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BMP4 regulation of sensory organ development in the chick inner ear

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Chapter 1

Introduction

All living organisms have developed highly specialized structures that are receptive to mechanical forces originating either from the surrounding environment or from within the organism itself. Among the most elaborate and greatly efficient of such structures are the mechanotransducers responsible for sensory awareness, for example, those facilitating touch, balance proprioception and hearing.

In vertebrates, the inner ear has evolved as one of those specialized structures, and contains mechanosensory cells mediating the animal response to multiple sensory inputs, including sound, balance and acceleration. Remarkably, all these functions rely in a limited number of mechanosensory cell types, collectively known as *hair-cells*, which are located in specialized epithelial structures named *sensory organs* (Eatock, Newsome. 1999).

The structure of a mature vertebrate inner ear has a complex three dimensional organization, consisting of an array of ducts filled with a special fluid, the endolymph, and lined by specialized epithelial tissues. These epithelial filled-tubes are surrounded by a bony capsule that largely follows the intricate contour of the membranous labyrinth, from which it is separated by a space filled with the perilymph, a fluid that is similar in composition to cerebral spinal fluid (Bissonnette, Fekete. 1996). The epithelium lining the membranous labyrinth can be subdivided by its function in sensory and non-sensory (Swanson, Howard, Lewis. 1990). The discrete sensory regions where the mechanosensory hair-cells are located in a highly-ordered pattern together with a specialized group of epithelial cells named *supporting cells* comprise the sensory organs of the inner ear. Those regions are highly innervated by sensory neurons of the VIIIth cranial ganglia, and these three major cell types: hair-cells, supporting cells and sensory neurons are considered as the basic unit of all sensory organs in the vertebrate inner ear (Lewis, Leverenz, Bialek. 1985; Torres, Giraldez. 1998)

In spite of having the same basic components, there are differences in the structure, anatomical location and function of the various sensory organs of the ear. Their number varies between animal species, and in the chick inner ear, there are a total of eight sensory organs: one auditory, known as the basilar papilla (organ of Corti in mammals), and seven vestibular organs that include three crista ampullae, two maculae, one lagena, and one macula neglecta (Wu, Oh. 1996; Torres, Giraldez. 1998).

The gross anatomy of the mature inner is similar in all vertebrates, and it is subdivided according to its morphology and function into a dorsal *vestibular* part, and a ventral *auditory* component. The vestibular part is turn subdivided in several compartments: the three *semicircular canals* that are orientated in

nearly orthogonal planes (anterior, lateral and posterior), the *endolymphatic duct and sac* and two sacs: the *saccule* and the *utricle*. The auditory component is restricted to the *cochlear duct* (*basilar papilla* in birds) (rev. in (Bissonnette, Fekete. 1996).

The three dimensional arrangement of the fluid ducts and the resident sensory receptor organs is critical for the vestibular and auditory function of the inner ear, and its development derives from extremely complex morphogenetic processes.

Gross Anatomy of the Inner Ear

Since we used the chick as a model system for this work, I shall use it here as an example to illustrate the anatomical distribution and morphology of the major components of the inner ear (Fig.1-1).

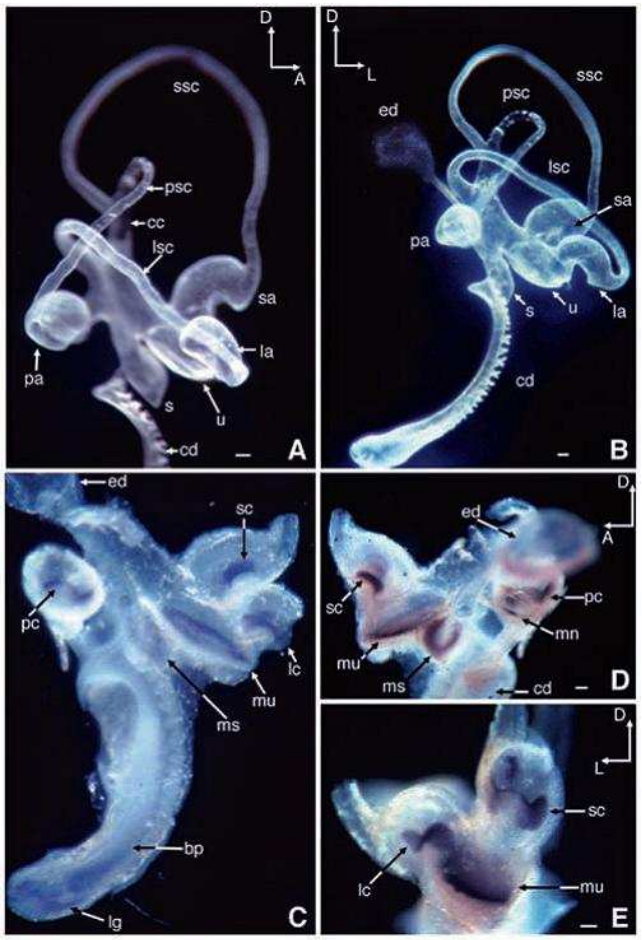

Fig. 1-1. Normal anatomy of an E9 right inner ear. Membranous portions of inner ears were either filled with paint (A,B) or stained with anti-HCA antibodies (C,D,E), and are shown in lateral (A), postero-lateral (B,C), medial (D) and an anterior (E) views. The inner ear in B is tilted dorsally to better reveal the lateral canal. In A and B, there are two ampullae at the anterior part of the inner ear (sa and la) and only one in the posterior (pa). The superior ampulla (sa) is the only one in a vertical position and the other two are in a horizontal position (A). The superior semicircular canal (ssc) is in a sagittal plane, the posterior semicircular canal (psc) is in a transverse plane and the lateral semicircular canal (lsc) is in a horizontal plane (A and B). The posterior canal (psc) has the most superior and anterior insertion point on the commom crus (cc in A). The superior canal (ssc) has a posterior and a slightly more ventral point of insertion on the common crus and the lateral canal has the most ventral and posterior point of insertion. The endolymphatic duct (ed) has a dorso-posterior projection and the cochlear duct (cd) has a ventro-posterior projection. All eight sensory organs revealed by anti-HCA staining are shown in C, D and E: superior crista (sc in C,D,E), lateral crista (lc in C,E), posterior crista (pc in C,D), maculae utriculi (mu in C,D,E) and sacculi (ms in C,D), basilar papilla (bp in C), lagena (lg in C) and macula neglecta (mn in D). The superior (sc in E) and the posterior (pc in C,D) cristae have a W-shaped pattern and the lateral crista (lc in E) has a V-shaped pattern. The patch of staining above the W-shaped pattern of the superior crista (sc) in E is an artifact resulting from trapping within the gelatinous material of the cupula. D, dorsal; A, anterior; L, lateral; ed, endolymphatic duct; la, lateral ampulla; pa, posterior ampulla; s, saccule; u, utricle. Scale bars, 100 mm; bar in A also applies to C.*From: Development 125, 11-20, Wu et al (1998)*

The auditory component of the inner ear is the cochlear duct (cd). In birds this structure is known as *basilar papilla*, and it is a relatively straight tube that extends medially and ventrally, rather than a coiled structure as in higher vertebrates. In addition, both the proximal and the distal ends of the cochlear duct point towards the posterior part of the body, forming an arc-shaped structure. In most vertebrates, only auditory hair cells are located in this ventral structure. However in the chick, two sensory organs are located in the basilar papilla: the auditory sensory basilar papilla, and the vestibular macula lagena, which is thought to function in linear acceleration detection (rev in (Bissonnette, Fekete. 1996).

The vestibular component consists of two connecting sacs, the utricle and the saccule (u and s, see Fig 1-1.), each of them containing a sensory maculae (macula utriculi and macula saccularis, mu and ms in Fig. 1-.1), and three semicircular canals: the superior (ssc), posterior (psc) and lateral (lsc). Each semicircular canal is situated in a different plane, roughly orthogonal to each other. The superior canal, the largest of the three, is in the sagittal plane (ssc in Fig.1-.1). The posterior canal forms a right angle with the superior canal and is, therefore, in the transverse plane (psc in Fig.1-1.). Finally, the lateral canal is located in a horizontal plane (lsc in Fig.1-.1).

Each canal ends in the region called common crus (cc in Fig.1-.1) on one side, and a swelling on the other side known called the *ampulla.* There are two ampullae, the superior and the lateral, located anteriorly (sa and la in Fig.1-1), and one posterior ampulla, situated posteriorly (pa in Fig.1-.1). Each ampullae contains a sensory cristae (superior cristae, lateral cristae and posterior cristae). The superior ampulla is positioned vertically while the other two ampullae are positioned horizontally (Fig.1-1).

Finally, another tube-shaped structure is located dorsally: the *endolymphatic duct and sac* (EDS). This is a non-sensory organ of the inner ear that is connected to the endolymphatic compartment, which is filled with endolymph, a potassium-rich fluid that bathes the apical side of inner ear sensory cells (see below). The functions ascribed to the endolymphatic sac are the regulation of the volume and pressure of endolymph, the immune response of the inner ear, and the elimination of endolymphatic waste products by phagocytosis (Couloigner, et al. 2004)

Sensory function of the inner ear: how the sensory organs work.

A remarkably feature of the inner ear is that it accomplishes its functions by using a relatively limited number of mechanotransducer sensory cell types, collectively called hair cells. These are highly specialized epithelial cells that utilize a group of derived microvilli, referred to as *stereocilia*, to detect pressure waves induced through either sound or motion (reviewed in (Eatock, Fay, Popper. 2006). However, significant variations in hair cell morphology exist both between different sensory epithelia, and even within a single epithelium. For example, vestibular epithelia contain Type I and Type II hair cells, which are

characterized by differences in their morphology, electrophysiology and innervation. Similarly, cochlear epithelia in both birds and mammals contain two distinct hair cell types, inner and outer hair cells in mammals, tall and short hair cells in birds. As is the case for vestibular hair cells, cochlear hair cell types can be distinguished based on their morphology and physiology (Eatock, Fay, Popper. 2006).

In addition to hair cells, each sensory patch also contains a variable number of non-sensory cells, collectively known as *supporting cells*. In many sensory organs the population of supporting cells seems largely homogenous, with no consistent morphological or molecular heterogeneities. However, in the mammalian cochlea, at least four unique types of supporting cell can be identified, indicating that there is diversity within supporting cells (Fekete. 2000) (Kelley. 2006).

Sensory organs are comprised of a population of several thousand hair cells arranged in a circular or oblong patch. However, there are examples of morphological specializations, such as the elongated auditory sensory epithelia found in some reptiles, and all birds and mammals (Eatock, Newsome. 1999). Sensory epithelia lie upon a sheet of extracellular matrix, a basal lamina, and also have a prominent extracellular structure associated with their apical surface, which varies is different sensory organs: a cupula, an otoconial membrane or a tectorial membrane. The supporting cells seat on the basal lamina, and their lateral membranes surround the hair cells, projecting up to the surface of the epithelium. Hair cells do not contact the basal lamina, and they are isolated from one another by the supporting cells. At the apical surface of the epithelium, the supporting cell processes form tight and adherens junctions with each other and with the hair cells.(Nayak, et al. 2007)

Hair cells have a highly specialized bundle of modified microvilli on their apical surface (the hair bundle), and it is this feature that enables them to detect mechanical stimuli and transduce them into electrical signals. In fact, the mechanical force stimulating the sensory hair-cells is generated by the movement of the fluid filling the membranous labyrinth: the endolymph. This fluid is produced within the inner ear by a specialized region of the epithelium: the *stria vascularis* (*tegmentum vasculosum* in birds). The endolymph has a high concentration of K^+ and a low concentration of Na^+, which is essential for the process of transduction by hair-cells. The ionic gradients established between the endolymph and the intracellular compartment generates the electromotive forces that depolarize the hair-cell membrane upon opening of mechano-sensitive K+ channels (Eatock, Fay, Popper. 2006).

The mechanically sensitive hair bundle bends back and forth in response to stimuli that are directed to it. Auditory stimuli induce a vibration of the structure on which the hair cells sit (the basilar membrane). Vestibular stimuli cause displacement of acellular structures overlying the hair cells (otolithic membrane in the saccule and utricle, responsible for linear-acceleration detection; cupula in the semicircular canals, responsible for rotational detection), resulting in bundle deflection. An excitatory deflection of a hair bundle directly opens transduction channels, which admit cations and depolarize the hair cell.

Inhibitory deflections close transduction channels and hyperpolarize the cell. These changes in membrane potential in turn increase (depolarization) or decrease (hyperpolarization) neurotransmitter release from graded synapses on basolateral surfaces of hair cells. Post-synaptic afferent fibres of the VIIIth cranial nerve innervating hair cells transmit their signals to the large cochlear and vestibular nuclear complexes of the brainstem's *medulla* and *pons*. The cochlear nuclei initiates a complex network of rostral projections though the *pons*, midbrain, and thalamus into the cerebral cortex. The vestibular nuclei send information to the cerebellum, oculomotor system and spinal cord. Auditory hair cells are innervated also by efferent projections that carry signals from the brain into the ear, and influence cochlear function in a feedback loop (efferent neurons, rev.in (Eatock, Newsome. 1999).

Overview of Inner Ear Development

The inner ear derives from a population of ectodermal cells forming the *otic placode*. This is a transient thickening of the ectoderm adjacent to the rhombomeres 4 to 6 of the developing hindbrain that gives rise to virtually all cell types of the membranous portion of the inner ear (rev. (Torres, Giraldez. 1998). The otic placode becomes visible typically once the first 5-10 pairs of somites have been generated, depending on the animal species. Then the placode invaginates to form the *otic cup* and the *otic vesicle*, the, ellipsoid-shaped structure lined by a pseudo-stratified epithelium (see (Torres, Giraldez. 1998) (Ohyama, Groves, Martin. 2007).

Otic induction

The existence of a pre-placodal territory, adjacent to the neural plate that has a common potential to generate sense organs and cranial ganglia, was proposed by Jacobson in 1966, on the basis of a set of elegant experiments where the ectoderm adjacent to the neural plate was rotated at specific stages of development (Jacobson. 1966). An initial set of genes (*Foxi*, *Msx* and *Dlx)* identify an ectodermal domain from the neural plate and the epidermis. This pre-placodal domain is segregated from the neural crest domain, and expresses a specific set of genes. Both the positioning of the pre-placodal ectoderm and its capacity to express the specific *Six/Eya/Dach* cassette seem to require interactions between the presumptive pre-placodal domain and the surrounding tissues (rev in (Streit. 2007). The transit from a pluripotent ground state to one in which otic fate is specified requires a round of interactions that position and specify the fate of individual placodes (Ohyama, Groves, Martin. 2007; Streit. 2007). This notion of sequential rounds of interactions is classical, and was anticipated by Yntema (1950) and Jacobson (1966) (Jacobson. 1966).

The otic field becomes progressively committed to the otic fate, eventually reaching an irreversible state of determination. This state can be defined as the property of the otic primordium to develop into the membranous labyrinth and to generate the cellular phenotypes of the adult organ independently of the embryonic environment. This is fully achieved at the otic vesicle stage (Waddington, 1937; Jacobson, 1963; Swanson et al., 1990; Gallagher et al., 1996). Once the otic vesicle acquires the state of determination it undergoes a

period of intense proliferative growth. Cell proliferation of the otic vesicle is under the control of growth factors. Among the growth-factors involved, those belonging to the insulin family, insulin and insulin-like growth factors, play an important role in otic development (Sánchez-Calderón et al., 2007). Following the proliferative period, the otocyst enters a differentiation phase, during which extensive morphogenic events take place to shape the final organ (reviewed by Bok et al., 2007).

Otic Patterning

The regional (or axial) polarity needed to develop the membranous labyrinth of the ear has long been recognised as providing the basis for inner ear function. Classical transplantation experiments showed that rotated otic placodes produce enantiomorphic twins (Harrison 1945; Yntema 1955), which somewhat resemble the symmetric ear of the hagfish. This reversal in polarity will happen along the anterior-posterior axis alone until the dorso-ventral axis become fixed somewhat later during otic cup formation. Yntema (1955) suggested that the fixation of polarity in the ear rudiment may be a local expression of a general body polarity. The early patterning of the ear is also set, at least in part, by interactions between the ear and the surrounding tissues, particularly the neural tube. The first sign of otic regionalisation is that of the establishment of the otic proneural and non-neural fields (Alsina et al., 2004). But axial polarity extends to further complexity in the regionalisation of the otic vesicle, when establishing the different domains of the inner ear. The regional specification of the otic vesicle depends on Wnt, FGF and SHH signals emanating from the neighbouring neural tube, and there is a general consensus that the anterior-posterior axis is fixed before the dorso-ventral, and that the otic placode is at some stage equipotent as to the anterior-posterior identity. However the process of patterning is concomitant to growth and complex morphogenetic movements, and implicates many gene network interactions, all of which is far from being understood (see rev. by Whitfield & Hammond, 2007, Schneider-Maunoury & Pujades, 2007).

Cell fate commitment during sensory organ development

The different cell types that composed the sensory organs are originated from the otic vesicle epithelium, and their commitment to a specific lineage follow a stereotyped temporal sequence: the auditory (cochlear) and vestibular neurons are the first cell types to be specified in the inner ear of chick and mice (Adam et al., 1998). Later on, sensory hair-cells and supporting cells are formed at specific locations (see below). Current evidence suggests that neurons and sensory cells share a common progenitor (Satoh and Fekete, 2005). In mouse, CRE-LoxP fate mapping of the expression of *Neurogenin1* (*Neurog1*) delineate a particular domain that contributes to both neurones as well as to macular epithelia (Raft et al., 2007). Moreover, a recent fate map and labelling experiments have shown that both sensory organs and their innervating neurons arise from the same domains of the otic placode (Bell et al., 2008)

In the chick, neuronal progenitors can be detected in the otic field as early as otic placode/cup stage, and only in the rostral aspect of the otic placode as

revealed by the expression of *Neurog1* (Alsina et al., 2004). It is now clear that the bHLH gene *Neurog1* determines neuronal fate with downstream transcription factors such as *NeuroD* playing various roles in differentiation (Bertrand et al. 2002). Otic neuroblasts delaminate from the ventral aspect of the otic cup forming the statoacoustic ganglion (SAG), which is also known as also known as cochleovestibular ganglion (CVG), and corresponds to the VIIIth cranial nerve. As development proceeds, this ganglion splits into the cochlear and vestibular ganglia (rev. in Sánchez-Calderón et al., 2007).

Within the CVG, all neurons are originated from the otic placode, and most Schwann cells that are of neural crest origin. Some neuroblasts within the CVG undergo through a period of cell proliferation before they become postmitotic, start to differentiate and begin to innervate the inner ear epithelia (D'Amico-Martel and Noden, 1983). The basic plan for the innervation of the ear is believed to consist of an initial gross projection of sensory fibres to the sensory epithelia, followed by the selection of synaptic contacts and the maintenance of established connections (Fekete and Campero, 2007). Diffusible factors produced by the sensory epithelium are thought to mediate this process, so that cochlear and vestibular neurons are trophically dependent on their targets (Fekete and Campero, 2007; Sánchez-Calderón et al., 2007).

In parallel, within the otic vesicle, regions of the epithelium are specified as prosensory patches that will generate the sensory organs: the *cristae*, *maculae* and auditory epithelium (organ of Corti in mammals, *basilar papilla* in chick, see above). A number of genes are differentially expressed identifying these prosensory domains. The list includes signalling molecules such as *BMP4* and *FGF10*, transcription factors such as *Sox2,* and elements of the Notch signalling pathway such as *Jagged1* and *Lunatic Fringe*. Functional data suggest that Notch signalling pathway, *FGFR1* and *Sox2* are important for prosensory specification (reviewed in Kelley, 2006). Following the specification of the prosensory domains, individual cells develop as either hair-cells or supporting cells. It is commonly accepted that the hair-cell/supporting cell fate decision requires lateral inhibition through the Delta-Notch mechanism, which results in the characteristic cellular pattern of ear sensory epithelia. Expression of *Jag2* ligand and *Delta-like1* ligand (*Dll1*) in developing hair-cells leads to the activation of Notch1 receptor and expression of the down-stream targets *Hes1* and *Hes5* in neighbouring cells, which, in turn, drives their development as supporting cells (Lanford et al., 1999; Riley et al., 1999; Murata et al., 2006; Daudet and Lewis, 2005;)

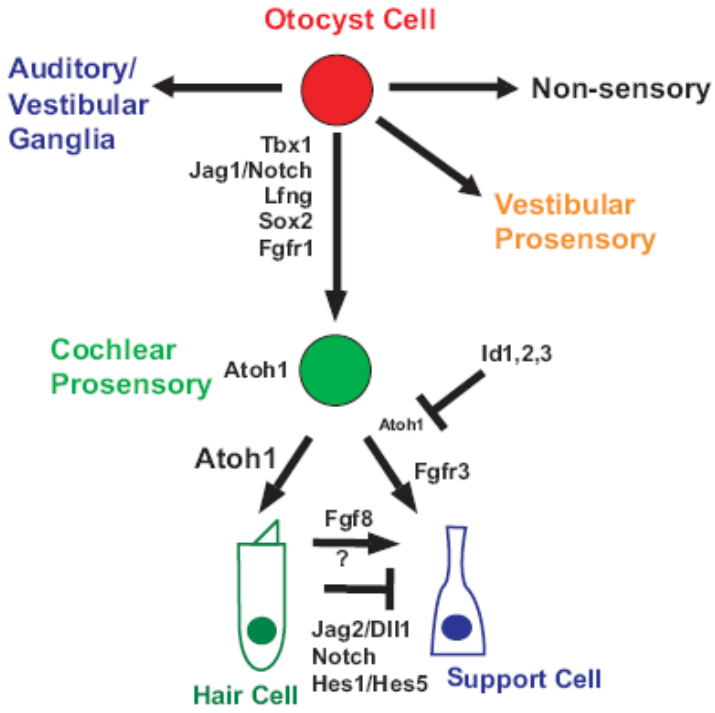

Fig. 1-2. Determination of cell fates in the organ of Corti. Cells located within the otocyst can develop along one of four different pathways. As cochlear prosensory cells, as closely related vestibular prosensory cells, as cells that will give rise to neurons in the auditory and vestibular ganglia or as non-sensory epithelia. Cells that will develop as cochlear prosensory cells initially express a number of genes that have been shown to play a role in prosensory specification, including Tbx1, Jag1, Lfng, Fgfr1 and Sox2 (see text for details). Following prosensory specification, all prosensory cells express Atoh1 leading to the initiation of a hair cell specification program. At the same time, prosensory cells also express Ids1, 2 and 3 which act to inhibit Atoh1 activity. Id expression is subsequently down-regulated in cells that will develop as hair cells, leading to an increase in the level of Atoh1 expression and the initiation of expression of the notch ligands, Jag2 and Dll1. Expression of notch ligands leads to activation of the Notch1 and the downstream target genes HES1 and HES5, in neighboring cells. The presence of HES genes along with continued expression of Ids leads to loss of Atoh1 expression. At the same time, developing hair cells produce inductive signals, including activation of the Fgf signaling pathway, that recruit surrounding cells to develop as supporting cells. While Fgf signaling clearly plays a role in the development of some types of pillar cells, other unidentified inductive signals (indicated by "?") are also assumed to exist
From: Kelley MW, (2007) ***Int. J. Dev. Biol. 51****: 571-583*

In addition to those mentioned above, members of the BMP family of proteins are expressed in prosensory patches and it ahs been speculated on their possible role in the development of the sensory elements of the inner ear (Oh, Wu, Chang, Barald). The present work addresses this problem and, therefore I shall review with some detail this family of proteins, including their structure, molecular pathways involved in cell signaling, and know functions in the inner ear and other developing model systems.

BMP signalling pathway

This thesis focus on the function of this signalling pathway in the development of the inner ear, so we present here a brief overview of it (see also Fig. 1-2)

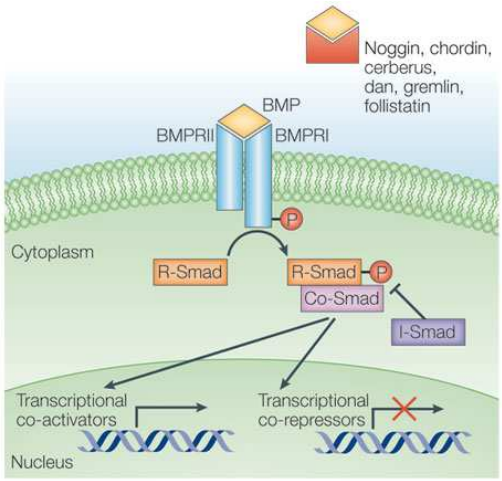

Figure 1-3. BMP signalling pathway. Bone morphogenetic protein (BMP) ligands bind to the BMP receptors BMPRI and BMPRII, and BMPRII then phosphorylates and activates BMPRI. Phosphorylated BMPRI subsequently phosphorylates receptor-activated Smad proteins (R-Smads), which associate with common mediator-Smad (co-Smad) and enter the nucleus, where they regulate gene expression. The Smad proteins regulate promoter activity by interacting with transcriptional co-activators or co-repressors to positively or negatively control gene expression. The BMP signal can be blocked by extracellular antagonists, such as noggin, which bind BMP ligands and prevent their association with the BMP receptors, as well as by intracellular proteins, such as inhibitory Smads (I-Smads), which prevent the association between R-Smads and co-Smads.
From: Xiao et al, 2007. Biochem. Biophys. Res. Commun. 26;362(3):550-3.

Bone morphogenetic proteins (BMPs)

Bone morphogenetic proteins (BMPs) belong to the Transforming Growth Factor (TGFβ) superfamily of growth factors. This comprises seven genes in Drosophila melanogaster and at least 30 genes in mammals, including 3 TGFβ isoforms, 4 activin-β-chains, the protein nodal, 10 bone morphogenetic proteins (BMPs) and 11 growth and differentiation factors (GDFs) . Members of the TGF-b superfamily are essential for large number of biological processes during development and adulthood, and have been implicated in various pathological processes, all of which has been subject of numerous reviews (Massague. 1998; Massague, Chen. 2000; Massague, Wotton. 2000; Miyazawa, et al. 2002; Shi, Massague. 2003; Chen, Zhao, Mundy. 2004; Massague, Gomis. 2006) .

BMP proteins were initially named for their ability to induce ectopic bone formation (Urist. 1965; Wozney, et al. 1988), but there is now strong genetic and experimental evidence that these molecules regulate biological processes as diverse as cell proliferation, apoptosis, differentiation, cell migration and cell-fate determination (Massague. 1998). Moreover, the vertebrate BMPs are involved in the development of nearly all organs and tissues, including the

nervous system, somites, limbs, lung, kidney, skin, and gonads, as well as in critical steps in the establishment of the basic embryonic body plan (reviewed in(Hogan. 1996)).

Depending on the level of amino acid sequence homology, BMPs can be further subdivided into two groups; BMP2/4 and BMPs5-8. The BMP2/4 group, which includes the first described invertebrate BMP (Dpp) (Padgett, St Johnston, Gelbart. 1987) is the best characterized having representative members in both bilateral and ancestral radial metazoans (Lelong, Mathieu, Favrel. 2001).

Like all members of the TGF-b superfamily, BMPs are synthesized as large precursors, which are processed and proteolytically cleaved to yield carboxy-terminal mature protein dimers. Each monomer comprises several extended β-strands interlocked by three conserved disulfide bonds that form a tight structure known as “cystine knot” (Sun and Davies 1995, see below). Within a subfamily, these ligands form a homodimeric or heterodimeric complex that bind to, and initiate signal transduction through a family of transmembrane serine/threonine kinases (Reviewed in (Massague, Chen. 2000)

BMP receptors

Receptors for BMP proteins are transmembrane serine/threonine kinases that belong to the TGF-b receptor family. All members of this family are classified, based on their structural and functional properties, into two subfamilies: type I and type II receptors. These receptors cluster separately in two distinct polyphyletic groups. Type I receptors form a group that is related to, but that excludes type II and viceversa. Within each sub-group, receptors are clustered according to the type of ligand they bind to. Thus, type I and type II receptors are divided into three categories: binding Activins, BMP and TGF-b.(Massague. 2000)(Shi, Massague. 2003)

For BMP proteins, there are three Type-I receptors identified: Alk2, Alk3 (BMPRIa) and Alk6 (BMPRIb), and three Type-II receptors: BMPRII, ActRII and ActRIIB. There is also an alternative splice variant of BMPRII, which lacks most of the C-terminal tail and is expressed in C2C12 cells for example (Massague. 2000).(Shi, Massague. 2003)

Unlike TGF-b family members, BMP proteins bind with higher affinity to Type-I receptors, and only with low affinity to Type-II receptors (Rosenzweig et al., 1995; ten Dijke et al., 1994). Different BMPs bind with different affinity to the three type I receptors; BMP-4 preferentially binds to BMPR-IA and -IB, BMP-7 binds with higher affinity to ALK-2 and BMPR-IB than to BMPR-IA and Growth and Differentiation Factor (GDF)-5 interacts most efficiently to BMPR-IB among type I receptors. This supports the concept that the affinity of the BMPs to the receptors is important for the activation of the signal transduction and leads to the hypothesis that some BMPs prefer specific receptors for their signaling. It is also known that different cells express different receptor combinations and downstream signaling molecules that contribute to the diversity of BMP signalling responses (Shi, Massague. 2003; ten Dijke, et al. 2003)

The mechanism of receptor activation is thought to occur by binding of the dimeric ligand to the extracellular domains of both types of the receptors. This induces a close proximity and a productive conformation for intracellular kinase domains of the receptors, facilitating the phosphorylation and subsequent activation of the type I receptor. The type II receptor kinases are thought to be consitutively active, although the regulation of this process is unclear. In this way, the Type-I receptor, which is also a serine threonine kinase, is activated by the Type-II receptor by phosphorylation at the GS-Box a juxtamembrane domain enriched in glycines and serines. As mentioned above, this domain is conserved in all BMP Type-I receptors (Shi, Massague. 2003).

The activated type I receptor initiates intracellular signaling by phosphorylating specific downstream components, including the nuclear effector Smad proteins. The L45 loop regions in the kinase domain of type I receptors were found to be important determinants for signalling specificity and binding of specific Smad proteins (Feng and Derynck, 1997, Chen and Massagué, 1999; Persson et al., 1998).

Intracellular signaling via Smad proteinS

Activated TGF-b type I receptors initiate intracellular signalling by phosphorylating different members of the Smad family of proteins. The Smad proteins are homologs of the *Drosophila* protein, *mothers against decapentaplegic* (MAD) and the *C. elegans* protein SMA, and members of this family are classified according to their functions (rev in (Massague, Seoane, Wotton. 2005).

Upon activation of type I receptors a subgroup of “receptor regulated Smads” (R-Smads) are recruited and activated by phosphorylation. While Smad1, Smad5 and Smad8 are phosphorylated by BMP-type I receptors, Smad2 and Smad3 are phosphorylated by TGF-β and activin receptors (Attisano and Wrana, 2002; Massagué, 1998). Phosphorylation occurs at the two extreme serine residues in the carboxy-terminal SSV/MS motif (Abdollah et al., 1997; Kretzschmar et al., 1997; Souchelnytskyi et al., 1997). Activated R-Smads form heteromeric complexes with Smad4, a common partner of the TGF-b and BMP pathways (therefore named Co-Smad), and these complexes are translocated into the nucleus. Within the nucleus these Smad complexes regulate the transcription of target genes in cooperation with co-activators and co-repressors (Derynck et al., 1998) (Massague, Seoane, Wotton. 2005).

