and again observed overexpression of *Delta1* after 6hrs (5/5). Three experiments of qRT- were performed to quantify the *Delta1* induction. Embryos showing strong GFP in the presumptive otic region were selected for RNA

extraction and proceded for RNA quantification by qRT-PCR. From three independent experiments performed for qRT-PCR, we have seen once a slight increase in *Delta1* mRNA levels (for *GADPH*, CT=1 and EP=1; *Sox3*, CT=1 and EP=6.65; *Delta1*, CT=1 and EP=1.23) in the electroporated side as compared to the non-electroporated side of the embryo (see Graph 2).
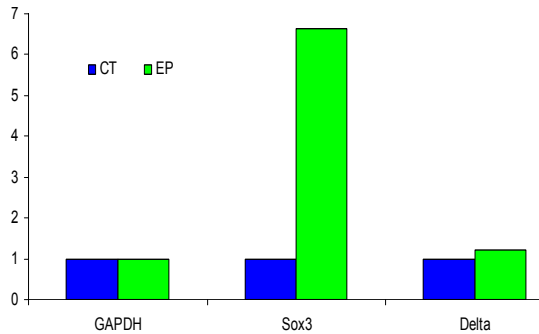
Graph 2: Relative qRT-PCR with slightly increased levels of *Delta1* transcripts in the electroporated tissue

In order to assess if the induction of *Delta1* was a rapid event, electroporated embryos were sacrificed after 3hrs post-electroporation. In those embryos, induction was not observed (0/5; Figure 17E). In order to see when Sox3 protein is translated after electroporation, we electroporated Sox3 and sacrificed embryos after 1-hr and 2-hrs of incubation (data not shown). Just after 2 hrs of post-electroporation, GFP-Sox3 signal was seen as strong as the one observed after 6hrs (8/10) suggesting that Sox3 protein is translated before 2-hrs after electroporation. The fact that *Delta1* was not induced until 6-hrs of post-electroporation suggests that *Delta1* induction by *Sox3* might not be direct.

We next sought to determine the temporal specification of neurogenic and non-neurogenic chick otic domains. For this, chick embryos were electroporated at HH10 and HH12 stage embryos and were allowed to develop for 6 hrs and assayed for the expression of *Delta1* (Figure 18A

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and K respectively). Comparing the effects of misexpression of *Sox3* at different stages it was seen that maximum *Delta1* induction was obtained when the embryos were electroporated at 5-9ss stages especially in the posterior non-neural domain. Re-specification of the two domains with respect to *Delta1* expression was still seen at HH 10 stage embryos (6/7) suggesting that at this stage the otic placode is still competent to respond to the external factors (Figure 18A-G).

Taken together, these results suggest that from HH9 to HH12 the otic ectoderm progressively loses the ability to ectopically activate neurogenesis. Thus, there is a tight temporal relationship between age of the embryo and ability to induce *Delta1*. We can postulate that the neural territory is not specified until stage HH12 but after that the otic ectoderm is specified in proneural and non-neural domain as a result, *Sox3* can no longer re-specify the whole otic territory into neural.
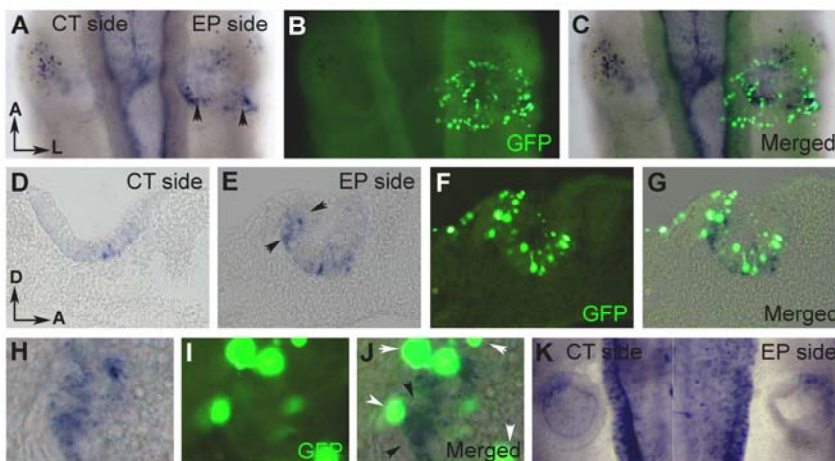
Figure 18: Temporal specification of the neurogenic territory
A-J) Chick embryos electroporated with EGFP-Sox3 at HH 10 stage in the otic placode A) whole mount ISH with *Delta1*. B) GFP. C) Merged image of ISH and GFP. D-E) Sagittal sections. D) Control side E) Electroporated side. H-J) Higher magnification of the electroporated side of the section. White arrows indicate GFP cells that do not show any *Delta1* induction whereas black arrows show *Delta1* induction. K) Chick embryo electroporated with EGFP-Sox3 at HH 12 stage in the otic cup.

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Next, we wanted to see whether *Delta1* induction was maintained for a long period. For this we electroporated embryos at stage 5-9ss and left the embryos to incubate for 20hrs after *Sox3* electroporation. In this case, very few cells as compared to GFP showed ectopic *Delta1* (3/6; Figure 19) indicating that these *Delta1* positive cells might have gone into a more differentiated state or are lost (will be described later in results and discussion).
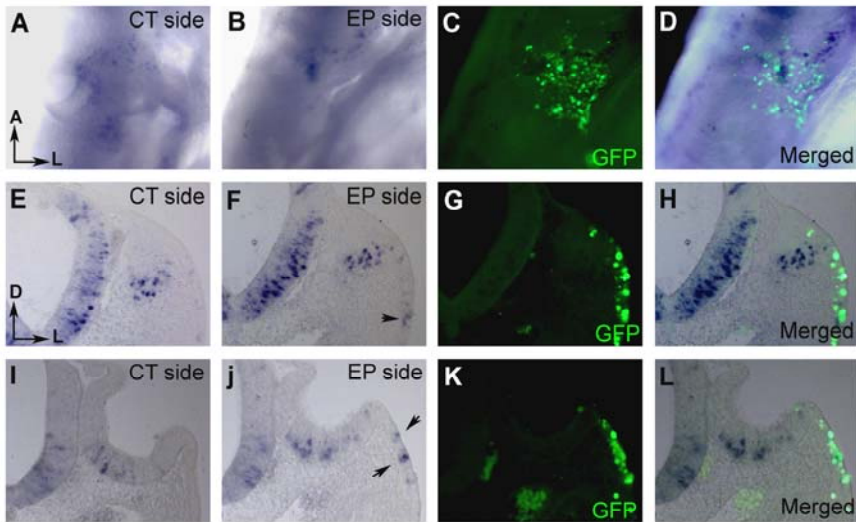
Figure 19: *Delta1* induction at HH10 and analysis at 20hrs post-electroporation
A-D) Whole mount ISH with *Delta1* after 20 hrs Sox3 electroporation. A) control side. B) electroporated side. E-L) transversal sections of the electroporated embryos. Black arrows indicate the *Delta1* ectopic induction. A: anterior, L: lateral, D: dorsal, CT: control, EP: electroporated, GFP: green fluorescence protein.

4.2.4. Role of Sox3 overexpression on Hes5

An important transcriptional output of Notch signaling is the up-regulation of the bHLH transcription factors *Hes1* and *Hes5* (Louvi and Artavanis-Tsakonas, 2006). These are known to be DNA-binding transcriptional repressors at specific recognition sequences to which they

bind (N-boxes and ESE-boxes), being different from the E-boxes recognized by proneural proteins (Davis and Turner, 2001).
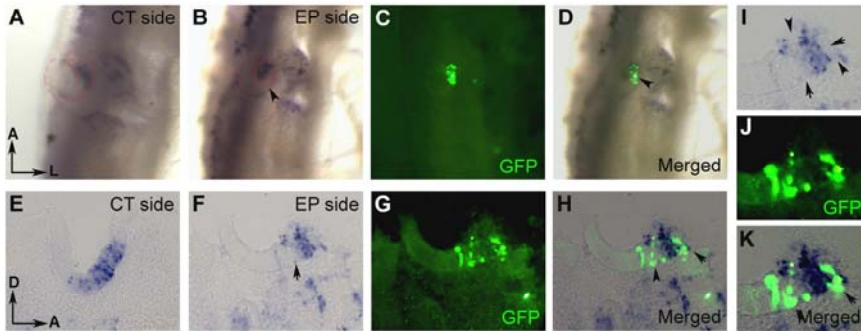
Figure 20: *Hes5.2* **inhibition after 20hrs of** *Sox3* **electroporation**
A-D) Lateral view of whole mount embryos ISH with *Hes5.2* after *Sox3* electroporation. E-H) Sagittal sections of the embryo. I-K) higher magnification of the sections (40X) A: anterior, L: lateral, D: dorsal, CT: control, EP: electroporated, GFP: green fluorescence protein.
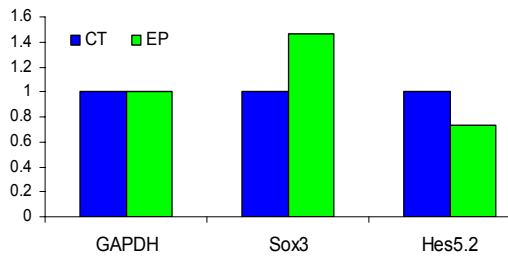
Graph 3: qRT-PCR of *Hes5.2* **expression after** *Sox3* **electroporation**

In the neural tube of a mouse, *Hes5* has been shown to be directly regulated by the Notch pathway in vivo (de la Pompa *et al*., 1997; Lutolf *et al*., 2002). Therefore, we decided to explore how *Delta1* induction participate in the cascade of events in the Notch pathway that regulate neuronal production. For this, we electroporated full-length *Sox3* at HH8-9 stage embryos in the presumptive otic placode and harvested the embryos at 20hrs for the analysis of *Hes5.2* expression. A down-regulation of *Hes5.2* was seen only in the electroporated side (3/5; Figure 20A-K). Detailed analysis of the sections showed that most of the *Hes5.2*

inhibition was cell autonomous manner (Figure 20A-H) as none of the GFP positive cells showed any *Hes5.2* expression. The results were further confirmed by qRT-PCR (Graph 3). *Hes5.2* RNA quantity was reduced in the electroporated side of the embryos as compared to non electroporated side (for *GAPDH*, CT=1 and EP=1; *Sox3*, CT=1 and EP=1.46; *Hes5.2*, CT=1 and EP=0.73). These results suggest that higher levels of *Sox3* in the neurogenic domain convert cells to *Delta1* positive precursors whereas the same cells are down-regulated for the expression of *Hes5.2*.

4.2.5. Ngn1 induction in response to Sox3 overexpression

Neurogenin1 (*Ngn1*) is a neuronal determination gene whose inactivation leads to reduced number of otic neurons (Kim *et al.*, 2001; Liu *et al.*, 2000; Ma *et al.*, 1998, 2000). Detailed temporal studies of the sequence for the activation of proneural genes during otic neurogenesis revealed the following temporal sequence for the onset of gene expression: *FGF10*> *Ngn1*/ *Delta1*/ *Hes5*> *NeuroD*/ *NeuroM* (Alsina *et al.*, 2004). As *Delta1* induction by *Sox3* seemed not to be direct, we reasoned that *Sox3* could be initially activating *Ngn1* which later induce *Delta1* induction. Electroporated embryos were analyzed for *Ngn1* expression after 3 and 6 hrs of *Sox3* overexpression. In all cases, ectopic *Ngn1* induction was observed (3 hrs (5/5), 6hrs (9/9); Figure 21). However, embryos electroporated with vectors expressing either N2EGFP alone (6/6) or pCDNA empty vector (3/3) also showed ectopic expression of *Ngn1* indicating that the *Ngn1* probe was hybridizing non specifically with part of the expression vector.
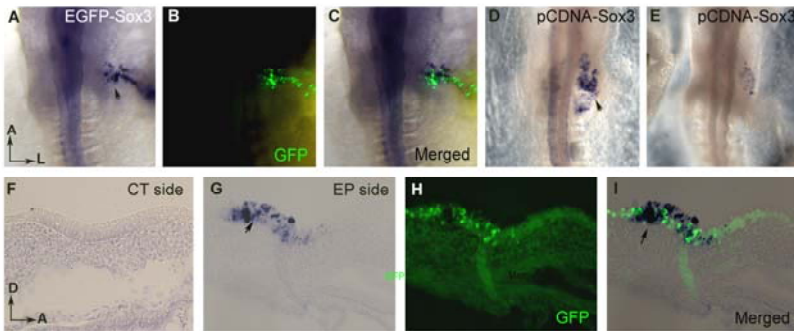
Figure 21: *Ngn1* **induction after 3hrs and 6hrs of EGFP-***Sox3* **full length electroporation**
A-C) EGFP-Sox3 in the right side of the embryos and incubation for 6 hrs D-E) Electroporation with pCDNA-Sox3 and incubation for 3 and 6hrs respectively F-G) Sagittal section of electroporated embryos. A: anterior, L: lateral, D: dorsal, EP: electroporation, CT: control. J) Relative qRT-PCR showing *Ngn1* induction after 6 hrs of post EP incubation.
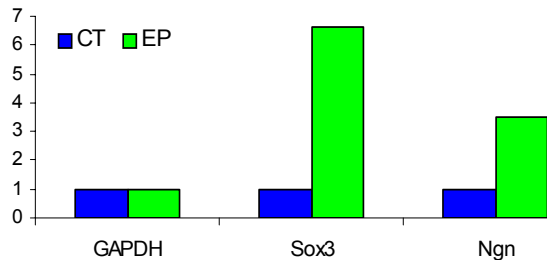
Graph 4: Relative qRT-PCR showing *Ngn1* induction after 6hrs of post EGRP-*Sox3* electroporation incubation

Thus, from those experiments it was not possible to conclude whether *Sox3* overexpression was able to induce *Ngn1* prior to *Delta1* induction. Quantification of the effects of electroporation by qRT-PCR presented an increase of *Ngn1* mRNA levels (for *GADPH*, CT=1 and EP=1; *Sox3*, CT=1 and EP=6.65; *Ngn1*, CT=1 and EP=3.48) in one of three independent experiments conducted for qRT-PCR (Graph 4).

4.3. Sox3 can induce early markers but not later markers of neuronal diffentiation

NeuroD expression is initiated at HH12 after *Ngn1* and *Delta1* expression (Alsina *et al.*, 2004) and labels neuroblasts before and after delamination to the CVG. In order to see the shift of the ectopic neural precursors to the next differentiation state, the electroporated embryos were analyzed for the expression of *NeuroD* after 20hrs and 40hrs of *Sox3* electroporation. Some embryos showed ectopic *NeuroD* expression outside the otic ectoderm after 20hrs (5/10; Figure 22) whereas only fewer number of embryos showed ectopic *NeuroD* induction within otic ectoderm in the posterior domain (2/10; Figure 22L). When the same analysis was carried out after incubating embryos for 40hrs after electroporation some embryos displayed ectopic *NeuroD* induction outside otic ectoderm (7/14), and only one presented expanded *NeuroD* expression within the posterior non-neural otic epithelium after 40hrs of electroporation (Figure 23). However, as GFP was never found after 40hrs it was impossible to assess at those stages if ectopic *NeuroD* cells were *Sox3* positive.

The fact that only fewer cells expressed differentiation markers may be due to, i) high levels of *Sox3* which do not allow further differentiation, ii) few *NeuroD* positive cells observed at 20hrs post-electroporation may undergo cell death due to lack of required external factors or due to its specification in an ectopic place. For this reason, cell death in the otic vesicle was assayed by TUNEL technique and compared the electroporated side of otic vesicles to the non-electroporated side. An increased number of TUNEL positive cells were found in the electroporated side as compared to the non electroporated side (Figure 23F-G) suggesting that ectopic neural cells were excluded from the non-neural ectoderm by induced cell death.