R-Smads and Smad4 share two highly conserved domains, termed MH1 and MH2, which are separated by a less conserved Pro-rich linker region. Except for Smad2, which cannot bind DNA directly owing to a small insert encoded by an extra exon15, the MH1 domains of the R-Smads and Smad4 are responsible for DNA binding, tipically to GTCT containing sequences, also termed Smad binding elements (SBEs). In addition, BMPR–Smads have been reported to bind to GCAT motifs in Xvent 2B and mouse Dlx3 promoters, GCCG-rich containing sequences in Smad6, and SBE and GGCG elements in Id1 gene promoters (Massague, Seoane, Wotton. 2005). Robust BMP-induced activation of Id1 promoter requires the cooperation of three distinct sequence motifs, i.e.

SBE, GGCG and CAGC. Multimerization of all three elements found in Id1 promoter is needed to generate a highly sensitive BMP/Smad specific enhancer (Korchynskyi and ten Dijke, 2002) (Rev. (ten Dijke, et al. 2003)

The direct affinity of Smads to DNA is relatively weak (Shi et al., 1998), and Smads appear thus to require cooperation with other DNA binding factors to efficiently bind to promoters of target genes (Hata et al., 2000). It has been shown that the MH2 domain mediates Smad–receptor interactions, Smad–Smad interactions and Smad interactions with transcription factors, co-activators and co-repressors (Massague, Seoane, Wotton. 2005).

Finally, the linker region is phosphorylated by kinases such as mitogen-activated protein kinases (MAPKs), glycogen synthase kinase-3β (GSK-3β) and cyclin-dependent kinases (CDKs), and is thus thought to integrate inputs from other signalling pathways (rev in (Massague. 2003; Eivers, Fuentealba, De Robertis. 2008).

Modulation of Bmp signaling

In order to achieve coordination between different cells or within a single cell, the different processes regulated by extracellular molecules must be finely regulated. In fact, BMP signalling has been shown to be tightly regulated at multiple levels: intracellularly, at the membrane site, and extracellularly .(Massague. 1998).

Extracellular Modulation

BMPs have been proposed as morphogens in multiple contexts and for such a role, the exact concentration of active ligands is important for rendering a particular biological effect. Extacellular regulation of ligand binding to its receptors is probably the the best characterized way of modulating BMP signalling during development ((Massague. 2000)). The active local concentration and consequently the extent of action of BMP molecules is controlled, at least in part, by the influence of extracellular modulators, whose biological roles have been extensively reviewed (Balemans and Van Hul, 2002; Harland, 2001; Massague´ and Chen, 2000),

An increasing number of these natural antagonists is being identified in vertebrates and invertebrates, and they appear to have evolved independently given the lack of primary sequence conservation. In vertebrates, the list of BMP antagonists already comprises more than seven proteins, including noggin, chordin, chordin-like, follistatin, FSRP, the DAN/Cerberus protein family, and sclerostin (Balemans and Van Hul, 2002). Studies in Drosophila demonstrated the presence of similar antagonists in invertebrates. The Drosophila short gastrulation (sog) is the homologue of chordin, and affects dorsal–ventral patterning by antagonizing dpp and screw, the homologues of, respectively, vertebrate BMP-2/-4 and BMP-5/-6/-7/-8 (Holley et al., 1995; Neul and Ferguson, 1998). In both vertebrates and invertebrates, other regulatory factors are involved in the BMP/dpp-screw pathway. Secreted zinc metalloproteinases antagonize the activity of chordin/sog, including Drosophila Tolloid (Tld),

Xenopus Xolloid (Xol), and human BMP-1 (Marques et al.,1997). Another extracellular factor, twisted gastrulation (tsg), which is conserved among vertebrate and invertebrate species, influences this proteinase cleavage and modifies the interactions between chordin/sog and BMP/dpp-screw by binding to the complex. The formation of this ternary complex leads to a more efficient inhibition of BMP/dpp activity (Oelgeschla¨ger et al., 2000).

Membrane Receptor Modulation

Another way of regulating BMP signalling has been described in Xenopus, by the protein BAMBI, which is a pseudoreceptor for members of the TGF-b superfamily that shows high sequence similarity to Nma, its mammalian homologue. BAMBI/Nma is structurally related to type I serine/threonine kinase receptors in the extracellular domain, but it lacks the intracellular serine/threonine kinase domain (Onichtchouck et al., 1999; Grotewold et al., 2001). The pseudoreceptor antagonizes the effects of TGF-b, Activin, and BMPs by stably associating with serine/threonine kinase receptors, thus preventing the formation of active receptor complexes (Onichtchouck et al., 1999).

Intracellular regulation

BMP-induced activation of R- and Co-Smads is negatively regulated by multiple mechanisms. There are at least two members of the Smad family of proteins, Smad6 and Smad7 that account fur such a function, and are referred as Inhibitory Smads (I-Smads). The molecular mechanisms of action of this proteins are complex, and they are described in general terms here. Extensive reviews about the subject are available (Massague, Seoane, Wotton. 2005)

Both, Smad6 and Smad7, can antagonize the Smad pathway by competing with R-Smads for interaction with the activated type I receptor. Smad6 has also been shown to compete with Smad4 for complex formation with the phosphorylated Smad1. Whereas Smad7 acts as a general inhibitor of TGF-b family member signaling pathway, Smad6 preferentially blocks BMP signalling. I-Smads are potently induced by TGF-b family members, and may thus participate in a negative feedback loop to control the intensity and duration of TGF-b signalling (Massague 2005).

Another mechanism by which I-Smads inhibit Smad signalling is via receptor degradation. Smad7 constitutively interacts with HECT-domain ubiquitin ligases Smurf2 and Smurf1. Upon recruitment of the Smad7/Smurf complex to the activated receptor, Smurf1 or Smurf2 induces receptor degradation via proteasomal and lysosomal pathways. In addition to the I-Smads, Smurfs have also been found to interact with R-Smads, thereby targeting R-Smads for ubiquitin-mediated degradation via the proteasome pathway. Smurf-mediated degradation of R-Smads decreases cellular competence to TGF-b family induced responses(Massague, Seoane, Wotton. 2005).

Inhibition of the Smad pathway can also occur via growth factor induced phosphorylation of the linker region of R-Smads, inhibiting the ligand-induced

nuclear accumulation of R-Smads. Activation of Erk by EGF, HGF or activated Ras can induce the phosphorylation of S/TP or PXS/TP motifs in the linker region (Calonge and Massagué, 1999; Kretzschmar et al., 1997a,b, 1999, de rob 2008).

BMP pathway in inner ear development

Most of the studies about BMP functions in the inner ear have focused on BMP4. This is mostly based on the remarkable feature of BMP4 expression during chick inner ear accompanying the development of the eight sensory organs (Oh et al., 1996; Wu and Oh, 1996). BMP4 expression was the first transcript to be mapped to prosensory patches and it is widely accepted as an early marker of the development of all sensory organs in chick (Oh et al., 1996; Wu and Oh, 1996, Cole et al 2000). Although this is not the case for all sensory organs in other species, Bmp4 expression is conserved in the development of vestibular cristae among several vertebrate species, including zebrafish, frog, chicken and mouse (Hemmati-Brivanlou and Thomsen, 1995, Morsli et al., 1998, Mowbray et al., 2001, Wu and Oh, 1996). In spite of these observations, the function of BMP4 in the development of the sensory elements of the ear had remained intriguing.

Moreover, at least four members of the BMP family have been reported to be expressed during inner ear development in the chick: Bmp5, Bmp7, Bmp4 (Oh, Johnson, Wu. 1996; Wu, Oh. 1996) and BMP2 (Chang et al. 2002), and different studies have analyzed the role of BMP signalling in various aspects of inner ear development (Chang et al., 1999; Chang et al., 2002; Gerlach et al., 2000). In this section I shall provide a brief summary of the current knowledge about the expression and function of Bmps in the development of the vertebrate inner ear, focusing in the chick as a model system.

Expression of BMP signalling pathway components in the develpoping inner ear of the chick

BMP4

Detailed analysis of Bmp4 mRNA expression was carried out in a seminal paper by Oh and Wu (1996) describing the time and location of sensory organ generation in the chick inner ear. Those results, summarized here, were the basis for investigating the roles of BMPs in sensory organ generation, providing fundamental information for the analysis of our results.

Bmp4 is detected in the otic placode of chick embryos at stage 10 but, as soon as the otic placode starts to invaginate, Bmp4 is expressed in the medial and posterior margin of the invaginating placode (stage 11, E1.5). When the otic placode deepens to form the otic cup, Bmp4 transcripts are found in the rim of the otic cup. At stage 16, the otic cup is closing rapidly and it is closed completely by the beginning of stage 17 (E2.5). The Bmp4-positive area at the ventral rim of the otic cup expands at the beginning of stage 16 (26 somites), and during this stage 16 (28 somites), two concentrations of the hybridization

signal appear, a posterior focus and an anterior streak. Both of these concentrations are more apparent by stage 17 within the otic epithelium, and also at low levels in the periotic mesenchyme (Gerlach et al., 2000). By stage 19 (E3), two principal foci of Bmp4 hybridization signals are evident in the otocyst, one anterior and another posterior. Whereas more Bmp4 expression sites appear in other parts of the otic epithelium, the two initial foci positive to Bmp4 persist and remain in the same relative positions within the otocyst throughout development. These two anterior and the posterior patches correspond to the areas that give rise to superior and posterior crista ampullaris, respectively (Romanoff, 1960; Von Bartheld et al., 1991).

At stage 20, a new area of BMP4 expression appears in the medial part of the otocyst, and it corresponds to the beginning of the presumptive macula sacculi, a pattern that becomes more apparent by stage 21 (E3.5). By stage 22/23, the presumptive lateral crista ampullaris appears in the anterolateral wall of the otocyst. By stage 24 (E4), the presumptive macula utriculi also appears and has a broad and diffuse domain of Bmp4 expression located between the macula sacculi and the lateral crista ampullaris. Bmp4 transcripts are also present in the mesenchyme surrounding the dorsolateral portion of the inner ear.

By stage 27 (E5), the inner ear undergoes a substantial expansion in the dorsoventral dimension, and most of the sensory organs in the inner ear are already discrete entities based on Bmp4 mRNA distribution. Expression of Bmp4 is found at the lateral crista, maculae utriculi, and sacculi. In addition, Bmp4 transcripts are detected in the mesenchyme surrounding the primordium for the anterior and posterior semicircular canals. In the basilar papilla, the Bmp4-positive area forms V-shaped domain, with a short anterior arm and a longer posterior arm joined ventrally at the lagena. All three Bmp4-positive areas, anterior, posterior, and the weak middle one, continue to elongate, with the V-shaped pattern almost disappearing by E9. At E5, Bmp4 expression in the vestibular sensory tissue is only interrupted by the *cruciatum*, which is a non-sensory epithelium present only in the superior and posterior cristae but not in the lateral crista (Dohlman, 1964; Landolt et al., 1975). Bmp4 expression persists in the dorsolateral surrounding mesenchyme, and the adjacent otic epithelium also becomes positive to Bmp4, eventually becoming the roof of the ampulla.

By E7, crista ampullaris are differentiated and exhibit the typical dome-shaped morphology. Interestingly, Bmp4 transcripts sre then concentrated in supporting cells, except at the periphery of the cristae where Bmp4 mRNA still spans the entire epithelium. Similarly, at E6-6.5, Bmp4 transcripts are distributed across the entire sensory epithelium of the lagena and the macula neglecta, but localize to supporting cells at E7.

Bmp4 finally disappears from the different vestibular organs being undetectable in the macula sacculi by E9, and in the macula utriculi and macula neglecta by E12. In the ampulla Bmp4 expression becomes very weak at E12 and disappears by E16, when also disappeared from the lagena.

In contrast to the expression of Bmp4 in the supporting cells of the vestibular organs, Bmp4 transcripts are concentrated in the hair cells of the basilar papilla. This occurrs by E12, and this pattern is much more evident by E16, and it continues at least until hatching (Wu and Oh, 1996).

BMP5

Bmp5 gene expression in the chick otocyst is associated with presumptive sensory areas, but it is very transient between E2 and E4. Bmp5 mRNA is not detected in the developing inner ear until stage 13 (E2). Bmp5 expression is similar to that of Bmp4, being present in the dorsal and posterior margins of the otic cup. By stage 16 (E2.5), when the otic cup is almost completely closed forming the otocyst, the Bmp5 expression in the posterior rim become restricted to one focus similar to that of Bmp4 (Wu and Oh, 1996). In addition, Bmp5 expression is high at the anterodorsal rim, but it disappears after the otocyst is formed. Bmp5 expression in the posterior otocyst remains only until stage 22 (E3.5). This posterior BMP5-positive area overlaps with that of Bmp4, These results indicate that Bmp5 is expressed only transiently in the primordium that eventually gives rise to the posterior crista, cochlea, lagena, and macula neglecta (Wu and Oh, 1996).

In addition, there is a strong Bmp5 expression in the first branchial furrow, which persists at least until stage 24 (E4). This branchial furrow eventually gives rise to the external auditory meatus, and this expression domain seems to be conserved in birds and mammals (Wu and Oh, 1996)

BMP7

Among the three Bmps characterized so far, Bmp7 is the earliest to be expressed in the inner ear. It also shows the widest expression in the otic epithelium up to day 4, including sensory and non-sensory tissue. As sensory organs mature, Bmp7 expression shows different patterns, depending on the vestibular or auditory regions. In the latter, Bmp7 segregates away from the sensory patches, whereas in the basilar papilla, becomes restricted to the sensory tissue (Wu and Oh 1996).

Bmp7 mRNA is detected in the otic placode, particularly in the epithelium next to the hindbrain. As the otic placode invaginates to form the otic cup, Bmp7 expression concentrates in the dorsal and posterior portions of the otic cup. This expression is similar to that of Bmp4 and Bmp5, but is much broader and stronger than the other two.

By stage 16 (E2.5), the majority of the otic cup is positive for Bmp7 except for the ventral portion of the otocyst. Hybridization signals show somewhat of a gradient, with stronger signal at the rim of the otic cup. At stage 24 (E4), the absence of Bmp7 mRNA in the ventral area of the otocyst persists in the anteromedial area of the otocyst, probably corresponding to the neurogenic domain (Alsina 2004). Worth mentioning, from stage 8 (otic placode stage) to stage 23, Bmp4- and Bmp5-positive areas are always found within a subset of the Bmp7-positive region (Wu and Oh, 1996).

By E4 (stage 24), the majority of the otocyst is positive for Bmp7, and the presumptive sensory organs fall within this expression domain, with the only exception of part of the macula utriculi that is containes by the negative anteromedial area. At this age, there are two Bmp7-negative areas within the otocyst: an anteromedial area and a dorsolateral area. The latter normally gives rise to the semicircular canals, whereas, as mentioned above, the anteromedial area includes part of the macula utriculi and the neurogenic domain.

In general, as sensory organs matured, Bmp7 expression becomes segregated from the main vestibular sensory organs of the inner ear. By E5, Bmp7 expression is downregulated from the macula sacculi and the three cristae, and this segregation was more extensive at E7. Bmp7 positive areas overlap with Bmp4 all around the peripheral portion of the crista.
At E7, Bmp7 expression in the maculae sacculi and utriculi is almost absent, and completely gone by E9. Similarly, Bmp7 expression in the macula neglecta initially overlaps with that of Bmp4, and it is segregated from the sensory tissue at E7, to disappear by E9. The epithelium lining the semicircular canals is positive for Bmp7 at E7, and it disappears by E12. At E8, Bmp7 transcripts remain concentrated in the side wall of the ampulla. A small portion of the epithelium in the periphery of the cristae is positive for Bmp7 and overlapped with Bmp4.

Bmp7 shows a different pattern from Bmp4 during the maturation of the auditory basilar papilla. Instead of segregating away from the sensory regions, Bmp7 expression is restricted to the sensory basilar papilla as development continues. Bmp7 expression extends across the entire epithelium at E12 and become restricted to supporting cells by E16. This is in contrast with Bmp4 expression that follows a complementary expression pattern, being expressed in hair cells (see above, Wu and Oh 1996). During this period, from E12 to E16, the basilar papilla already exhibits the spatula shape of a mature papilla (Cotanche and Sulik, 1985), and hair cell generation is complete (Katayama and Corwin, 1989, 1993). This distribution, Bmp4 in hair cells and Bmp7 in supporting cells of the papilla, remains at least until hatching (Wu and Oh 1996).

Bmp2

Unlike Bmp4 and -7, Bmp2 is not expressed in the otic cup or the newly formed otocyst (Chang et al., 1999). However, starting at E3.5, Bmp2 is expressed in the canal outpouch, from where the semicircular canals are generated. Bmp2 expression is not detected in the center of the canal outpouch, which is fated to be resorbed but toward the ends of the canal outpouch, in those regions that are destined to form the canals (Chang et al., 2002). Once the canals are formed, by E7, Bmp2 expression displays a similar pattern as in younger ages although with more robust expression in the outer rim of the canal. Bmp2 was also expressed at low levels in the endolymphatic duct, but there is little or no expression in the mesenchyme surrounding the canal pouch. At later stages of development, Bmp2 is also expressed in the otic capsule (Chang et al., 2002).

BMP receptors and Smads

Although there is no such a detailed analysis of the expression of any of the BMP receptors or BMP activated R-Smads throughout inner ear development as there is for the ligands, partial information is available from the literature and from functional studies (Dewulf et al., 1995, Chang et al., 2002, Chang et al., 1999). For instance, BMPRIA (Alk3) has been reported to be ubiquitously expressed in otic epithelium and surrounding mesenchyme from day 3-5, up to E6 (Chang et al., 2002). Similar results were also reported for mouse inner ear (Dewulf et al., 1995). BMPRIB (Alk6) expression, however, has not been detected in the chick inner ear until E6. BMPRIB expression is highest in the mesenchyme surrounding the developing canal pouches, although the two ends of the canal epithelial are also weakly positive. Hybridization signals are found in close association with epithelia at the two ends of the canal pouch, but not with the epithelia in the central region of the canal pouch, which may have implications for the process of canal development (Chang et al., 2002).

BMP activated R-Smad have only been studied in relation to the semicircular canal formation at stages when sensory patch formation is already accomplished. Chang et al (2002) reported that Smad1 is expressed ubiquitously in the entire canal pouch otic epithelium at E6, but its expression in the surrounding mesenchyme is low. Later, in canal development, Smad1 expression is also detected in the otic capsule. In contrast, Smad5 expression is strong in mesenchymal cells that are bordering the canal pouch epithelium, but is weak in the canal epithelia. Smad8 hybridization signal has not been detected in the otic epithelium or adjacent mesenchyme at either E6 or E9 (Chang et al., 2002).

In mouse, only one report showed expression of inhibitory Smads 6 and 7 in the periotic mesenchyme, related to a role for TGF-b/ BMP signalling in otic capsule development (Liu et al, 2007, see below). To our knowledge, data about expression of other Smad family members in the chick inner ear are not available.

Noggin

Noggin has been described to be expressed early in the periotic mesenchyme of the developing chick inner ear (Chang et al., 1999; Gerlach et al., 2000). At early stages of otic development (stages 11-15), Noggin mRNA is localized in the anterior and posterior periotic mesenchyme adjacent to the otic pit but not in the otic epithelium. Between stages 12 and 15, periotic mesenchyme cells immediately adjacent to the pit show high levels of noggin. However, in stage 17-20 embryos, Noggin mRNA is not detected in the periotic mesenchyme.

Starting at E4, Noggin is expressed weakly only in the ventral tip of the cochlear duct, in a region that does not overlap with the sensory region expressing Bmp4, and this expression remains at least until E6.5.

BMP functions during in inner ear development

The functions of BMPs in the development of the chicken inner ear had been explored by blocking BMPs activity in vivo using avian retrovirus encoding Noggin, dominant negative or constitutively active forms of BMP receptors, beads carrying Noggin protein, and by implantation of Noggin-expressing cell lines (Chang et al., 1999; Chang et al., 2002; Gerlach et al., 2000). The effects reported so far are morphogenetic malformations associated with growth defects of semicircular canals and their associated sensory organs. Given the general inhibition of BMP signalling by Noggin, and because several Bmp genes are expressed in the developing inner ear, including Bmp2 and Bmp7, specific roles for Bmp4 in inner ear development cannot be extrapolated unambiguously from these results. In addition to this, these studies did not analyzed in detail the cellular effects of BMPs during sensory organ formation, and on what specific role(s) they may play in the development of sensory cells. While this project was in progress, two papers appeared addressing some of these issues. They will be included in the discussion chapter (Li, et al. 2005) (Chang et al., 2008).

In addition to its role in inner ear morphogenesis, Bmps are involved in patterning the surrounding mesenchymal cells that will develop into the bony labyrinth. Chang et al. (Chang et al., 2002) have compared the phenotypes of the otic capsule in ears that were infected with an avian retrovirus encoding either constitutively active or dominant-negative forms of the Bmp type IB receptor (Bmpr1b). Ectopic expression of constitutively active Bmpr1b induced cartilage overgrowth, whereas a dominant-negative form resulted in cartilage loss in the otic capsule. Inhibition of cartilage formation also occurs in response to treatment with Noggin, a secreted inhibitor of Bmp (Liu et al., 2002). In both cases, it is not clear whether the inhibition of cartilage development results from failure of mesenchyme to form cartilage precursor cells or from the resorption of initiated cartilage.

Dan is another Bmp antagonist (see above) involved in patterning during ear development. Dan is synthesized in the chick otic placode and at later stages in the medial wall of the otocyst, close to the hindbrain rhombomeres (Gerlach-Bank et al., 2002). Exogenous Dan has effects similar to Noggin, but also has profound effects on the endolymphatic duct (ED/ES) including gross structural abnormalities in or deletions (Gerlach-Bank et al., 2004). Adding exogenous BMP4 together with Dan rescues semicircular canal loss and prevents the effects on ED/ES, demonstrating that the effects of Dan are mediated through BMPs. Inhibition of Dan protein translation using antisense oligo morpholinos electroporated into the otic epithelium of chick embryos causes overgrowth of the ED/ES at the expense of canal structures. These experiments indicate that Dan may help to partition the otic epithelium of chick embryos into the SCC and the ED/ES fields. However, Dan is only expressed in extra-otic tissue in the mouse, showing that its role differs between mammals and birds.

In order to understand the possible functions of BMP4 in the development of the sensory elements of the ear at a cellular level, we focused our attention in some of the key regulators of sensory cell fate specification known up to now. They

belong to the bHLH family of transcription factors and I shall provide a brief introduction to this family of proteins, focusing on the Atoh1 gene and the Id sub-family of proteins, which are of particular interest fo this project.

The Helix-loop-Helix family of proteins

The helix-loop-helix (HLH) family of transcription factors comprises more than two-hundred members, which have been identified in organisms from yeast to man (reviewed in Littlewood and Evan, 1995; Massari and Murre, 2000). In metazoa, HLH proteins function in the coordinate regulation of gene expression, orchestrating cell cycle control, cell lineage commitment and cell differentiation. An essential role has been established for a number of HLH proteins in the development of haemopoietic, myogenic, pancreatic and neurogenic mammalian cell lineages (Norton. 2000).

Four main groups of HLH protein can be distinguished on the basis of the presence or absence of additional functional domains. The highly conserved HLH region comprises two amphipathic a helices, each 15-20 residues long, which are separated by a shorter intervening loop that has a more variable length and sequence. The HLH domain primarily mediates homo- or hetero-dimerisation, which is essential for DNA binding and transcriptional regulation. Nearly all HLH proteins possess a region of highly basic residues adjacent to the HLH domain, which facilitates binding to DNA containing the canonical ‘E box’ recognition sequence, CANNTG. Some HLH proteins also bind the related ‘N box’ sequence, CACNAG (Massari, Murre. 2000).

Proneural Genes

A particular group of the bHLH family of proteins was characterized by their function in the early steps of neural development in Drosophila, and named proneural genes (García-Bellido, 1979). Molecular analysis led to the isolation of the first two groups of proneural genes, as-c genes: achaete (ac), scute (sc), lethal of scute (lsc) and asense (ase) (Villares and Cabrera, 1987). A further proneural gene was isolated in a PCR-based screen to identify bHLH sequences, the Drosophila gene atonal (ato) (rev. In Bertrand et al., 2002)

In vertebrates bHLH genes homologous to Drosophila proneural genes *as-c* and *ato* have been extensively characterized. Even though their roles extend beyond the process of neurogenesis, they are still generally known as “proneural genes” (Bertrand et al, 2002). The vertebrate asc family includes ash1, which is present in all species analysed (for example, Mash1 in mouse, Cash1 in chick, Zash1 in zebrafish and Xash1 in Xenopus), and three other genes that, curiously, have each been found in only one class of vertebrates (Mash2 in mammals, Xash3 in Xenopus and Cash4 in chick). The number of vertebrate genes that are related to Drosophila ato is larger, but only two of them Atoh1 and Aoth5 (formerly known as Math1 and Math5 in the mouse) have a bHLH domain similar enough to that of ato to be considered as orthologues. Other vertebrate ato-related genes can be grouped into distinct families, for example, the neurogenin (Ngn) family, the NeuroD family and the Olig family. They are characterized by the presence of family- specific residues

in their bHLH domain indicating that different members in each family share biochemical properties that distinguish them from other neural bHLH proteins. (Bertrand et al, 2002).

Neurogenin, NeuroD and Olig proneural genes are indeed key regulators of vertebrate neurogenesis as they regulate all features inherent to the process of neuronal differentiation. First, they modulate the transition from a proliferating neural progenitor to a post-mitotic neuron, generally by activating the expression of cyclin-dependent kinase (CdK) inhibitor and promoting cell cycle exit (Farah et al., 2000; Ohnuma et al., 2001; Bertrand et al., 2002; Kageyama et al., 2005; Nguyen et al., 2006). Proneural proteins also coordinate the acquisition of both generic and specific neuronal characters, as they trigger the expression of other transcription factors that regulate pan-neuronal and subtype specific characters (reviewed in Bertrand et al., 2002). Neurogenins have a similar proneural function to that of their Drosophila counterparts, whereas other proneural bHLH, such as NeuroD, are involved in specifying neuronal fates or in neuronal differentiation and survival, but have not conserved proneural role (Bertrand et al., 2002; Cau et al., 2002). Thus, vertebrate neurogenesis is driven by proneural genes in individual neural progenitors that promote full neuronal differentiation as a result of the induction of a cascade of downstream bHLH genes.

As described above, during inner ear development, proneural genes (Neurogenin, NeuroD) seem to play an important role in neuronal cell fate specification. In this work we focused our attention in the process of hair cell generation, in which another proneural gene, Atoh1, plays an important role.

The bHLH protein Atoh1 and inner ear hair-cell specification

The factors that specify cells to develop as either hair cells or supporting cells are still poorly understood, and most probably remain to be discovered. However, recent work has identified the basic helix–loop–helix (bHLH) transcription factor Atoh1 as a key regulator of hair cell development (reviewed in Kelley 2006).

Atoh1 is initially expressed in all inner ear sensory epithelia at developmental stages coincident with, or soon after, terminal mitosis (Chen P, 2002, Bermingham, N. 1999, Lanford, 2000). Deletion of Atoh1 leads to a complete loss of hair cells, whereas overexpression of Atoh1 in the embryonic cochlea is sufficient to induce cochlear progenitor cells to develop as hair cells (Bermingham, N. 1999, Jones, J. M. 2006). Similarly, overexpression of Atoh1 in the postnatal saccular macula induces hair cell formation, probably through the transdifferentiation of supporting cells (Zheng, J. L.2000). Surprisingly, ectopic expression of Atoh1 is sufficient to induce hair cell formation in non-sensory cells located near the organ of Corti in embryonic, postnatal and adult inner ears (Zheng, J. L.2000, Woods 2004, Kawamoto, K. 2003). In embryonic or early post natal tissue, Atoh1 is able to induce ectopic cells comparable to endogenous hair cells, in terms of the expression of hair-cell specific markers and the development of a stereociliary bundles (Zheng, J. L.2000, Woods 2004).

Very recently, Atoh1 was introduced into precursor hair cells in the inner ear of mice at day 11.5 of embryonic development in utero, and the authors could show that by embryonic day 18.5 the transfected cells had differentiated into hair cells and could be seen among untransfected, natural hair cells. Those transfected cells displayed their characteristic bundles, and they also seemed to form the correct neural contacts with afferent nerve fibres, thus allowing the newly formed cells to interact with the auditory nervous system. Additionally, by mechanically stimulating the hair bundle while recording using electrophysiological methods, they showed that the cells transfected with *Atoh1* were capable of mechano-electrical transduction, demonstrating that the cells are indeed functional auditory sensory cells (Gubbels, et al. 2008).

Altogether, the existing data suggest that Atoh1 behaves as a master gene for hair cell specification, being both necessary and sufficient to specify hair cells in prosensory patches, and outside sensory fated domains.

Inhibitors of differentiation and DNA binding proteins (Ids)

Id proteins are a particular subfamily of the basic helix-loop-helix (bHLH) familiy of proteins characterized for sharing the helix-loop-helix domains, but lack the DNA binding domain (rev. in Norton 2000). The first Id protein identified (Id1) was named for its ability to inhibit the binding to DNA of bHLH transcription factors (Benezra 1991), Id proteins physically interact with other transcriptional regulators, principally those of the bHLH type. Since Id proteins lack the basic domain such ID-bHLH heterodimers are unable to bind to DNA, and hence ID proteins act as dominant negative regulators of bHLH proteins (Benezra et al., 1990; Garrell and Mondolell, 1990; Ellis et al., 1990). Since most bHLH proteins positively regulate sets of genes during cell fate determination and cell differentiation, the term ‘ID’ conveniently alludes to the ability of these proteins to inhibit both DNA binding and differentiation (Norton 2000).

Transcriptional inhibition by Id proteins is mediated via inhibition of DNA binding of bHLH or other activator proteins at E boxes (CANNTG), N boxes (CACNAG), or Ets sites (GGAA/T) present in the promoter regions of regulated genes (reviewed in Zebedee and Hara, 2001).

Id regulation of cellular differentiation

The first direct genetic evidence for a role of Id proteins in regulating cellular differentiation came from mutational studies of the Drosophila emc locus. This gene encodes for a helix-loop-helix protein that forms heterodimers with bHLH target proteins and prevents them from binding DNA and functioning transcriptionally. Loss and gain of function mutants of emc in Drosophila showed that emc inhibits the functions of Daughterless and achaete-scute bHLH proteins, which are involved in sex determination and neurogenesis (Campuzano, 2001).