If *Sox3* induces cell death one can presume that the size of the ganglion or the otic vesicle after 20hrs of *Sox3* electroporation should not be same. For this we compared the total number of sections obtained after electroporation from otic vesicles of both sides of an embryos and the number of sections showing ganglion in each side. Average numbers of sections obtained for the otic vesicle in control side and electroporated sides were compared. Our analysis showed that only 7.1% reduction was obtained in electroporated side whereas when the same analysis was done for the size of the ganglion, a 38.95% reduction was observed.

These results suggest that the overall size of the otic vesicle was not changed much but the size of ganglion is reduced in the electroporated side of the embryo after *Sox3* overexpression.
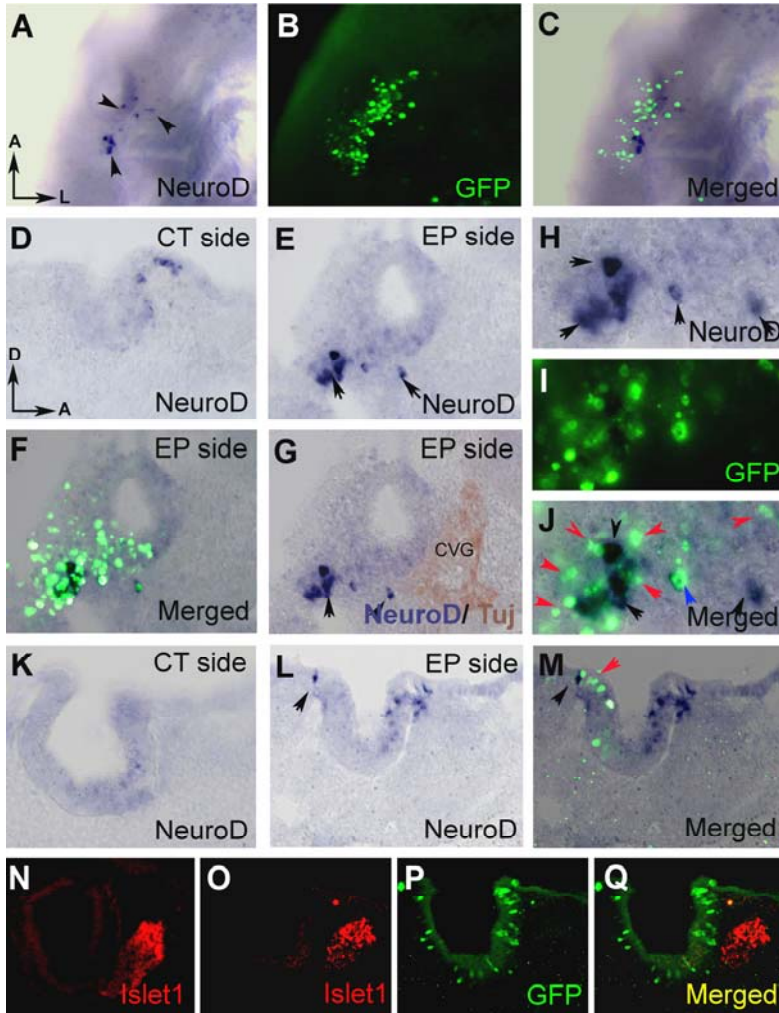
Figure 22: *NeuroD* induction after 20hrs of EGFP-*Sox3* electroporation

A-C) Whole mount ISH with *NeuroD* of embryo electroporated with EGFP-*Sox3* and incubated for 20hrs. D-F) Sagittal section of one of the embryos showing ectopic *NeuroD* cells after *Sox3* electroporation. G) Immunostating with Tuj showing no Tuj positive cells in the places where *NeuroD* induction was seen. H-I) High magnification images of sagittal section of the embryos electroporated with *Sox3* and incubated for 20hrs. Red arrows indicate cells showing only GFP, black arrows cells indicate only Delta expression and blue arrows indicate cells that coexpress both genes. K-M) Another embryo electroporated with *Sox3* showing *NeuroD* ectopic expression. N-O) Immunostaining with Islet1 showing no induction after 20hrs of incubation A: anterior, L: lateral, D: dorsal, EP: electroporation, CT: control.

Several reasons can explain this reduction 1) *Sox3* itself might induce cell death directly, 2) The change in fate induced by *Sox3* is unwanted by the embryo and this induces cell death, 3) Bylund *et al* (Bylund *et al* 2003) has showed that overexpression of all *SoxB1* genes leads to inhibition of neuronal differentiated cells as down-regulation of *SoxB1* genes is a required step for neuronal differentiation. It is possible that at long run, high levels of *Sox3* inhibits the process of differentiation and that total number of neuronal cells differentiating and delaminating is reduced.
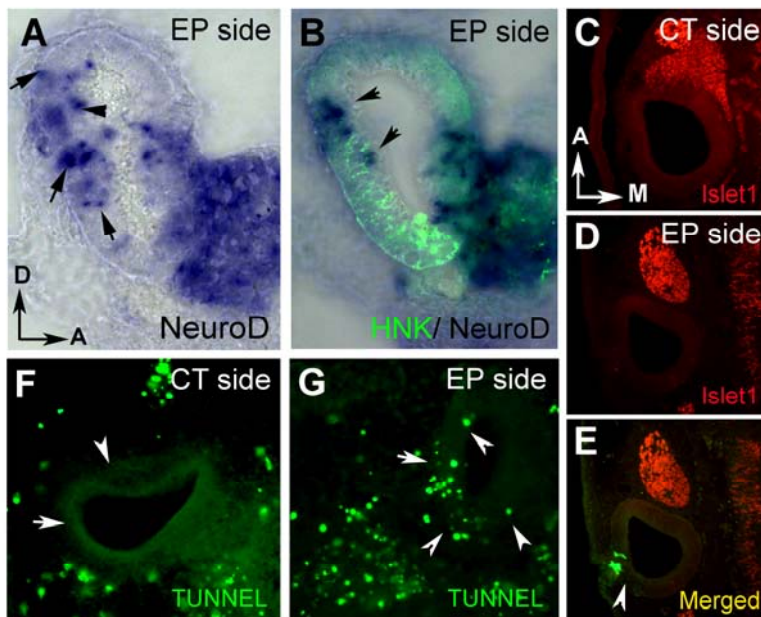
Figure 23: Example of *NeuroD* induction after 40hrs of EGFP-*Sox3*
A-B) Sagittal section of the embryos electroporated with *Sox3* and analyzed for the expression of *NeuroD* after 40hrs. C-E) Coronal section of the embryos electroporated with *Sox3* and analyzed for the expression of Islet1/2 after 40hrs. No ectopic Islet1/2 positive cells are seen F-H) Immunostaining with TUNEL showing increased cell death in the EP side of the embryos (G) as compared to the non-electroporated side (F) A: anterior, L: lateral, D: dorsal, EP: electroporation, CT: control.

Next, the ability of ectopic neuronal cells to transit into more differentiated cells was analyzed by determining the appearance of expression of Islet1. The LIM homeodomain transcription factor Islet1 is

expressed in few cells located at the sites of delamination and in those that have delaminated (Adam *et al*, 1998; Camarero *et al*, 2003). The LIM homeodomain gene family of transcription factors is known to be crucial for specification of neuronal identity in neural tube-derived neurons. When the expression of Islet1 after incubating 20hrs (0/9) and 40hrs (0/13) of *Sox3* electroporation was analyzed by immunohistochemistry, no ectopic Islet1 expressing cells were observed (Figure 22N-Q and Figure 23C-E respectively). This is in agreement with our above results in which ectopically neural induced cells did not progress to late differentiation markers. Furthermore, the shape of the ganglion was similar to the control non-electroporated side of the *Sox3* electroporated embryo (Figure 22 and Figure 23).
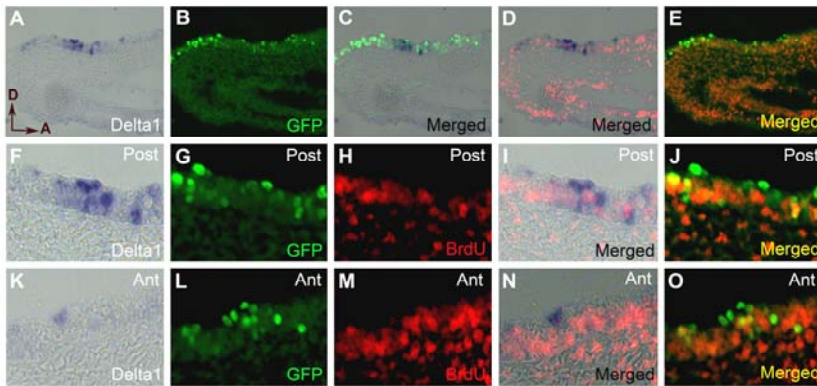
Figure 24: Role of *Sox3* in proliferation
All sections are sagittal A-E) ISH of electroporated embryo with *Delta1* expression and immunnostained for BrdU F-J) High magnification of the posterior domain of electroporated embryo. K-O) High magnification of the anterior domain of electroporated embryo.

In order to study whether *Sox3* induces cell proliferation, we incubated electroporated embryos in the presence of BrdU during 30 minutes before scarifying embryos. Both anterior and posterior domains were carefully examined for the co/expression of BrdU and GFP or BrdU and *Delta1*. Detail analysis showed that mostl of the GFP positive cells were BrdU positive. In addition, severalof the *Delta1*-positive cells were co-

expressing BrdU suggesting that in this case the *Delta1*-expressing cells are still in a proliferative state.

4.4. Role of Sox3 *in the regulation of patterning genes expressed in the Non-neural territory*

4.4.1. Sox3 regulates Lmx1b expression in the otic territory by inhibiting *its expression*

The neurogenic and non-neurogenic domains of the otic cup display a specific combination of transcription factors (Abello *et al.*, 2007). It has been proposed that the interaction of sensory and non-sensory epithelium is crucial for proper otic development. However, interaction between genes of neurogenic and non-neurogenic domain has not yet been studied. Therefore, after gain of function of *Sox3* gene at pre-otic placode stages, the expression of *Lmx1b* was analyzed. *Lmx1b* is expressed very early in the otic placode and progressively gets excluded from the neurogenic territory, thus it was reasoned that *Sox3* could influence *Lmx1b* regionalization by inhibiting its expression. *Lmx1b* is a transcription factor of the LIM family, homologous to the *Drosophila* apterous gene. Apterous/*Lmx1* genes are involved in the specification of the dorsal limb character in *Drosophila* and vertebrates (Blair *et al.*, 1994; Riddle *et al.*, 1995; Blair, 1995; Vogel *et al.*, 1995b). *Lmx1b* in mice has been shown to play a fundamental role in specifying the roof plate, as well as regulating isthmus development (Adams *et al.*, 2000; Chizhikov and Millen, 2004). For this, *Sox3* was misexpressed in the posterior pre-otic region at 5-9ss stages and the embryos were analyzed for the expression of *Lmx1b* after 20hrs of incubation.
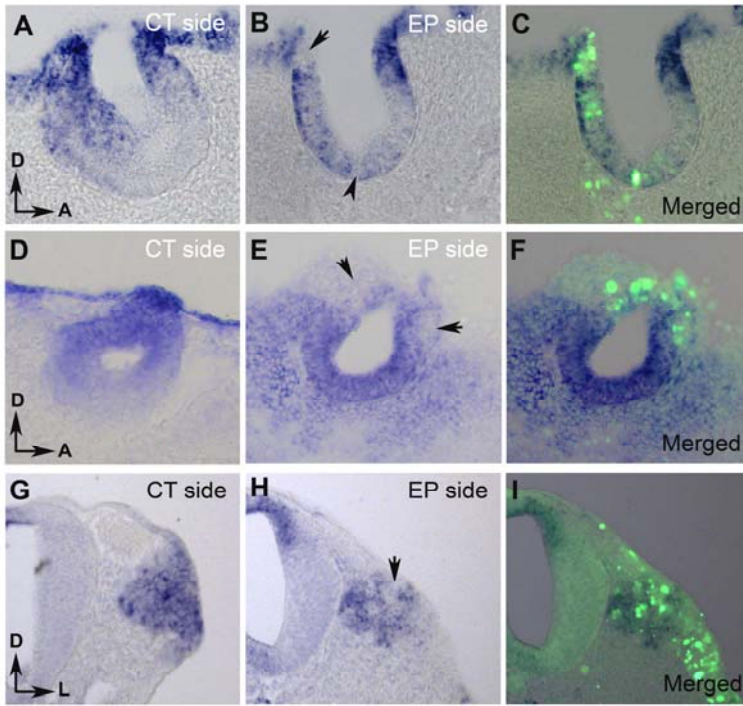
Figure 25: Role of *Sox3* in regulating *Lmx1b* expression
A-F) Sagittal sections showing Lmx1b inhibiton in a cell autonomous manner. G-I) transversal section showing inhibition of *Lmx1b*.
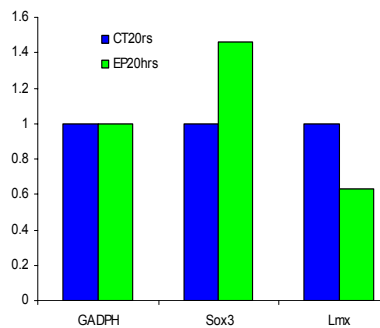
Graph 5: qRT-PCR displaying *Lmx1b* down-regulation after 20hrs

Sox3 overexpression caused a dramatic inhibition of *Lmx1b* expression in the electroporated otic vesicle after 20hrs of incubation (10/10; Figure 25). Close analysis of the sections revealed that *Lmx1b* inhibition was cell autonomous as only GFP positive cells showed *Lmx1b* inhibition. This

effect was not seen after 6hrs of incubation (4/4; data not shown). The ISH results were further confirmed by qRT-PCR analysis (Graph 5) by reduced expression of *Lmx1b* (for *GADPH*, CT=1 and EP=1; *Sox3*, CT=1 and EP=1.46; *Lmx1b*, CT=1 and EP=0.63).

In addition to *Lmx1b*, *Tbx1* expression is also confined to the non-neurogenic territory and in mice *Tbx1* negatively regulates neurogenesis (Vitelli 2003, raft 2004, xu 2007). This led us to explore the effect of *Sox3* misexpression on *Tbx1* expression. When *Sox3* was overexpressed, the expression of *Tbx1* was not affected after incubating embryos for 20hrs of post-electroporation (5/5; Figure 26A-D). We further confirmed the results with qRT-PCR and have found same levels of RNA (for *GADPH*, CT=1 and EP=1; *Sox3*, CT=1 and EP=1.46; *Tbx1*, CT=1 and EP=0.99) in both otic cups (electroporated and non electroporated) (Graph 6).
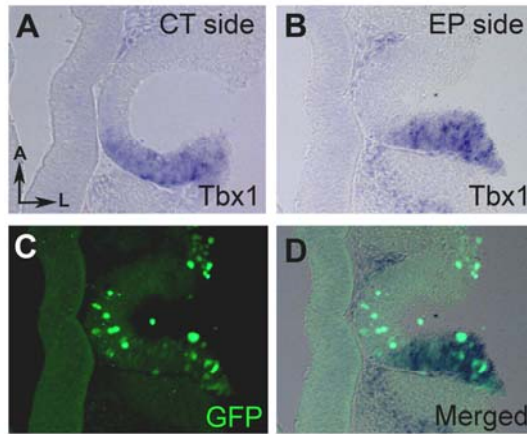
Figure 26: Effects of *Sox3* overexpression on *Tbx1*
A-D) Coronal sections showing no difference in *Tbx1* expression after *Sox3* electroporation.
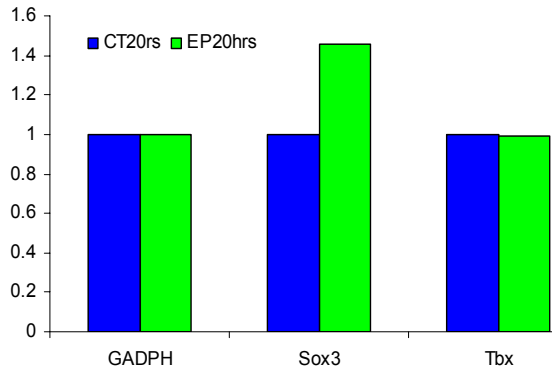
Graph 6: qRT-PCR showing *Tbx1* after *Sox3* electroporation

In summary, these experiments show that gene mapping to the proneural and non-neural regions are regulated in a distinct manner. Some of the posterior genes, such as *Lmx1b* are regulated by genes that are expressed in the proneural domain but others like *Tbx1* are not.

4.4.2. Ectopic Sox3 is not involved in regulating Pax2 expression during otic development

Several studies have demonstrated that in chick one of the earliest genes expressed in the presumptive otic-epibranchial region is the *Pax2* gene. *Pax2* appears in the ectoderm at 4 somites and progressively increases its expression to all cells that will become otic cells (Groves and Bronner-Fraser, 2000). *Pax2* gene belongs to a family of transcription factors containing paired-box homeodomain. All *Pax* genes play important roles in many aspects of organogenesis. But studies on mice mutants of *Pax3* and *Pax2* have shown gross defect in inner ear development (Epstein *et al.*, 1991; Torres *et al*, 1996). Although *Sox3* appears after *Pax2*, some laboratories have suggested that both genes reflect the process of otic commitment (Groves and Bronner-Fraser, 2000).

Previous studies have shown ectopic induction of early placodal markers *Pax6* and *Eya1* expression after *Sox3* injection in *Medaka* fish and the

formation of lens-like and otic vesicle-like structures were displayed at ectopic locations in the embryonic ectoderm (Koster *et al.*, 2000). A similar approach was conducted in chick by *Sox3* overexpression in order to see whether *Sox3* was able to regulate *Pax2* expression.

In order to test the ability of *Sox3* to induce extra placodal tissue, *Sox3* was electroporated in a broad ectodermal region and the expression *Pax2* was analyzed. In the *Sox3* overexpression experiment no difference in the extension of *Pax2* expression was seen when large area of the ectoderm was electroporated (0/10) (Figure 27) and comparing the two sides of the embryo.
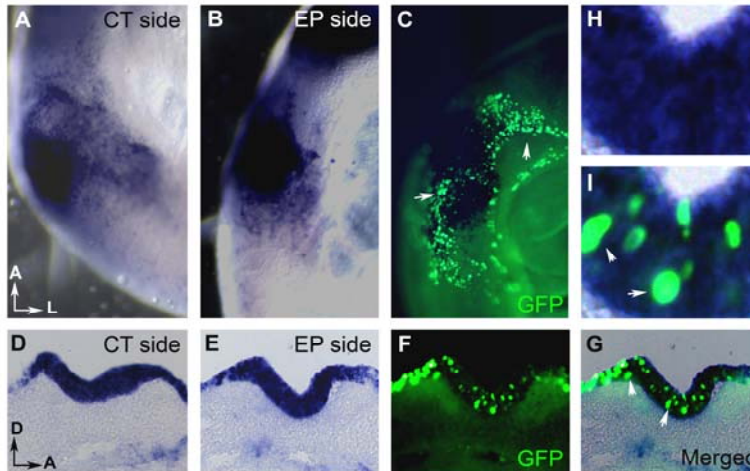
Figure 27: Overexpression of *Sox3* has no effect on *Pax2* expression
A-C) Whole mount ISH with *Pax2* of *Sox3* electroporated embryos. D-G) Saggital section showing no induction or inhibition of *Pax2*. H-I) High magnification images. A: anterior, L: lateral, D: dorsal, EP: electroporation, CT: control.
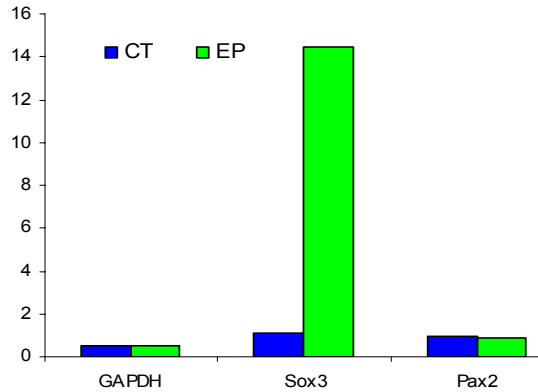
Graph 7: qRT-PCR of *Pax2* after *Sox3* electroporation

Detailed analysis of otic sections showed that in the otic cup there was neither a *Pax2* induction nor inhibition in the electroporated side of the embryo when comparing with the non-electroporated side (Figure 27A-G). We further confirm our results with qRT-PCR experiments (Graph 7) and have seen no difference in the expression level of Pax2 (for *GADPH*, CT=0.5 and EP=0.51; *Sox3*, CT=1 and EP=14; *Pax2*, CT=0.93 and EP=0.9).

4.5. Effects of Sox3 on morphology

In the experiments presented above, some morphological differences between *Sox3* electroporated and non-electrporated placodes, cups and vesicles were observed, only after sectioning. Embryos were either transversely or sagittally sectioned through the electroporated embryos. In several cases ectodermal thickenings were observed in embryos electroporated with *Sox3*. When these embryos were analyzed at otic placode cup stages after 6-8hrs of *Sox3* electroporation cell masses inside the otic cups or thickenings of the otic ectoderm (11/15) were observed at the site of GFP (Figure 23A-H). These cell masses were arranged in a disorganized fashion.

Furthermore, such thickenings were also seen outside the otic domain. *Sox3* electroporated in or near the optic cup showed ectodermal thickening at the site of GFP (Figure 28I-L) suggesting that *Sox3* might be involved in placodal induction but because of the absence of other factors complete development ceases afterwards. No induction of *Pax2* was observed in ectopic thickening in the optic region.
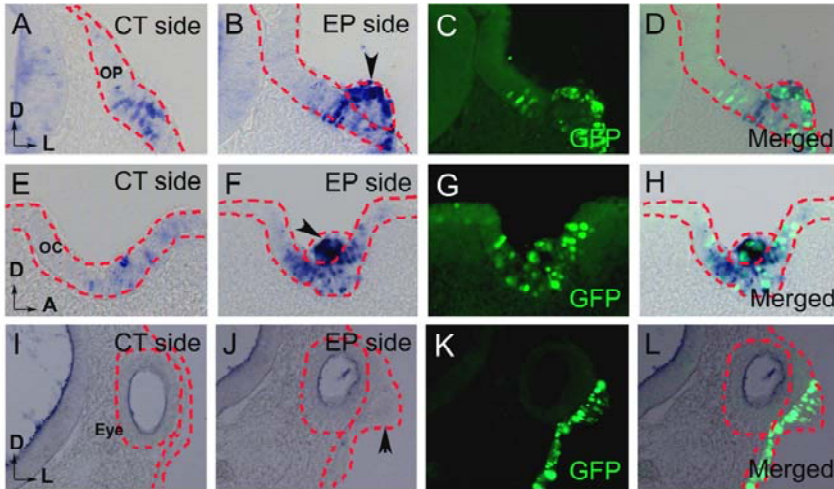
Figure 28: *Sox3* and morphology
A-D) Transversal sections showing ectodermal thickening E-H) Sagittal sections showing ectodermal thickening inside otic cup. I-L) Ectodermal thickening near optic cup. A: anterior, L: lateral, D: dorsal, EP: electroporation, CT: control, oc:otic cup, op:otic placode.

Later on, at otic vesicle stages, the shape of the vesicle was dramatically different from the control side (data not shown). The electroporated otic vesicle presented a developmental delay in otic vesicle formation (Data not shown). Significant different phenotypes observed were: 1) The cup of the electroporated side was wider. 2) The orientation of the otic cup was modified and rotated to the most latetro-ventral part rather than to the dorsal side. 3) The size of the otic cup in some instances was smaller as compared to the control side of the same embryo, 4) The ectodermal thickening or cell masses were found at early stages. These findings are in

accordance with the results from Abu-Elmagd (Abu-Elmagd *et al.*, 2001) in which *Sox3* electroporation in the epibranchial arches induced ectodermal thickenings.

Thus, it is possible that the ectodermal thickenings observed in our experiments may reflect the role of *Sox3* in inducing placodal fate.

CHAPTER II. ROLE OF LMX1B IN OTIC DEVELOPMENT

In the previous section the role of *Sox3* in conferring neural fate to the anterior otic territory was analyzed. *Sox3* in addition to regulating the expression of neurogenic genes was also able to inhibit the expression of a patterning gene such as *Lmx1b* but not other genes such as *Tbx1* and *Irx1*. The role of *Sox3* in down-regulating *Lmx1b* could be crucial for the restriction of *Lmx1b* expression in the posterior territory. However, the role of *Lmx1b* in ear development has not yet been addressed neither the signaling pathways that settle the early expression of *Lmx1b* in the otic territory. The following section will try to cover some of these aspects, although some of the results are still preliminary and need further work for full understanding.

4.6. Lmx1b is expressed in complementary fashion to Islet1

Homeobox genes are key regulatory genes involved in regional specification in organisms as diverse as *Drosophila* and vertebrates (McGinnis and Krumiauf, 1992; Finkelstein and Boncinelli, 1994; Manak and Scott, 1994). One such family, the LIM homeobox genes, consists of transcription factors with a typical cysteine-histidine-rich LIM domain and a homeodomain (Dawid *et al.*, 1998). LIM domain genes function as regulators of cell fate in a variety of different tissues and organisms (Freyd *et al.*, 1990; Karlsson *et al.*, 1990; Taira *et al.*, 1994; Tsuchida *et al.*, 1994; Lundgren *et al.*, 1995; Shawlot and Behringer, 1995; Way and Chalfie, 1998). A member of vertebrate LIM homeobox gene family, *Lmx1b* (German *et al.*, 1992) has been shown to be essential for establishing the dorsoventral patterning of the vertebrate limb (Riddle *et al.*, 1995; Vogel *et al.*, 1995). Additionally, the expression pattern of *Lmx1b* during ear development was described by Giraldez (Giraldez,

1998) and an essential role for *Lmx1b* in mice limb and kidney development is already well established (Chen *et al.*, 1998). In a similar manner to the detailed expression profile of *Sox3* performed at preplacodal stages (between 5 to 10 somites), *Lmx1b* expression profile in the otic ectoderm at these stages was also performed. Low levels of *Lmx1b* are detected, but in few scattered cells of the otic placode at 5-6ss. *Lmx1b* expression is strongly expressed adjacent to the neural tube and broadly expressed in the preotic territory from 7-8 to 9-10ss. The relative broad expression of *Lmx1b* is progressively refined but confined to the posterior region of the presumptive otic ectoderm at the level of R5-R6 after 9-10 ss. It is interesting that the high expression of *Sox3* is confined in the otic epithelium at the level of R4-R5, while *Lmx1b* is expressed at the level of R5-R6 very early before otic placode is morphologically visible. At otic cup stages the *Lmx1b* expression is completely complementary to the neurogenic otic domain in chick (Abello *et al.*, ms in preparation).

At otic vesicle stages (HH17) *Lmx1b* expression was confined only to the non-neural domain (Figure 29A-C). The immunostaining of Islet1 was carried out in order to study the degree of complementarily between both domains. Islet1 was expressed in the ganglion and very few cells appear in the otic epithelium at this stage. Although Islet1 positive cells are expressed mostly in the ganglion, *Lmx1b* expression is all around the otic epithelium except the domain which is immediate adjacent to the islet expressing ganglion (Figure 29D-F).
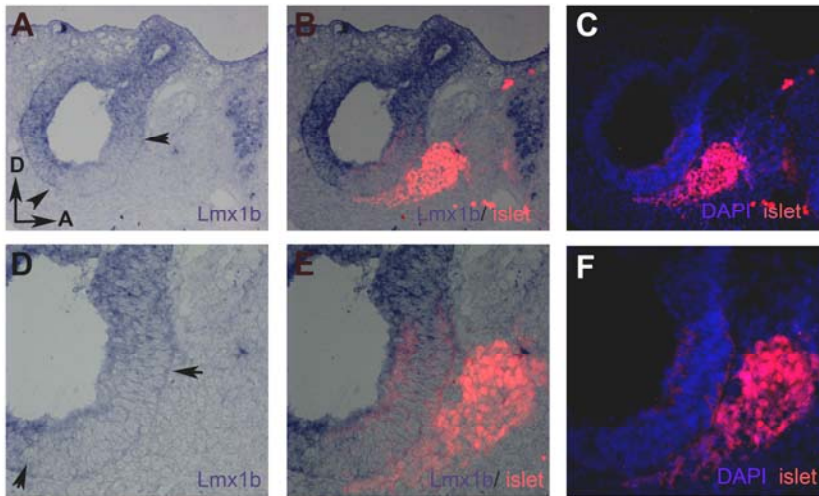
Figure 29: *Lmx1b* **and Islet1 complementary expression at otic vesicle**
A-C) sagittal section of an otic vesicle of a HH17 embryo showing complementary expression of the two genes D-E) High magnification images of sagittal section showing *Lmx1b* expression is strictly confined to the non-neural otic domain and is completely devoid of proneural domain.

4.7. Analysis of the role of Lmx1b in Ootic neural/non-neural specification

4.7.1. Checking LmxMO functionality

In order to assess the role of *Lmx1b* during inner ear development, a loss of function strategy was followed by using specific morpholinos (Gene tools Inc.) against *Lmx1b* transcript. In addition, overexpression of *Lmx1b* in the inner ear was also assessed to better understand the genes under the regulation of *Lmx1b* in the chick inner during early development (stage 5-9ss) to late developmental stages (HH12-15).

Loss of function experiments in chick have been carried out in several ways. In some instances, dominant negative construct for the desired transcription factor is electroporated and described its opposite effects to the electroporation of full-length constructs. In addition, the electroporation of siRNA for the desired genes has been shown to

efficiently inhibit gene function in chick. However, recent data have indicated that at early stages of chick development, electroporation of siRNA can lead to unspecific effects and to the activation of the p53 pathway. In this report, electroporation of siRNA was compared to the electroporation of morpholinos and the latter was concluded to be more efficient in inhibiting gene function. In our study we have used this latter strategy and specific morpholinos for the *Lmx1b* gene (Gene tools, Inc) were electroporated in the otic ectoderm of 5 to 7 somite stage embryos.

In order to check the functionality of the *Lmx1b* specific morpholinos, embryos were electroporated in the isthmic organizer (IsO) where the role of *Lmx1b* has been already characterized. Injection of Lmx1b.1MO and Lmx1b.2MO in zebrafish by O'Hara *et al.* (O'Hara *et al.*, 2005) resulted in loss of *Wnt1* and *Fgf8* expression at the mesaencephalic and metaencephalic region (MMR). On the other hand, overexpression of both Lmx1b.1 and Lmx1b.2 induced ectopic expression of *wnt1* and *fgf8* suggesting a requirement for Lmx1b.1 and Lmx1b.2 at the isthmus to maintain the expression of *wnt1*, *wnt3a*, *wnt10b*, *pax8*, and *fgf8* in zebrafish (O'Hara *et al.*, 2005). Furthermore, loss of Fgf8 was observed in *Lmx1b*$^{-/-}$ mice embryos at the midbrain hindbrain boundary (MHB) (Gua *et al.*, 2007) and misexpression of *Lmx1b* in chick embryos was shown to be able to induce ectopic expression of *Fgf8* (Matsunaga *et al.*, 2002).