In mammalian cell culture systems, differentiation of various cell lineages has been shown to be accompanied by downregulation of vertebrate Id gene

expression, while overexpression of Id proteins within these systems, including keratinocytes, myoblasts, myeloid precursor cells, mammary epithelium, and preadipose cells, was shown to inhibit their ability to differentiate under appropriate conditions (reviewed in Lasorella et al., 2001). More recently, in vivo studies using targeted expression of Id genes to thymocytes, intestinal epithelia, and B-lymphocytes of mice have demonstrated inhibition of cellular differentiation in these systems (Lasorella, Uo, Iavarone. 2001; Ruzinova, Benezra. 2003)

Id proteins and the cell cycle

Several lines of evidence suggest tha Id proteins play a critical role in promoting G1-S cell cycle transition. First, Id3 was cloned as a mitogen-induced early response gene and, Id1, Id2, and Id3 are considered mitogen responsive (Lasorella et al., 2001). Typically, quiescent cells express low/undetectable levels of Id genes. However, following mitogenic stimulation Id expression is rapidly induced (within 1-2 hours) as part of a cascade of 'delayed' early response genes Moreover, antisense Id constructs were shown to delay cell cycle re-entry in serum-stimulated NIH 3T3 cells these cells, while microinjection of anti-Id antibodies led to late G1 withdrawal from the cell cycle (Lasorella et al., 2001). One way to explain this function of Id proteins is that following the mitogenic signal, Ids downregulate the expression of immediate early genes (e.g. c-fos and egr-1) by antagonising the factors responsible for up-regulation of the immediate-early-gene expression (Yates et al., 1999).

Secondly, Id2, Id3 and Id4 (but not Id1) are substrates for CDK2-dependent phosphorylation during late-G1/early-S phase (Hara et al., 1997; Deed et al., 1997). Phosphorylation of Id2 and Id3 alters their bHLH dimerisation specificity, or subcellular localization, and has been suggested to be important for transitions at the G1–S boundary, since mutants of Id2 and Id3 that lack CDK2 phosphorylation sites elicit S phase arrest and cell death (Deed et al., 1997).

Thirdly, Id2 has been found to reverse cellular growth inhibition by the retinoblastoma protein (pRb) through direct interaction with pRb, p107, and p130 via its HLH domain (reviewed in Lasorella et al., 2001). Moreover, Id2 had been shown to interact genetically with pRb since Id2/pRb double knockout animals display partial suppression of Rb null-associated embryonic lethality (Lasorella et al., 2000). This suggests that Id proteins can inactivate Rb pathway either through direct interaction with pRb or through altered expression of genes that regulate Rb phosphorylation and, ultimately, function. Other cell cycle regulatory proteins involved in the pRb pathway have been shown to be affected by Id expression, and analysis of the promoter regions of the cell cycle inhibitory proteins p15, p16/Ink4a, and p21 (Pagliuca et al., 2000; Prabhu et al., 1997) has demonstrated activation by E-proteins, which is abrogated by Id proteins.

Finally, recent studies have shown that Id1 and Id3 are also involved in the regulation of p27 protein, a cyclin-kinase-inhibitor in vitro (Everly et al., 2004), and in Xenopus neural crest progenitors in vivo (Kee and Bronner-Fraser, 2005). Moreover, Id3 has been recently shown to be involved in the

transcriptional repression of p27, which was crucial for cell cycle progression in a wounding model of human dermal fibroblasts. These experiments suggested also that Id3 is involved in controling the early mRNA degradation phase, and p27 mRNA stability at the G1/S (Chassot, et al. 2007).

In summary, ID proteins can function at multiple stages in cell cycle control by modulating the transcription of several known target genes, in some instances by directly interacting with non bHLH proteins.

Id proteins and apoptosis

Id genes have been also shown to promote apoptosis in a variety of experimental models. Transgenic mice with targeted Id1 expression in T cells showed a 96% reduction in the total number of thymocytes due to massive apoptosis (Kim et al., 1999). In addition, apoptosis is induced by overexpression of Id1 in dense mammary epithelial cell cultures (Parrinello et al., 2001) and Id1 in neonatal and adult cardiac myocytes (Tanaka et al., 1998), Id3 in B-lymphocytes (Kee et al., 2001) and Id4 in an astrocyte-derived cell line (Andres-Barquin et al., 1999). However, little is known about the molecular mechanisms involved in Id induction of apoptosis.

Id proteins in development

Many studies have shown that Id proteins play several important roles during development (rev. in (Yokota. 2001) (Ruzinova, Benezra. 2003)).

First, in situ hybridization analyses of Id gene expression have demonstrated widespread expression of Id1, Id2, and Id3 throughout the developing organism from early gestation through birth. Considerable overlap in expression patterns of Id1 and Id3, and distinct expression of Id4 (limited to the nervous system) has been observed in mouse and Xenopus (Yokota. 2001). Expression of Id1-4 has been also studied in the early stages of chick embryos, being expressed dynamically in several sites of the embryo, and having largely complementary expression patterns during early nervous system development (Kee, Bronner-Fraser. 2001a; Kee, Bronner-Fraser. 2001b; Kee, Bronner-Fraser. 2001c).

Several knockout animal models have been generated, providing functional data about the role of Id genes in development (Rev.in (Perk, Iavarone, Benezra. 2005). Deletion of the Id1 gene alone fails to produce an obvious phenotype, while mice null for Id2 possess defects in immunity due to a lack of lymph nodes and Peyer's patches and a severely reduced population of natural killer (NK) cells and Langerhans cells. Mice null for Id3 show defects in B cell proliferation and humoral immunity (Hacker et al., 2003; Yokota, 2001). Given the overlapping expression patterns of Id1 and Id3 during mouse embryogenesis, it has been suggested that redundant functions exist between Id1 and Id3 proteins. Consistent with this idea, mice that are null for both Id1 and Id3 are embryonic lethal (E13.5) with aberrant neuronal differentiation and angiogenesis (Lyden et al., 1999). Specifically, neuroblasts prematurely withdraw from the cell cycle with enhanced expression of neural-specific differentiation markers. In addition, these mice display vascular malformations

in the forebrain and an absence of branching and sprouting of blood vessels into the neuroectoderm. Although the expression patterns of neuronal and endothelial differentiation markers is altered in Id1/Id3 double null embryos, the specific targets of Id genes in these systems remain to be elucidated (Lyden et al., 1999, Perk 2005).

Regulation of Id

The Id proteins themselves are regulated at the post-translational level. In addition to cell-cycle-linked phosphorylation (see above), intracellular levels of Id proteins are regulated through the ubiquitin-proteasome degradation pathway (Bounpheng et al., 1999). In common with most other proteins encoded by early response genes, Id proteins rapidly turnover in the cell, having a reported half-life of 20-60 min, depending on the cell type. Id4 is apparently much less sensitive to inhibitors of the 26S proteasome pathway, and, although its degradation is dependent on ubiquitin-activating enzyme activity, this Id family member might also be degraded through an alternative pathway (Norton, 2000).

Heterodimerisation with bHLH proteins extends the half-life of Id3 (and also enhances the degradation of its heterodimeric bHLH protein partner); Id proteins might therefore be less susceptible to degradation by the 26S proteasome pathway in the heterodimer state (Deed et al., 1996; Bounpheng et al., 1999a).

Id proteins are also regulated by subcellular localisation. Although Id proteins do not possess nuclear localisation signals, their bHLH protein partners possess an efficient nuclear localisation signal, and in vitro experimentos of co-transfection of cells with constructs expressing Id and bHLH proteins leads to sequestration of the Id protein into the nucleus, which suggests that heterodimerisation regulates the subcellular distribution of IDs (Deed et al., 1996). The physiological relevance of such a mechanism has yet to be established.

Finally, many studies have suggested that transcriptional regulation of Id genes play a key role in their function. In diverse mammalian cell lineages, Id expression responds to several cell surface ligand-receptor interactions, including mitogens, and members of the TGF-b family of proteins (rev. in Norton et al., 1998, Ruzinova and Benezra 2003). We shall focus here in the transcriptional regulation of Id genes by BMP proteins.

Transcriptional regulation of Id genes by BMP

Although the expression of Id genes is induced by various stimuli, including some growth factors, BMPs are one of the most important regulators of Id genes, and induction of the Id1, Id2 and Id3 genes in response to BMP treatment has been shown in a range of diverse cell lines and embryonic stem cells (rev. in Miyazono 2002, Ruzinova 2003). Figure 1-4 illustrates the regulation of Ids by BMP and some of their known functions.

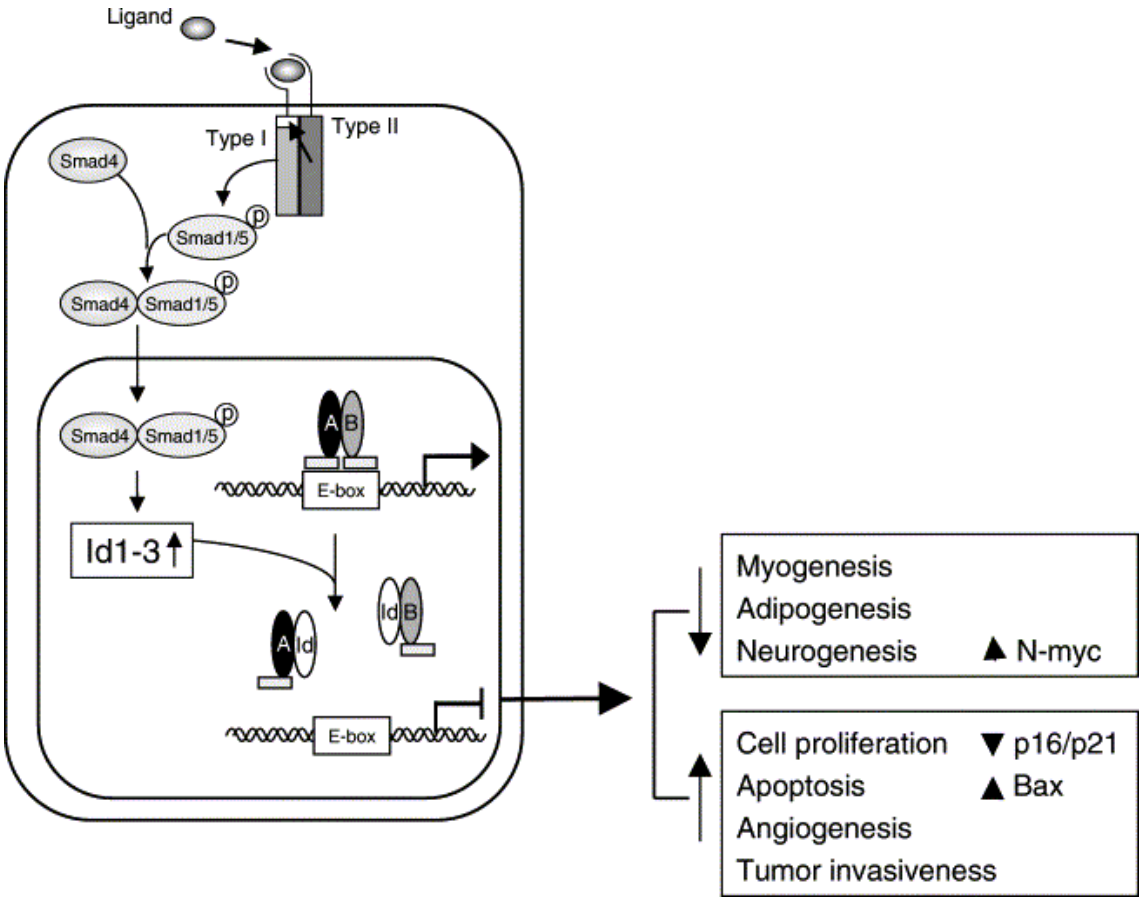

Fig. 1-4.. BMPs strongly induce Id1-3 expression in a Smad-dependent manner. Id proteins act as dominant negative inhibitors of basic helix–loop–helix (bHLH) transcription factors. Effector function of Id1 in BMP receptor-induced biological responses. bHLH transcription factors can be divided into two main categories. The class A bHLH transcription factors, also known at E proteins, that are ubiquitously expressed and the class B bHLH proteins that show a tissue-restricted pattern of expression. bHLH proteins bind to specific DNA sequences known as E-boxes. Dimerization is essential for DNA binding and transcriptional activity of the bHLH proteins. In general, class B bHLH proteins form heterodimers with class A. Id proteins bind to both class A and class B bHLH proteins and inhibit their ability to bind DNA and can inhibit myogenesis, adipogenesis, neurogenesis and promote cell proliferation, apoptosis, angiogenesis and tumor invasion.
From: ten Dijke, et al, 2003. Molec and Cellular Endocrinology. 211, 105-113.

Id1 is a direct BMP target gene whose expression can be upregulated by BMPs in the absence of de novo protein synthesis, and requires Smad1 or Smad5 (Korchynskyi, O. and ten Dijke, P. (2002) (Lopez-Rovira, T. et al. (2002) (Katagiri, T. et al. (2002). Accordingly, elements conferring BMP responsiveness to the Id1 promoter have been identified as Smad-binding elements (SBEs) and a GC-rich region, which both bind BMP-associated Smad1 and Smad5 (ten Dijke, et al. 2003) .

Although the regulation of Id1 by BMPs has been investigated in more detail, there is also evidence that Id2 and Id3 are regulated by these factors. Sequence analysis of the Id3 promoter revealed potential BMP-responsive elements with similar organization to that found in the Id1 promoter. Because

the expression pattern of Id3 during development is virtually identical to that of the Id1 gene, it is likely that BMP-induced Smads coordinately regulate expression of both of these genes (Ruzinova, Benezra. 2003).
During embryogenesis, the expression of the Id genes overlaps with the expression of BMP2 and BMP4 in many sites, and misexpression of BMP4 during embryonic development induces ectopic expression of Id3 (Yanagisawa, M. et al. 2001). Moreover, BMPs can repress neuronal and myogenic cell fate, and this regulatory activity has been tied to their ability to upregulate expression of the genes encoding Id proteins. In vitro, neural progenitors exposed even briefly to BMP2 fail to undergo neurogenesis and upregulate expression of Id1, Id3 and Hes-5 (Nakashima, K. et al. 2001).

Finally, Id genes have shown to be direct targets of BMP in undifferentiated embryonic stem cells (Hollnagel 1999), ehere they play a critical role in BMP-mediated suppression of ES cell differentiation, and maintenance of self renewal and pluripotency (Qi-Long Ying, cell 2003)

Id proteins and hair cell development

As described below, the generation of hair cells requires the activity of the bHLH factor Atoh1 (Bermingham et al., 1999; Zheng and Gao, 2000). However, the factors that regulate Atoh1 transcription and activity during inner ear development are essentially unknown. The Atoh1 gene contains an E-box consensus binding site in its enhancer region that is related to its autoregulation through binding of Atoh1/E-protein heterodimers (Helms et al., 2000), this autoregulatory enhancer being active in developing hair cells (Woods et al., 2004). Moreover, in some model systems the activity of bHLH genes such as Atoh1 must be actively inhibited to prevent premature differentiation (Bertrand et al. 2002). Given that the function of Ids in various systems is to suppress binding of specific bHLH transcription factors, such as Atoh1, to E-proteins, it seemed likely that Ids could regulate hair cell development through the negative regulation of Atoh1 autoregulatory activity.

While this thesis project was in progress, a study was published analyzing the possible role of Id in the regulation of Atoh1 expression in the developing mouse cochlea. The authors showed that prior to hair cell formation, three Id genes — Id1, Id2 and Id3 — are broadly expressed throughout the floor of the cochlear duct including the domain of Atoh1 expression. As development continues, Id expression was specifically downregulated in developing hair cells, suggesting that loss of Id function relieves an inhibition of Atoh1 activity in those cells. Consistent with this hypothesis, prolonged expression of Id3 in sensory progenitors inhibited hair cell formation, suggesting that indeed, downregulation of the Id family is a key step in hair cell development (Jones et al 2006).

Whether this is a general mechanism that also operates in the vestibular sensory organs was not determined in that study, and this was one aspect addressed in the present work. Another important issue not yet addressed is the way in which Id genes are regulated in the sensory organs of the inner ear. Understanding the regulation of Id genes in progenitor sensory cells would

ultimately lead to a better understanding of the molecular networks regulating hair cell formation.

Msx as BMP targets

Among the genes involved in development, msh/Msx form one of the most highly conserved families of homeobox-containing genes and respond to and correlate with BMP activation (Ramos and Robert, 2005). The vertebrate muscle segment homeobox (Msx) genes encode homeodomain transcription factors characterized as transcriptional repressors and are related to the Drosophila muscle segment homeobox (msh) gene (Davidson, 1995). In Drosophila, msh expression is first detected in the mesoderm of the developing somatic musculature of the embryo and later in the central nervous system (CNS) and in specific muscles. The mouse Msx genes consist of three physically unlinked members, Msx1, Msx2 and Msx3, which share 98% homology in the homeodomain (M. Ekker et al.1997). Msx1 and Msx2 are expressed during embryogenesis, in overlapping patterns, at many sites of epithelial–mesenchymal inductive interactions, such as limb and tooth buds, heart, branchial arches and craniofacial processes, but also in the roof plate and adjacent cells in the dorsal neural tube and neural crest. Msx3, however, is expressed exclusively in the dorsal aspect of the neural tube in the mouse, caudally to the isthmus. Msx genes from cephalochordates or ascidians are strongly expressed in the neural plate, suggesting an ancestral function for these genes in chordate and vertebrate neural-tube patterning (Ramos and Robert, 2005).

Studies on Xenopus and zebrafish embryos suggest that the neural crest is specified at the border of the neural plate at a specific concentration in a BMP gradient, which exactly coincides with an increased level of activation of the BMP downstream targets Msx genes Msx1 is required to induce early neural crest cell markers and is considered an upstream factor in the genetic cascade of neural-crest specification. Msx function requires other co-factors to activate this cascade, and WNT signalling also has proved necessary to induce both neural-crest formation and Msx1 expression. In line with this result, synergetic effects of WNT and BMP take place for Msx1 and Msx2 induction in cell-culture conditions.

A variety of studies support the notion that the roof plate cells produce signalling molecules, including BMP, that are required for the specification and proliferation of dorso-lateral cells of the neural tube. Exogenous BMP4 and BMP7 can induce a dorsal fate in neural tube explants and also Msx1 expression (Liem et al., 1997) whereas BMP4 can induce ectopic Msx1 expression in lateral explants of the telencephalon. Accordingly, application of noggin to the dorsal neural plate down-regulates Msx1.

Increasing evidence both in chick and mouse suggests that Msx1, Msx 2 and Msx3 genes have distinct roles. First, in several in vivo and in vitro conditions, Msx1 and Msx2, together with Bmp4 or Bmp7, control and promote cell death during neural-crest-cell, neuronal-precursor and eye formation. By contrast, Msx3 cannot induce apoptosis in the neural tube. Secondly, enhancement of

BMP signalling or of Msx1, but not Msx3, expression induces roof plate cell fate and represses the transcription of neuronal differentiation genes in early stage chick embryos. By contrast, at later stages of neural-tube development, dorsal progenitor cells lose their competence to generate roof plate cells in response to BMP signalling and, instead, form dorsal interneurons. These suggest that these two Msx family members can mediate distinct aspects of BMP signalling in the dorsal neural tube.

How do BMP signals regulate the expression of MSX and how do these genes in turn control the different aspects of neural development are questions that still remain largely unexplored. Preliminary results show that the targeted disruption of Smad4 causes a reduction of Msx2 expression and an increase of Msx3 expression in embryonic stem (ES) (Sirard et al., 2000). It is also known that Msx1 inhibits neuronal differentiation, at least in part, through repression of regulatory genes, such as those that encode basic helix-loop-helix (bHLH) and paired homeodomain transcription factors. Several consensus sites for MSX1 binding are present in the enhancer region of Atoh1and Ash1 (Liu et al., 2004)

BTG genes

During development, cell proliferation and cell differentiation are tightly coordinated through signalling pathways that ultimately regulate the expression of cell cycle control genes. Btg/Tob genes are a newly characterized family of cell cycle modulators and studies in different model systems suggest that they act as antiproliferative genes (Guehenneux et al. 1997). Btg genes can promote cell differentiation, and they can also regulate apoptosis and cellular senescence (Matsuda et al. 2001, Tirone. 2001, Duriez et al. 2004, Lim. 2006). Btg/Tob genes encode for a group of structurally related proteins, characterized by the presence of two novel and highly conserved domains (“antiproliferative” boxes A and B) located in the first 120 residues of the protein (Guehenneux et al. 1997, Guardavaccaro et al. 2000). This gene family is conserved across phylogeny, with two members identified in invertebrates: the gene Fog-3 in C.Elegans (Chen et al. 2000) and the mRNA with accession number AF177464 in D.Melanogaster. Also, a structurally related gene has been characterized in amphioxus: the AmphiTOB gene (Holland et al. 1997). In vertebrates, the whole family is composed of at least six members that have been named differently in different species: Btg1, Btg2 (Tis21 in mouse and PC3 in rat), Btg3 (also named ANA for Abundant in Neuroepithelium Area), Btg4 (PC3B), Tob (Tob1) and Tob2 (Bradbury et al. 1991, Fletcher et al. 1991, Matsuda et al. 1996, Rouault et al. 1996, Guehenneux et al. 1997, Yoshida et al. 1998, Ikematsu et al. 1999). It has been proposed to name the family as APRO (standing for AntiPROliferative) (Matsuda et al. 2001), but most studies and genetic databases continue using the BTG/Tob nomenclature, that will be adopted in this report.

In spite of having similar N-terminal sequences and activities, BTG proteins are smaller than Tob proteins and both groups are generally considered as subfamilies (Matsuda et al. 2001, Tirone. 2001, Jia and Meng. 2007). The Btg2 gene was the first member to be identified, as an immediate response gene in

mouse and rat, in two different cellular contexts. The mouse gene was isolated in TPA stimulated NIH 3T3 fibroblasts and was named Tis21 (for TPA-induced sequence) (Fletcher et al. 1991), whereas the rat homologue was isolated as an immediate response gene to NGF stimulation in neural crest-derived PC12 cells (Bradbury et al. 1991). Shortly after, the human Btg1 (B-cell-translocation gene 1) was cloned from a chromosomal translocation observed in a lymphoid malignancy (Rouault et al. 1992). Human BTG1 and BTG2 proteins share 66% sequence identity, and the only significant difference between the two proteins is a 10 amino-acid insertion in the carboxy-terminal part of BTG1 (Duriez et al. 2004).

Overexpression of Btg/Tob members in different cellular models have assigned them antriproliferative and/or pro-differentiative functions (reviewed in Tirone. 2001, Duriez et al. 2004). Btg1 has been shown to modulate avian myoblast differentiation in vitro (Marchal et al. 1995, Rodier et al. 1999), and Btg2 can promote differentiation and survival of cultured-neural cells (Corrente et al. 2002, el-Ghissassi et al. 2002). Overexpression of Btg2 during mouse embryonic development in vivo regulates cell cycle exit and differentiation of mouse cerebellar progenitors (Canzoniere et al. 2004). Furthermore, Btg2 is expressed at the onset of neurogenesis in neuroepithelial cells, concomitantly with the switch from proliferative to neurogenic divisions (Iacopetti et al. 1994, Iacopetti et al. 1999, Hammerle et al. 2002). Knock out mice for Btg1 are not yet available and Btg2-/- mice have minor defects in vertebral patterning (Park et al. 2004). Morpholino injection of Btg genes in Xenopus embryos disrupts some aspects of anterior neural development and notochord differentiation (Sugimoto et al. 2005, Wessely et al. 2005, Sugimoto et al. 2007). It has been suggested that some redundant function may exist between different members of the family (Park et al. 2004, Wessely et al. 2005).

Information about developmental expression of Btg genes is still fragmentary and limited to individual genes in specific developmental processes in amphioxus, xenopus, zebrafish and mouse (Iacopetti et al. 1994, Holland et al. 1997, Iacopetti et al. 1999, Buanne et al. 2000, Chen et al. 2000, Saka et al. 2000, Sakaguchi et al. 2001, Thisse et al. 2001, Hammerle et al. 2002, Canzoniere et al. 2004, Park et al. 2004, Thisse and Thisse. 2004, Sugimoto et al. 2005, Wessely et al. 2005, Feng et al. 2007, Sugimoto et al. 2007), and there is no detailed description of the expression pattern of any member of the family in the chick embryo. Moreover, there is no available data about Btg gene expression in the inner ear of any animal species.

To gain insights on the function of these genes during development, and since Btg1 and Btg2 are the more closely related genes in the family, we sought to analyse and compare their expression profile during early stages of chick development, and specifically during inner ear development.

Aims

Bone morphogenetic proteins (BMPs) are diffusible molecules involved in a variety of cellular interactions during development. In particular, Bmp4 expression accompanies the development of the ear sensory organs during patterning and specification of sensory cell fates, and it has been shown to play a role in inner ear development and morphogenesis. However, there is no understanding of the cellular effects of BMP4 in prosensory progenitors, and about its role in the process of sensory fate specification. The present thesis project was aimed at exploring the effects of BMP-signaling on the development of hair-cells, using the chick inner ear as a model.

The specific aims proposed were:

1- Analyze the cellular effects caused by addition of BMP4 in a model of isolated chick otic vesicles in culture, measuring parameters of cell proliferation, cell death and sensory cell fate specification.

2- Analyze the cellular effects caused by inhibition of BMP4 signaling in a model of isolated chick otic vesicles in culture, measuring parameters of cell proliferation, cell death and sensory cell fate specification.

3- Analyze the expression in the innear ear of downstream targets of BMP signalling, in particular, analyse the members of Id gene family.

4- Analyze the regulation of Id genes by BMP signalling in the inner ear.

5- Analyze the expression of genes involved in the process of terminal differentiation, in particular, Btg1 and Btg2 genes

6- Analyze the regulation of Btg1 and Btg2 gene by BMP signalling in the inner ear:

Chapter 2

BMP-SIGNALING REGULATES THE GENERATION OF HAIR-CELLS

Bmp4 and prosensory genes in the otic vesicle

Several *Bmp*s are expressed in the early otic placode and vesicle, and *Bmp4* has been shown to foreshadow the prospective sensory patches of the otic vesicle (Cole et al., 2000; Oh et al., 1996; Wu and Oh, 1996). Our first aim was to study the correspondence between the pattern of expression of *Bmp4* and the onset of specification of hair-cells. For this purpose we carried out experiments by *in situ* hybridization of chick embryos from E2 to E4, where the BMP4 was analyzed along with the expression of prosensory genes.

The bHLH protein *Atoh1* and is necessary and sufficient for hair-cell differentiation in vertebrates and it will be used here as the earliest readout of hair-cell fate (Bertrand et al., 2002; Woods et al., 2004). *Atoh1* was not detected until E4 (HH23-25) when it was distinctly expressed in two patches that were located one anterior and the other posterior, dorsal to the equator of the otocyst (Fig. 1Aa,b). They correspond to the nascent anterior and posterior *cristae*, which are the earliest vestibular patches to develop (Cole et al., 2000; Wu and Oh, 1996). *Atoh1* transcripts occurred in single cells that were spaced, reflecting the typical arrangement of hair-cells that results from lateral inhibition (inset of Fig.1Ab). As shown in Figure 1Ac-d, *Bmp4* anticipated the expression of *Atoh1*, and it was expressed in the E3 otic vesicle (HH20) at two distinct anterior and posterior patches (Fig. 1Ac) to then co-localize with *Atoh1* by E4 (HH24) (Fig. 1, compare Ab with Ad). The expression of *Bmp4* showed sharp boundaries and extended throughout most cells of the domain (Fig. 1Ae).

Bmp4 and *Lunatic fringe* (*Lfng*) are expressed during the development of prosensory patches (Cole et al., 2000; Sanchez-Calderon et al., 2004). One example of the expression of *Bmp4* and *Lfng* in E4 otocysts is illustrated in Figure 1Ba and 1Bb, and compared with *Fgf10* in 1Bc. The expression of *Bmp4* and *Lfng* at the posterior *crista* (pc) and *macula utriculi* (mu) is indicated in all three examples. As shown in Figure 1Bc, *Fgf10* was also expressed at these territories, and all three genes showed similar expression patterns. A detailed study of the *Fgf10* expression will be presented elsewhere, the purpose here being merely to support the use of *Lfng* and *Fgf10* to asses the regional specification of the sensory domain with independence of *Bmp4* expression (see below).

BMP4 inhibits hair-cell output in the otic vesicle

Otic neurons and sensory hair-cells are born sequentially, but within a domain that is in both cases positive to *Lfng* and *Fgf10*, hair-cells being anticipated by *Bmp4* expression. This poses the question of what is the role of BMP4 in the specification of sensory fate. We analyzed this problem by studying the effects of exogenous BMP4 and inhibition of BMP-signalling on *Atoh1* expression -as the earliest readout of hair-cell development-, using isolated otic vesicles grown

in culture for 18h. Note that these experiments mimic an ectopic gain of function of BMP4 in the whole otic vesicle and the inhibition of endogenous BMP activity.