The chick Lmx1bMO was injected in the IsO by electroporation at stage as early as 1-4ss and left the embryos to develop for 24 hrs. After 24 hrs of incubation the embryos were sacrificed and embryos showing fluorescein in the IsO region were selected for further analysis. When the embryos were analyzed for the expression of *Fgf8* (Figure 30A-B) and *Wnt1* (Figure 30C-D), both genes presented a clear inhibition in the electroporated area of the IsO region confirming the ability of morpholino to inhibit *Lmx1b*.
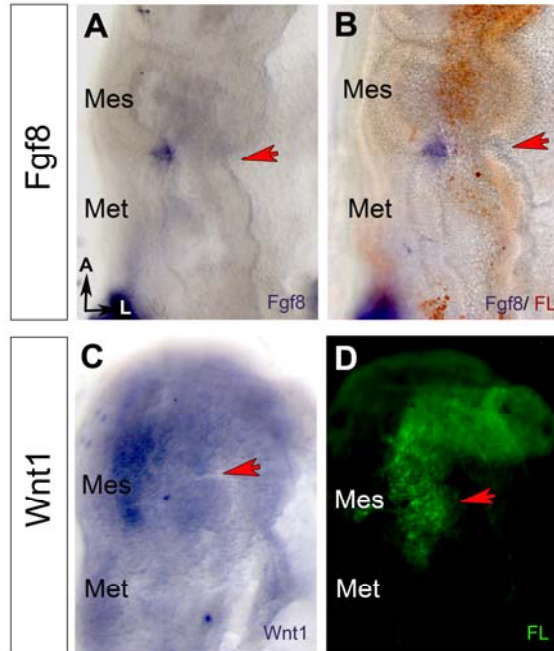
Figure 30: Checking Lmx1bMO functionality
A-B) Whole mount ISH with *Fgf8* of embryos electroporated with
Lmx1bMO at 1-4ss in the neural tube and incubated for 20hrs thereafter.
C-D) Whole mount ISH with *Wnt1* of embryos electroporated with
Lmx1bMO at 1-4ss in the neural tube and incubated for 20hrs. Red
arrows indicate inhibition of the expression of the gene only on one side
showing Flourescein. Mes: Mesencephelon, Met: Metencephelon, FL:
Flourescein, A: anterior, L: lateral

4.7.2. Role of Lmx1b in neurogenesis

As *Lmx1b* is expressed very early in presumptive otic placode at 6ss stage
and is regionalized in complementary fashion to the proneural domain at
around 9-10ss stage (Abello *et al.*, 2007), the ability of *Lmx1b* in
regulating the expression of genes that play important role in neurogenesis
was explored.

Fgf10 is expressed restricted to the proneural territory and appears later
than *Sox3* but before *Neurogenin1*.

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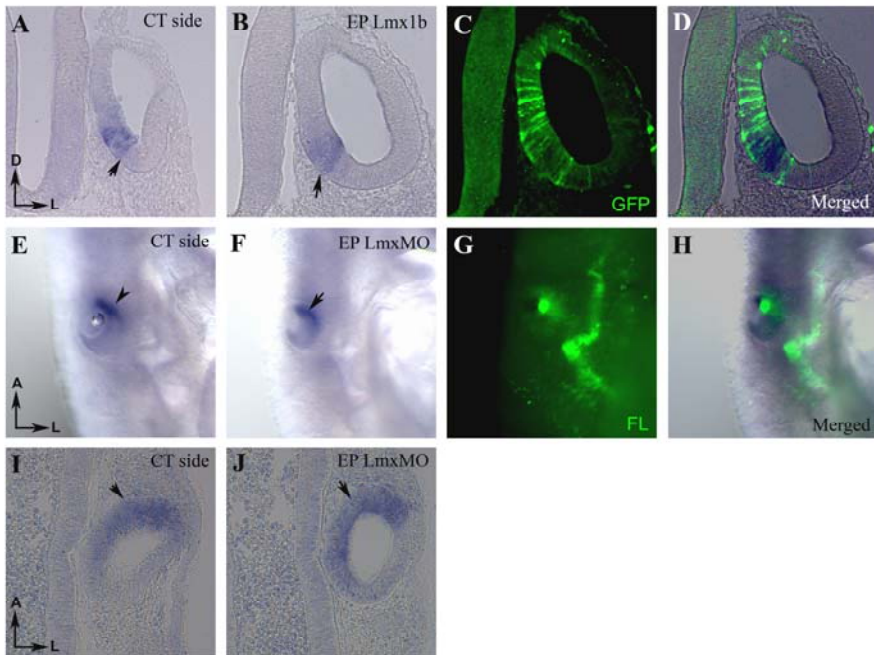
Figure 31: Role of *Lmx1b* in regulating *Fgf10* expression
A-D) Transversal section showing ISH with *Fgf10* of embryos electroporated
with *Lmx1b* at 5-9ss in the presumptive otic placode and incubated for 20hrs
thereafter. E-H) Whole mount ISH with *Fgf10* of embryos electroporated with
Lmx1bMO at 5-9ss in the presumptive otic placode and incubated for 20hrs
thereafter. I-J) Coronal sections of the same embryos electroporated with
Lmx1bMO at 5-9ss CT: control, EP: electroporated, FL: Flourescein, A:
anterior, L: lateral

For the gain of function of *Lmx1b*, we electroporated the full-length
Lmx1b in the presumptive otic placode at HH10 stages and left the
embryos for 20hrs. Analysis with *Fgf10* was carried out by in situ
hybridization. Figure 31A-D shows transversal sections of control side
and electroporated side of an embryo with *Fgf10* expression in the ventral
proneural territory. In the electroporated side with full-length *Lmx1b* no
difference was seen in the expression intensity or expression domain size
of *Fgf10* (0/3). Thus, our results suggest that *Lmx1b* does not regulate
Fgf10 expression and the neurogenic domain is established correctly even
if high levels of *Lmx1b* are present in the anterior territory. These results
are in accordance with our previously described results in which Notch

signaling was able to extend the expression domain of *Lmx1b* and keeping the size of proneural domain unchanged (Abello *et al.*, 2007).

We further wanted to see the effect of Lmx1bMO on *Fgf10* expression regulation. We use the same strategy for the loss of function of *Lmx1b*. For this we electroporated the same stage embryos with Lmx1bMO and left the embryos to develop for 20hrs afterwards. When we analyzed the expression of *Fgf10* in Lmx1bMO electroporated embryos (0/2), no difference was seen between the two sides of electroporated embryo (Figure 31E-J). Again, the inhibition of *Lmx1b* did not expand the expression of *Fgf10* confirming the above results of gain of function.

As *Lmx1b* up regulation and down-regulation did not show any effect on *Fgf10* expression, we further wanted to analyze the effect of *Lmx1b* on *NeuroD* expression. Again, we electroporated the Lmx1MO in the presumptive otic ectoderm at stage 5-9ss and left the embryos to develop for 20hrs afterwards. When we analyzed the expression of *NeuroD* in LmxMO electroporated embryos (0/5), no difference was seen between the control and electroporated otic vesicles of the embryos (Figure 32).
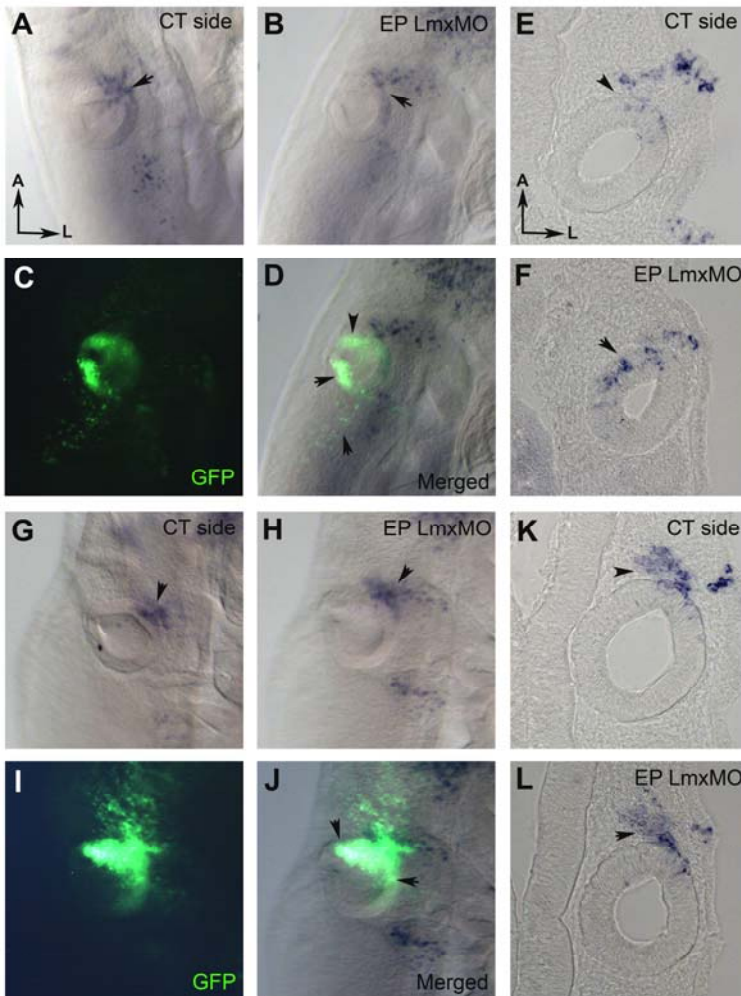
Figure 32: Role of *Lmx1b* in regulating *NeuroD* expression
A-D) Whole mount ISH with *NeuroD* of embryos electroporated with Lmx1bMO at 5-9ss in the presumptive otic placode and incubated for 20hrs thereafter. E-F) Coronal sections showing of Lmx1bMO electroporated embryos G-J) Whole mount ISH with NeuroD of embryos electroporated with Lmx1bMO at 5-9ss in the presumptive otic placode at anterior domain and incubated for 20hrs thereafter K-L) Coronal sections of the same embryos electroporated with Lmx1bMO CT: control, EP: electroporated, FL: Flourescein, A: anterior, L: lateral.

In Figure 32C fluorescein can be seen in the anterior as well as posterior domain and we did not see any ectopic *NeuroD* cells in the posterior non-neural domain (Compare Figure 32A and B). No ectopic *NeuroD* positive

cells were identified in the posterior non neural domain when the sections were analyzed coronally (Compare Figure 32E and F). Whereas, Figure 2I shows an excess of fluorescein in the anterior domain without an increase of *NeuroD* cells in the normotopic domain of *NeuroD* expression (Compare Figure 32G and H) and the coronal section also showed equal size of the *NeuroD* domain (Compare Figure 32K and L).

In conclusion, these results indicate that *Lmx1b* does not play any role in regulating genes that are expressed in the otic anterior neurosensory domain.

4.7.3. Role of Lmx1b in chick otic patterning

Next, we decided to investigate whether *Lmx1b* could regulate genes from the non-neurogenic domain. In the chick otic placode the expression of *Hairy1* appears at HH11, after *Lmx1b*, suggesting a possible role of *Lmx1b* regulating *Hairy1* expression.

For this the full-length *Lmx1b* or Lmx1bMO was electroporated in the presumptive otic ectoderm at stage 5-9ss and left the embryos to develop for 20hrs afterwards. When we analyzed the expression of *Hairy1* in *Lmx1b* electroporated embryos, *Hairy1* expression in the two otic cups was not different hence no ectopic nor enhancement of the *Hairy1* expression was observed (0/4) in the normal expression domain in any of our experimental embryos (Figure 33A-H).

Similarly, the loss of function experiment was conducted to check if the loss of *Lmx1b* had any effect on the expression of *Hairy1*. When the embryos were electroporated with Lmx1bMO (0/2), no difference was seen between the two sides of electroporated embryo (Figure 33I-N). Furthermore, coronal section (Figure 33M-N) show same size of expression domain with same intensity.
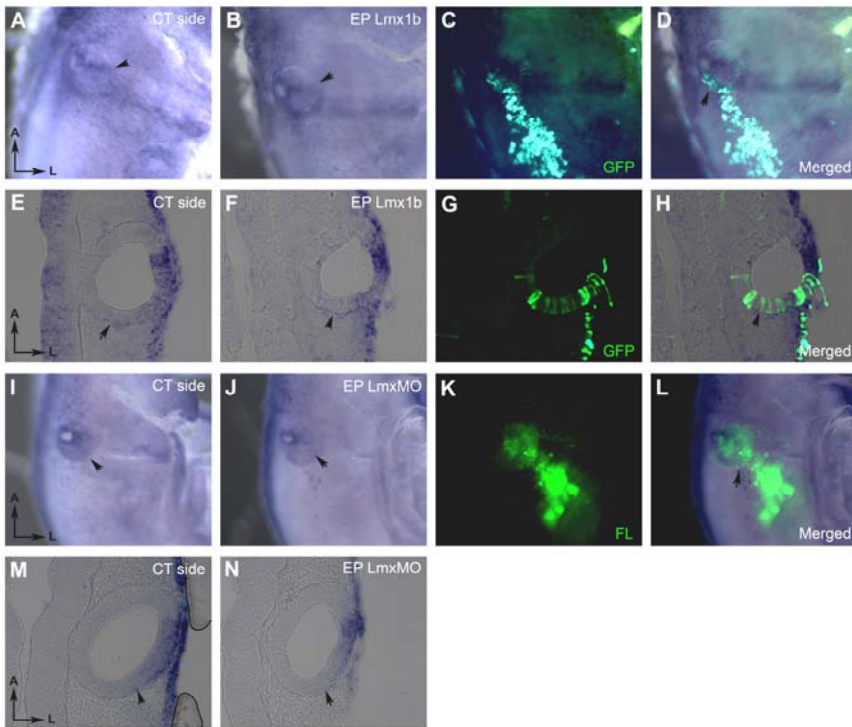
Figure 33: Role of *Lmx1b* in regulating *Hairy1* expression
A-D) Whole mount ISH with *NeuroD* of embryos electroporated with *Lmx1b* full-length at 5-9ss in the presumptive otic placode and incubated for 20hrs thereafter. E-H) Coronal cryostat sections of embryos overexpressing *Lmx1b*. I-L) Whole mount ISH with *Hairy1* of embryos electroporated with Lmx1bMO at 5-9ss in the presumptive otic placode and incubated for 20hrs thereafter. M-N) Coronal sections of the same embryos electroporated with lmxMO CT: control, EP: electroporated, FL: Flourescein, A: anterior, L: lateral

From these results we can conclude that *Lmx1b* is not involved in regulating *Hairy1* expression in chick otic development neither is required for the maintenance of its expression. It is possible that there are other signals which might regulate both genes at the same time.
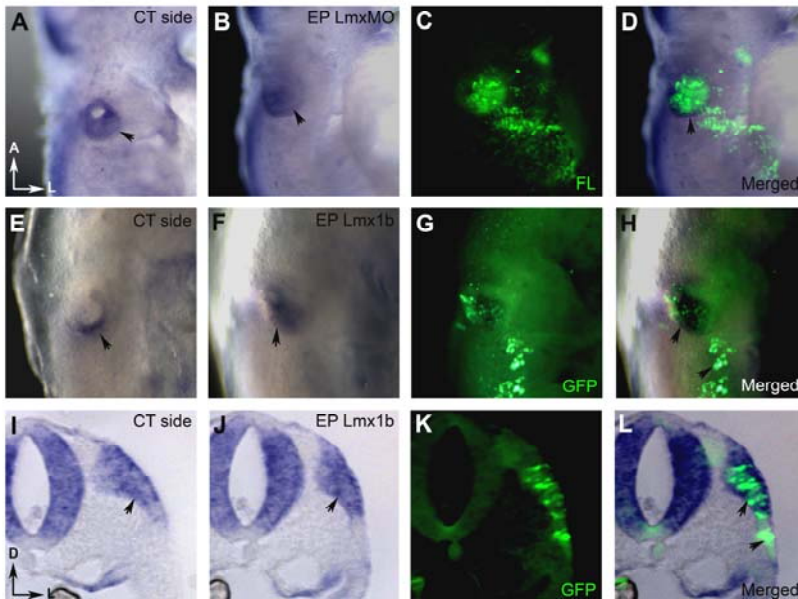
Figure 34: Role of *Lmx1b* in regulating *Irx1* expression
A-D) Whole mount ISH with *Irx1* of embryos electroporated with
Lmx1bMO at 5-9ss in the presumptive otic placode at posterior as well
anterior domain and incubated for 20hrs thereafter. E-H) Whole mount
ISH with *Irx1* of embryos electroporated with Lmx1bMO at 5-9ss in the
presumptive otic placode mainly at posterior domain and incubated for
20hrs thereafter I-L) Transversal sections showing no difference in the
expression of *Irx1* after *Lmx1b* electroporation CT: control, EP:
electroporated, FL: Flourescein, A: anterior, L: lateral.

Another important patterning gene that is expressed at the same time
when *Lmx1b* is expressed in an overlapping manner is *Iroquois* (*Irx*)
(Abello *et al.*, 2007). When we electroporated either full-length *Lmx1b* or
Lmx1bMO in the presumptive otic ectoderm at stage 5-9ss and examined
the expression of *Irx1* after incubating 20 hrs of post electroporation, no
difference was seen in the expression profile of *Irx1* in two sides of an
electroporated embryo. Figure 34A-D shows whole mount in situ with
Irx1 after electroporating Lmx1bMO in the right side of the embryo.
Although the fluorescein was seen in the region where *Irx1* is expressed,
the expression domain or the expression intensity of the *Irx1* did not

present difference between the control and the electroporated side of the same embryo (0/2).

Similarly, the gain of function experiment was conducted to check if the gain of *Lmx1b* has any effect on the expression of *Irx1*. When the embryos were electroporated with *Lmx1b,* no difference was seen between the two sides of electroporated embryo (0/3; Figure 34E-L).

4.7.4. Lmx1b role in Pax2 expression

 Previous studies have shown that *Pax2* is one of the earliest genes expressed in the presumptive otic placode (Groves and Bronner-Fraser 2000) and that *Lmx1b* expression followed afterwards. *Lmx1b$^{-/-}$* embryo showed a down-regulation of *Pax2* in the midbrain-hindbrain boundary (MHB) at the 3-somite stage whereas the expression was lost completely by E9.5 indicating that *Lmx1b* is required for maintaining *Pax2* expression (Gua *et al.,* 2007). These observations suggest that *Lmx1b* might play an important role in regulation.

To check whether *Lmx1b* plays role in maintaining the expression of *Pax2* in otic development as is seen in MHB development we used the same strategy of gain of function and loss of function experiments.
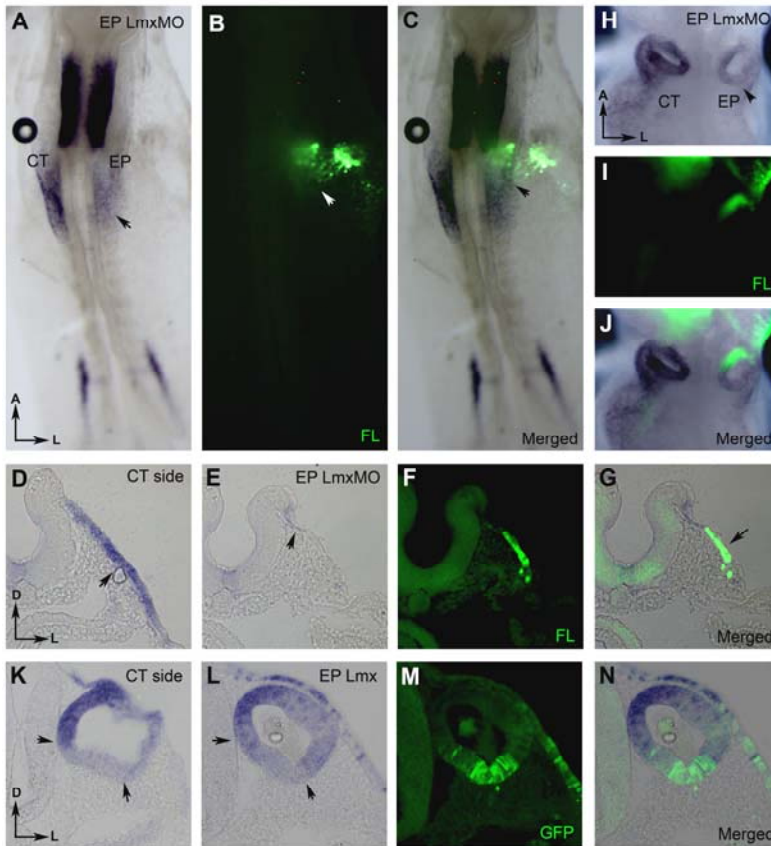
Figure 35: Role of *Lmx1b* in regulating *Pax2* expression
A-C) Whole mount ISH with *Pax2* of embryos electroporated with Lmx1bMO at 5-9ss in the presumptive otic placode and incubated for 6hrs thereafter. D-F) Transversal sections showing inhibition of *Pax2* expression after Lmx1bMO electroporation. H-J) Embryo showing whole mount ISH with *Pax2* expression after electroporation with Lmx1bMO at 5-9ss in the presumptive otic placode. K-N) Transversal sections of embryo showing no difference in the expression of *Pax2* after *Lmx1b* electroporation CT: control, EP: electroporated, FL: Flourescein, A: anterior, L: lateral

When we electroporated Lmx1bMO in the presumptive otic ectoderm at stage 5-9ss and examined the expression of *Pax2* after incubating 6hrs an inhibition in the expression of *Pax2* was seen only in the electroporated side (5/8) and *Pax2* expression was normal in the control non-electroporated side (Figure 35A-F). Otic ectoderm failed to get thickened in the electroporated side (compare Figure 35D and E). Embryos

electroporated with Lmx1bMO and incubated for 20hrs afterwards showed inhibition in the expression of *Pax2* only in the electroporated side (3/3) and *Pax2* expression was normal in the control non electroporated side (Figure 35H-J) although otic cup is developed normally and the morphology was not disturbed. Transversal sections showed cell autonomous inhibition of *Pax2*. These observations suggest that blockade of *Lmx1b* down-regulates the expression of *Pax2* and also delays otic development when the morpholino effects are checked only after 6hrs of incubation. After 20hrs of post-electroporation of Lmx1bMO the otic vesicles did not display any morphological defects suggesting that the maintained expression of *Pax2* was not required for growth and early morphogenesis of the otic vesicle.

Similarly, the gain of function experiment was conducted to check if the *Lmx1b* play role in *Pax2* induction. When *Lmx1b* was overexpressed in the otic vesicle some expansion of the *Pax2* domain was observed. In addition, when the embryos were electroporated with *Lmx1b* outside the *Pax2* ectodermal domain, in one example increased *Pax2* in the ectoderm was observed (1/3; data not shown). These experiments indicate that *Lmx1b* is able to regulate *Pax2* expression. This is in agreement with what has been described previously in the MHB.

4.8. Role of BMP signaling in the regulation of Lmx1b and Sox3 expression

Bone morphogenetic proteins (BMPs) are members of the transforming growth factor beta (TGFβ) superfamily and were first identified for their ability to induce ectopic bone formation (Wozney *et al*., 1988; Celeste *et al*., 1990; Kingsley, 1994; Reddi, 1994; Jones *et al*., 1991). The BMPs have been implicated in embryonic axis specification in many organisms, including *Xenopus*, chicken, and mice (see review by Hogan, 1996). In

addition to BMP ligands, it has been shown that locally expressed BMP antagonists play critical roles in the patterning and morphogenesis of rudimentary organs (Hogan 1996; Merino *et al.* 1999; Lu *et al.* 2001).

Previous work in our laboratory has addressed the role of FGF in the neural fate in the otic placode. In these studies, FGF signaling has been shown to be essential for the appearance of *Sox3* expression in the otic-epibranchial territory. Moreover, its restriction to the anterior territory is mediated by a local source of *Fgf8* in the anterior ectoderm (Abello *et al.*, mn in preparation, see introduction). During CNS development it has been shown that BMP signaling plays an opposing role to FGF pathway in the establishment of a neural fated ectoderm. However, an early role of BMP in the regulation of a neural and non-neural territory has not been addressed in the otic placode. BMP4 signaling pathway has been reported to regulate sensory specification and hair-cell differentiation. Because of the fact that 1) *Lmx1b* is expressed complementary to *Sox3* in the otic field, 2) *Lmx1b* is regulated by BMP signaling in roof plate (Chizhikov and Millen, 2004) and, 3) BMP7 is expressed in the posterior otic territory, we have analyzed the early role of BMP signaling in *Lmx1b* expression during inner ear development.

Several approaches have been followed for this purpose. On one hand, embryos were cultured in the presence of BMP signaling inhibitors such as Noggin (an antagonist of BMP ligand) and Dorsomorphin (6-[4-(2-Piperidin-1-yl-ethoxy)phenyl]-3-pyridin-4-yl-pyrazolo[1,5-a]pyrimidine), also known as compound C (Hao *et al.*, 2008), which selectively inhibits BMP type I receptors, on the other hand BMP signaling was activated by the electroporation of a constitutive active form of the BMPR1 at early stages of otic development.

4.8.1. Blockade of BMP signaling by Noggin and Dorsomorphin treatment

Chick embryos were cultured in the presence or absence of Noggin at 5-10ss stages and cultured for 6hrs. We then analyzed and compared the expression of *Lmx1b* between embryos cultured in the presence or absence of Noggin.

Down-regulation of *Lmx1b* expression was observed in all stages with strongest effects in the embryos that were cultured in the presence of Noggin (1μg/ml) at stages as early as 5-6ss (2/4) and 7-8ss (4/5). In contrast control embryos cultured in the absence of Noggin did not show reduction in *Lmx1b* expression either at 5-6ss (0/3) nor 7-8ss (0/2) stages (Figure 36C and D). When explanted embryos from 7-8ss were also cultured in the presence of Dorsomorphin (10μM) or DMSO, a stronger inhibition of *Lmx1b* was observed (Figure 36A and B). Interestingly, the posterior domain of *Lmx1b* was inhibited in a stronger fashion than the most medial expression domain, suggesting that higher levels of BMP are present in the hindbrain that sustain medial *Lmx1b* expression or that other signals maintain *Lmx1b* expression in that area.

The *Msx1* and *Msx2* genes have been proposed as downstream targets of BMP signaling in a variety of experimental situations (Vainio *et al.*, 1993, Hogan, 1996; Maas *et al.*, 1996). In the limb mesenchyme of conditional mutants of BMP, loss of *Msx1* expression was documented at 10.75 dpc whereas expression of both *Msx1* and *Msx2* mesenchyme is significantly diminished at 11.5-11.75 dpc, (Ovchinnikov *et al.*, 2006). When the expression of *Msx1* was analyzed after culturing the embryos in the presence or absence of Noggin for 6hrs at 9-10ss stages, a down-regulation of *Msx1* was observed (Figure 36E and F) in the experimental embryos (1/1) but not in control embryos (1/1). These experiments suggest that the BMP directly regulates the expression of *Lmx1b* and its presence is important for the maintenance of *Lmx1b* expression.

We have mentioned above that *Sox2* and *Sox3* are among the first transcription factors to be expressed in a spatial and temporal pattern consistent with the acquisition of neural fate in the ectoderm (Okuda *et al.*, 2006). Ectopic BMP signaling leads to the expansion of prospective non-neural ectoderm at the expense of prospective neural ectoderm (Kudoh *et al.*, 2004, Rentzsch *et al.*, 2004; Delaune *et al.*, 2005).
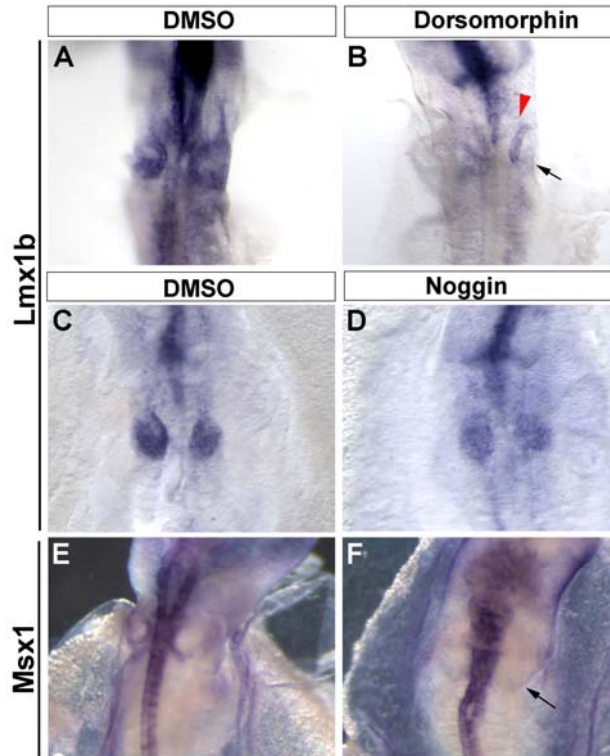
Figure 36: Effect of BMP inhibition on *Lmx1b* regulation
A-B) Embryos of 7-8 ss cultured for 24 hr in the presence of DMSO or Dorsomorphin (10μM). C-D) Embryos of 7-8 ss for 6hr cultured in the presence of DMSO or Noggin (1μg/ml) A-D In situ hybridization for *Lmx1b*. E-F) 9-10ss embryos incubated in the presence of DMSO or Noggin for 6hrs and ISH with *Msx1*.
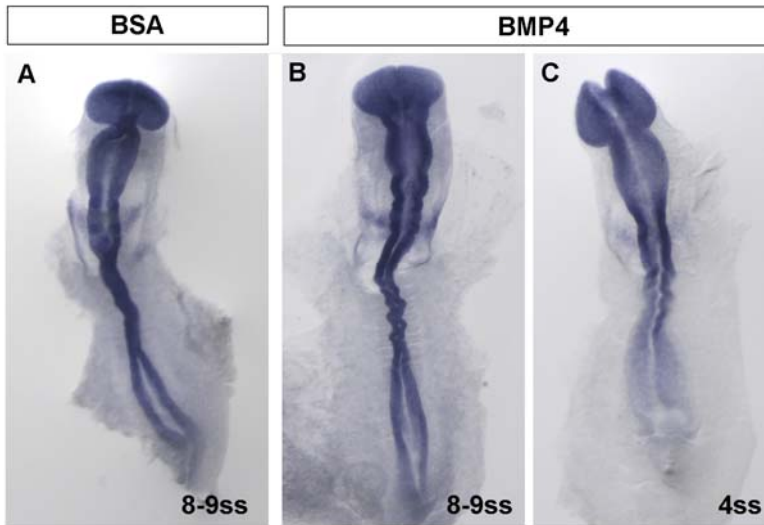
Figure 37: Role of BMP in regulating *Sox3* expression
No difference in the expression of *Sox3* was observed after culturing embryos in the presence of BMP4. A: anterior, L: lateral.