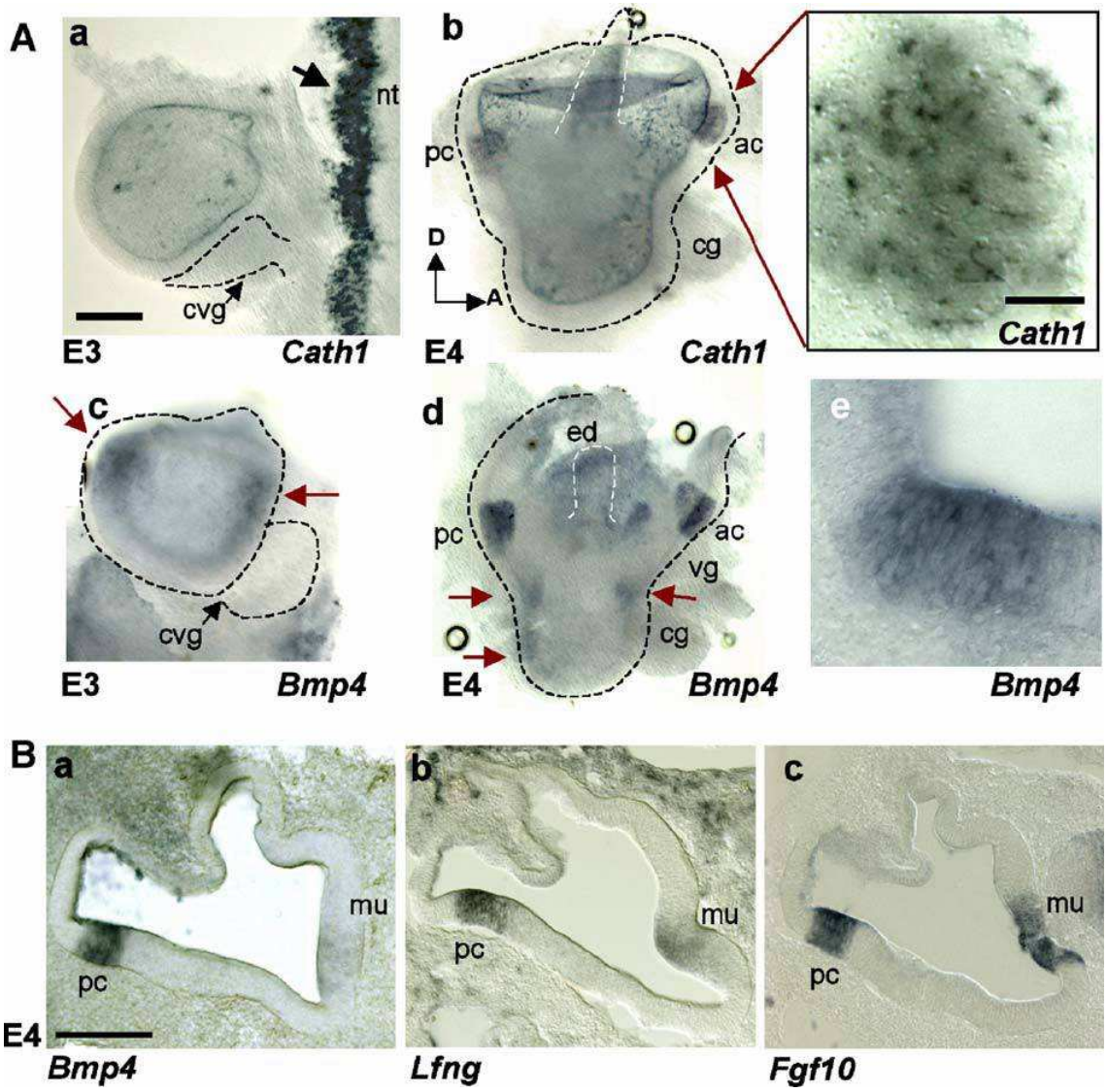

Figure 1. Expression profile of Bmp4, Cath1 and prosensory genes.
A, Whole-mount *in situ* hybridization for *Bmp4* and *Cath1* on otic vesicles at E3 and E4. All vesicles are oriented dorsal up and anterior right except in (a) where the otic vesicle was rotated 45º clockwise with respect to bars. (a) Note *Cath1* expression in the dorsal aspect of the neural tube (black arrow) but not in the otic vesicle in E3 (HH19). (b) *Cath1* expression in the anterior and posterior *cristae* (ac, pc) of an E4 (HH24) otocyst. The inset shows the anterior *crista* at higher magnification. Note the salt and pepper pattern of expression of *Cath1* on a flat view of the epithelium. (c) *Bmp4* expression in E3 (HH19) otic vesicle showing two early patches of expression (red arrows). (d) *Bmp4* expression in an E4 otocyst, showing labeling in the anterior and posterior *cristae*. Note also the nascent ventral expression domains that correspond to the *maculae* and *papila basilaris* (red arrows). (e) Higher magnification of the posterior *crista* showing that *Bmp4* was ubiquitously expressed within the sensory patch. **B**, Expression of *Bmp4*, *Lunatic fringe* (*Lfng*) and *Fgf10* genes in E4 otocysts. Para-saggital sections of E4 otocysts bisecting the vestibular cavity, illustrating the expression of *Bmp4* (a), *Lfng* (b), and *Fgf10* expression (c) at the posterior *crista* (pc) and the *macula utriculi* (mu). Note that the expression domains of all three genes are similar. ov, otic vesicle; cvg, cochleo-vestibular ganglion; cg, cochlear; vg, vestibular; ac, anterior *crista*; pc, posterior *crista*; mu, *macula utriculi*; bp, *basilar papilla*; ed, endolymphatic duct; nt, neural tube; A, anterior; D, dorsal. Scale bars in A panel: 200µm, and 20µm for insets. Scale bar in B panel:100 µm.

Figure 2a-c shows *Atoh1* expression in the anterior *crista* in E4 (HH22) otic explants grown in control (a), in the presence of exogenous BMP4 (b) or Noggin (c). The frame, and hence the surface area was identical for all three photographs. Otic vesicles in control conditions (a) developed *Atoh1*-positive patches that were very similar to those developed *in vivo* (compare (a) with Fig. 1Ab). Otic vesicles incubated with BMP4 did also develop sensory patches, but those patches were smaller than in control. Note that patches although smaller, still expressed *Atoh1* with a salt-and-pepper pattern (Fig. 2b, e). The incubation with Noggin, on the contrary, produced very large *Atoh1*-positive patches (Fig. 2c, f). The bar diagrams of Figure 2d compares the size of *Atoh1*-expressing domains in control, BMP4 and Noggin. The increased size of *Atoh1*-positive patches occurred at a constant cell density indicating an increase in the number of *Atoh1*-expressing cells. The photomicrographs in Figure 2e-f are from optical sections after flat mounting the explants, and show that the spacing of *Atoh1*-positive cells within the plane of the epithelium was similar in both conditions. This suggests that neither BMP4 nor Noggin interfered with the cellular or molecular mechanisms that generate the singling out and spacing of hair-cells.

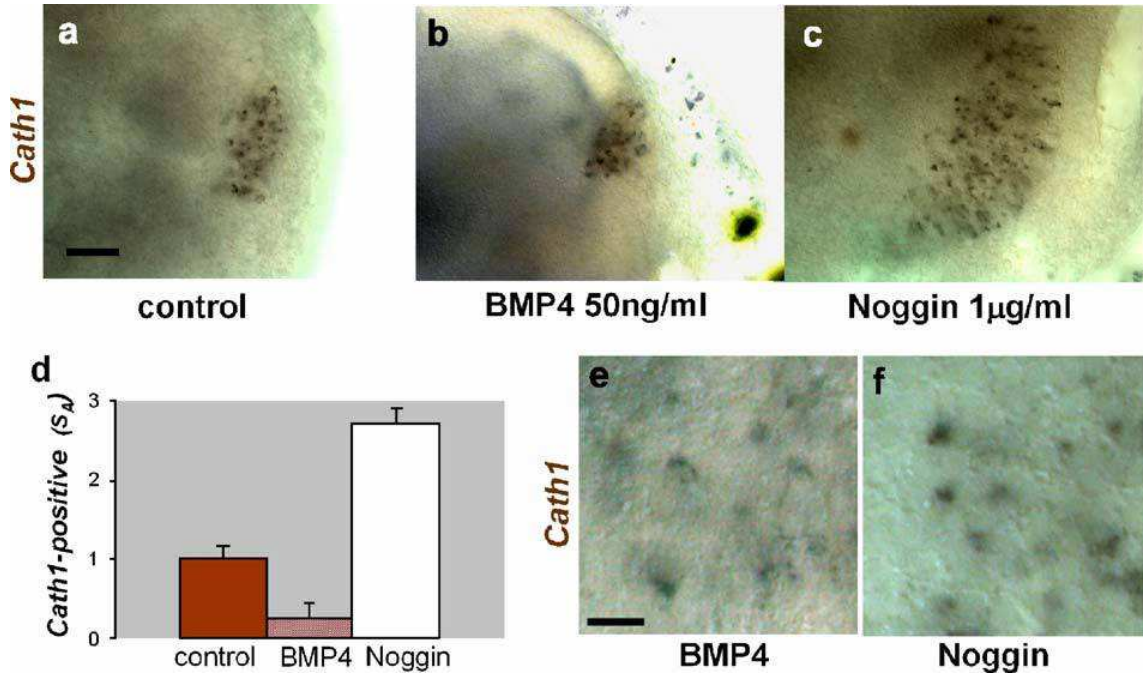

Figure 2. BMP4 reduced sensory hair-cell output.
BMP4 and Noggin modified the number of *Cath1*-expressing cells. E4 (HH22) otic vesicles were isolated and incubated for 18 hours in control (a), 50 ng/ml BMP4 (b), and 1µg/ml Noggin (c), and processed for *Cath1 in situ* hybridization. Photomicrographs correspond to identical magnifications of the anterior sensory patch as identified by *Cath1*-expression. Note the reduction of the size of the patch with BMP4 and its enlargement with Noggin. Photomicrographs in (e) and (f) show a detail of two representative *Cath1*-positive patches from otic vesicles incubated with BMP4 and Noggin, respectively. The size of the patches was measured planimetrically and plotted in (d). Values are control : BMP4 : Noggin = 1±0.2 (n=9) : 0.2±0.2 (n=10) : 2.7±0.2 (n=9). Difference BMP4 against control p≤ 0.08, and Noggin against control p≤ 0.01. Scale bar: 50µm in (a), 10 µm in (e) and (f).

We further studied the reversibility of the effects of BMP4 by incubating otic vesicles first with BMP4 and then returning to control medium. Under these conditions, BMP4 reduction in *Atoh1*-positive cells persisted after returning to control medium, suggesting that the effect of BMP4 was irreversible (size of the patches normalized to control were: control =1.00±0.25; BMP4 = 0.04±0.36; n=5). Summarizing, these results indicate, first, that BMP4 decreases the number of hair-cells. Secondly, that there is an endogenous BMP activity that negatively regulates the number of *Atoh1*-positive cells. Thirdly, that the effect of BMP4 occurs probably at early stages of hair-cell generation. And finally, that BMP4 acts in an irreversible manner (see below cell death).

The experiments that follow studied the effects of BMP4 on *Lfng*, *Fgf10* and *Bmp4,* genes that mark early the otic prosensory domain (Fig. 3). Cultured E4 (HH22) otic vesicles expressed *Lfng* and *Fgf10* at the anterior and posterior *cristae*, and at the ventral domain that anticipates *macula* and *papila basilaris* (Fig. 3a, d). When incubated with BMP4, the expression of *Lfng* was also present at the anterior and posterior *cristae* (see red arrows), however the *cristae*, were much smaller with BMP4 than in control (Fig. 3b, n=9/11 otic vesicles). On the contrary, incubation with Noggin expanded *Lfng* at the *cristae* (see red arrows in Fig. 3c, n=9/9). Similarly, the expression of *Fgf10* at the *cristae* (Fig. 3d-f red arrows) was either very small or suppressed by BMP4 (Fig. 3e), and the size of the *cristae* increased dramatically with Noggin (Fig. 3f, red arrows, n=10/10 for BMP4 and 6/6 for Noggin). Experiments done on E3 otic vesicles gave similar effects, and *cristae* did not develop at all with BMP4 (not shown). The ventral expression domain of *Lfng* and *Fgf10* persisted after BMP4 (Fig. 3e) and also in E3 otic vesicles. Recall that at these stages (E3-E4, HH19-22), *Lfng* and *Fgf10* are also expressed within the neurogenic domain (Cole et al., 2001; Alsina et al., 2004). On the other hand, *Bmp4* is strongly expressed at the *cristae*, its expression being delayed at the ventral domain (Fig. 1 and Cole et al., 2001). Since there was no evidence for ectopic sensory patches after BMP4 or Noggin, the indication is that neither the gain nor the loss-of fuction of the BMP-pathway resulted in re-specification of otic epithelium at the stages under study.

We examined also the effects of BMP4 on *Bmp4* expression and, surprisingly, the observation was that BMP4 reduced *Bmp4* endogenous expression (Fig. 3g-i). Control otic vesicles expressed *Bmp4* at the two anterior and posterior patches, as well as at other dorsal domains related to the endolymphatic sac (black arrow in Fig. 3g-i). Incubation with BMP4 dramatically suppressed *Bmp4* expression in otic vesicles (Fig. 3h). The expression of *Bmp4* in the presence of Noggin, however, was intense and distinct at the two patches that correspond to the anterior and posterior *cristae* (Fig. 3i, red arrows). Note that *Bmp4* was also induced at the endolymphatic duct (ed) by Noggin (black arrow in Fig. 3i). Taken together, these results indicate that the regulation of BMP4 activity modulates the size of the prosensory domain, the expression of *Bmp4* being negatively regulated by its own activity (see discussion).

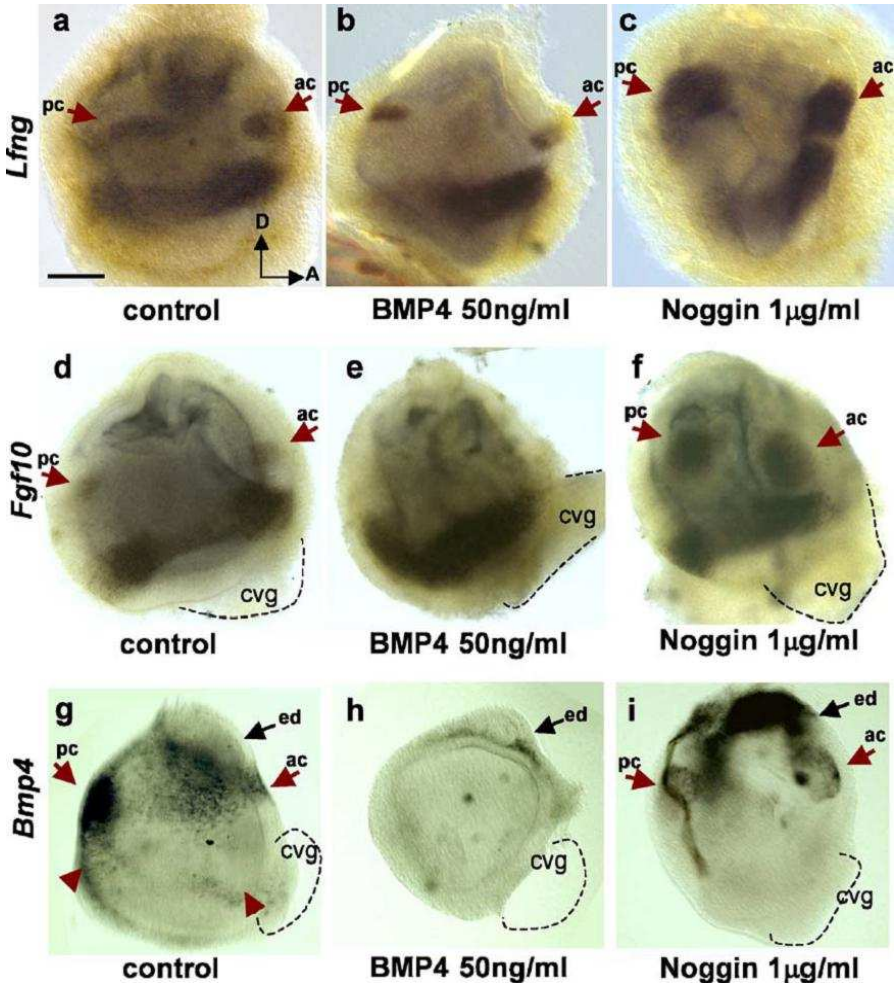

Figure 3. Effects of BMP4 and noggin on prosensory genes Lfng, Fgf10 and Bmp4. (a-c), Expression of *Lfng* after addition of BMP4 or Noggin in E4 (HH22) otic vesicles. (a) *Lfng* expression in control medium showing the two *cristae* (red arrows) and the ventral expression domain (*maculae* and *papila basilaris*, see Cole et al., 2000). (b) Incubation with BMP4 (50 ng/ml) reduced the size of the *cristae*. (c) Noggin (1µg/ml) induced the expansion of *Lfng* positive patches. (d-f) A similar experiment probed for *Fgf10*. (d) In control, the anterior and posterior *cristae* expressing *Fgf10* just appeared to be visible (red arrows). (e) BMP4 maintained an intense expression of *Fgf10* at the ventral domain, but was weaker or absent at the *cristae*. (f) On the contrary, Noggin exaggerated *Fgf10* expression at the *cristae* (red arrows). (g-i) BMP4 reduced *Bmp4* expression in the otic vesicle. (g) Control otic vesicles showed two characteristic domains of *Bmp4* expression (red arrows). Note also that weaker expression was detected at more ventral aspects corresponding to the initiation of *maculae* (anterior arrowhead) and *papila basilaris* (posterior arrowhead). (h) BMP4 resulted in a complete suppression of *Bmp4* expression in the otic vesicle and (i) the opposite result was found with Noggin (red arrows). Note also the expression at the endolymphatic duct (ed, black arrow) (Cole et al., 2000). A: anterior, D: dorsal, cvg, cochleo-vestibular ganglion; ac, anterior cristae; pc, posterior cristae; ed, endolymphatic duct/sac. Scale bar: 100 µm.

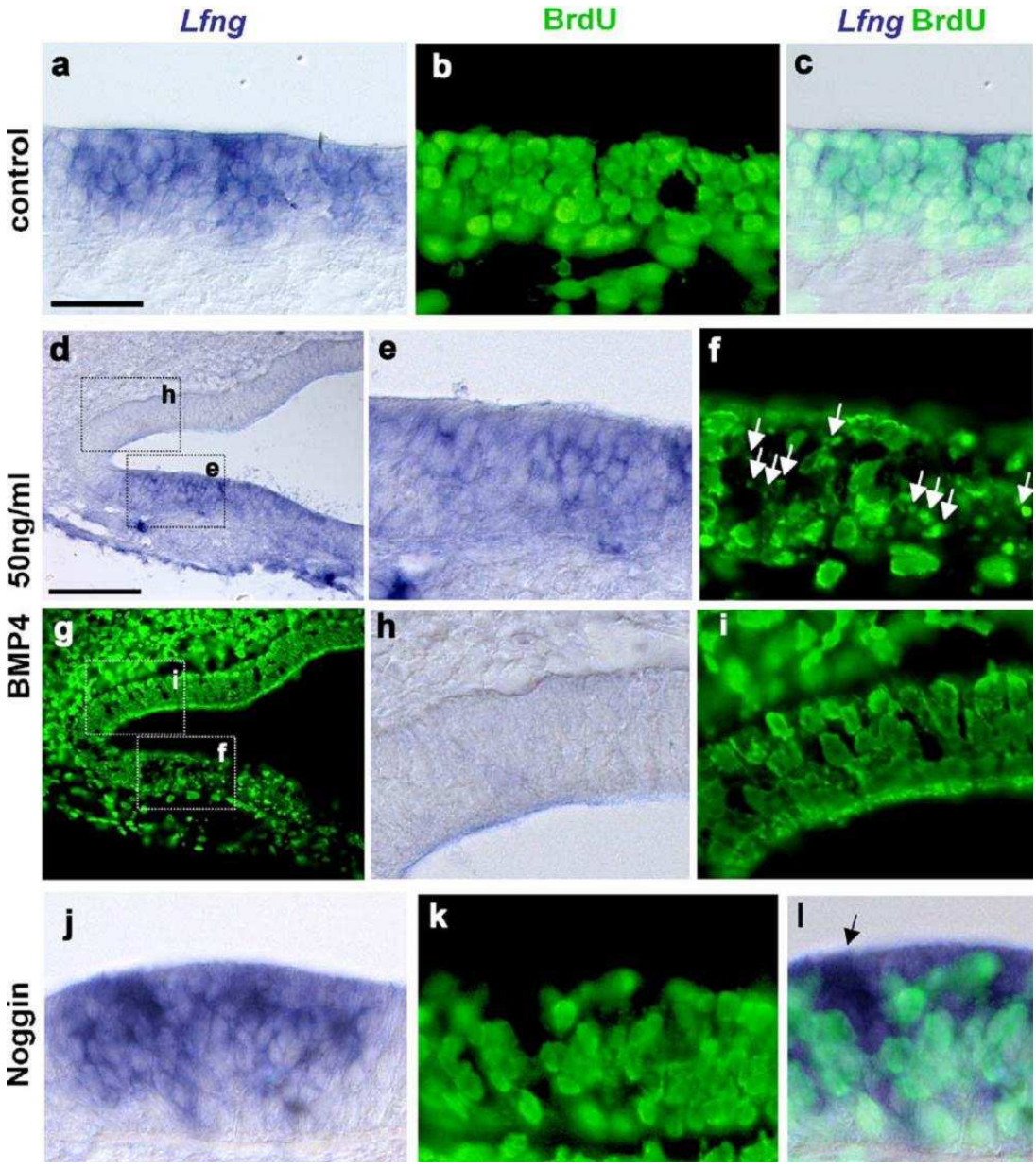

Figure 4. BMP4 reduced cell proliferation within the prosensory patches.
HH22 otic vesicles were grown in control culture medium, with BMP4 (50 ng/ml) or Noggin (1µg/ml) for a period of 18 hours and exposed throughout the culture to BrdU. Prosensory patches were identified by the expression of *Lfng*. (a) Section of a control otic vesicle expressing *Lfng* at the prosensory patch and (b) the corresponding section showing the BrdU labeling. (c) Overlay image. Most *Lfng* cells have incorporated BrdU in culture. (d-i) *Lfng* expression and BrdU-labeling after BMP4 treatment. (e) High magnification of the *Lfng* prosensory epithelium. (f) Disperse BrdU-labeled cells were detected in the corresponding prosensory patch. Note that some of the BrdU labeled cells show fragmentation of the nuclei (white arrows). (h) Magnification of a non-sensory epithelium. (i) In the non-sensory epithelium, as in control, there was high density of BrdU-positive cells. (j-l) *Lfng* expression and BrdU-incorporation after incubation with Noggin. (k) High density of BrdU-labeled cells. (l) Most *Lfng* cells show BrdU-incorporation in the overlay, but with a stripe of silence. Scale bar in (a) corresponds to 25 µm and holds for (a-c), (e-f, h-i), and (j-l). Scale bar in (d) and (g) is 50 µm.

BMP4 reduced the number of progenitors within the prosensory domains

The reduction in the number of *Atoh1*-positive cells along with that of the size of the prosensory patches induced by BMP4, and the converse effect of Noggin, could be related in principle to the rate or the number of dividing sensory progenitors. This possibility was investigated by examining cell proliferation with bromo-deoxi-uridine (BrdU) incorporation within the prosesory domain. Figure 4 illustrates the effects of BMP4 and Noggin upon continuous 18-hour-exposure to BrdU in HH22 otic vesicles. This allowed us to analyze DNA-synthesis and assess the spatial distribution of proliferating cells within the prosensory domain as labeled with *Lfng*. In control otic vesicles, the proliferative activity was intense along the otic epithelium as it was within the prosensory domain (Fig. 4a-c). Figure 4b-c shows also that in control conditions *Lfng*-positive cells incorporated BrdU, indicating that they were actively proliferating throughout the incubation period. Incubation with BMP4 (50ng/ml) induced a strong and restricted loss of BrdU incorporation into nuclei within the prosensory patches labeled by *Lfng*, (Fig. 4d-i). Density of BrdU-positive nuclei measured as percent of labeled nuclei was: control = 58±8.1 (n=4); BMP4 = 34±4.9 (n=3), $p<0.02$; Noggin = 45±7.1 (n=3) $p<0.16$). Note that neighboring domains that were negative to *Lfng* showed intense proliferative activity (Fig. 4h-i, compare f and i), indicating that the effect of BMP4 was selective to prosensory domains. After BMP4 addition, prosensory patches contained condensed nuclei and nuclear debris that where also positive for BrdU (Fig. 4f), indicating that the loss of proliferative activity was related to apoptotic death of cellular progenitors. Noggin, on the other hand, did not change the overall proliferative activity within sensory patches (Fig. 4j-l). However, upon Noggin treatment, groups of cells within *Lfng*-positive patches seemed to be silenced and to arrest DNA replication (Fig. 4k-l), suggesting that BMP-inhibition would favor cell-cycle withdrawal at particular spots (see below). In summary, BMP4 reduced cell proliferation within prosensory patches.

Effects of BMP signaling on hair-cell production and hair-cell specification

Given that BMP4 reduced the number of *Atoh1*-positive cells along with the loss of active progenitors within prosensory domains, we wanted to further analyze these effects and to test whether the expansion of *Atoh1* after BMP-inhibition was a result of an increased output of progenitors, a favoured cell specification, or both. Otic vesicles were exposed to continuous BrdU-labeling and analyzed for BrdU-incorporation and *Atoh1* expression. Otic vesicles (HH22) were incubated with BrdU and BMP4 or Noggin for periods of 6 hours (Fig. 5 a-d, g-h) or 18 hours (Fig. 5e, f, and i-l). Incubation with BMP4 during 6h resulted in small patches with few *Atoh1*-positive cells accompanied by condensed nuclei and apoptotic bodies (Fig. 5c,d, see arrowhead and Torchinsky et al., 1999). In the presence of Noggin patches were large with rounded BrdU-positive nuclei indicating actively proliferating cells intermingled with cells expressing *Atoh1* (Fig. 5a, b). Interestingly, after the 6h incubation with Noggin we were able to find cells that were positive to *Atoh1* and to BrdU (see Fig. 5g, h), suggesting that either *Atoh1*-positive cells are able to proliferate, or that *Atoh1* is expressed after S-phase and about cell division. Comparison of the maximum diameter of

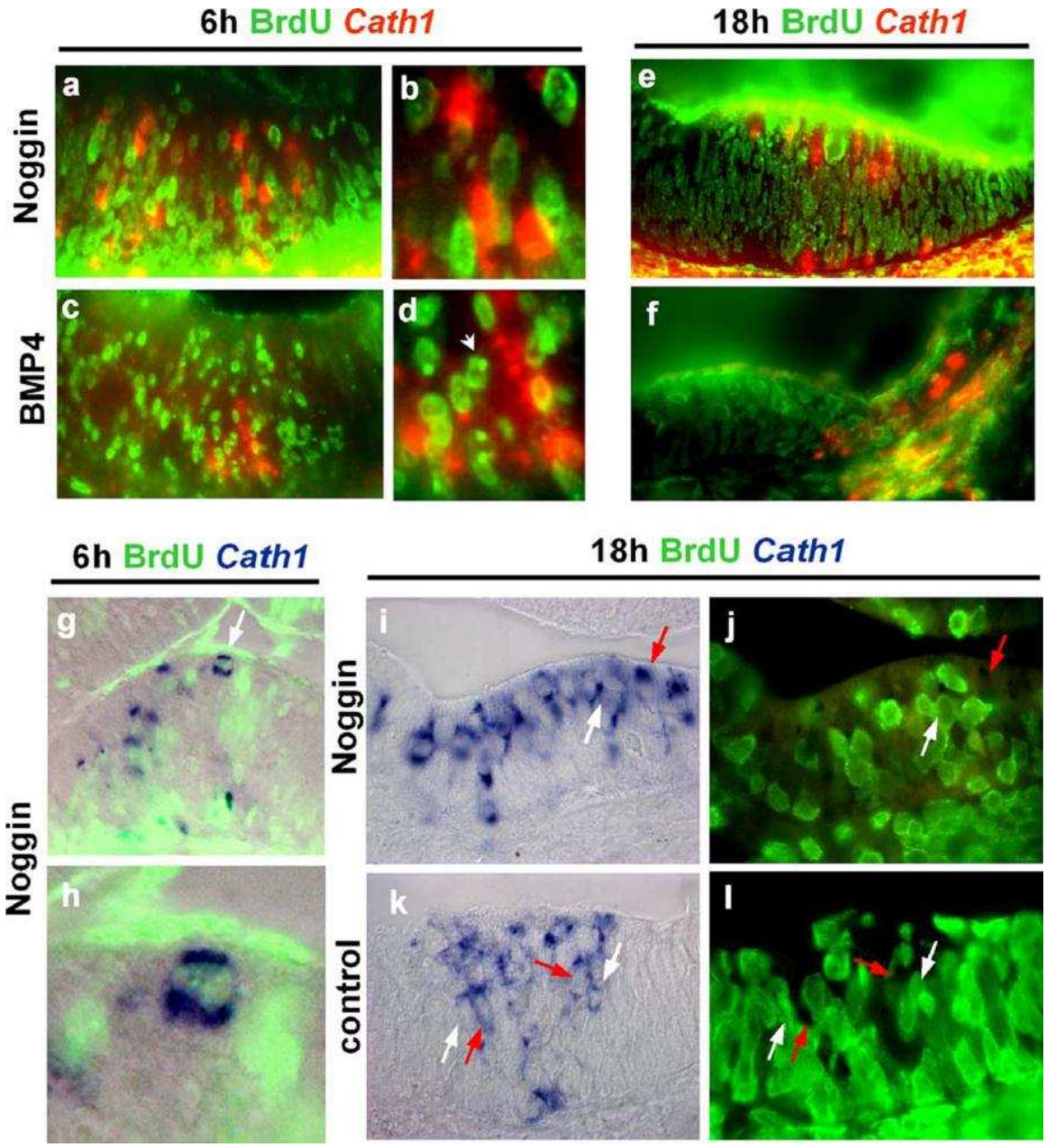

Figure 5. BMP4 and Noggin caused opposite effects on the determination of hair cell precursors.

Cell proliferation determination of hair cell precursor fate measured by BrdU incorporation and the expression of *Cath1* in HH22 otic vesicles. (a) A patch corresponding to an otic vesicle after 6 h with Noggin. Note the high number of *Cath1* positive cells (fluorescent red) in between BrdU-positive cells (green). (b) Higher magnification image of (a). (c) *Cath1*-positive patch after 6 h with BMP4. (d) At high magnification condensed BrdU-.positive nuclei and apoptotic bodies were apparent (white arrowhead). (e,f) Photomicrographs of patches from otic vesicles incubated with BMP4 or Noggin for 18 hours. (g) Same experiment than in (a) but *Cath1*-expression was detected with purple chromogenic substrate to better identify co-localization of in situ hybridization reaction and BrdU-incorporation. (h) Higher magnification of the cell labeled with an arrow in (g). Note the expression of *Cath1* and BrdU nuclear staining. (i, j) Section of a sensory patch with *Cath1* expressing cells after 18 h of culture with Noggin (i), and the corresponding BrdU-incorporation image in (j). (k, l) Similar but for a patch corresponding to a control otic vesicle. White arrows point to a *Cath1*- and BrdU-positive and red arrow points to a *Cath1*-expressing cells that did not incorporate BrdU during the culture.

Atoh1-positive patches after 6 hours (see methods) gave an increase of about 60% for Noggin above control levels, (control = 40.2±10; Noggin = 70.2±8; (µm), n=7; p<0.01) Similarly, although not statistically significant, BMP4 tended to reduce the size of the patches within the 6-hour period (BMP4 = 20.9±6; (µm), n=5; p<0.28) This suggests that, at least in part, the effects of BMP and BMP-inhibition on hair-cell production was very rapid, and did not require completion of cell-division (<6h).

This notion was supported further by the following experiment in which we compared the percentage of *Atoh1*-positive cells that incorporated BrdU in 18h-incubations in control and with Noggin (Fig. 5 i-l). This should give an estimate of the fraction of hair-cells that were born in culture (both *Atoh1* and BrdU-positive) and of those that have been specified in the explant without cell division (*Atoh1*-positive, BrdU-negative). After 18h in culture with BMP4, it was difficult to find *Atoh1*-positive cells in sections of isolated otic vesicles (Fig. 5f), picnotic nuclei and epithelial disorganization being similar to that shown above. Noggin-treated vesicles, on the other hand, showed the typical array of ordered *Atoh1*-positive cells and cell proliferation activity within the epithelium (Fig. 5e). Double staining for *Atoh1* and BrdU-incorporation was clearer when *Atoh1* was visualized with a purple precipitate and BrdU with immunofluorescence than with double fluorescence (Fig. 5i-l). In control medium, 35%±5 of the cells were positive both for *Atoh1* and BrdU (n=6 sections from 3 patches), indicating that only one third of the cells was actually born in culture. With Noggin, this figure was slightly lower, but not significantly different, 21±13% (n=6 sections from 3 patches) (Fig. 5i-l), suggesting that at least in part the increase in *Atoh1*-positive cells after Noggin must arise from cell-specification, and not from newly nascent precursors.

BMP and cell death

The reduction of cell proliferation induced by BMP4 could be related to the impaired survival of progenitors. To explore this possibility we analyzed cell death using the TUNEL technique, after BMP4 and Noggin-incubation. Culture experiments were also performed in presence of BrdU. A typical experiment is shown in Figure 6a-d that illustrates TUNEL positive cells in control (a), and in the presence of 20 and 50 ng/ml BMP4 (b and c) or 1 µg/ml Noggin (d). A small but detectable apoptotic cell death occurred in control otic vesicles (a), as it does in normally developing otic vesicles (Leon et al., 2004). The incubation with BMP4 in the absence of serum (b and c) induced an increase in cell death at the equator of the otic vesicle, particularly strong at two domains located anterior and posterior (arrows in c). Noggin reduced cell death in cultured otic vesicles (d). The effects of BMP4 on cell death were prominent within the sensory domains, as shown in Figure 6e-g. Serial sections of BMP4-treated E3.5 (HH22) otic vesicles were assayed for either *Lfng* or TUNEL. As shown with arrowheads in Figure 6e-g, the *Lfng*-positive domains (Fig. 6e) correlated with cell death domains (Fig. 6f), and along with the decreased BrdU incorporation (Fig. 6g). BMP4-induced apoptosis required cells to enter the cell-cycle, as revealed by studying the density of TUNEL-positive cells induced by BMP4 in the presence of 10mM hydroxy-urea (HU), a compound that reduces DNA-synthesis by inhibiting the ribonucleoside diphosphate reductase. BMP4-

induced apoptosis was reduced by 10mM HU (BMP4 = 1.0±0.23 n=10, BMP4+HU = 0.21±0.12 n=8, p<0.01; control values were 0.06±0.10 n=8). These results reinforce the idea that the deleterious effects of BMP4 occurred on actively proliferating cells.

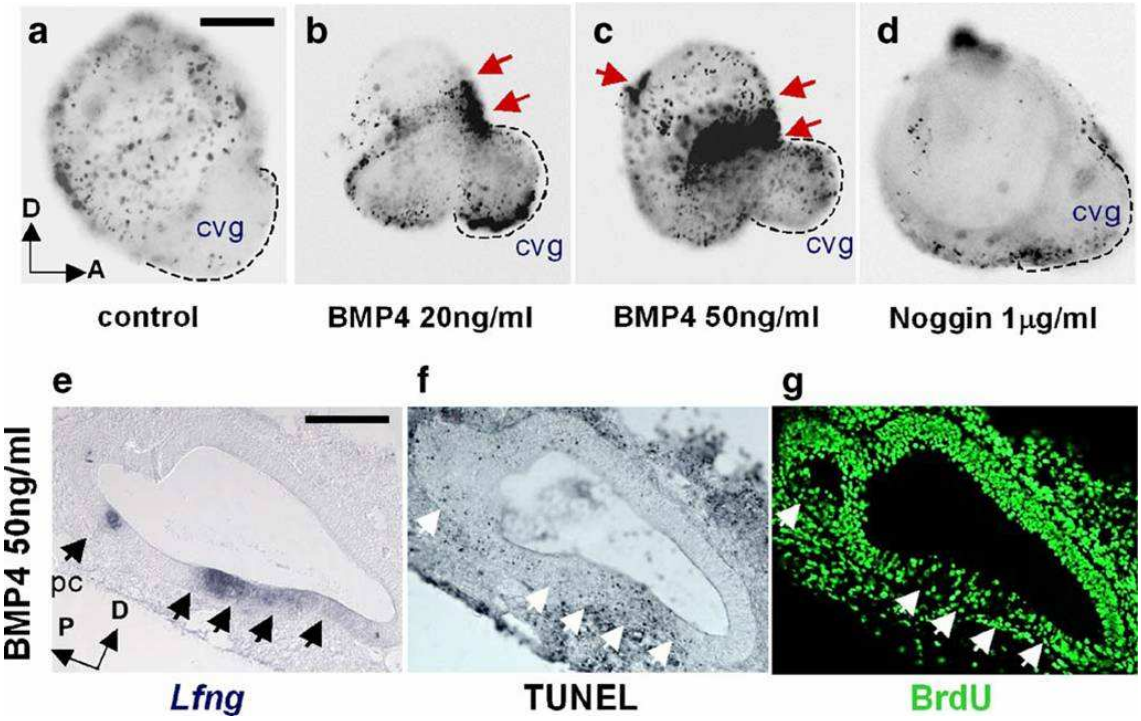

Figure 6. BMP4 induced cell death in the prosensory domain of the otic vesicle. HH19 otic vesicles were isolated and incubated for 18 hours in control (a), 20 ng/ml BMP4 (b), 50 ng/ml BMP4 (c), and 1μg/ml Noggin (d), and processed for TUNEL assay. Arrows in (b) and (c) indicate areas of high density of TUNEL labeled cells after BMP4 treatment. Alternate serial sections of E3.5 otic vesicles after 18 hours of culture with BMP4 and BrdU were processed for either *Lfng* expression or TUNEL. (e) Para-sagital section of an otic vesicle incubated with BMP4 showing two patches of *Lfng* expression (pc and *papila basilaris* arrowheads). (f) The corresponding alternate serial section processed for TUNEL. Note positive cells within the *Lfng* expressing domains (arrowheads). All tissue surrounding the epithelium has been hided to enhance the epithelial distribution of the TUNEL signal (g) BrdU incorporation of the same section as shown for TUNEL. Note that BrdU incorporation was reduced at the TUNEL positive domains (arrowheads). A, anterior, D, dorsal, P, posterior; cvg, cochleo-vestibular ganglion, pc, posterior *cristae*. Scale bar=200μm

BMP4 induces Msx1 but not Msx2 in the sensory cristae

There is no direct evidence for a link between the BMP-signaling cascade and cell death. However, it has been postulated that Msx genes may mediate different aspects of BMP signaling during development (see Discussion). Figure 7 shows experiments where the expression of Msx1 and Msx2 was explored after a 6-hour incubation of otic vesicles with BMP4 or Noggin. Msx1 was expressed in the vestibular cristae (arrows) and in the endolymphatic duct (ed) in control vesicles (Fig. 7a). Its expression was restricted to the otic epithelium (Fig. 7d-f). Msx1 was induced by BMP4 at the presumptive cristae (Fig. 7b upper arrows, and Fig. 7e) and the basilar papilla (Fig. 7b) and it was

suppressed by Noggin (Fig. 7c, f). Expression of Msx2, however, was fainter than that of Msx1 in control medium (Fig. 7g) and there were no signs of increased expression after BMP4 (Fig. 7h). An effect of BMP4 could be detected however in the mesenchymal tissue attached to the dorsal aspect of the otocyst (Fig. 7h), and Noggin, like with Msx1, was able to suppress Msx2 expression. These experiments show that Msx1 but not Msx2 correlate with the activity of BMP-signaling in the otic vesicle.

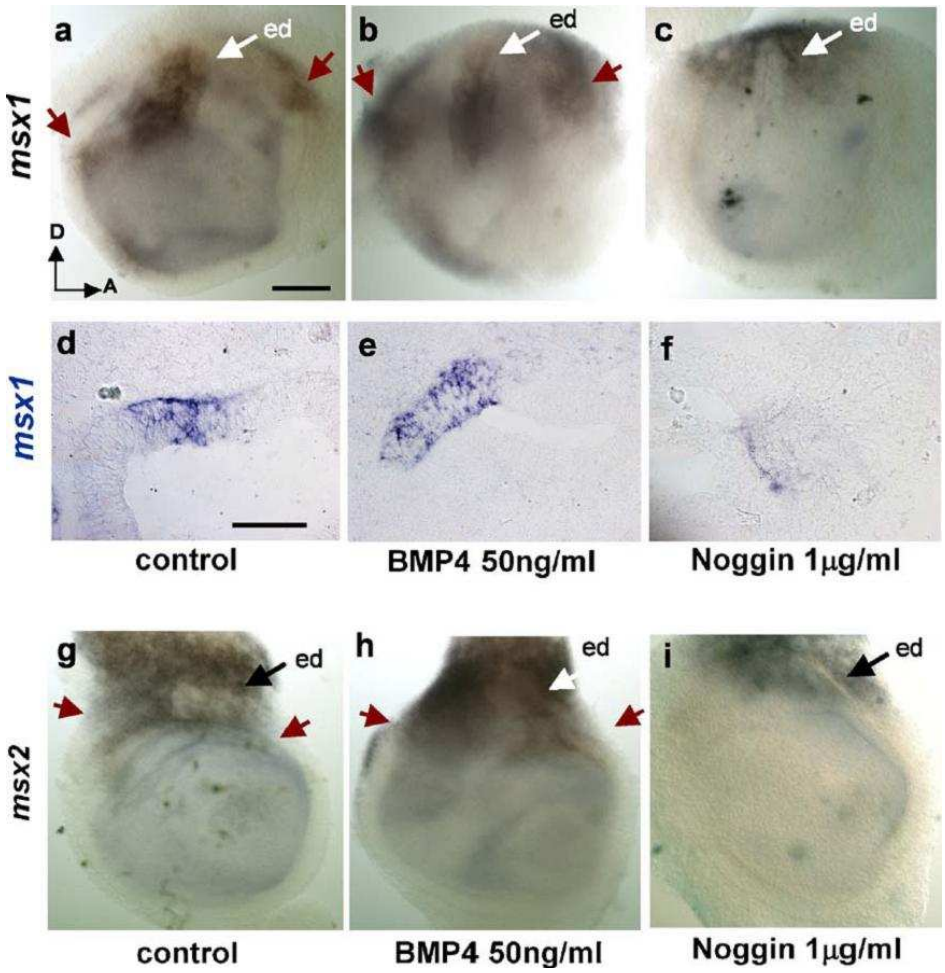

Figure 7. Effects of BMP4 and Noggin on Msx1 and Msx2
(a-c) E3.5 otic vesicles were isolated and grown in culture for 6 h with control medium (a), or with addition of 50 ng/ml BMP4 (b) or 1µg/ml Noggin (c) and assayed for *Msx1* expression. (a) Low levels of *Msx1* expression were detected in the two *cristae* (red arrows) and the endolymphatic duct (ed). (b) Otic vesicle with increased expression of *Msx1* at the *cristae* and the endolymphatic duct (ed) after incubation with BMP4. (c) Noggin abolished *Msx1* expression at *cristae* and endolymphatic duct. (d-f) Representative sections of otic vesicles showing that in all conditions *Msx1* expression was restricted to the otic epithelium. (g-i) Experiment probed for *Msx2.* (g) In control conditions, *Msx2* was faintly expressed at the *cristae* (red arrows). (h) *Msx2* expression was not substantially increased by BMP4 (red arrows), (i) Noggin reduced *Msx2* expression in all domains. cvg, cochleo-vestibular ganglion; ed, endolymphatic duct/sac. Scale bar=100 µm (a-c, and g-i), and 50 µm (d-f).

Chapter 3

BMP-SIGNALING REGULATES ID1-3 GENE EXPRESION IN THE DEVELOPING INNER EAR

We have shown that BMP4 regulates the generation of hair cells by a mechanism that includes the regulation of cell fate acquisition, cell proliferation and the survival of sensory progenitors. In order to further analyse the molecular mechanisms by which BMP4 exerts its effects, we sought to explore possible targets of the BMP pathway. The Id family of genes has been identified as direct transcriptional targets of BMP4, and they are known to regulate many developmental processes including the transition between self-renewal and cell differentiation (see Introduction). The experiments that follow show the expression pattern of Id1-4 genes throughout ear development and their regulation by BMP signalling.

First, we analyzed by ISH the expression of Id1-3 genes in the otic region during the stages of sensory organ formation, between embryonic day3 (E3, HH18-20) and day 7 (E7, HH29-30). Secondly, we analysed the possible regulatory link between BMP4 and Id gene expression in the inner ear. For that purpose, we used two different approaches to measure Id expression after the modulation of the BMP signalling pathway both *in vivo* and in cultured otocysts.

Id genes are expressed in prosensory regions of the otic epithelium

The experiments that follow describe first the expression pattern of the Id1-4 genes during the period of sensory patch formation, at embryonic day 3, and then that occurring during the differentiation of the sensory organs, E5 and E7. The former period can be characterised by the expression of Bmp4 in the prosensory regions, but not yet that of Atoh1, whereas the latter is characterised by the onset and establishment of Atoh1 expression in the sensory patches.

Figure 3-1 shows the expression of all four Id genes in HH19 embryos. It also shows that of Bmp4 and Atoh1 for comparison. As mentioned before, at this stage Bmp4 begins to be expressed as two distinct patches in the otic epithelium at the equator of the otocyst (Fig. 3-1 A, A' A''). In parallel Id1, Id2, and Id3 genes were highly expressed in the periotic mesenchyme surrounding the regions of Bmp4 expression (Figs. 3-1 B-D, B'-D'). Expression of Id4 in those areas could not be observed in whole mount preparations (Fig. 3-1 E-E'). Cryosectioning of these embryos revealed that all four Id family members were expressed in the otic epithelium, including the prosensory patches, and in the non-sensory epithelium of the lateral wall of the otocyst (Fig. 3-1 B''-E''). However, the medio-ventral wall of the otocyst was consistently devoid of Id expression (see Fig. 3-1C'')

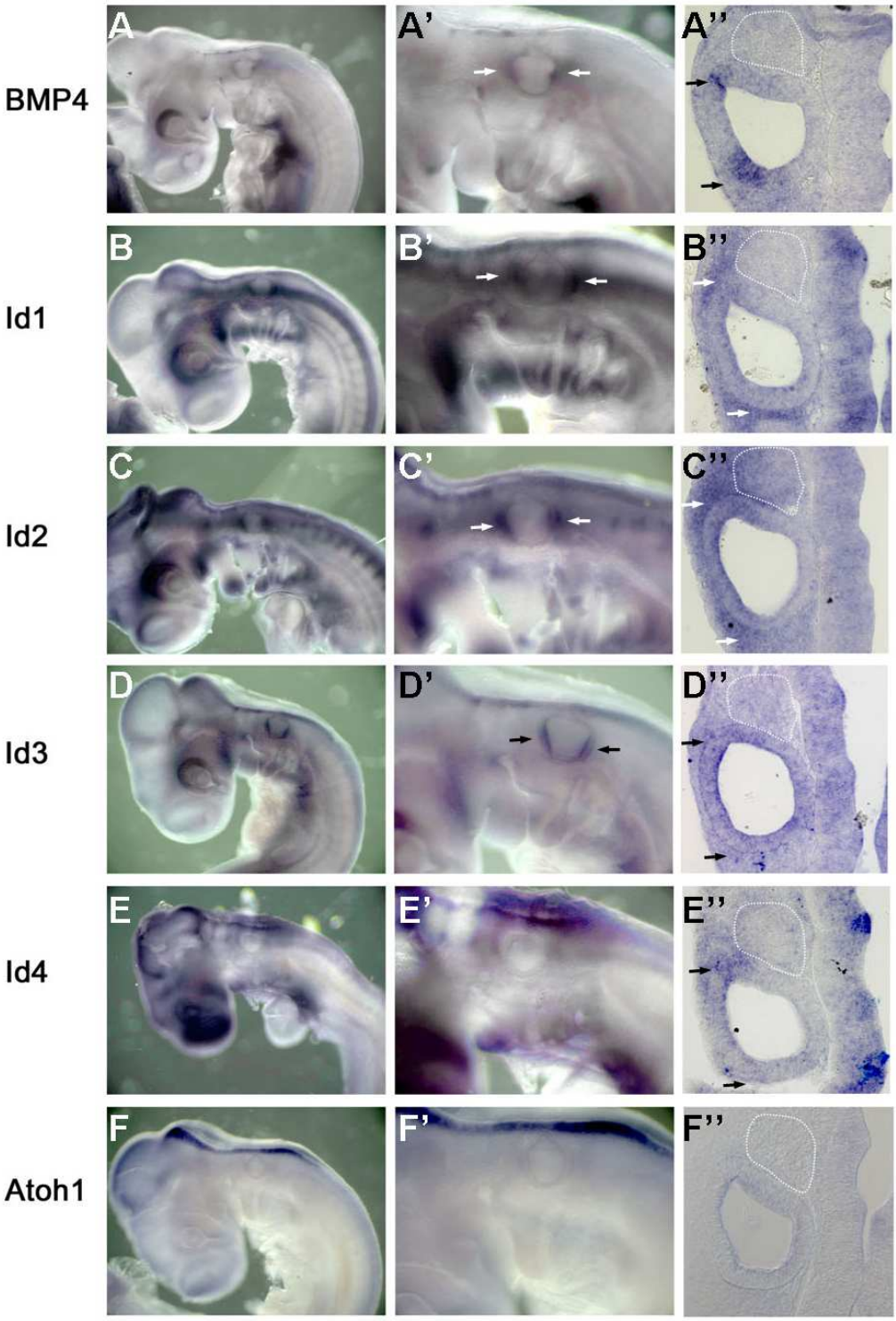

Figure 3-1. Expression of Id genes at E3. Embryos at embryonic day 3 (HH18-19) were processed for ISH detection of the genes indicated on the left. A-F: Lateral views of the cephalic region. A'-F': magnified view of the same embryos illustrating the expression in the otic region. A''-F'': 20 µm coronal sections showing the left otic vesicle and the statoachoustic ganglion (with lines).Anterior is to the top, medial to the right. Arrows indicate the sites stronger gene expression.

Despite the overlapping pattern of the different Ids, some differences in the intensity and/or the extent of expression among different genes were observed. Id1 and Id2 expression was remarkably high in the mesenchyme adjacent to the prosensory patches and surrounding the cochleo-vestibular ganglion (CVG). However, while Id2 was expressed in cells of the CVG, Id1 was completely excluded from it (Fig. 3-1 B'' and C''). Id3 was also detected in the periotic mesenchyme, although its expression was weak at HH19 (Fig. 3-1 D', D''). In some embryos it was necessary to perform an extra step of ISH development after sectioning in order to detect the Id3 mRNA in the otic epithelium (see methods). This may explain why it has been previously reported that Id3 was downregulated in the HH19 otocyst (Kee, Bronner-Fraser. 2001c), Id4 was also weakly expressed in the otic region at these stages (Fig. 3-1 E'), but extra development of cryostat sections revealed its expression in the anterior and posterior sensory patches, as well as in the lateral wall of the otocyst (Fig. 3-1 E''). As mentioned in chapter 2, Atoh1 expression was not detected at this stage (Fig. 3-1 F-F'').

From stage HH21 to 29, the size, anatomic location, and three dimensional characteristics of the otic vesicle preclude a simple analysis by whole mount ISH. For this reason, we performed ISH in cryostat sections, combining the serial reconstruction with the use of known molecular markers, either in the same or in adjacent sections. In this way we could precisely identify the anatomic structures where Ids were expressed in the inner ear.

Figure (3-2 A) shows a schematic diagram exemplifying the typical analysis carried out for the analysis of Id gene expression pattern during these stages. First, coronal sections covering the entire otocyst, in this case of an HH21 embryo, were probed with Id2 riboprobe (Fig. 3-2 A, left panel). After ISH, the sections were processed for double immunohistochemistry (on the same slides) using antibodies against Prox1 and TUJ1, and counterstained the nuclei with DAPI (Fig. 3-2 A, middle pannel). Those antibodies have been well characterized as markerks of prosensory patches (Prox1 and Tuj1), and nerve fibers and the CVG (Tuj1) (Stone, Shang, Tomarev. 2003) (Molea, Stone, Rubel. 1999). To better analyze and illustrate gene and protein expression patterns, we performed the serial reconstruction of the otic vesicles (Fig. 3-2 A, rigth).

The main features of Id2 expression at this stage are illustrated in the following figures (Fig. 3-2 B-G). Note that in this panel the ISH hybridization signal is shown in the red chanel, as the original image was first inverted and then pseudocoloured to better visualize it in comparison with the immunofluorescence signal for Prox1 (see materials and methods). Using this type of analysis, we found that that Id2 expression was localized to the periotic mesenchyme in the dorsal regions of the otocyst (Fig. 3-2 B). A few ventral sections from that, Id2 expression was maintained in the periotic mesenchyme, and begun to be detected in the otic epithelium, concentrated in two discrete regions: an anterior domain corresponding to the anlagen of the anterior and lateral crista, and a broader posterior patch, that anticipates the posterior crista. This was confirmed by the co-labeling with the Prox1-antibody, and is illustrated in the merged image in (Fig. 3-2 C). The posterior domain was continuous from

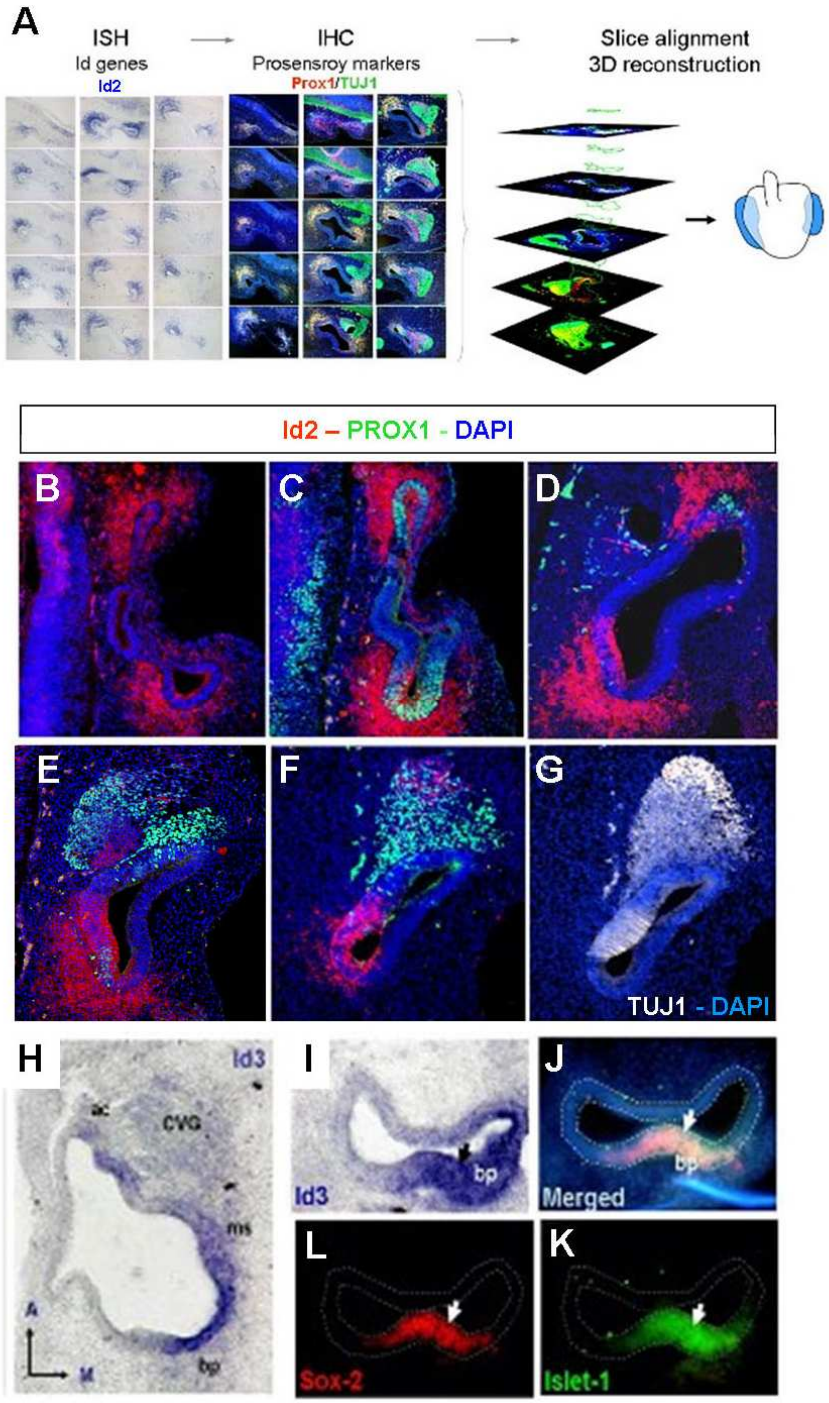

Figure 3-2. Analysis of gene expression of Id2-3 genes at E3-5.
A: Diagram of the procedure followed. Left panel, coronal sections through the entire otocyst were processed for ISH, in this case Id2 is shown. Middle panel: after ISH, double immunofluorescence was carried out in the same sections, utilizing known markers of sensory progenitors, in this case Prox1 and Tuj-1. Right panel: serial reconstruccion of the entire otocyst allowed precise identification of the sites of Id expression.
B-G: coronal sections from dorsal (B) to ventral (F), showing the right otocyst of an HH21 embryo, processed as described in A. Anterior is to the top, medial to the left. B-F: Merged images of Id2 expression (pseudocoloured in red), Prox1 (green) and DAPI staining of nuclei (Blue). G: the same section than F, showing Tuj-1 expression pseudo-coloured in white.
H: 20 µm coronal section of an HH22 embryo processed for Id3, showing the right otocyst approximately at the equatorial plane. Anterior is to the top, medial to the right. I-J: Coronal sections of the same embryo shown in H, at a more ventral plane. The image has been rotated, and anterior is to the left, and lateral to the top. I: Section processed for ISH with Id3 probe. J-K: the adjacent section to I was double immunolabled with anti-Islet1 (K) and anti-Sox2 (L) antibodies. J: Merged imaged of Islet-1, Sox2, and DAPI staining.

dorsal to ventral, and extended more ventrally than the anterior one, as it can be observed by the red signal in more ventral sections (Fig. 3-2 D-F). While Id2 remained expressed in the otic epithelium in the posterior region at the middle level of the otocyst, it was restricted to the surrounding areas of the epithelium where a few Prox1 positive cells were located in the anterior pole (Fig. 3-2 D). Even more ventrally, Id2 expression was absent from the anterior region, where some Prox1 positive cells were still observed, while remained in the restricted in the posterior (Fig. 3-2 E). Given the localization, and the fact that Prox1 is expressed in neuronal progenitors and in the CVG (Fig. 3-2 E-F) (Stone, Shang, Tomarev. 2003), those cells expressing Prox1 but not Id2 are likely to be neuronal progenitors rather than prosensory precursors (see discussion). In the ventral tip of the otic vesicle, the cochlear duct begins to elongate, and Id2 was highly expressed in the posterior anlagen of the auditory sensory epithelium, as can be identified by the Tuj-1 positive signal (Fig. 3-2 G). A similar pattern was obtained for Id3 and is illustrated for a stage HH22 otocyst (Fig. 3-2 H-I). In this experiment, we labelled the prosensory patches and CVG in adjacent sections with Sox2 and Isl1 antibodies (Fig. 3-2 J, merged of both) (Neves et al.2007, Zeng et al 2001). A coronal section at level of the equator revealed Id3 expression in the broad posterior domain corresponding to the anlagen of the macula sacularis (ms), and the basilar papilla (bp), and in the anterior region where the macula utricularis will emerge (mu) (Fig. 3-2 H). A section through a more ventral level illustrates the Id3 expression in the presumptive basilar papilla (Fig. 3.2 I).Id3 expression domain included the region where proliferating sensory progenitors are located, as the double staining with Islet1 and Sox2 revealed (merged image, Fig. 3-2 J). The Individual signals for those antibodies are also shown (Fig.3-2 K-L).

In summary, Id genes are expressed in the otic epithelium at the initial steps of prosensory patch formation, including the areas defined by Bmp4 expression (Oh and Wu, 1996). Id expression domains are broader than the prosensory patches, and include the adjacent non sensory epithelium, where Bmp7 is also expressed (Oh and Wu 1996). Moreover, strong expression of Id1-3 genes was found in the periotic mesenchyme adjacent to the sensory patches.

Id genes are down-regulated from sensory progenitors that express Atoh1

As development proceeds, several morphological changes are evident in the otocyst. Between days 3 and 4, the round-shaped vesicle develops two distinct dorsal and ventral bulges, and the beginning of a hollow tube in the medial wall, that will become the endolymphatic duct (see introduction and Bissonnette and Fekete, 1996). The dorsal bulge (pars superior) is the area from where the semicircular canals and the utricle will emerge. The ventral bulge (pars inferior) will evaginate as a hollow tube on its distal part to form the cochlear duct. The proximal part of the pars inferior evaginates on its anteromedial side to generate the saccule anlage (Bissonnette and Fekete, 1996). Concomitantly, new prosensory patches emerge and become distinguishable at specific times and locations (Oh and Wu, 1996, Neves et al, 2007). Given the possible interactions between Id genes and Atoh1 in the sensory patches (Jones et al 2006), we analyzed Id and Atoh1 expression at similar stages.

By day 4, all presumptive sensory organs (except the macula neglecta) are disctinct entities that can be identified by Bmp4 and Sox2 expression (Wu and Oh 1996, Neves et al 2007). The first hair cells are generated at this stage, and several studies have analysed their order of appearance and differentiation within the different sensory organs (Bartholami, 1991, Von Bertheld 1993,). The order of hair cell formation in the chick inner ear seems to be well defined: vestibular organs mature before the auditory basilar papilla (Bartolami, Goodyear, Richardson. 1991)(Wu and Oh, 1996, Oh and Wu, 1996). Consistent with this, we have previously shown that Atoh1 expression is first expressed in the anterior and posterior cristae of the chick otocyst, during stages 23-24 (chapter 1). We now analyzed Atoh1 expression at day 5 (HH25), and this is shown in a series of transverse sections through the entire otocyst, in which each sensory organ is distinguished by Atoh1 expression (Fig. 3-3 A-F). We found that Atoh1 mRNA was still highly expressed in the anterior cristae (Fig. 3-3 A), in the macula utricularis (Fig. 3-3 B), in the lateral crista (Fig. 3-3 B, C), in the macula sacularis (Fig. 3-3 C, D) and in the posterior crista (Fig. 3-3 F). In addition, we found strong expression of Atoh1 in the paratympanic organ (Fig. 3-3 C), and in the dorsal margin of the hindbrain (Fig. 3-3 A-F). Noticeably, we did not detected Atoh1 expression in the basilar papilla or macula lagena at this stage.