In contrast, work in chick has shown that the earliest phase of neural induction occurs prior to gastrulation (Streit *et al.,* 2000) and that inhibition of BMP signaling is only required as a late step in the selection of neural fate (Linker and Stern, 2004; Stern, 2005). For this reason the influence of exogenous BMP4 on *Sox3* expression was analyzed by culturing embryos in the presence of soluble BMP4 or 5%BSA for 6hrs. When embryos of stage 8-9ss were cultured with high doses of BMP4, no inhibition of *Sox3* was detected (0/4) (Figure 37A and B). This could be due to the fact that BMP4 does not play role in the maintenance of *Sox3* expression at that stage but influences its establishment. However, when embryos of 4ss stage where cultured with BMP4, again *Sox3* expression was not modified. This results, suggests that BMP4 does not regulate *Sox3* establishment nor maintenance.

4.9. Overexpression of a constitutive form of the Alk3 dramatically expanded Lmx1b expression

We further verified our results by electroporating vector pCIG-caALK3 (*Activin-Like Kinase Receptor-3*), a constitutively active BMP receptor type 1A (Bmpr1a) under control of β-actin enhancer and CMV promoter. Bmpr1a encodes BMPR-IA, one of the type I receptors for BMP2, BMP4 and BMP7 (Yamaji *et al.*, 1994). This single-pass transmembrane serine-threonine kinase is ubiquitously expressed during embryogenesis and is required for the initiation of gastrulation in the mouse embryo (Mishina *et al.*, 1995a, b; Zhao, 2002). To address the question if gain of function of BMP has opposite effect on *Lmx1b* expression, chick embryos were transfected with Bmpr1a by electroporation into the presumptive otic ectoderm at stages 4-9ss and assayed for gene expression after culture for 20 hrs. pCIG-caALK3 plasmid contain an IRES Nuclear-GFP cassette. After 20hrs of electroporation, chick embryos were sacrificed and analyzed for the expression of *Lmx1b*. In contrast to the previous Noggin and Dorsomorphin experiment where *Lmx1b* was down-regulated just within 6 hrs of incubation, a dramatic expansion of *Lmx1b* was seen in the otic vesicles (Figure 38). *Lmx1b,* which is normally confined to the posterior non-neural domain, was expanded into the proneural domain and/or ectopic *Lmx1b* expression was seen outside or within otic domain (11/11) after pCIG-caALK3 electroporation. No expansion of Lmx1b was observed in embryos (0/2) that were electroporated with empty pCIG and incubated for 20hrs.
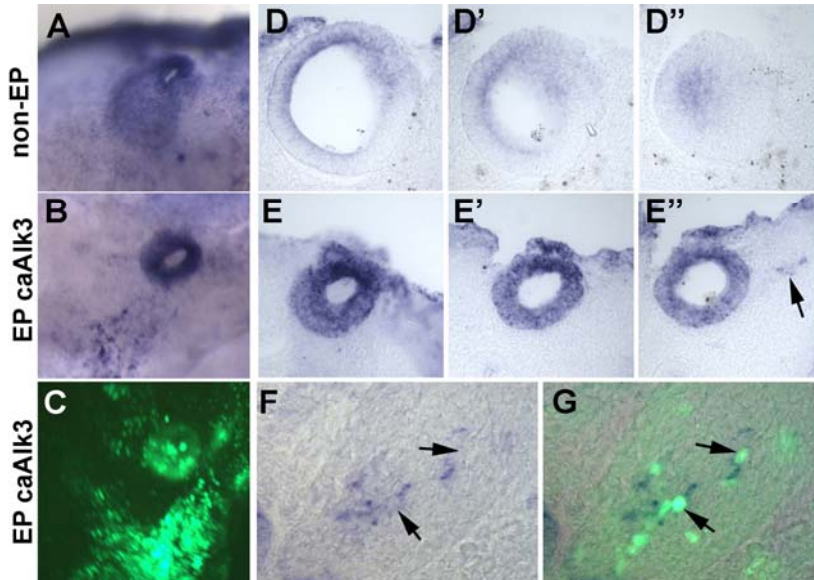
Figure 38: Effect of gain of function of BMP on *Lmx1b* expression
A-C) Whole mount of an embryo hybridized for *Lmx1b* probe after 20hrs of electroporation with pCIG-caALK3. D-D'') Sagittal section of CT side of the electroporated embryo and E-E'') sagittal section of the electroporated side of the embryos. F and G) higher magnification showing ectopic *Lmx1b* expressing cells it the ganglion. A: anterior, L: lateral, CT: control, EP: electroporated.

Interestingly, *Lmx1b* expressing cells after overexpression of pCIG-caALK3 were found in the CVG (Figure 38F-G). During normal development *Lmx1b* expressing cells are never observed in the CVG. The fact that delaminated neuroblasts do express in the CVG further indicates that ectopic BMP activity is able to induce *Lmx1b* expression in the neuogenic domain. However, the *Lmx1b*-expressing cells present in the neurogenic territory continue their developmental pathway and transit into neuroblasts that are able to delaminate into the CVG even in the presence of *Lmx1b* activation. This is in accordance with previous results in which exogenous BMP4 signaling does not block Sox3 expression, thus otic neural specification.

In conclusion, by either blockade or over activation of BMP signaling pathway it has been shown that *Lmx1b* expression depends on this

pathway for its induction. This is interesting because for the first time an early role of BMP signaling in otic development has been described in addition to its well characterized role in sensory specification. Furthermore this implies that during the early phases of otic placode development in which the proneural territory is established, a BMP signaling activity is present in parallel to the previously described FGF signaling.

4.10. Effect of overexpression of a constitutive form of the Alk3 on the expression of other genes

As *Lmx1b* was expanded into the proneural domain in response to misexpression of Bmpr1a we have also analyzed a possible effect on genes that are expressed in the proneural domain. When pCIG-caALK3 was electroporated at HH 8-9 and embryos were incubated for 20hrs and analyzed for the expression of *Fgf10*, no difference was seen in the expression of Fgf10 between the control and electroporated otic vesicles (Figure 39A-F). These results are in accordance with our previous experiments in which embryos cultured in the presence of DAPT (an inhibitor of Notch signaling) expanded the expression domain of *Lmx1b* (designated by the non neural domain) but the proneural domain was not reduced (Abello *et al*, 2007) and with our current results of *Lmx1b* overexpression. These results suggest that the proneural domain is established and maintained independently of the action of posterior genes such as *Lmx1b* or BMP activity. However, it should be noted that these analysis have been performed only after 20hrs of incubation and further defects in inner ear development and sensory specification due to ectopic *Lmx1b* or ectopic BMP activity have not been explored nor the final developmental paths followed by the *Lmx1b*-neuroblasts.

As in our gain and loss of function experiments of BMP, *Lmx1b* expression was shown as tightly regulated by BMP signaling, we explored

the expression of other genes like *Hairy1* whose expression is similar to the *Lmx1b* at many places in the chick. For this the embryos were electroporated with pCIG-caALK3 in the presumptive otic ectoderm at 5-9ss stages and incubated for 20hrs and analyzed for the expression of *Hairy1*. In our gain of function experiment, a small expansion and induction in the expression of *Hairy1* (Figure 39G-L) was seen in the electroporated embryos (2/4). These results suggest that BMP signaling besides regulating the expression of *Lmx1b* also regulates the expression *Hairy1*.
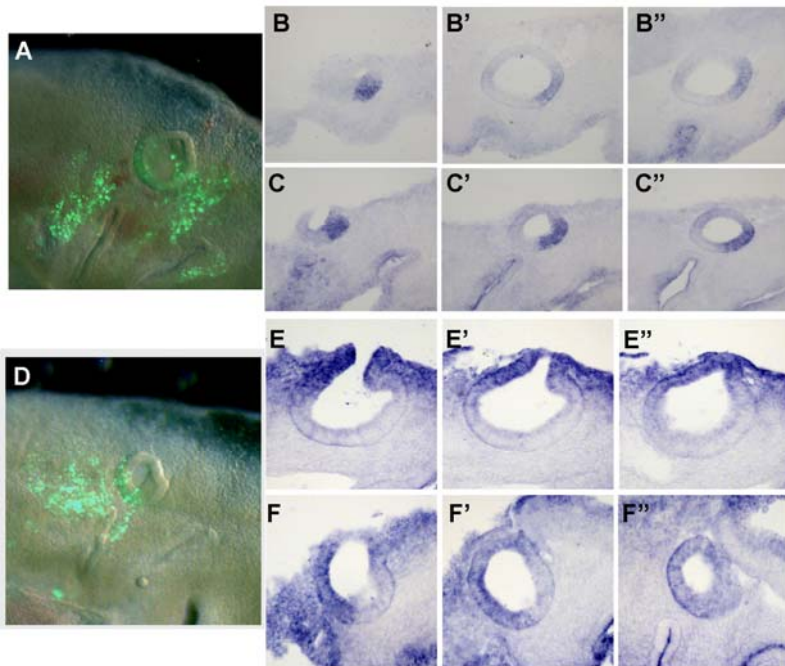
Figure 39: Effect of gain of function of BMP on *Fgf10* and *Hairy1* expression
A and D) whole mount ISH of two embryos with *Fgf10* and *Hairy1* respectively that were electroporated with pCIG-caALK3 and left for 20hrs for incubation. B-C'') sagittal sections of otic cups of control side of embryo (B-B'') or electrporated (C-C'') revealed with *Fgf10*. E-F'') sagittal sections of control (E-E'') or EP side of embryo (F-F'') stained for with *Hairy1* probe. D: dorsal, A: anterior, L: lateral, CT: control, EP: electroporated.

DISCUSSION

5. DISCUSSION

During the last 50 years, enormous efforts have allowed understanding the molecular mechanisms by which the ectoderm is induced to a neural fate and the CNS develops. In parallel, in several species it has been described how the cephalic PNS derives from neural crest and cranial placodes as well as the signals that induce them. Despite these advances, little still is known on how neural competence and commitment is acquired in the cranial placodes. Is the process of neural fate in the placodes a default of placodal fate? Which genes mediate neural fate and induce proneural genes?

In the present work, I have studied the early events of otic regionalization and acquisition of neural fate of the otic primordium focusing on the roles played by *Sox3* and *Lmx1b* in this process. Our laboratory has shown previously that the first event of inner ear regionalization is the establishment of a neural competent domain at the onset of the otic placode formation. The otic placode is formed by two cell populations: one anterior characterized by the expression of *Sox3*, and another posterior exhibiting higher levels of *Lmx1b*.

Gain of function experiments of *Sox3* gene in the preotic territory show that *Sox3* is responsible for the commitment of the otic ectoderm to a neural fate and induces *Delta1* expression. The ectopic neural fated cells however do not transit to a fully differentiated state reinforcing the idea that high levels of *Sox*3 maintain otic cells in a progenitor/precursor state. Interestingly, the experiments also showed that otic neural specification leads to a down-regulation of *Lmx1b* but not other non-neurogenic genes, revealing a novel genetic interaction between *Sox3* and *Lmx1b*. BMP signaling which has been reported to have a role in sensory development is required at early stages for the induction of *Lmx1b* expression in the

otic field. A model is proposed in which neurogenic and non-neurogenic territories are established by a mechanism that concomitantly depends on anterior FGF signaling to promote *Sox3* and BMP activity to induce *Lmx1b* caudally. Progressively as the anterior otic territory commits to a neural fate, *Lmx1b* is further excluded from the neurogenic territory. In addition, we show evidences that *Lmx1b* function is required for maintenance of *Pax2* expression in the inner ear.

Sox3 and Sox2 are expressed in a distict manner in the otic field and Sox3 can induce Sox2.

It was shown previously that *Sox3* expression appears in the otic field (Groves and Bronner-Fraser, 2000) and in a common epibranchial placodal domain (Ishii *et al*., 2001). Detailed analysis of *Sox3* expression by removing the underlying mesoderm and endoderm has revealed that indeed *Sox3* expression initially appears broadly in a common otic and epibranchial territory and progressively gets restricted to an anterior band from 7 to 10ss embryo that encompasses the epibranchial and most anterior otic field. On the other hand, *Sox2* expression is very weak at those preotic stages and does not present an anterior restriction in the otic placode. The expression profile presented here further indicates that *Sox3* and *Sox2* may display distinct functions during otic development. This is in agreement with expression profiles of both SoxB1 proteins conducted by Neves and colleagues at otic cup and otic vesicle stages that revealed that both *Sox3* and *Sox2* co-exist in the neurogenic territory but only *Sox2* is present later on in the sensory epithelium (Neves *et al*., 2007). In mouse, *Sox2*-deficient mice like *light coat and circling* (*Lcc*), and *yellow submarine* (*Ysb*), show hearing and balance impairment (Kiernan *et al*., 2005). Thus, although both genes share high similarity, in the inner ear may play distinct roles.

At preotic stages high levels of *Sox3* precede expression of *Sox2* in the otic placode. During CNS formation, the chick *Sox2* and *Sox3* genes are expressed in similar but not with identical patterns. *Sox3* is expressed throughout the epiblast before primitive streak formation and becomes progressively restricted to the forming CNS, while *Sox2* appears in the presumptive neural ectoderm at the time of neural induction before the CNS becomes morphologically apparent, and its expression is then restricted to the CNS as it develops (Rex *et al.*, Rogers *et al*, 2009). It is tempting to suggest that in the otic placode, *Sox3* could also be delineating the ectoderm competent to respond to inducing signals. At this moment it is difficult to discriminate if restriction of *Sox3* to the anterior placodal domain reflects the restriction of competence to respond to neural inducing signals or whether reflects restriction of neural committed cells from a pan-competent domain. Graft experiments at different times with neural inducing signals should address this issue by mapping the exact temporal window of otic neural induction. So far, it has been determined by blockade of FGF signaling either at 5-6ss or at 7-8ss, that the process of otic induction is dissociated of neural induction, suggesting that neural induction goes from 7ss onwards while otic induction has already finished at that stage (Abelló *et al*, mn in preparation).

On the other side, restriction of *Sox3* expression to the anterior field but not *Sox2* seems to be a pre-requisite for the acquisition of a neurogenic fate in the anterior territory. The anterior enhancement of *Sox3* expression has been shown to be in part mediated by the appearance of *Fgf8* in the same ectodermal band (Abelló et al., mn in preparation). In a similar manner, it was already reported that, *Fgf8* is able to induce *Sox3* expression in the chick epiblast and it is necessary for *Sox3* expression in zebrafish epibranchial placodes (Streit *et al.*, 2000; Nikaido *et al.*, 2007).

In *Xenopus*, overexpression of *Sox3* was able to directly activate *Sox2* and *gem* in naïve ectoderm, while *Sox2* was unable to do so (Rogers *et al.*, 2009). In zebrafish embryos, injection of MO-Sox3 caused a dramatic down-regulation of *Sox2* expression in the CNS (Dee *et al.*, 2008). During placode neural development, the relationship between both genes has yet not been analyzed and here I present evidence that overexpression of *Sox3* in the otic ectoderm is able to induce *Sox2*, following a similar logic than during CNS development. Taken together, the results suggest that *Sox3* is the earliest gene to be mediating neural commitment in the otic placode and that its regionalization may be important for the final emergence of a neurogenic and non-neurogenic otic epithelium.