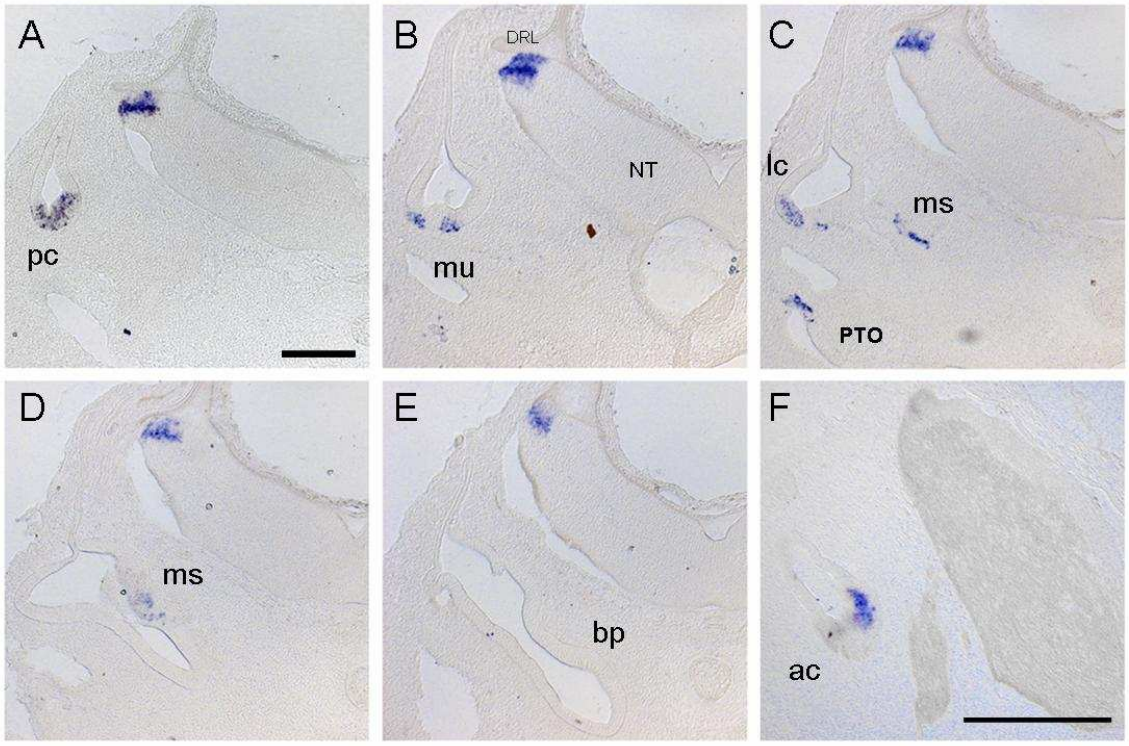

Fig. 3-3. Atoh1 expression at E4-5
A-F: Transversal sections throughout the developing inner ear at embryonic day 5, from posterior (A) to anterior (F). Dorsal is to the top, medial to the right. Sections were processed for ISH detection of Atoh1 mRNA (blue signal), and expression in the different sensory organs is indicated. A: expression in the posterior crista (pc). B: expression in the macula utricularis (mu). C: expression in the lateral crista (lc), the macula sacularis (ms) and the paratympanic organ (PTO). D: expression in the macula sacularis (ms). E: the site of the sensory basilar papilla is indicate (bp) and devoid of Atoh1 signal. F: higher magnification view of the anterior crista (ac). Expression in the dorsal rombic lip (DRL) is present in sections A-E. Scale bars: 50 µm.

We then turned our attention to the analysis of the expression of Id genes in relation to Atoh1 expression in the vestibular sensory patches (Fig. 3-4). Double ISH hybridization experiments with Atoh1 and Id2-3 probes showed that the expression of these genes was downregulated in the regions were Atoh1 was strongly expressed (Fig. 3-4 A-B'). This is illustrated by transversal sections at stage HH27 showing the absence of Id3 expression in the lateral crista and both utricular and sacular maculae (lc, mu and ms, Fig. 3-4 B-B'). A high magnification view of the lateral cristae showed also that the non-sensory epithelium surrounding the crista exhibited high levels of Id3 mRNA (Fig. 3-4 B). In contrast, within the dome-shaped sensory tissue Atoh1 was highly expressed, as revealed by double ISH hybridization developed with a red fluorescent precipitate (Fig. 3-4 B').

Similar results were obtained after staining the same sections with an antibody against MyoVIIa, to reveal the prescence of differentiated hair cells (Hasson T, 1997). As an example, the maculae sacularis, that was devoid of Id expression (Fig. 3-4 C, and magnified in Fig. 3-4 D) showed many differentiating hair cells (Fig. 3-4D'), The diagram in Fig. 3-4 E illustrates the localization in the inner ear of vestibular and sensory organs at this stage, and is possible to visualize that at this stage the macula sacularis is located nearby the developing auditory basilar papilla. It is interesting to note, that Id3 was expressed in the elongated cochlear duct (see above Fig. 3-4A, and Fig. 3-4C). Identical pattern was observed in the whole inner ear for Id2, and it is exemplified showing the proximal region of the cochlear duct (Fig. 3-4F). The area positive for Id3 and Id2 was identified as the prosensory auditory region with Islet-1 antibody (Fig. 3-4F). Merged images in which Id2 ISH was pseudocoloured in blue, showed that Id2 was indeed expressed in auditory progenitors at this stage (Fig. 3-4F').

In summary, Id2-3 were dowregulated from vestibular organs in the regions where Atoh1 become highly expressed. However, they remained expressed in the auditory progenitors. These results suggest that Id withdrawal from the sensory patches follows the dorsal to ventral sequence of maturation of the sensory organs mentioned above. This dynamic expression was similar for Id2-3 and Id1 (not shown), suggesting common regulatory mechanisms for Id1-3 gene regulation in sensory progenitors.

It is interesting to note that the downregulation of Id expression from the sensory patches at stages of hair cell differentiation implies that they must be somehow segregated from Bmp4 expressing regions (Bmp4 is typically expressed in the sensory patches). This segregation is directly shown at a later stage (E7) (Fig. 3-5 A-B'). This figure illustrates two examples of the comparison between Id2-3 expression patterns with that of Bmp4. While Bmp4 expression was strong in the sensory region of the lateral crista, and in the roof of the ampula (Fig. 3-5 A), the adjacent section probed for Id2 showed a complementary pattern (Fig. 3-5 B). A triple staining for Id2 mRNA, MyoVIIa and 3A10 (Fig. 3-5 B') showed that Id2 expression was absent from the hair cells, but maintained high.in the adjacent tissues. Similarly, the two Bmp4 positive sensory regions of the posterior crista (Fig. 3-5 C) were devoid of Id3 expression, which was confined to the non-sensory epithelium (Fig. 3-5 D).

Again, triple staining confirmed the presence of innervated hair cells in this sensory organ (Fig. 3-5 D').

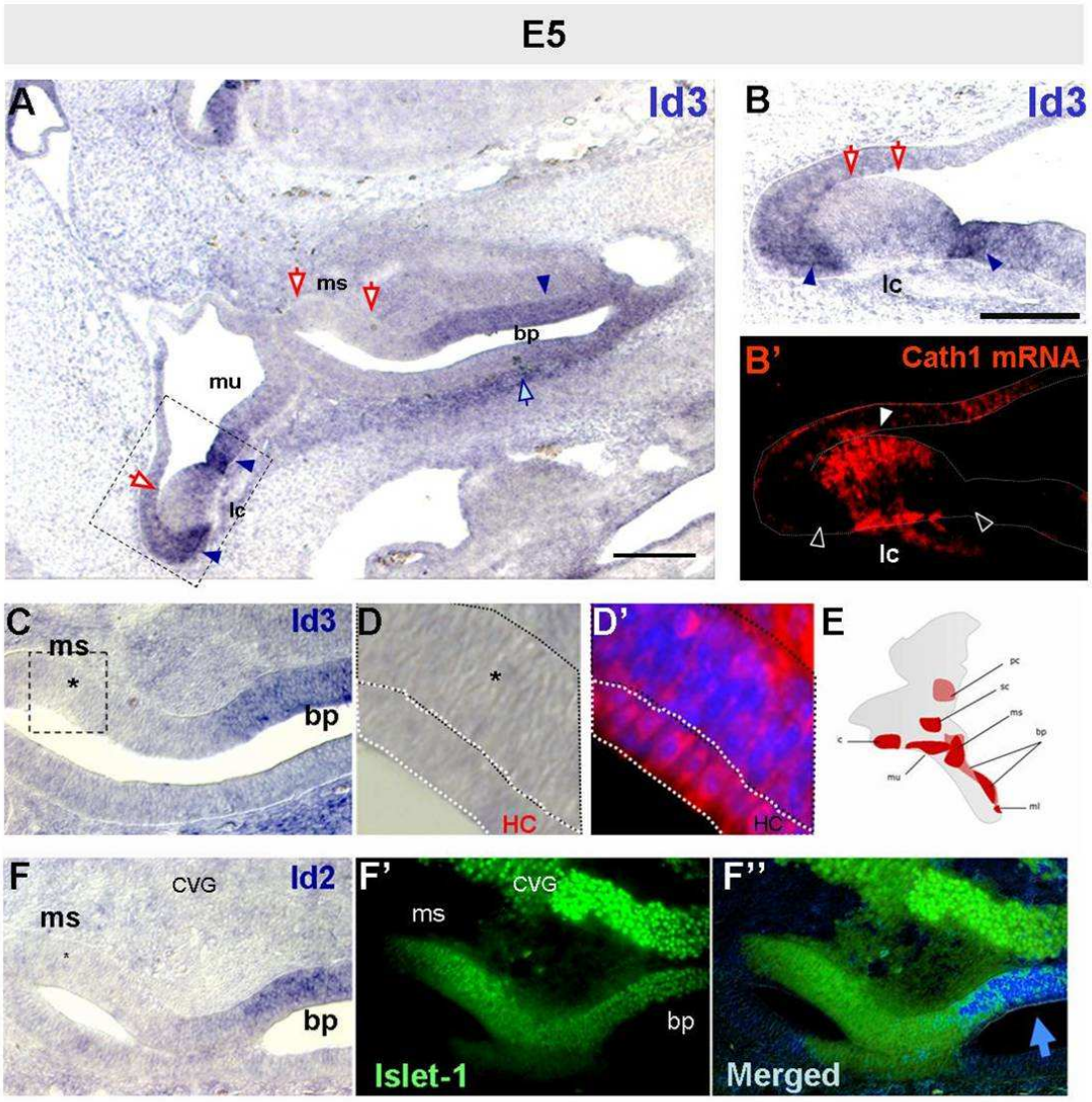

Figure 3-4. Expression of Id2-3 at E5
A-D, F-F'': Transversal sections of the inner ear at E5. E Schematic representation of the inner ear at this stage, indicating the locations of the differenti sensory organs at this stage. A:panoramic view shows Id3 expression. Blue arrows indicate expression of Id3 in the otic epithelium of the auditory basilar papila (bp) and adjacent to the lateral crista (lc). Blue empty arrowhead indicates Id3 expression in the periotic mesenchyme of thebasilar papilla. Red arrows indicate the vestibular sensory patches (ms, mu, lc, blue arrow), where Id3 is not expressed. B and B' are details of the dotted box in A. B: blue arrows indicate the high expression of Id3 in the cells surrounding the lateral crista. Red arrows indicate the dome-shape sensory tissue, where Id3 expression is low. B'': The same section was processed for double ISH, and Atoh1 mRNA was detected with a red fluorescent precipitate. White arrowhead indicates sites of atoh1 expression, and empty arrowheads indicate the Id3 sites of expression.
C: Detailed view of the transversal section in A, showing Id3 expression in basilar papilla (bp), but not in the macula sacularis (ms). D-D': detail of the dotted region indicated in C. D: Id3 was not detected in this area. D': immunolabeling with MyoVIIa antibody (red) revealed the location of hair cells (HC).

We then examined the basilar papilla at E7, when hair cell differentiation has already started in this organ (Fig. 3-5 E-E''). A transversal section showing the distal region of the basilar papilla showed that Id3 expression was concentrated in the tip of the duct (Fig. 3-5 E). Double staining with MyoVIIa antibody, revealed two regions where hair cells are present, corresponding to the distal basilar papilla (bp) and to the macula lagena (ml) (Fig. 3-5 E''). The merged image showed that, as it was the case for vestibular organs, Id3 levels were downregulated in the regions where hair cells are present (Fig. 3-5 E''). As shown above for the cristae, Id3 expression was high in the adjacent non-sensory epithelia separating the basilar papilla from the lagena (arrowheads in Fig. 3-5 E). A similar pattern was also observed for Id1 and Id2 (not shown).

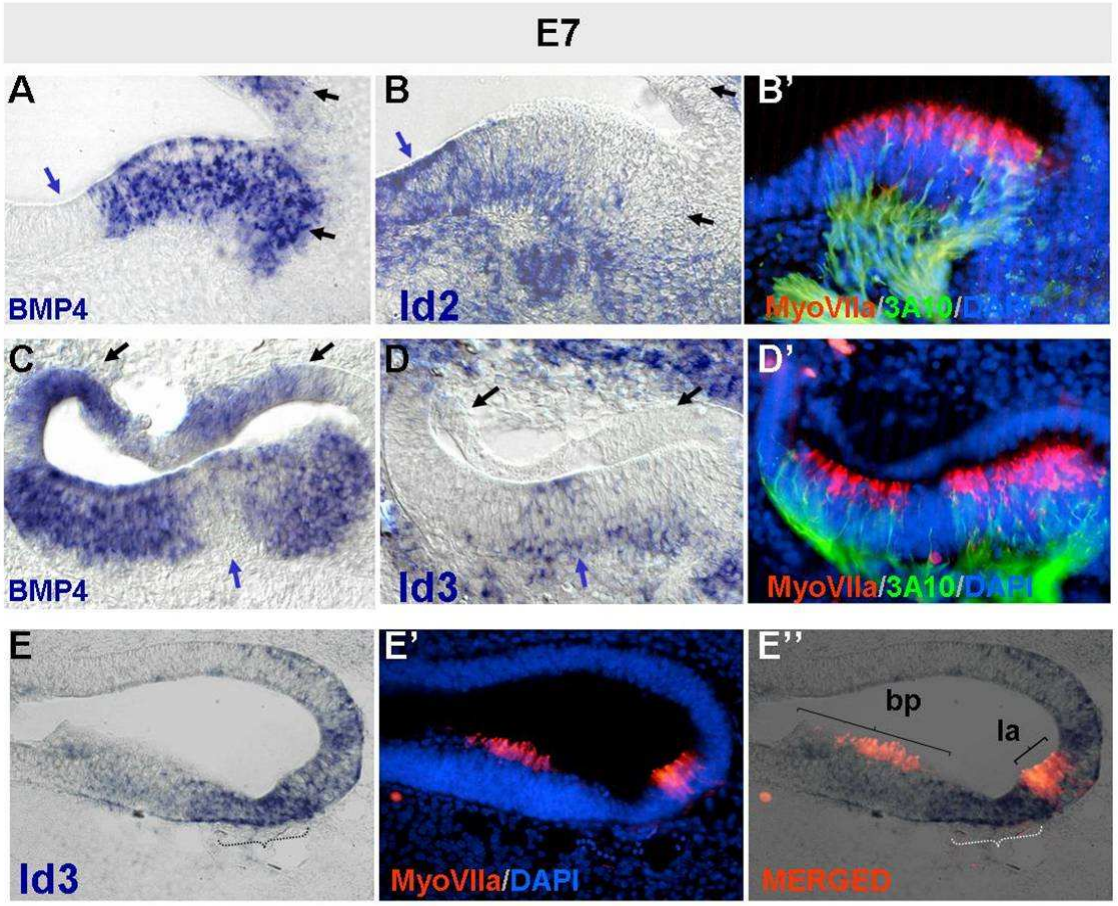

Figure 3-5. Expression of Id2-3 at E7

A-E': Transversal sections of the inner ear at E7. A: transversal section at the level of the lateral crista processed for Bmp4 ISH (blue). Black arrows indicate strong Bmp4 signal in the sensory lateral crista, and in the roof of the ampula. Blue arrow indicates the non-sensory epithelium devoid of Bmp4 signal. B': Section adjacent to A, showing Id2 ISH signal. Arrows indicate the same relative positions than A to compare. B'': the same section as B was labelled with anti-MyoVIIa (red) and 3A10 (green) antibodies, and nuclei counterstained with DAPI (blue). C: transversal section processed for Bmp4 at the level of the posterior crista. Black arrows indicate strong Bmp4 signal in the roof of the ampula. Blue arrow indicates the non-sensory epithelium known as *cruciatum* devoid of Bmp4 signal. D: Section adjacent to C, showing Id3 ISH signal. Arrows indicate the same relative positions than A to compare. D': the same section as D was labelled with anti-MyoVIIa (red) and 3A10 (green) antibodies, and nuclei counterstained with DAPI (blue) E-E'': Transversal sections at the most distal part of the cochlear duct triple stained for Id3 ISH (E), MyoVIIa immunofluorescence (red E') and DAPI (blue in E'). The merged image show the location of the auditory hair cells in the basilar papilla (bp) and the vestibular hair cells in the macula lagena (la). The white braquet indicates the strong region of Id3 expression between those sensory organs.

Altogether, our data are similar to those reported from studies in the mouse cochlea, with Id1-3 gene expression being downregulated from Atoh1 positive hair cells, consistent with a role of Id proteins acting as negative regulators of Atoh1 (Jones et al 2006). However, the initial co-expression of Ids with Bmp4, and their segregation at later stages raises interesting questions about the possible regulation of these genes by Bmp4.

BMP signalling regulates Id expression in the inner ear

In order to analyze the role of Bmp signalling in the regulation of Id genes, we performed studies like the ones described in chapter 1, where isolated otic vesicles were grown in culture and treated with BMP4 and BMP-inhibitors.

A typical experiment is shown for Id2 in Fig. 3-6. First, we analyzed the cultured vesicles by whole mount ISH (Fig. 3-6 A-C). Control otic vesicles showed Id2 expression being concentrated in two regions of the otocyst, one anterior and one posterior (Fig. 3-6 A), as it was described in vivo (see above). Culturing of E3 otic vesicles in the presence of BMP4 for 4 hours, produced a strong and ubiquitous induction of Id2 expression (Fig. 3-6 B, n=10/10, 3 different experiments). On the contrary, the incubation with Noggin during the same period abolished Id2 expression in all vesicles examined (Fig.3-6 C, n=10/10, 3 experiments). The same results were obtained for Id1 and Id3 (n=10/10, at least two experiments for each gene, not shown), suggesting again that similar regulatory mechanisms operates on these genes in sensory progenitors (see discussion).

Crysostat sections of cultured otic vesicles confirmed that in a control condition, Id2 was expressed in discrete locations of the otic epithelium, and its surrounding mesenchyme (Fig. 3-6 D, D'). The addition of BMP4 was able to induce Id2 expression in both, epithelium and mesenchyme (Fig 3-6 E, E'), while the incubation with Noggin caused a striking downregulation of Id2 expression (Fig 3-6 D). Only a few vesicles (n=2/10 for this experiment) retained a weak residual signal for Id2 after Noggin treatment, mostly located in the mesenchyme immediately adjacent to the epithelium (arrows in (Fig 3-6 F').

In order to quantify these effects in the epithelium and in the mesenchyme separately, we adapted a method to quantify the area of Id expression (see materials and methods). Briefly, several otic vesicles were serially sectioned and reconstructed (one per group is shown in Fig. 3-6 G). We set up a common intensity threshold for all pictures (using control epithelia without signal), and analyzed them using the ImageJ software, measuring the area above that threshold. The results were plotted in a graphic, in which epithelial and mesenchymal area values of Id expression are shown in different columns (Fig. 3-6 H). In this way, we confirmed that BMP4 addition during 4h caused an expansion of Id2 expression that includes nearly all the epithelium and mesenchyme, whereas Noggin had the opposite effect.

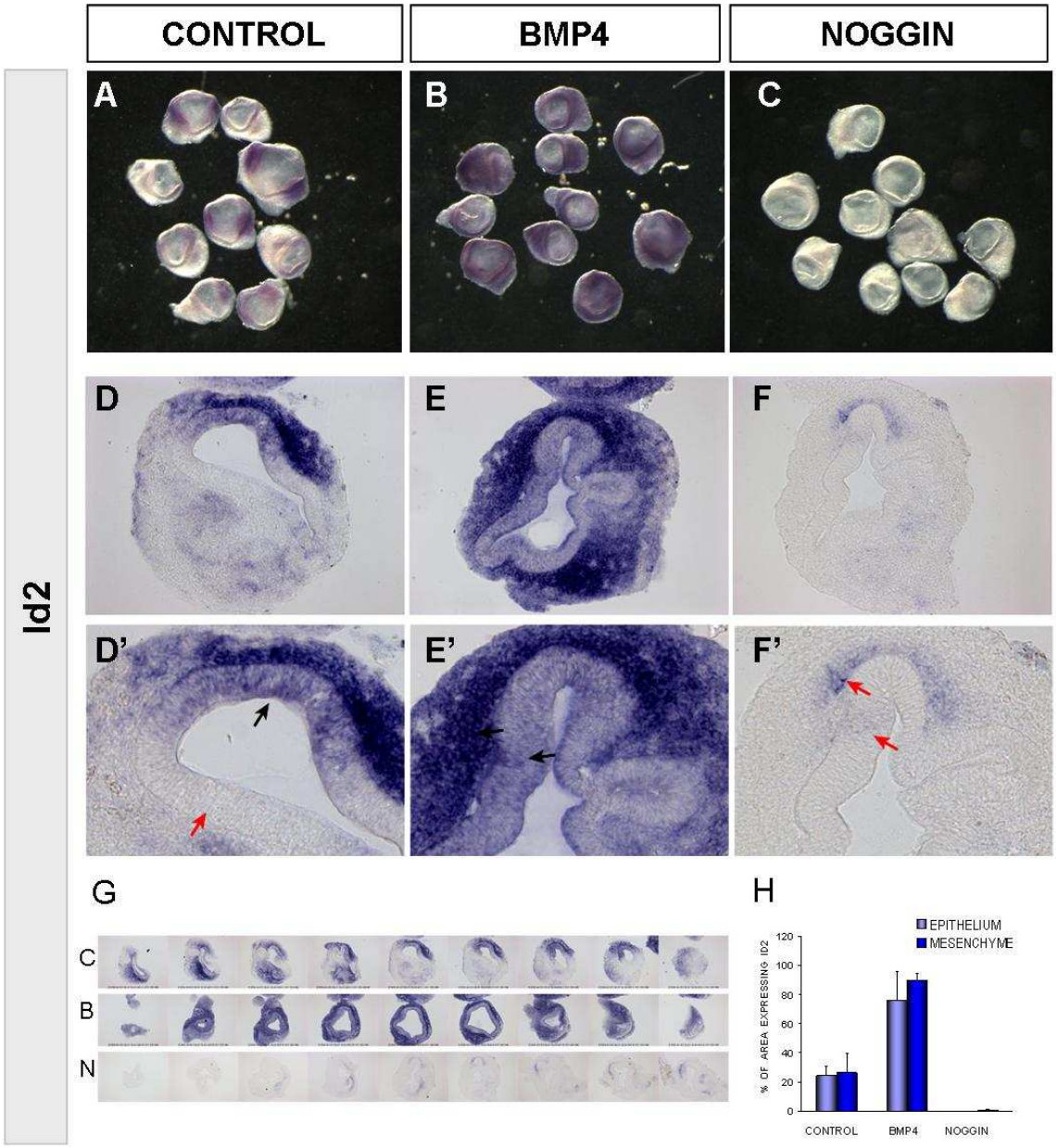

Figure 3-6. BMP regulation of Id2
A-C: Whole mount ISH for Id2 mRNA of isolated otic vesicles. Vesicles were isolated at HH20, and cultured for 4 h either in control media (A), media plus 100ng/mL of BMP4 (B) or Noggin 1µg/ml (C).
D-F': 20µm crystat sections of cultured otic vesicles. D'-F': higher magnification view of the vesicles shown in D-F. Black arrows indicate sites of strong Id2 expression.
G: Serial sections covering an entire otic vesicle, showing one example for each condition. H: Quantitative representation of the effects of BMP4 and Noggin addition in Id2 expression surface. Values for the columns represent the percentage of surface area positive for Id2 ISH (see matherials and methods). Dark blue correspond to mesenchymal expression and pale blue epithelial.

In summary, all regions of the otic epithelium and periotic mesenchyme responded to a short exposure of exogenous BMP4 with the induction of Id genes. Conversely, blocking Bmp signalling with Noggin was sufficient to rapidly and completely downregulate Id genes. These results suggest that an endogenous activity of BMP signalling is responsible for the normal expression of Id genes in the otic vesicle and periotic mesenchyme, and indicate a fast turnover of Id mRNAs, that are degraded in the absence of Bmp signal. Then Id1-3 expression is dependent on the continuous binding of BMP molecules their receptors.

The regulation of Id genes by BMP4 in the otocyst was further demonstrated by quantitative Real Time PCR. For these experiments, otic vesicles were cultured in the same conditions as described above, but at the end of the incubation period total RNA was extracted and processed for this type of analysis (see diagram in Fig. 3-7 A, and matherials and methods). As illustrated in Fig. 3-7 B , Bmp4 induced the expression of Id1-3 after 4 hours of incubation, reaching a maximum of six-fold increase for the Id1 mRNA. The effect was selective as judged by the lack of effect on other genes that also regulate the differentiation state of the progenitor cells, like Sox2 and NeuroD (Fig. 3-7 B). The induction of Id genes by Bmp4 was very rapid, and increased levels of Id1-3 transcripts were detected already at 1 h of incubation (Fig. 3-7 C), and in a dose-dependent manner (Fig. 3-7 D). In all cases, Noggin addition resulted in a strong reduction of Id expression (Fig. 3-7 B). These data further indicates that Ids are direct target genes for Bmp4 in the inner ear.

The fact that Bmp4 induced Id expression so rapidly in all regions of the otocyst implies that BMP receptors must be present ubiquitously in the otic epithelium, making all the tissue competent to respond to the BMP signal. This is consistent with data reporting a broad distribution of BMPRI-a in the otocyst (Chang et al., 2002). On the other hand, Noggin experiments demonstrate that the activation of Bmp receptors is required for the endogenous expression of Ids in the otic epithelium and periotic mesenchyme at this stage.

Upon BMP binding to its receptors, a series of phosphorylation events transduce this signal to the receiving cell. Although several pathways have been shown to be activated by BMP molecules, the main mechanism involved in the cell response to them is mediated by phosphorylation of Smad1-5-8 proteins, which finally results in regulation of gene expression (see introduction). In order to gain insights on which of this pathways was relevant for the Id response to BMP4 in the otocyst, we performed a series of experiments that are described in detail below.

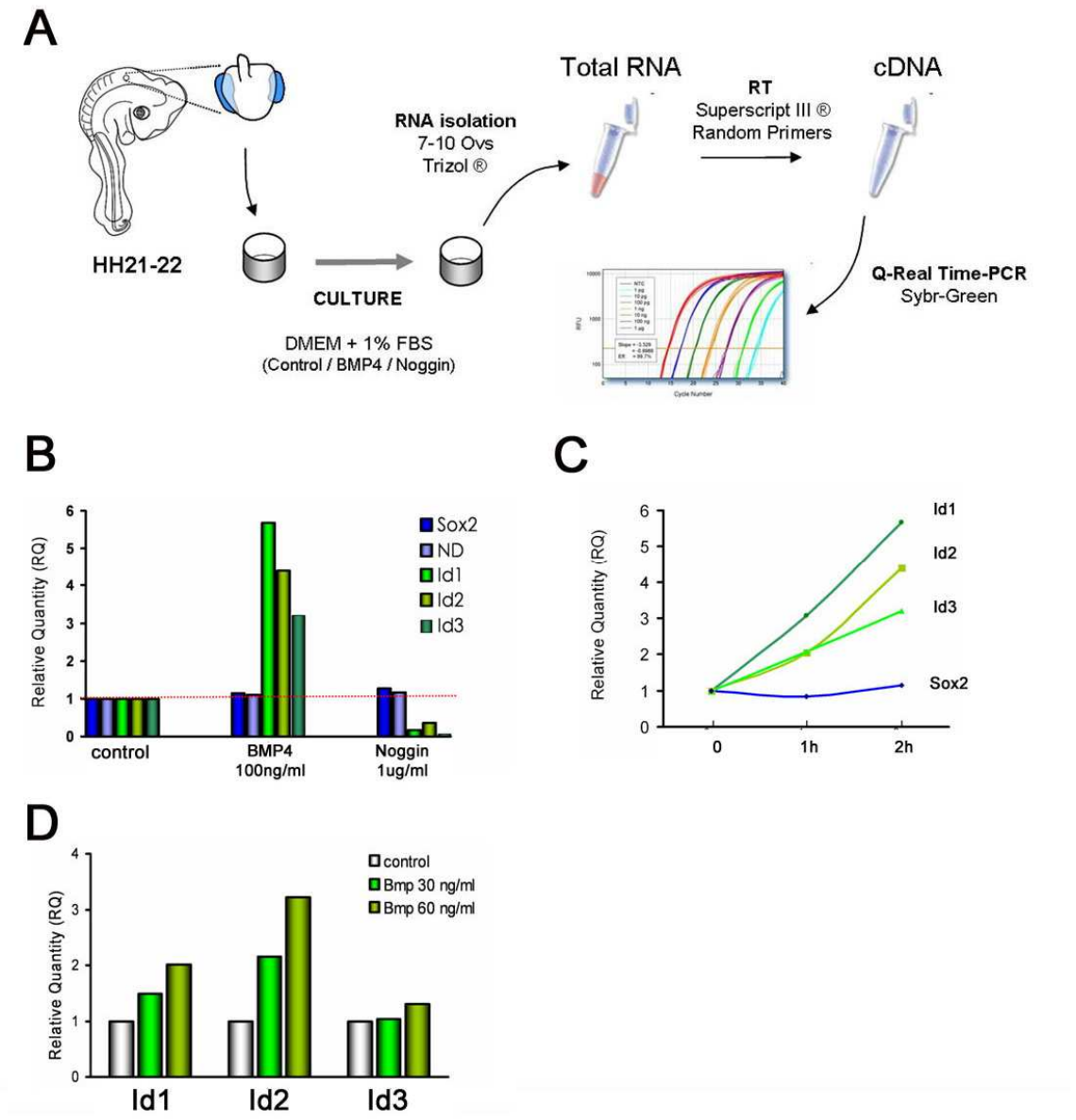

Figure 3-7. BMP induction of Id1-3 measured by Quantitative Real-Time PCR
A: Diagram illustrating the procedure for qRT-PCR: otic vesicles were isolated from HH21-22 embryos, and cultured either in control conditions, or in the presence of BMP4 or Noggin. Total RNA was isolated using the Trizol reagent, an purified RNA was retrotranscribed with the Superscritpt III DNA polymerase, utilizing random primers. Real time PCR was carried out using the SyberGreen approach, and the relative quantity of expression was estimated using specific primers for Id genes (and others), using GAPDH and 18S as calibrators.
B: Incubation of otic vesicles for 4 h with 100 ng/ml BMP4 produced an increased in the relative quantity of Id1-3 mRNA. Id1 showed the highest degree of induction (6 fold) with respect to control (1). Incubation with Noggin (1µg/ml) almost completely abolished Id1-3 expression. Other genes analyzed, as Sox2 and NeuroD did not show variations upon these tratments.
C: Id1-3 were rapidily induced upon BMP4 addition. The same experiment than B was performed, but cultures were stopped at earlier time-points. The graph shows the result of relative expression of Id1-3 and Sox2 genes after 1-2 hour of incubation with 100 ng/ml BMP4. Id genes expression was increased already at 1 h post-treatment, whereas the levels of Sox2 expression did not change.
D: The graph represents the relative expression of Id1-3 at 1 hour of incubation with the indicated doses of BMP4.