Sox3 mediates the acquisition of neural fate in the otic ectoderm

Sox3 knock-down in inbred mice is lethal as early as gastrulation but in outbred mice Sox3 display mild defects such as craniofacial abnormalities and reduced size and fertility (Rizzoti *et al.*, 2004). Thus, work on SoxB1 function during neural development has been mainly addressed in zebrafish and *Xenopus*. Overexpression of *Sox3* leads to expansion of neural ectoderm and concomitant N-tubulin and *Ngn1* expansion (Dee *et al.*, 2008; Rogers *et al.*, 2009). In *Xenopus*, induction of neural markers is evident at tailbud stages but not at HH14 where a down-regulation of *Ngn1* was observed, suggesting that is a consequence of promoting neural fate but not a direct regulation of *Sox3* on *Ngn1* expression. Our results of overexpression of *Sox3* in the otic ectoderm reveal that overexpression of *Sox3* in the otic fated ectoderm can promote *Delta1* expression, but most probably this effect is indirect; *Delta1* expressing cells being detected only after 6hrs but not 3hrs post-electroporation even if Sox3 protein is already present shortly after 1hr of electroporation. Taking the published data and our results presented here, it can be postulated that *Sox3* initially

induces *Sox2* which in turn activates neurogenesis, leading to the activation of *Delta1* in *Sox3*-expressing cells only after 6hrs. Overexpression of SoxB1 proteins in the neural tube indicated that SoxB1 genes inhibited neural markers (Bylund *et al.* 2003). However, recent data in *Xenopus* suggests that primary neurogenesis is not inhibited by *Sox3* overexpression but actually delayed (Rogers *et al.*, 2009). In Bylund report early markers of neuronal fate barely decreased, while NeuroM and NeuN showed a dramatic reduction when *Sox3* was electroporated suggesting an impairment of neuronal differentiation by *SoxB1* genes. Those experiments differ from the presented here as overexpression of *Sox3* was performed in an already specified neurogenic epithelium. In zebrafish and *Xenopus*, *Sox3* was overexpressed before neural induction stage and in both cases expansion and/or ectopic neural tissue was observed (Dee *et al.*, 2008; Rogers *et al.*, 2009). In those experiments *Sox3* delays differentiation by keeping cells in a precursor undifferentiated state but *Sox3* cannot override the neurogenic pathway leading eventually to increased number of neural tissue. This is in agreement with our results in which, overexpression of *Sox3* induces *Delta1* expression but the ectopic cells rarely transit into differentiation measured with neuronal markers such as *NeuroD* and Islet1. Detail analysis of the induction of Delta1 on cells overexpressing *Sox3* indicate that only the cells with less Sox3-GFP protein or that have already begun down-regulating the fusion protein do activate *Delta1*. Thus, *Delta1* is induced when high levels of Sox3-GFP start to diminish. Another point to address is that at 40hrs post-electrporation, Sox3-GFP protein was completely down-regulated (data not shown) but very few cells did express *NeuroD* ectopically. The explanation for this result may be due to lack of proper trophic factors in the non-neurogenic domain. FGF10 has been shown to be required for otic neuronal differentiation (Alsina *et al.*, 2004) and lack of FGF10 in the non-neurogenic territory could account for the observed cell death of the

ectopic *Delta1*-positive cells generated by *Sox3* overexpression. Further experiments by overexpressing *Sox3* together with FGF10 should resolve this question. In parallel, it cannot be obviated that several patterning genes are expressed in the non-neurogenic domain, thus ectopic *Sox3/Delta1* cells might enter into conflict with *Tbx1*, *Hairy1* or either any unidentified gene. In the mouse, *Tbx1* expression is found exclusively in the posterior half of the otic cup (Vitelli *et al*., 2003, Raft *et al*., 2004, Arnold *et al*., 2006). Interestingly, in *Tbx1* null mutants, the expression domains of genes such as *Ngn1*, *NeuroD1*, *Lfng* and *Fgf3* lose their normal anterior restriction in the otocyst and expand into more posterior regions, while are significantly reduced in a transgenic mouse line carrying multiple copies of human TBX1 (Raft et al., 2004). On the other side, *Hairy* and *Enhancer of Split homologs* (*Hes/Her*) act as prepattern genes in the zebrafish midbrain-hindbrain boundary, mouse olfactory placode and the inter-proneural stripes of the Xenopus and zebrafish neuroectoderm by repressing neural fate *(Cau et al*., 2000; Geling *et al*., 2003; Bae *et al*., 2005*)*. And finally, the placode is probable receiving constant information from surrounding tissues for proper patterning. It is well known, that signals from the adjacent neural tube are essential for DV patterning and at some extend to AP patterning in chick. Rotation of the neural tube and notochord of E1.5 chick embryos such that the DV axis was inverted relative to the otic vesicle led to the acquistion of ventral identity in dorsal regions. Anteroventral markers, including *Lfng* and *NeuroD*, became expressed anterodorsally, adjacent to the rotated notochord and floorplate; a ventral marker (*Otx2*) expanded dorsally and expression of the dorsal marker *Gbx2* was lost (Bok et al., 2005), being Shh an essential ventralizing factor (Liu et al., 2002; Riccomagno et al., 2002; Bok et al., 2005).

One of the puzzling results is that *Hes5.2* activation at 6hrs was not detected adjacent to the *Delta1*-induced cells in the posterior territory

suggesting that the generation of *Delta1* cells is independent on lateral inhibition. In contrast, when *Hes5.2* expression is analyzed after 20hr a clear down-regulation of *Hes5.2* is found in the Sox3 electroporated cells in the neurogenic territory. Interestingly, mapping of the Notch1-intracellular domain (N1ICD) have shown that Notch pathway is only active in the neurogenic domain (Del Monte *et al.*, 2007). Therefore, in that domain conversion of cells to *Delta1* dramatically switches off *Hes5.2* expression in the same cell. When the effects on *Sox3* overexpression on the neurogenic territory was analyzed by comparing the size of the ganglion, similar results to Bylund et al are found, consistent with a delay on neuronal differentiation.

The fact that overexpression of *Sox3* promotes neurogenic phenotype in the caudal otic epithelium before HH12 but not after indicates that the non-neurogenic territory is not fully specified before this stage. This would be in agreement with recent experiments suggesting that the final regionalization of the otic placode is probably not fixed until the otic cup stage. Otocyst rotations and recent work on otic cup rotations in the AP axis suggest that, in spite of the regionalized expression of various genes along the AP axis, the AP regionalization is not totally fixed at 16 ss, and re-specification may take place after otic placode formation (Wu *et al.*, 1998; Bok *et al.*, 2005). Only after patterning is fixed, segregation of territories could allow the control of local cell fate decisions or govern morphogenetic processes and growth.

Lmx1b expression is inhibited by Sox3

SoxB1 proteins can function as either activators or repressors depending on the context. For example, *Sox2* activates expression of δ-crystallin, Nestin, and Pax6 (Ambrosetti *et al.*, 1997; Donner *et al.*, 2007; Kamachi *et al.*, 2000; Kamachi *et al.*, 2001; Tanaka *et al.*, 2004; Wilson and

Koopman, 2002) and can also activate and repress expression of itself, Oct-4, FGF-4, Nanog, and UTF1 to control the stem-cell state (Boer et al., 2007; Botquin et al., 1998). The interaction with different partner proteins (Kamachi *et al*., 2000; Wilson and Koopman, 2002) and post-translational modification, such as SUMOylation has been postulated as a possible mechanism for modulating SoxB1 gene activation or repression functions. By overexpressing *Sox3* during neural induction, *Sox3* inhibited *zic1* expression and *Xvent2* (Dee *et al*., Rogers *et al*., 2009). *Xvent2*, an epidermal marker dependent on BMP signaling, however was not directly inhibited by *Sox3* but instead down-regulated through the activator functions of *Sox3* in promoting neural fate (Rogers et al., 2009).

We also explored the role of *Sox3* in regulating genes that are expressed in the non-neural domain in order to assess the relationship between both compartments. *Sox3* overexpression dramatically inhibited *Lmx1b* transcription. The effects were evident after 20hrs of post-electroporation but not before, suggesting that *Sox3* does not regulate *Lmx1b* directly. It is possible that *Sox3* induces neural fate in the posterior domain and activation of neural genes leads to *Lmx1b* inhibition. However, electroporation of either Sox3-EnR or Sox3-VP16 constructs would allow us to discriminate whether *Sox3* is acting as a repressor or activator when *Lmx1b* is down-regulated. Furthermore, electroporation of *Delta1* or *Ngn1* at preotic stages could give a hint on the molecular mechanism behind *Lmx1b* regulation. The fact that *Sox3* can down-regulate *Lmx1b* highlights the mechanism by which the diffuse *Lmx1b* expression is progressively excluded from the neurogenic domain and patterning between neurogenic and non-neurogenic domains is refined.

In the neural tube, *Lmx1b* maps in the roof plate, floor plate, and in the midbrain-hindbrain boundary, all domains of no active neurogenesis (Giraldez, 1998; Yuan and Schoenwolf, 1999). However, the relationship between *Sox3* and *Lmx1b* has not been addressed in the neural tube.

In contrast to the down-regulation of *Lmx1b,* another posterior gene like *Tbx1* was not modulated by *Sox3* overexpression. This data indicates that *Tbx1* does not depend on neural fate and *Sox3* activity. Therefore, it can be postulated that *Tbx1* expression depends in distinct signals from the ones regulating *Lmx1b*. Experiments conducted in zebrafish suggest that *Tbx1* could be established by retinoic acid from the posterior somites (MR, unpublished results). Blockade of FGF in chick suppressed *Sox3* expression and concomitantly expanded *Lmx1b* expression but not *Tbx1* (Abelló et al., unpublished results) reinforcing the relationship between *Sox3* and *Lmx1b*. From the above results one could hypothesize that the specification of the neurogenic and non-neurogenic domains are independent but final *Lmx1b* expression is dependent on the neurogenic activity. In Dreher mutant, loss of *Lmx1a* expands *Sox2* sensory domains, further supporting an interaction between the two compartments for the final refinement of patterning (Nichols *et al., 2008*).

Sox3 does not regulate Pax2 expression but induces ectoderm to a placodal phenotype

As revisited by (Begbie and Graham, 2001) the placodes have for long been defined as focal thickenings found in the vertebrate head that give rise to cranial sensory structures, with some but not all common features. In *Medaka* fish, ectopic *Sox3* expression leads to ectopic expression of *Pax6* and *Eya1* in embryonic ectoderm and causes ectopic lens and otic vesicle formation (Koster *et al.,* 2000). In chick, *Sox3* overexpression induced ectodermal thickenings in the otic epibranchial field (Abu-Almagd *et al.* 2001). Thus, *Sox3* may mediate distinct roles depending on the stage, co-factors bound to the protein or to the dose of expression. In our hands, ectopic expression of *Sox3* was unable to ectopically induce *Pax2* but in several instances induced ectodermal thickenings that

expressed *Delta1* within the *Pax2* domain. When *Sox3* was overexpressed close to the otic vesicle (note close to the *Pax6* domain), ectodermal thickenings resembling ectopic lens placodes were generated. In chick, ovexpression of Sox2 together with Pax6 did generate ectopic lenses and *Pax6* and *Sox2* bind cooperatively to delta-crystallin promoter (Kamachi *et al.,* 2001; Kondoh *et al.,* 2004) Thus, Pax2/Sox3 may act together to specify otic fate, while Sox3 with other co-factor could mediate neurogenic functions.

In summary, we conclude that *Sox3* is able to give neural competence to the naive ectoderm by inducing *Sox2, Delta1* and to some extent *NeuroD* but not Islet or Tuj, which means that *Sox3* alone is not enough for complete differentiation and that other factors from non-neural domain or from neural tube are important for fully neuronal differentiation. Moreover, *Sox3* inhibits the expression of *Lmx1b* only but not other otic genes suggesting that final regionalization of *Lmx1b* depends on neural committment.

Role of Lmx1b in patterning and otic development

In the present study, we have shown that: (1) expression domains of *Lmx1b* and *Sox3* are gradually segregated before otic placode formation leaving expression of each gene within its territory non-neural and proneural otic domain until cup stage; (2) misexpression of *Lmx1b* has little effect on the expression pattern of *Pax2* in the otic territory; (3) silencing the expression of *Lmx1b* resulted in a dramatic down-regulation of *Pax2* expression; (5) *Fgf8* expression was repressed in *Lmx1b*MO injected embryos at the IsO but not in the otic region; (6) misexpression or silencing of *Lmx1b* does not affect the expression of patterning genes like *FGF0, Irx1* or *Tbx1*. The possible role of *Lmx1b* in the formation and maintenance of inner ear development is discussed below.

Nail patella syndrome patients (an autosomal dominant disorder due to mutation in LMX1B gene) have shown sensorineural hearing defects (Bongers *et al.*, 2005) and the deaf (otic vesicle expression) phenotype observed in homozygous dreher mouse mutants (autosomal recessive mutation in Lmx1a) (Failli *et al.*, 2002). Recent more exhaustive analysis on the developmental malformations due to *Lmx1a* mutation in the inner ear has revealed that newborn *Lmx1a* mutants have only three sensory epithelia: two enlarged canal cristae and one fused epithelium comprising an amalgamation of the cochlea, saccule, and utricle. Horizontal and anterior crista fused, whereas the posterior crista fuses with an enlarged papilla neglecta that may extend into the cochlear lateral wall (Nichols *et al.*, 2008).

In chick, *Lmx1b* instead of *Lmx1a* gene is present in the developing inner ear. *Lmx1b* is one of the earliest genes expressed at the presumptive otic ectoderm after *Pax2* expression and before the otic placode is morphologically visible. From a medial row of cells adjacent to the closing neural tube, progressively cells concentrate to the caudal and medial otic region with some scattered cells in the anterior domain. Complementary expression of *Lmx1b* and genes from the neurogenic territory is more evident at otic cup/vesicle stage.

One of the approaches followed in this work to knock-down *Lmx1b* function has been the use of morpholinos specific for *Lmx1b* transcript. Not too many reports using this technique in chick have been reported but for example it has been used to successfully block *Pax7* expression (Basch *et al.*, 2006). Recently, it was reported that for young embryos (before HH11) the use of morpholinos has less deleterious effects than RNAi electroporation (Voiculescu *et al*, 2008; Mende *et al.*, 2008).

Specific MO-Lmx1b electroporated in the MHB were able to down-regulate *Fgf8* and *Wnt1*, two genes previously reported to depend on *Lmx1b* activity in the isthmus, demonstrating their effectiveness in

blocking *Lmx1b* function (Guo *et al.*, 2007). When MO-Lmx1b were electroporated in the otic field, a dramatic down-regulation of *Pax2* in the ectoderm and in the otic vesicles was seen. In Lmx1b-/- mice, the transcription factors *En1* and *Pax2* were down-regulated prior to the 4-somite stage in the MHB (Guo *et al.*, 2007). The results thus indicate that in the otic field *Lmx1b* may also be required for the maintenance of *Pax2* expression. Although not yet tested, it would be interesting to assess the long-term effects of *Lmx1b* inhibition on otic development to fully understand the role of *Lmx1b* in ear development and in parallel the consequences on *Pax2* inhibition. In some instances, loss of *Pax2* was paralleled with loss of placodal morphology, in agreement with the reported *Pax2* function in provinding placodal fate. However, in other cases in which down-regulation of *Pax2* was not complete, the otic placode did progress to otic vesicle stage. Thus, reduced amounts of *Pax2* do not compromise otic vesicle growth and closure.