BMP induced Id genes through Smad1-5-8 pathway

First, we treated otic vesicles with BMP4, and analyzed in parallel Id mRNA expression and the phosphorylation of BMP receptor-activated Smads. In order to localize in situ this two processes, we cryosectioned the otic vesicles and performed immunohistochemistry using an antibody that recognizes the phosphorylated form of Smad1-5-8. In control sections P-Smad immunoreactivity was strong in restricted regions of the otic epithelium that corresponded to the domains of higher endogenous Id2 expression (Fig 3-8 A-A''). In the merged image, Id2 expression signal was inverted and pseudocoloured as described above, and is shown in red. This result indicated that endogenous BMP activity is indeed present and localized in the early prosensory regions of the E3 otocyst, correlating with Id expression. When we incubated the otic vesicles with BMP4 for 4 hours, we observed again the increased Id2 expression (Fig. 3-8 B), together with a dramatic increase in P-Smad immunoreactivity all throughout the epithelium (Fig 3-8 B', B''), confirming the ubiquitous activation of the BMP pathway in the otic vesicle.

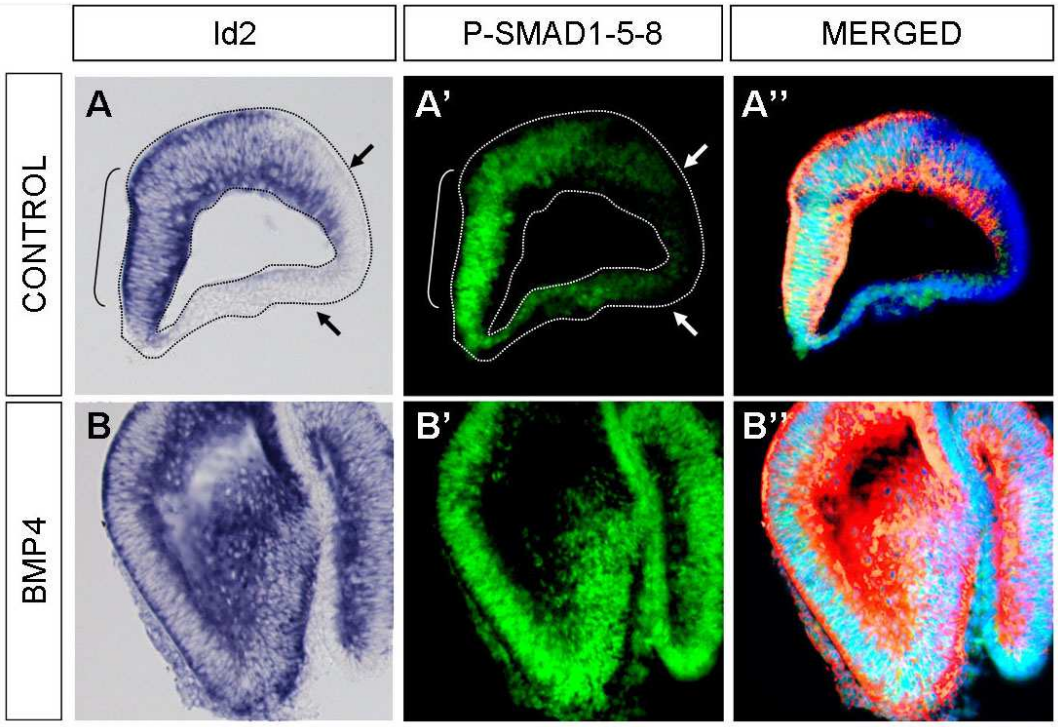

Figure 3-8. BMP4 induces phosphorylation of Smad1-5-8
A-A'': Cryostat sections of HH21 otic vesicles cultured during 4 hours in control conditions, and processed for ISH with Id2 probe. Black arrows indicate sites where Id2 was not detected. A': the same section was processed for immunohystochemistry using P-SMAD1-5-8 antibody (green nuclei). White arrows indicate the sites of low signal. Braquet in A-A' indicated the area of strong Id2 expression and P-SMAD1-5-8 immunoeactivity. A'': merged image, in which Id2 was pseudocoloured in red, and DAPI nuclear staininig is shown in blue.
B-B'': Cryostat sections of HH21 otic vesicles cultured during 4 hours in the presence of 100 ng/ml BMP4 and processed for ISH with Id2 probe. B': the same section was processed for immunohystochemistry using P-SMAD1-5-8 antibody (green nuclei). B'': merged image, in which Id2 was pseudocoloured in red, and DAPI nuclear staininig is shown in blue.

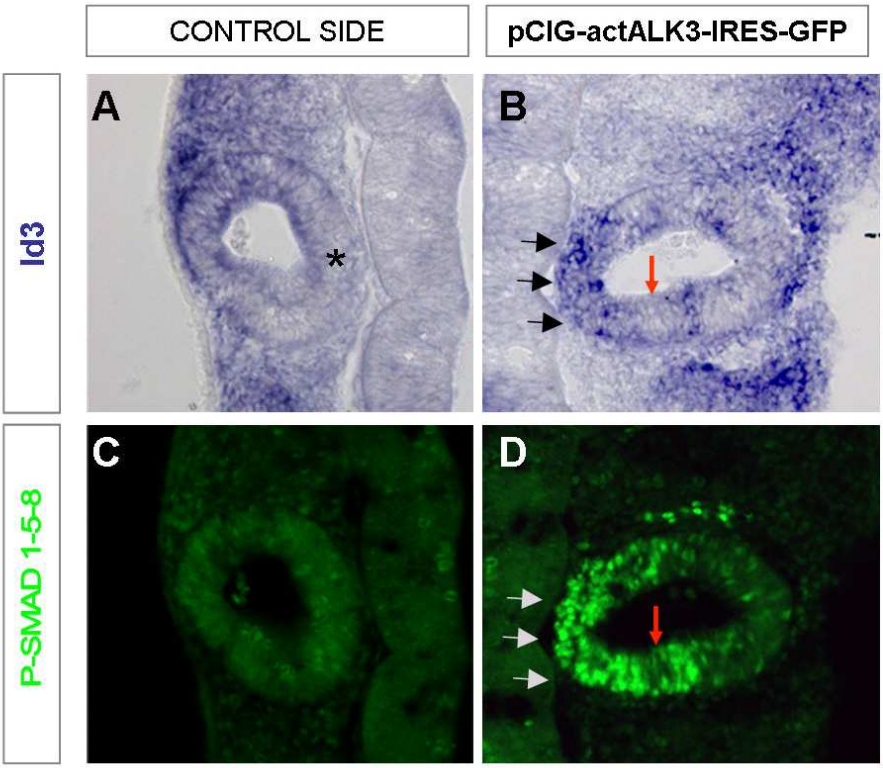

Figure 3-9. Overexpression of act-ALK3 induces phosphorylation of Smad1-5-8 and Id3
A-B: Coronal sections of an electroporated embryo. Electroporation was done at HH14, and the embryo allowed to developed in ovo for 6 hours. The embryo was processed for whole mount ISH with Id3 probe. A: Left otic vesicle that has not been electroporated. Asterisk indicated the medial wal of the otocyst that did not express Id3. B: Right otic vesicle, electroporated with pCIG-actAlk3-IRES-GFP. Arrows point to the medial wall of the otocyst were Id3 was ectopically induced. C-D: The same sections were processed for immunohystochemistry using P-SMAD1-5-8 antibody (green nuclei). White arrows in D indicate the medial wall were P-SMAD levels are greatly increased. Red arrow in B-D indicates a spot of low Id3 expression and low P-SMAD signal.

Secondly, we overexpressed in the otic epithelium a constitutively active form of BMPRI-a (act-ALK3), by in ovo electroporation. Given the fast response of Id genes in culture, we allowed the embryos to develop for 6 hours before fixing and processing for ISH or IHC. Electroporated otic vesicles exhibited a strong upregulation of the expression of Id1, Id2 and Id3 already at 6 h post-electroporation (n=3 for each gene, two different experiments). Electroporation with the same vector containing GFP but not the ALK3 produced no differences in Id1-3 gene expression, while strong GFP fluorescence was detected (n=10 at least three experiments, not shown). An example of the upregulation caused by the constitutively active ALK3 is illustrated in coronal cryostat sections probed for Id2 shown (Fig 3-9 A-B). The non-electroporated side showed the endogenous pattern of Id3, in the antero-lateral wall of the otocyst, being absent from the medio-ventral aspect (asterisk in Fig 3-9 A). In contrast, a section through a similar level of the electroporated side showed increased levels of Id3 mRNA ectopically expressed in the medial wall of the otocyst (arrows in Fig 3-9 B). Moreover, we analyzed those sections by immunohyscochemistry, using the anti-P-Smad1-5-8 antibody, and found that electroporation with act-ALK3 produced a dramatic increase in p-SMAD1-5-8 signal compared to non-electroporated (Fig 3-9 C, D), paralleling the increase in Id3 gene expression.

The electroporated vector contained an IRES-GFP sequence downstream the ALK3 construct, which allowed us to follow the electroporated cells. Comparison of the GFP signal with that of P-SMAD confirmed that those cells expressing GFP (and therefore ALK3*) are the same that have higher levels of P-SMAD1-5-8 (not shown). Therefore, increase in P-SMAD and Id gene transcription was cell autonomous, as it was observed only in those cells that were electroporated with ALK3* and not their neighbours (see red arrow in Fig 3-9 B and D). Electroporation with an empty vector expressing only GFP did not show any of these effects (not shown).

Finally, we cultured otic vesicles in the presence of Dorsomorphin, a compound that has been recently described as inhibitor of BMP activity that selectively blocks the BMP-induced Smad signalling pathway (Anderson, Darshan. 2008; Cuny, et al. 2008; Hao, et al. 2008; Yu, et al. 2008). Addition of Dorsomorphin to the culture media for 4 h resulted in down-regulation of Id expression (n=10/10, not shown). The effect was in all similar in all aspects to that produced by Noggin (see above), indicating that the endogenous BMP-dependent expression of Id genes in the otic vesicle was indeed dependent on Smad activation. Exposure to Dorsomorphin for 20 hours, in an experiment similar to that described in chapter 2-, also induced the expansion of sensory patches, suggesting that this Smad activation underlies both short and long term effects of BMP-inhibition (not shown).

Taken together, these experiments show that Id expression in the otic epithelium and mesenchyme is dependent on Smad1-5-8 phosphorylation.

Chapter 4

Differential expression of Btg1 and Btg2 in developing the Inner Ear

In the previous chapters we have shown that BMP4 regulates the cell fate and survival of sensory precursors in the otic epithelium of the inner ear, and we identified the Id1-3 genes as molecular targets of BMP signalling in otic progenitors. Expression of Id genes was dependent on the endogenous activation of the Smad signalling pathway, and ectopic expression occurred along the entire otic epithelia and surrounding mesenchyme when BMP4 was added in excess. However, prosensory progenitors showed a differential response in cell cycle progression and survival upon BMP addition. Therefore, we decided to explore genes regulating those processes that may be expressed differentially in sensory progenitors, and therefore contribute to their differential response to BMP signalling modulation.

Btg/Tob proteins belong to a novel class of cell cycle modulators that can regulate cell survival, cell cycle exit and cell differentiation (see Introduction). Several of the proposed cellular functions actions of Btg proteins prompted us to investigate these genes in otic progenitos. In particular, the functional links between members of the Btg family and BMP-Smad signalling pathway, including Btg2, which is also able to activate the Atoh1 promoter in other experimental models (see introduction). Information about developmental expression of Btg genes is still fragmentary in other species and there was no detailed description of the expression pattern of any member of the family in the chick embryo up to this work. Moreover, to the best of our knowledge, their expression during inner ear development has not been described in any animal species.

In this chapter we present expression data about Btg2 during the period of sensory organ development in the chick inner ear, and compare its expression to that of Btg1 at embryonic day 6, when sensory organs begin differentiation. In addition, and to gain insights on the function of these genes during chick development, we analysed and compared their expression profile in the whole chick embryo during early stages of development. Those results are presented separately in the Appendix II (“Btg1 and Btg2 gene expression during early chick development”), although some of the observations derived from that study are also mentioned in the final discussion.

Btg2 expression in the otic placode and cup

As it happens with Bmp and Id genes, expression of Btg2 along otic development was very dynamic. Btg2 mRNA was detected in the otic placode, at stage HH11-12 (Fig. 4-1 A-A’’). Btg2 was strongly expressed in other regions of the embryo (see Anex II), and only a few cells were detected in the otic placode, that was identified as an ectodermal thickening labelled with Pax2 and HNK1 antibodies (Fig. 4-1 A-A’’). Transversal sections showed that Btg2 expression was localized in the anterior region of the otic placode, including the

limits between epidermal and placodal ectoderm (Fig. 4-1 A), and was not expressed in the posterior otic placode (red arrow in Fig. 4-1 A'-A').

Btg2 mRNA was barely detected in the otic epithelia in the subsequent stages, but it appeared at very low levels and concentrated in a few positive cells in the anteroventral domain of the late otic cup, at stages HH15-16 (Fig. 4-1 B). This expression was difficult to observe in sections, and is better illustrated with a lateral view of partially dissected otic cups (Fig. 4-1 B). This expression of Btg2 corresponded in time and space whith delamination of otic neuroblasts to form the CVG (Alsina 2004).

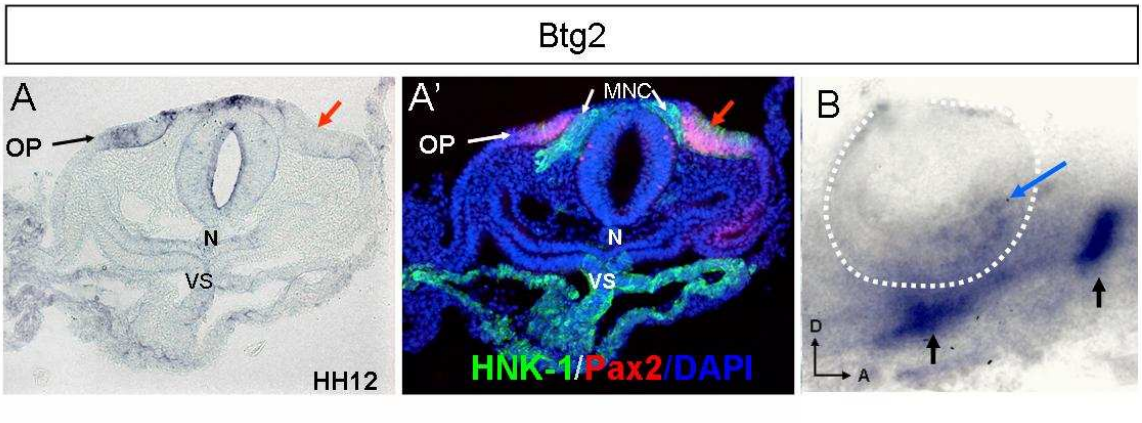

Figure 4-1. Btg2 expression in the otic placode and otic cup.
A-A: Transversal section through the otic placode at stage HH12. A: Black arrow indicates Btg2 expression in the left otic placode. Red arrow indicates the absence of Btg2 in the posterior region of the right otic placode. A: Immunostaining of the same section as A, with HNK1 and Pax2 antibodies. HNK1 labels migrating neural crest cells. Note that the green cells ingressing next to the left otic placodes, and not the right one. This confirms that the section is oblique, the left one being more anterior thatn the one in the right. Pax2 labels the placodal epithelia.
B: Lateral view of the right otic cup of a stage HH15 embryo, processed for Btg2 whole mount ISH. Dotted line indicates the limits of the otic cup. The neural tube and part of the mesenchyme was removed, and the tissue was flatted before photographing. Black arrows indicate the expression of Btg2 in the epibranchial placodes (out of focus). Blue arrow indicates the expression of Btg2 in a few cells of the otic epithelium.

Btg2 expression in the early otocyst

At HH17-18, whole mount ISH showed high expression of Btg2 in several regions of the embryo, including the somites, the central nervous system and the olfactory and epibranchial placodes (Fig. 4-2 A). Expression in the otic region appeared very weak and restricted to the nascent CVG, although it was difficult to observe in whole mount preparations (Fig. 4-2 A-A'). Within the otic epithelia, Btg2 was restricted to an anteromedial domain (neurogenic), although since this expression was weak, in most of the cases this was only apparent at this stage upon dissection of the closing otocyst (Fig. 4-2 B). Only few hours later in development Btg2 was expressed in the ventral-medial region of the otic epithelium, again corresponding to the site of neuroblast delamination, and including cells of the CVG, as shown in a transversal section through the anterior otocyst at HH20 (Fig. 4-2 C). Btg2 was also expressed in the neuroepithelium of the hindbrain, with high levels in its most dorsal aspect,

being excluded from the floor plate (Fig. 4-2 C, see also Appendix II). At a similar stage HH21, a coronal section through the medio-ventral level of the otocyst illustrates the appearance of a posterior spot of Btg2 positive cells, very similar to that of Bmp4 or Id1-4 (Fig. 4-2 D, compare to Fig. 3-1 A). Moreover, cells in the CVG were also positive for Btg2 (Fig. 4-2 D), as well as the proliferative neuroepithelium of the neural tube.

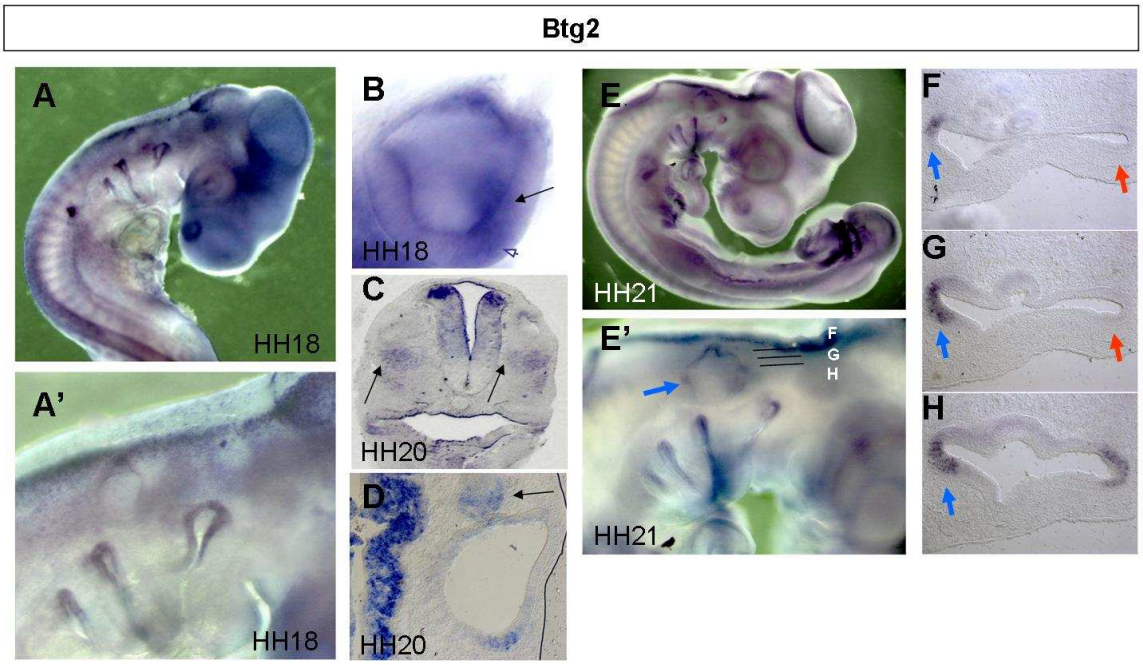

Figure 4-2. Btg2 expression at E3
A: Lateral view of an HH18 embryo processed for whole mount ISH with Btg2 probe. A': Enlarged view of the same embryo showing the otic region.
B: Medial view of an HH18 left otic vesicle. Dorsal is to the top, anterior to the right. ISH was performed in whole mount, and the otic vesicle dissected and flatted for photograph. Arrow indicates the ventromedial domain of Btg2 expression.
C: Transversal section through the anterior region of the otocyst of an HH20 embryo. ISH was performed in whole mount, and further developed in the section. Arrows point to the Btg2 positive cells located in the ventral aspect of both otic vesicles.
D: Coronal section of an HH20 embryo processed for ISH with Btg2. Image shows the right otic vesicle at the equatorial level. Anterior is to the top, medial to the left. Arrow points to the Btg2 expression in the SAG.
E: Lateral view of an HH21 embryo processed for whole mount ISH with Btg2 probe. E': Enlarged view of the same embryo showing the otic region. Blue arrow indicates Btg2 expression in the posterior region of the otocyst. Lines indicate the approximate levels of the coronal sections in F-H.
F-H: Coronal sections at the approximate levels indicated in B'. Blue arrow indicates the posterior patch of Btg2 expression. Red arrow of indicates the absence of Btg2 expression in the epithelium at that level.

As development proceeded Btg2 was expressed in the prosensory anterior and posterior patches, in the ventromedial neurogenic domain,and in cells of the CVG, which in some embryos could be observed in whole mount lateral views (Fig. 4-2 E-E'). Coronal sections through the otocyst evidenced Btg2 expression in the presumptive territory of the anterior and posterior cristae (Fig. 4-2 E'-

H).The posterior domain seemed to be more extended in the dorsal-ventral axis than the anterior, which is illustrated by three serial sections from dorsal to ventral (Fig. 4-2 F-H). This is similar to other genes that label sensory epithelia, including Id1-3 (see chapter 3), Bmp4 and Ser1 (Oh et al 1996, Cole et al 2000). Additionally, a few cells weakly positive for Btg2 were present in the dorsal-medial out-pocketing that will originate the endolymphatic duct (not shown, see Bissonette and Fekete 1996 and below).

Btg2 expression at early stages at embryonic days 4 and 5

In situ hybridization experiments in whole mount embryos at day 4-5 showed that Btg2 was still highly expressed all along the central nervous system, in the epibranquial and olfactory placodes, and in the myotome, but expression in the inner ear and other internal structures was not visible in whole mount preparations, and was only revealed upon histological sectioning (Fig. 4-3 A-C). At this stage, Btg2 mRNA expression was distinctively localized in the prosensory epithelium of vestibular cristae and maculae (Fig. 4-3 A), with a similar pattern to that of typical prosensory markers. Double staining with Tuj-1 antibody confirmed that areas of high Btg2 expression correlated with the innervated sensory regions, as it is exemplified for the lateral cristae and the two maculae in (Fig. 4-3, see inset Fig. 4-3 A'-A''). As it has been described for other markers of prosensory epithelium (Molea, Stone, Rubel. 1999). Btg2 was also expressed in the non-sensory epithelium of the endolymphatic apparatus (Fig. 4-3 A). Interesingly, Btg2 expression was only detected in a subpopulation of the SAG, while the majority of neurons of the SAG expressed high levels of Tuj1 but not Btg2 (Fig. 4-3 A). Given the ubiquitous expression of Btg2 in the SAG at earlier stages, this suggests that Btg2 was only transiently expressed, being downregulated from differentiating postmitotic neurons at HH23-24.

To further confirm that expression of Btg2 was restricted to prosensory progenitors, we performed ISH on transversal sections together with immunostaining of alternate serial sections using an antibody agains Sox2 as a sensory marker (Neves et al. 2007). Btg2 expression was restricted to sensory regions, as shown for the lateral crista in a transversal section at E5-5 (Fig. 4-3 C). Immunostaining with Sox2 in the adjacent section showed that Btg2 was indeed restricted to the Sox2 positive region (Fig. 4-3 D), and this is also shown at higher magnification Fig. 4-3 C'-D'.

In addition, we mapped Btg2 expression in transverse serial sections though the entire inner ear at E5, which is represented in the scheme in (Fig. 4-3 E), and found that Btg2 was expressed in all vestibular sensory organs. However, expression in the cristae seemed to be restricted to fewer cells than at previous stages. Btg2 expression in the crista was weaker also compared to more ventral sensory organs as the macula saculi, that retained high levels of Btg2 (see below). An example of the Btg2 expression in the anterior crista is shown in a transversal section at E5-5 (Fig. 4-3 F-F'). On possibility, is that Btg2 is downregulated as cells become postitotic and begin differentiation cells, as it happens in neural precursors of the neural tube (Iacopetti et al 1999). We have shown that hair cell generation in the cristae begins already at day 4 (see chapter 1 and 3), and by E5 numerous Atoh1 positive hair cells are present in

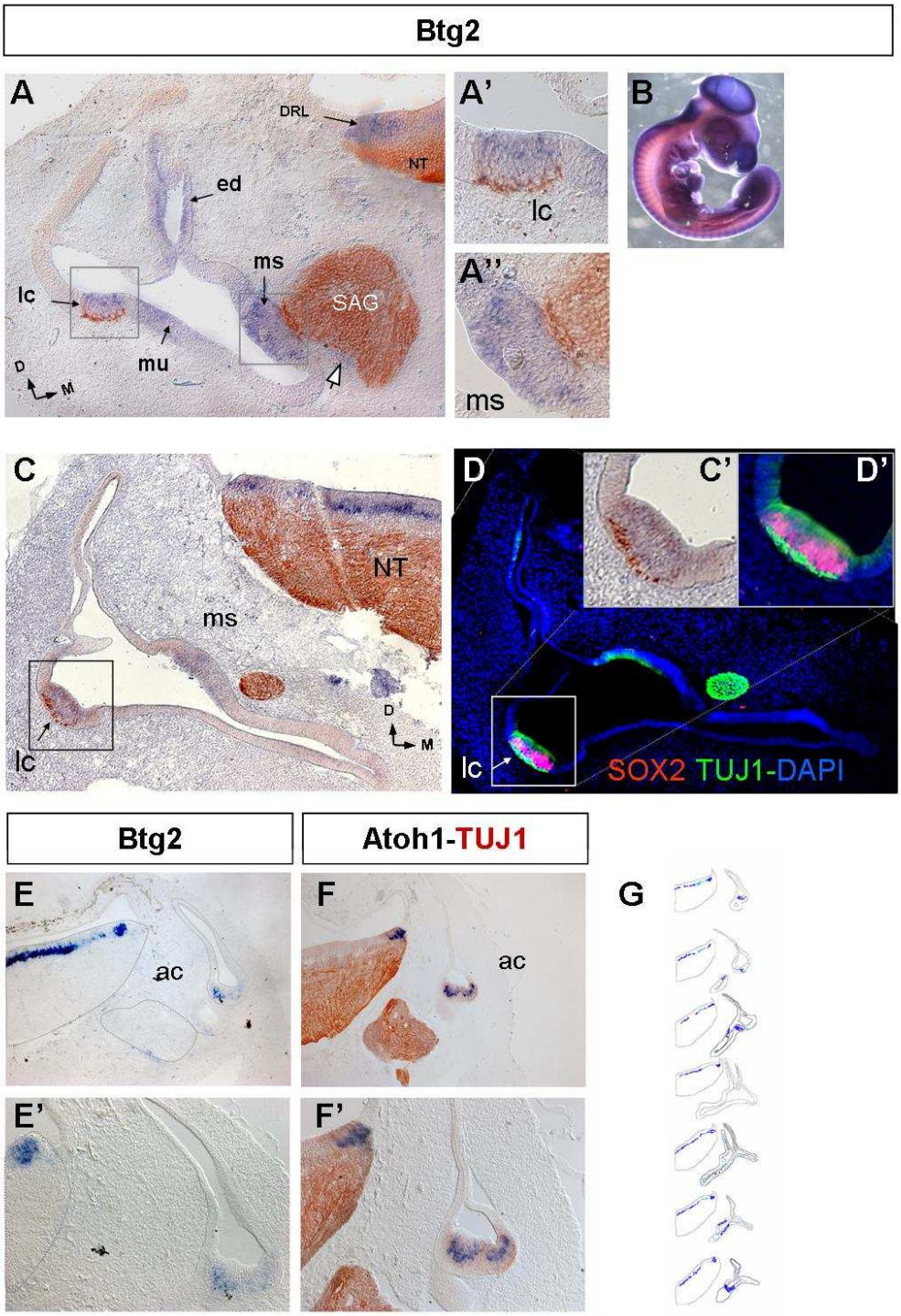

Figure 4-3. Btg2 expression at E4-5 and E5

A: Cryostat section through the inner ear at E4.5 processed for ISH with Btg2 probe. The section was double labelled with Tuj1 antibody (red signal). The plane of the section is not completely transversal, and the orientation is indicated at the bottom-left corner of the figure. Black arrows point to the sites of expression of Btg2 in the endolymphatic duct (ed), dorsal rombic lip (DRL) and in the vestibular sensory patches: lateral crista (lc), macula utricularis (mu), macula sacularis (ms). Empty arrow indicates cells expressing Btg2 in the SAG. A': Enlarged view of the lateral crista (lc). A'': Enlarged view of the macula sacularis (ms). B: Lateral view of an E4.5 embryo processed for whole mount ISH with the Btg2 probe.

C: Transversal section through the inner ear at E5 processed for ISH with Btg2 probe. The section was double labelled with Tuj1 antibody (red signal). The orientation is indicated at the bottom-right corner of the figure

D: Section adjacent to that shown in C, immunostained with anti-Sox2 (red signal) and TUJ1 (green signal) antibodies.

C'-D': enlarged view of the lateral crista from C and D respectively.

these organs. This is illustrated here for the anterior crista, showing in parallel with Btg2 transversal sections of stage-matched inner ears probed for Atoh1 (Fig. 4-3 G-G'). This shows that, Btg2 was expressed in the sensory organs concomitantly with Atoh1, but seems to be downregulated as more hair cells are generated (see below). Up to this stage, neither Btg2 nor Atoh1 expression was detected in the basilar papilla, but Btg2 expression was detected in the paratympanic organ (not shown).

Btg2 expression at early stages at embryonic day 6-5

Btg2 was analysed at later stages of development, when hair cells are already differentiating in the vestibular portion, and begin to form and differentiate in the auditory basilar papilla (Bartolami et al., 1991). A low low magnification view of a transversal section at E6-5, showed that expression of Btg2 in the cristae was greatly reduced, while remained high in the macula saculi, as shown in the (Fig. 4-4 A). Additionaly, we double stained this section on top of the ISH with anti-Islet-1 and MyoVIIa, and the merged imaged (where Btg2 signal was pseudocoloured in blue) is shown in Fig. 4-4 A'. This image revealed the presence of numerous MyoVIIa positive hair-cells in the lateral crista (green in Fig. 4-4 A') while Islet-1 staining indicated the prosensory auditory region of the basilar papilla (red in Fig. 4-4 A'). Btg2 was only detected in the macula sacularis, and at very low levels in the distal basilar papilla (see below). This downregulation from the lateral crista resembles the situation in the CVG where Btg2 was downregulated in differentiated TUJ1 positive cells. A similar situation was observed in the anterior cristae, although some cells expressing Btg2 could be still observed (Fig. 4-4 B). Here, the number and density of MyoVIIa positive cells seemed to be lower than in the lateral cristae (Fig. 4-4 B').

Remarkably, the expression of Btg2 was only detected in a few isolated cells within the basilar papilla, most of them located in its distal region (Fig. 4-4 A). This domain was populated by auditory sensory progenitors labelled with Islet-1 antibody (Fig. 4-4 A'). Hair cell differentiation was just initiated as evidenced by the few MyoVIIa positive cells that were not present in this section (not shown).