In contrast, no major differences on *FGF10*, *NeuroD* expression was observed after *Lmx1b* down-regulation, moreover there was not an evident expansion of the neurogenic territory, indicating that the function of *Lmx1b* is not linked to the regulation of the size of the neurogenic domain. However, in mice loss of *Lmx1a* can produce expansion of the sensory epithelium. In addition, the results indicate that neither *Hairy1* nor *Irx1* are regulated by *Lmx1b* directly. Taken together, *Lmx1b* is regulated by genes from the neurogenic domain but not vice versa and *Lmx1b* can regulate *Pax2* but not other otic genes such as *Hairy1* or *Irx1*.

The results on the gene interactions of *Lmx1b* with other otic genes were confirmed by overexpressing a full-length construct of *Lmx1b*. In those experiments *Pax2* cells were induced cell autonomously in the otic vesicle and adjacent ectoderm, although in the isthmus *Lmx1b* overexpression did not upregulate *Pax2* (Adams *et al.*, 2000). Moreover, no differences in the expression of *FGF10*, *Irx1* and *Hairy1* were found.

Role of BMP in the regulation of Lmx1b and Sox3

During vertebrate development the nervous system forms as a result of the ectoderm becoming divided into regions fated to become neural or non-neural (primarily epidermal) tissue. Current models for this process include a central role for BMPs in inhibiting neural fate such that cells only adopt a neural fate where BMP signaling is blocked or absent (Weinstein and Hemmati- Brivanlou, 1999; Munoz-Sanjuan and Brivanlou, 2002). In *Xenopus*, this is achieved on the dorsal side of the embryo by the production of BMP antagonists, such as Noggin, Follistatin and Chordin (Weinstein and Hemmati-Brivanlou, 1999; Munoz-Sanjuan and Brivanlou, 2002), which physically interact with BMP ligands to prevent activation of their receptors. However, FGF signals have also been shown to be an important factor in the acquisition of neural fate, with a predominant role in posterior regions (Storey *et al.*, 1998; Kudoh *et al.*, 2004, Rentzsch *et al.*, 2004). FGF signals have been suggested to have a neural inducing function independent of BMP signaling, but have also been shown to be capable of blocking BMP signaling by inhibiting the intracellular effector, Smad1 (Pera *et al.*, 2003, Sater *et al.*, 2003). Thus, it is increasingly clear that intercellular signals during early stages of development are likely to establish a 'pre-neural' state in the ectoderm prior to the time at which the cells of the ectoderm make a choice between neural and non-neural fates. However, BMP signaling and its inhibition, both via soluble antagonists and at an intracellular level by the FGF signaling pathway, still appears to play a central role in the final definition of neural and non-neural territories of the ectoderm (Dee *et al.*, 2007).

Is then BMP signaling involved in otic neural induction? Although the publication of several reports addressing the role of BMP signaling in

inner ear development, its implication of the early events of neural fate have yet not been followed. On the other hand, *Lmx1b* expression in the roof plate is induced by BMP signaling (Chizhikov and Millen, 2004). For this reason two main questions were addressed in this work which had not yet been analyzed: Does BMP signaling modulate the establishment of a proneural domain? Is *Lmx1b* induced by BMP signaling in the otic ectoderm?

At least 20 members of the large BMP family of proteins have been identified. BMP4 has mostly been implicated in regulation of neural induction, at the stages of otic induction and otic neural induction, BMP4 is expressed in the most lateral neuroectoderm and as the neuroectoderm folds to the dorsal portion of the whole neural tube. Thus, it is plausible its influence in the adjacent otic territory at the time of otic neural induction. *BMP7* turns up already in the caudal otic territory by 8ss and at otic cup stages together with *BMP5* is found in the posterior and dorsomedial epithelium in a similar pattern to *Lmx1b* (Oh *et al.*,1996).

As mentioned above, several members of the SoxB1 family, such as *Sox2* and *Sox3*, are among the first transcription factors to be expressed in a spatial and temporal pattern consistent with the acquisition of neural fate in the ectoderm (Okuda *et al.*, 2006) and the fact that ectopic BMP signaling leads to the expansion of prospective non-neural ectoderm at the expense of prospective neural ectoderm (Kudoh *et al.*, 2004, Rentzsch *et al.*, 2004; Delaune *et al.*, 2005), suggest a possible role of BMP in regulating *Sox3* expression in otic domain.

In *Xenopus*, after having identified a proximal regulatory region that recapitulated *Sox3* expression in the CNS, transgenic analysis demonstrated that Vent consensus binding sites were required for restriction of *Sox3* to the presumptive neural plate (Rogers *et al.*, 2007).

However, in chick inhibition of BMP signaling is only required as a late step in the selection of neural fate. Misexpression of BMP4 in the

prospective neural plate inhibits the expression of definitive neural markers (Sox2 and late Sox3), but does not affect the early expression of Sox3 (Linker and Stern, 2004; Stern, 2005). Furthermore, in ES cells acquisition of neural fate blocked by the FGF inhibitor PD184352 was not reverted when BMP4 signaling was exogenously blocked with Noggin, so it was concluded that FGF and Erk can act independently of attenuating BMP in this context (Stavridis *et al.*, 2007).

When embryos of 5-8ss where cultured in the presence of BMP4, expression of *Sox3* was not suppressed, indicating that BMP cannot override FGF signaling in inducing *Sox3*. In parallel, the fact that neuroblasts are present in otic vesicles after overxpression of caAlk3 at preotic stages further indicates that high levels of BMP cannot block neural fate acquisition. One should bear in mind that to directly test the inhibitory action of BMP on *Sox3* expression, embryos should be cultured in parallel with FGF singaling inhibitors and BMP antagonists such as Noggin.

Interestingly, *Lmx1b* expression was tightly regulated by BMP signaling. This is the first report that directly gives insight into the singaling pathway inducing *Lmx1b* in the otic filed. Gain of function of BMP resulted in a dramatic expansion of *Lmx1b* expression whereas *Lmx1b* was down-regulated by blockade of BMP activity by two different inhibitors. These results are in accordance with the previous reports in roof plate formation where *Lmx1b* was shown to be regulated by BMP signaling (Chizhikov and Millen, 2004). Another interesting result to highlight is the fact that ectopic induction of *Lmx1b* in the neurogenic territory did not prevent neuroblast formation and neuroblasts from the CVG were expressing *Lmx1b*. These results mean that neither *Lmx1b* expression nor high levels of BMP activity can suppress the neurogenic genetic program. This is partially in agreement with previous results from the laboratory in which

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expansion of *Lmx1b* into the neurogenic territory by Notch pathway did not suppress neurogenesis (Abelló *et al.*, 2007).

In conclusion, it has been shown that *Lmx1b* expression depends on BMP pathway for its induction. This is interesting because for the first time an early role of BMP signaling in otic development has been described in addition to its well characterized role in sensory specification. Furthermore, this implies that during the early phases of otic placode development in which the proneural territory is established, a FGF signaling force is acting in parallel with BMP signaling but BMP pathway probably is required for the regulation of *Lmx1b* expression but not for the expression of *Sox3*. Interestingly, we have not seen any other non-neural genes affected by BMPs.

Secreted molecules from the hindbrain such as Wnts from the dorsal neural tube and Sonic hedgehog (Shh) from the floor plate and notochord play important roles in establishing the DV axis of the inner ear (Liu *et al.*, 2002, Riccomagno *et al.*, 2002, Bok *et al.*, 2005, Riccomagno *et al.*, 2005). Another family of secreted molecules that may serve as signaling molecules for the inner ear patterning are BMPs, which are expressed in the roof plate of the hindbrain and dorsal ectoderm (Lee and Jessell, 1999). Since BMP and Shh pathways inhibit each other in the neural tube and inhibitors of BMPs, expressed in the notochord, also modulate Shh functions in the ventral neural tube (Liem *et al.*, 1995, Liem *et al.*, 2000, Patten and Placzek, 2002), it is possible that similar opposing interactions between Shh and BMPs occur in the inner ear.

The requirement of the neural tube for the formation of the otic vesicle has been extensively documented *in vivo* (Yntema, 1933, 1950; Waddington, 1937; Jacobson, 1963; Gallagher *et al.*, 1996) and *in vitro* (Represa *et al.*, 1991) whereas neural tube removal experiments suggest that the formation of the otic vesicle is strictly dependent on the presence of the neural tube until the 6ss (Giraldez, 1998). When the dorsal and

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ventral neural tube was removed, in the first case *Lmx1b* expression was abolished and in the second *Lmx1b* was up-regulated, indicating that the dorsal neuroectoderm is able to induce and maintain the expression of *Lmx1b*, whereas the ventral neural tube down regulates *Lmx1b*. In parallel, blockade of Shh abolishes otic neurogenic markers. It would be interesting to check if suppression of Shh can indeed up-regulate *Lmx1b* expression, either directly or indirectly by suppressing *Sox3/Ngn1* expression

Based on all the evidence provided above, a model for early otic patterning is proposed (Figure 40) in which sustained FGF signaling in the anterior otic-epibranchial territory is the driving force for maintaining high levels of *Sox3* and promoting neural character. In parallel, BMP signaling from the dorsal neural tube initially and from the caudal territory later on would regulate *Lmx1b* expression in the otic ectoderm. The final patterning of the otic cup would result in additional inhibition of *Lmx1b* in the neurogenic domain by *Sox3* expression.
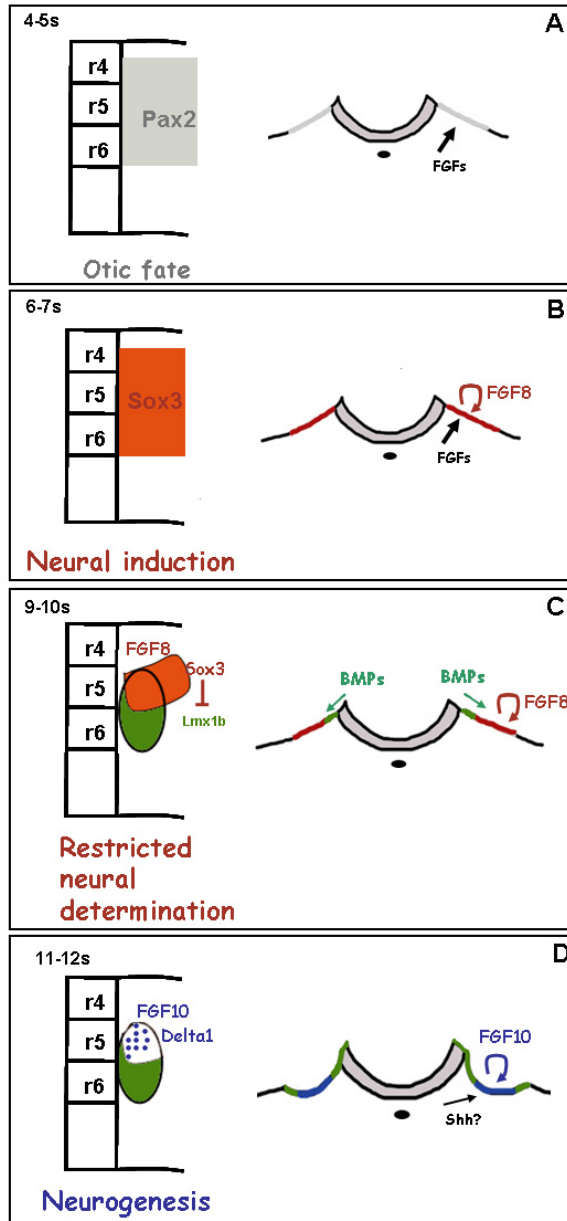
Figure 40: Proposed model for sequential otic neural determination

A) In chick, *FGF19* from the underlying mesoderm is essential for otic induction. *Pax2* expression appears at 4-5ss in a broad otic-epibranchial region. B) *Sox3* at 6ss is expressed broadly, while *FGF8* turns on only in an ectodermal anterior band. C) *FGF8* sustains *Sox3* in the anterior territory, while posterior

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Sox3 expression is gradually lost.C) BMPs from the dorsal neural tube induce *Lmx1b* in the medial and caudal otic region D) *FGF10* expression is restricted in the anterior otic domain where neurogenesis starts. The otic placode invaginates and is influenced by ventral signals (presumably Shh from notochord).

CONCLUSIONS

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6.1. Role of Sox3 in proneural specification

1. Both *Sox2* and *Sox3* are expressed initially in a broad domain but *Sox3* is regionalized in the proneural domain by HH10 stage.

2. Overexpression of *Sox3* can induce neuronal precursor cells by the ectopic expression of *Sox2* and *Delta1*.

3. Ectopic neuronal precurosor cells undergo initial differentiation expressing *NeuroD* but cannot accomplish full differentiation and induce cell death.

4. The non-neural otic region is not fully specified until HH12 stage.

5. *Sox3* overexpression inhibits *Hes5.2* expression in the neurogenic domain but cannot activate *Hes5.2* in cells adjacent to the ectopic *Delta1*-induced cells.

6. *Sox3* regulates the expression of *Lmx1b* but does not regulate the expression of other otic genes like *Tbx1 or Pax2*.

7. *Sox3* can induce placodal thickenings within the *Pax2/Pax6* expression domain.

6.2. Role of Lmx1b in otic patterning and development

1. *Lmx1b* is expressed in the posterior non-neural domain in complementary fashion to the Islet1 domain

2. *Lmx1b* does not have a major impact of *Pax2* induction but is required for *Pax2* maintenance.

3. *Lmx1b* does not regulate expression of genes that are expressed in the proneural domain nor the expression of otic patterning genes like *Irx1* or *Hairy*1

4. *Lmx1b* expression in the otic field is regulated by BMP signaling.

5. BMP signaling does not regulate the expression of *Sox3* in chick suggesting its role in late step of neural specification.

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8. ABBREVIATIONS

As	achaete-scute
As-c	achaete-scute complex
ato	atonal
atoh	atonal homologue
ash	achaete-scute homologue
AP	anterior-posterior
AV	anterior-ventral
BAC	bacterial artificial chromosome
bHLH	basic Helix Loop Helix
BMP	Bone Morphogenetic Protein
BrdU	5-Bromo-2´-deoxyuridine
BSA	Bovine serum Albumin
CNS	Central Nervous System
CVG	Cochleovestibular Ganglion
DIG	Digoxigenin
Dl	Delta
DNA	Deoxi-ribonucleic acid
dpc	days post-coitus
dpf	days post fertilization
DV	Dorsal-ventral
E	embryonic day
ED	endolymphatic duct
EGF	Epidermal Growth Factor repeats
EP	Electroporated
Espl	Enhancer of Split
EST	Expressed Sequence Tag
FAD	Formamide
FBS	Feotal Bovine Serum
FGF	Fibroblast Growth Factor
fISH	Fluorescent in situ Hybridization
Fluo	Fluoresceine
GFP	Green Fluorescent Protein
hrs	hours
Hes	Hairy and Enhancer of Split
HH	Hamburger and Hamilton
Hh	Hedgehog
ISH	in situ Hybridization

Jag	Jagged
LFng	Lunatic Fringe
min	minutes
MAM-l1	Master-minnd-like1
N	Notch
Neurog or Ngn	Neurogenin
NICD	Notch IntraCellular Domain
o/n	over night
PBS	phosphate buffered saline
PFA	Paraformaldehyde
PNS	Peripheral Nervous System
PPE	Preplacodal ectoderm
RFP	Red fluorescent protein
RNA	Ribonucleic acid
r	rhombomere
RT	Room Temperature
ss	somite stage
Shh	sonic hedgehog
t	time
TM	transmembrane
SOP	Sensory organ precursor
PSA	