In summary, Btg2 was expressed in neural progenitors of the CVG, and in prosensory progenitors of the vestibular cristae and maculae. In vestibular organs Btg2 onset of expression preceded that on Atoh1, it was maintained upon the onset of Atoh1, expression, and it was downregulated as sensory organ differentiation progressed.

However, the observation that Btg2 was not expressed in early auditory progenitors was puzzling. Given that Btg family members play similar functions in other systems and they are functionally redundant (Park et al, 2004), we wondered whether another member of the family could be accomplishing "Btg functions" in those early progenitors of auditory sensory hair cells. Btg1 is the closest related member to Btg2 in terms of gene sequence and protein functions (Matsuda et al 2001). In order to determine its possible role in the auditory progenitors, we analysed Btg1 mRNA expression at E6-5. Btg1 was indeed expressed in the basilar papilla and also in other sites of the inner ear, with a distinct expression pattern. These results are described in detail below.

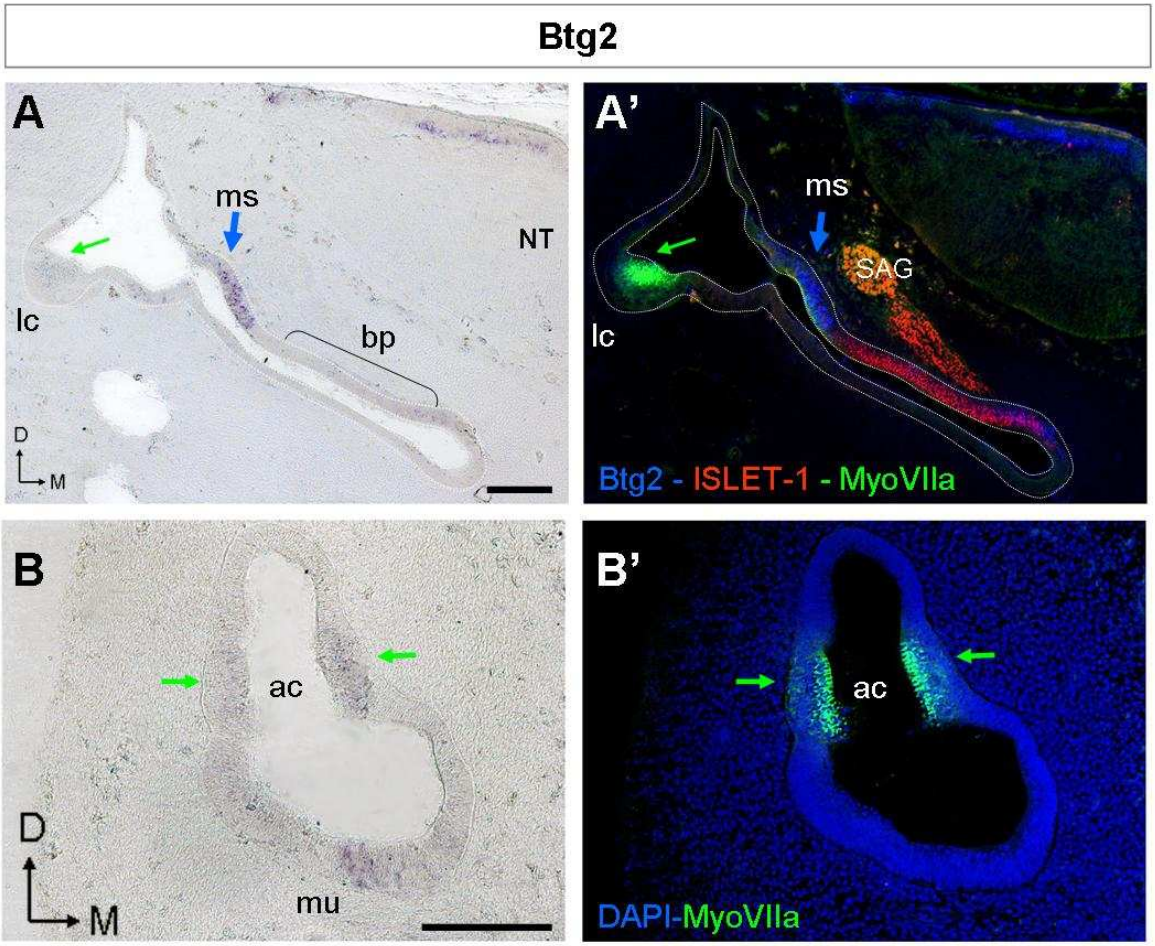

Figure 4-4. Btg2 expression at E6-5
A-A': Transversal section through the inner ear at E6-5. A: Shows ISH with Btg2 probe. Blue arrow indicates expression in the macula sacularis. Green arrow indicates the lateral crista.
A': The same section was processed for IHC with anti-Islet1 (red signal) and MyoVIIa (green signal). ISH for Id2 is shown in pseudocolour (blue). Blue arrow indicates expression of Btg2 in the macula sacularis. Green arrow indicates the hair cells in the lateral crista
B-B': Transversal section through the inner ear at E6-5 at the level of the anterior crista (ac), processed for Btg2 ISH (A') and Myo VIIa IHC (B'). Green arrows point to the sensory tissue of the anterior crista. B: Bright field image showing Btg2 ISH. Note that image is shown at higher magnification than A-A', and orientation is indicated at the bottom-left corner. B':Immunofluorescence image showing MyoVIIa expression in hair cells (green signal).

Differential expression of Btg1 and Btg2 at embryonic day 6-5

Adjacent transversal sections through the basilar papilla showed that Btg1 and Btg2 were expressed in a few cells in the distal part, probably corresponding to the macula lagena location (Fig. 4-5 A-B). Expression in the rest of the cochlear duct was very weak for both genes, and only sparse and weakly-positive cells were detected (Fig. 4-5 A-B). Surprisingly, Btg1 was strongly expressed in the SAG, where Btg2 is abstent (see below). In addition, Btg1 was also expressed in a U-shaped fashion in the surrounding condensing mesenchyme that will form the otic capsule (Fig. 4-5 B).

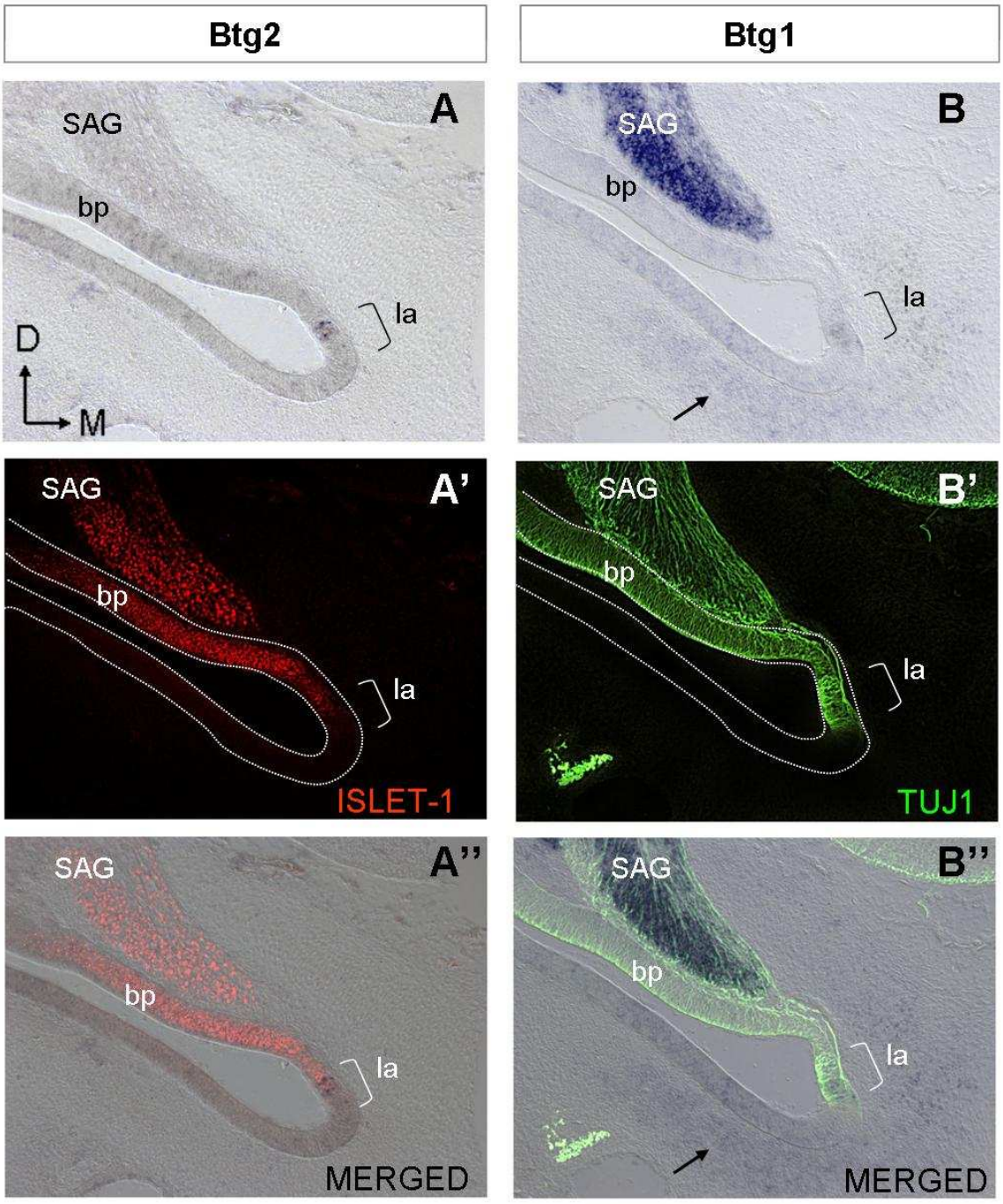

Figure 4-5. Differential expression of Btg2 and Btg1 at E6-5
A-B'': Transversal sections through the distal basilar papilla at E6-5. Orienation is shown in A.
A-A'': Section processed for Btg2 ISH (A) and Islet1 IHC (A').
B-B'': Adjacent section processed for Btg1 ISH (B) and TUJ1 IHC (B').
A''-B'': merged images. Arrow in A'' indicates expression of Btg1 in the periotic mesenchyme.
Abbreviations: basilar papila (bp), Statoachoustic Ganglion (SAG), macula lagena (la)

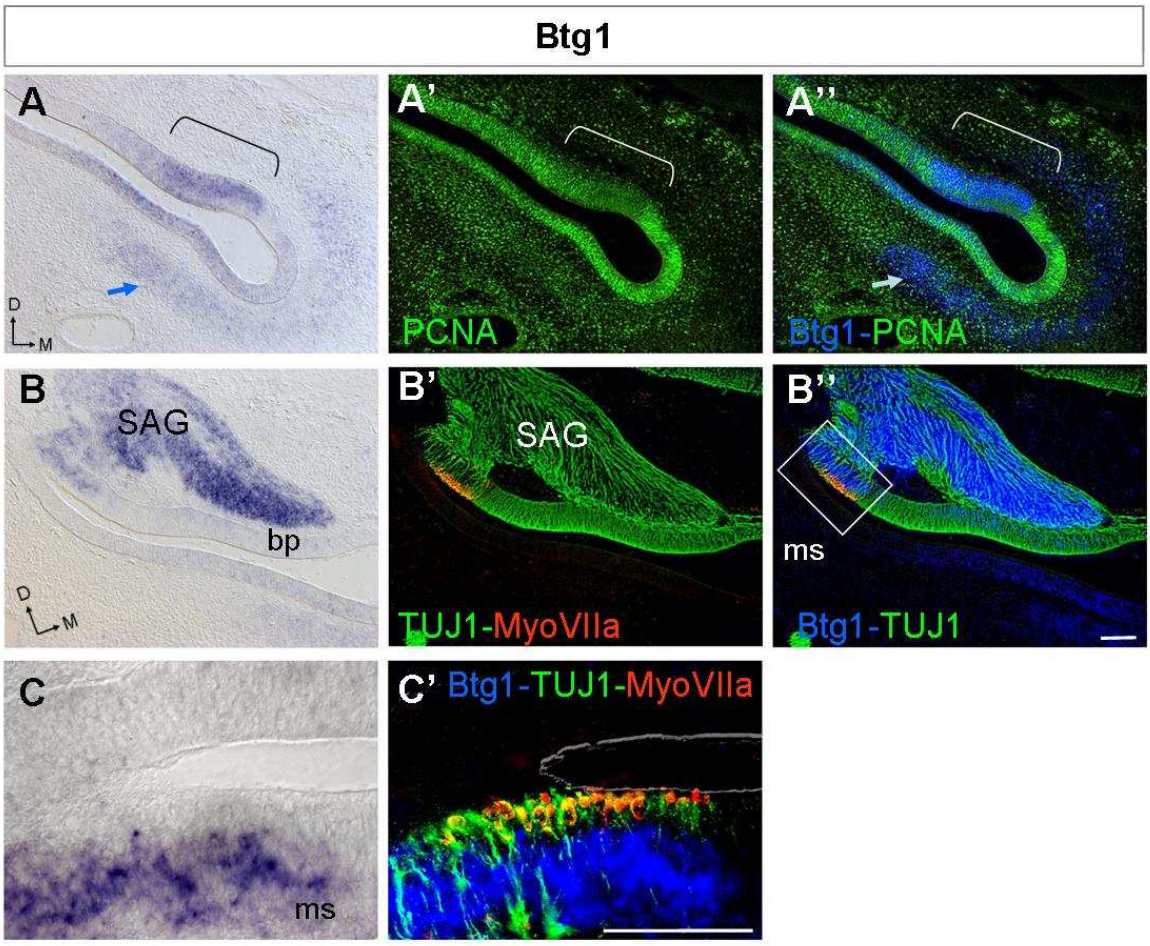

Figure 4-6. Btg1 expression at E6-5
A-A'': Transversal sections through the distal basilar papilla at E6-5. Orienation is shown in A. ISH signal for Btg1. Blue arrow points to expression in the periotic mesenchyme. Braquet indicates the area of Btg1 expression in the otic epithelia. A': The same section was processed for PCNA IHC (green nuclei). Note that the otic epithelia indicated by the braquet shows lower levels of PCNA immunostaining. A'': Merged image, with Btg1 pseudocoloured in blue.
B-B'': Transversal sections through the proximal basilar papilla and macula sacularis at E6-5. Orienation is shown in B. ISH signal for Btg1. B': The same section was processed for IHC with TUJ1 (green) and MyoVIIa (red) antibodies. B'': Merged image, with Btg1 pseudocoloured in blue.
C-C': High magnification view of the area indicated in B'', corresponding to the macula sacularis. Note that the image was rotated, in order to show the hair cells facing up (red signal C').

At E6-5, proliferating auditory sensory progenitors are present along the proximal-distal axis of the basilar papilla, and can be labelled by different markers including Bmp4, Sox2 and Islet-1 (Oh and Wu, 1996, Neves et al 2007). We took advantage of that, and double labelled those sections with antibodies for Islet-1 (Btg2 section, Fig. 4-5 A') and Tuj1 (Btg1 section Fig. 4-5 B'). This allowed us to localize not only the sensory progenitors in the basilar papilla but also neuronal cells in the SAG. The merged images confirmed that the few Btg1 and Btg2 positives cells corresponded to the macula lagena, and that the sensory auditory region was devoid of Btg1-2 expression (Fig. 4-5 A'-B'). Moreover, we could also determine that the strong Btg1 expression was located in sensory neurons of the SAG (Fig. 4-5 A'-B'). It is worth mentioning that we also stained these sections for MyoVIIa, and found no positive cells in

this region (not shown), consisitent with the delayed order of differentiation in the basilar papilla compared to vestibular organs (see MyoVIaI expression in the lateral crista in Fig. 4-4, and see below). Also, Islet-1 positive cells located in the proximal part of the papilla were devoid of Btg1-2 expression (not shown).

In spite of this, analysis of serial sections through the inner ear at E6-5, showed that Btg1 was expressed in a group of cells located in the distal portion of the auditory basilar papilla (Fig. 4-6 A). Btg1 expression was concentrated in the cells that were in contact with the basal lamina, and seemed to be excluded from cells located towards the lumen of the duct (Fig. 4-6 A).

Hair cell appearance and differentiation in the basila papilla is thought to occur first in the medial surface of this structure, and both, cell cycle exit and early differentiation has been shown to occur first in the distal region (Katayama, Corwin. 1989)(Bartolami et al 1991). This suggests that Btg1 occurred in the site of hair cell generation. Other genes have been shown to be differentially expressed in the distal region of the basilar papilla at similar stages, like FGF-19 (Sanchez-Calderon, et al. 2007b), and as mentioned above, staining of adjacent sections showed only few sparse cells expressing low levels of Btg2 (not shown).

Then, Btg1 probed sections were double labelled with the the proliferating cell-antigen PCNA, as a way to estimate the proliferative activity of the epithelium (Hall and Woods 1990).). The results indicated that the proximal domain (where slet-1 positive cells are located, see above Fig. 4-5 A') retain their proliferative capacity as evaluated by strong PCNA staining, whereas a spot of reduced signal was present in the distal area (Fig. 4-6 A'). Merged images confirmed that this area of reduced PCNA immunoreativity corresponded to the area of Btg1 expression (Fig. 4-6 A''),

Expression of Btg1 in the basilar papilla suggested that this gene was upregulated in the sensory patch upon hair cell generation. Moreover, Btg1 seemed to be excluded from the layer of luminal cells, where hair cells are located. Indeed, this was the case in the vestibular part of the inner ear at E6-5, where MyoVII positive cells were already visible. Strong Btg1 expression was found in the macula saculi, where it was also excluded from luminal cells (Fig. 4-6 B). Immunostaining those sections with MyoVIIa and TUJ1 demonstrated that those cells correspond to differentiating hair cells in the innervated macula

(Fig. 4-6 A'). The merged image showed that Btg1 was also highly expressed in the vestibular portion of the SAG, and confirmed that it was excluded from hair cells (Fig. 4-6 B'). This is also illustrated at high magnification in Fig. 4-6 C-C'.

Adjacent sections probed for Btg2 showed that it was not expressed in the ganglion (not shown), while it was maintained high in the macula (see above). In addition, analysis of serial sections at this same stage, revealed that Btg1 was expressed at low levels in the macula of the utricle, and only limited expression was detected in the three sensory cristae (not shown).

Chapter 5

Discussion

The experiments described in this thesis were aimed at studying the function of BMP4 during the early stages of development of the inner ear sensory organs. For this purpose, we performed functional experiments of gain and loss of function of BMP4, and studied the expression pattern and regulation of potential target genes, including members of Id and Btg gene families that were analysed in detail.

The resuts show that *Bmp4* anticipates the nascence of sensory patches and *Atoh1* expression, and that BMP-inhibition by Noggin increased the size of the sensory patches, together with the number of *Atoh1*-positive cells. The excess of BMP4 induced cell death and reduced the number of sensory progenitors, suggesting that a tight regulation of BMP activity in the prosensory patches is critical for survival of this progenitor population. These experiments unveiled an endogenous BMP4 activity that maintains otic progenitors in an undifferentiated state and which modulation results in the regulation of hair cell production.

The analysis of the Id family of proteins revealed a highly dynamic expression pattern in the developing inner ear, and their regulation by the BMP pathway. Id1-3 genes are expressed in the sensory patches anticipating Atoh1, and they are downregulated in hair cells at the time of hair cell differentiation. Id1-3 genes are also expressed in the periotic mesenchyme adjacent to the prosensory patches, and in the epithelia surrounding the vestibular cristae at stages of hair cell differentiation. Experiments with cultured otic vesicles showed that the expression of Id1-3 was rapidly induced by BMP4 in a dose-dependent manner, being abolished by Noggin. Dorsomorphin blocked endogenous Id expression, indicating that Smad activity is required for Id1-3 expression. Moreover, overexpression of a constitutively active BMPRI-a in the otic epithelium increased the levels of P-SMAD and Id1-3 expression in a cell autonomous way. These experiments suggest that Id expression may be part of the mechanism by which BMP signalling maintains sensory committed progenitors in an undifferentiated state.

Finally, the analysis of the expression of the Btg2 in the developing inner ear shows that Btg2 is transiently expressed in the prosensory regions, anticipating Atoh1 expression. Then, it is downregulated from the sensory domains as they mature. Also, Btg2 is transiently expressed in the otic precursors of the SAG. In addition, a closely related gene, Btg1 was expressed in the sensory elements of the inner ear and in postmitotic neurons of the CVG.

Taken together, these results suggest that BMP4 and BMP-inhibition regulate the generation of hair-cells: a high BMP4 activity would drive progenitors out of specification and into cell-cycle and apoptosis, whereas suppression of BMP-activity would allow hair-cell specification and cell-cycle withdrawal. We

speculate that the balance between BMP4 and BMP-inhibition is important at terminal division, by selecting between hair-cell specification and the self renewal of precursors. Also, we suggest that members of the Id family of proteins, and the gene Btg2, are part of the intracellular machinery used by BMP in the regulation of sensory development.

Expression of BMP4 and prosensory genes during hair-cell specification

The basic functional unit of the ear consists of mechano-transducing hair-cells (HCs), supporting cells, and primary afferent neurons. Those elements develop in a stereotyped manner with variations among animal species. Cell fate determination in the inner ear is sequential and coordinated in a precise spatial pattern. First cells to be determined are the neuroblasts that delaminate from the otic epithelium, at otic cup and placode stages. Then sensory patches emerge and give rise to hair-cells (HCs) and supporting cells.

Gene expression studies showed that all presumptive sensory organs initially express *Bmp4* and members of the Notch signalling system, a pattern that although not exactly is conserved in mouse and fish (Cole et al., 2000; Mowbray et al., 2001; Oh et al., 1996; Sanchez-Calderon et al., 2004; Wu and Oh, 1996). Before studying the functions of BMP4, we studied the expression pattern of *Bmp4* along with that of unambiguous markers of early specification of hair-cells like *Atoh1*. Proneural genes are crucial in the specification and differentiation of neuronal and sensory precursors (Bertrand et al., 2002). *Atoh1* is a proneural gene that belongs to the Atonal-like family of basic-helix-loop-helix transcription factors (Bertrand et al., 2002). Using gain- and loss-of-function approaches in mouse and chick embryos, it has been well established that *Atoh1* is necessary and sufficient to generate hair-cells from otic sensory domains (Woods et al., 2004, 2008). We analyzed the expression of *Atoh1* in order to get an early readout of hair-cell specification and to describe its relation with BMP4 signaling. The results show that *Atoh1* expression first appears at the anterior and posterior *cristae* between day 3.5 and 4 of development (HH22 to HH24), and it is preceded by about one day by the expression of *Bmp4*, *Lfng* and *Fgf10*. Moreover, ISH experiments showed that potential BMP target genes like all four members of the Id family of genes and the gene Btg2, were all expressed in the prosensory anterior and posterior patches, foreshadowing *Atoh1* expression (see below).

BMP4 regulates the number of prosensory progenitors and hair-cells

The experiments reported in the first chapter show that culturing E3 otic vesicles for 20 h in the presence of BMP4 produced a reduction in the size of the prosensory patches, as evaluated by the expression domains of prosensory-expressed genes -like Lfng, *Fgf10* and *Bmp4*-. In addition, BMP4 treated vesicles also showed reduced number of cells expressing the proneural hair-cell determination gene Atoh1. In parallel, BMP4 increased cell death within the prosensory domains. On the contrary, BMP4-inhibition expanded sensory patches and the number of Atoh1-positive cells.

Our experiments point to the notion that BMP4 regulates the generation of hair-cells by acting through a mechanism that regulates proliferation and specification of hair cell progenitors. High levels of BMP4 reduce the number of prosensory precursors by inducing cell death on actively proliferating cells (see below), whereas low levels of BMP (Noggin) favours hair-cell specification.

The fact that Noggin induced more hair-cells suggests that endogenous BMP4 maintains a negative pressure on differentiation and on the generation of hair-cells. In this context, Wang et al., (2004) showed that null mice for *Hmx2* and *Hmx3* display altered expression of inner ear markers at E11.5 such as expansion of *Bmp4* and *Dlx5*, and the number of apoptotic bodies and mitotic figures in the utricular and saccular maculae at E15.5 are much higher in mutant embryos. BMP4 expansion, although with reduced expression in mutants, coincides with reduction of hair-cell population. It is tempting to suggest that the balance between BMP4 activation and BMP4-inhibition regulates the final size of sensory patches through the regulation of the pool of progenitors.

However, this is not the only effect of BMP4 in hair-cell production since BMP-inhibition by Noggin is able to increase the number of *Atoh1*-positive cells without the need of cell proliferation, as shown in both 6h and 18h incubation experiments. This indicates that BMP-inhibition also favours cell specification (see below, Id expression). The simplest explanation for these results is that BMP4 tends to prevent cell specification and drive cells to enter the cell-cycle, whereas anti-BMP signaling would allow cell-specification as illustrated in the diagram of Fig 5.1. Otic progenitors undergo several cycles of cell division and progressive specialization to generate first neurons and then sensory cells. A connection must exist between cell-division and cell fate, in one hand, and extrinsic and intrinsic factors in the other so to generate different cell types at the appropriate time and locations (Cayouette et al., 2003; Livesey and Cepko, 2001). The balance between BMP4 and inhibitory signals, and probably other growth factors, would set a steady-state whereby progenitor activity and cell specification is coupled to generate the adequate number of hair-cells. The function of BMP4 described here is reminiscent of that described in dorsal telencephalic progenitors, where exogenous BMP4 reduces the production of interneurons in dorsolateral wall explants and inhibited their increase by Shh. BMP signaling inhibition with a dominant-negative BMP receptor virus increased the production of interneurons, even if Shh was blocked. The control of progenitor cell populations by BMPs has been also reported in other developmental systems, as in the neural tube by Liu et al. (2004).

An alternative explanation for the observed effects of BMP4 and Noggin would be that they occur in different domains, so that BMP4 would induce cell-death *within* the sensory domain and inhibit the surrounding epithelium to become sensory. Hence, Noggin would act by recruitment of neighboring epithelium into sensory fate (see below Id). Further knowledge on the specific properties of sensory-competent and sensory-determined domains are necessary to further analyse this possibility.

In addition, it may be possible that BMP4 may have other effects beyond those analyzed here and not excluded by our experiments. The instructive role for BMP4 for the prosensory domain has not yet direct supporting evidence. Our experiments indicate that BMP4 is not able to re-specify the otic epithelium to sensory fate, but they also suggest that the pro-apoptotic effect occurs at the prosensory domains. Studies by Lim et al. (2005) with BMPR dominant-negative expression show that it does not disrupt diencephalic patterning, but caused craniofacial, eye, and neural tube closure defects, suggesting that patterning and regulation of cell survival by apoptosis may be dissociated for the BMP signal and at particular stages of development. We are aware that our experiments do not address the possible effects of BMP4 on early patterning or those on occurring during more advanced stages of cell differentiation, which are not exclusive with our observations and indeed very possible (Li, et al. 2005).

BMP4 effect in the endogenous expression of Bmp4

Exogenous BMP4 was able to down-regulate *Bmp4* expression after 24h in the presence of recombinant-BMP4. This opens the question of whether the observed effects of exogenous BMP4 or Noggin may be caused by the opposite changes in the transcription of the *Bmp4* gene. This possibility is unlikely because recombinant BMP4 or Noggin are acting ubiquitously on the explants, independently on BMP4 transcription. Therefore, the effects observed in our experiments are probably not related to endogenous transcription but to the added exogenous factors. The diminished expression of *Bmp4* could be caused by down-regulation of expression and/or also by death of Bmp4-expressing cells, but this problem was not further explored. In the mouse retina, forced expression of *Msx*2, a target of BMP-signaling, induce suppression of *Bmp4* expression and cell death (Wu et al., 2003).

On the other hand, there are several secreted BMP-binding proteins that sequester and antagonize the biological effects of BMPs. Noggin is expressed transiently in the tissue surrounding the otic cup and vesicle, but it does not co-express with *Bmp4* in the epithelium (Chang et al., 1999). In developing organs, BMPs and BMP antagonists are often detected in adjacent domains (Brunet et al., 1998; Reshef et al., 1998). Therefore, both autoregulatory loops and neighboring inhibition may balance BMP4 activity in the sensory patches, not excluding the possible action of Noggin on other BMPs.

In conclusion, we propose that BMP4 has a specific role in controlling the number of hair-cells within the sensory patches. This function is exerted through the regulation of the fate of cell progenitors that populate prosensory domains, which are induced to die by high levels of BMP4, or allowed to commit to sensory fate when BMP-signalling is blocked. The experiments probably reflect the behaviour of the system at two extremes, BMP4 suppression and BMP4 excess. Normal development, most probably, requires a delicate balance between BMP4 in one side, and counteracting factors like BMP-inhibitors and other growth factors in the other, in order to maintain the balance between uncommitted progenitors and cell determination.

Id genes are direct targets of BMP4 in the inner ear

The regulation of Id gene transcription by BMP has been extensively studied in many systems (Ruzinova and Benezra, 2003). Also, BMPs and Ids have been shown to be coexpressed in several tissues during development (Yokoda. 2001) but this functional link has not been explored before in the inner ear.

Recent studies have reported the expression pattern of Id genes at early stages of development in the chick, but nor they focused on the inner ear, neither they reached the stages of sensory organ formation ((Kee, Bronner-Fraser. 2001a; Kee, Bronner-Fraser. 2001b; Kee, Bronner-Fraser. 2001c). In mammalian species, it has been shown that transcripts for *Id1*, *Id2*, *and Id3* are expressed in the otic vesicle of mice as early as E11.5 and in the cochlea in rats at postnatal day 1 (P1) (Jen et at., 1997; Lin et al., 2003). During the progress of this thesis another study analyzed the expression and function of Id genes during the development of the mouse cochlea (Jones et al., 2006, see below).

Here we provide the first detailed description of Id genes in the chick otocyst during the initial stages of sensory organ formation, between E3 and E7. The analysis comprised both the auditory and the vestibular otic eoithelia and the comparison with known molecular markers of prosensory regions.

Id expression in proliferative prosensory progenitors

The results show that all four members of the Id family are expressed in the otocyst at with similar patterns throughout the developmemt of the inner ear. This similarity suggests that those Id genes may share regulatory mechanisms, as it has been proposed in other model systems (Hollnagel, et al. 1999), (rev. in Yokoda 2001). Whether Id4 expression also parallels that of Id1-3 in later stages remains to be determined. As demonstrated by in situ hybridization studies in other species, a general feature of Id gene expression during development is that the distributions of Id1, Id2 and Id3 are to a great extent overlapping, while Id4 shows a unique expression pattern during embryogenesis (Riechmann and Sablitzky, 1995; Jen et al., 1996, 1997, rev in Yokoda 2001). In agreement with this, only Id1-3 were found to be expressed in the mouse cochlea (Jones et al., 2006).

At embryonic day 3, Id1-3 are expressed in the otic epithelium in broad regions that include the prosensory patches, and precede by at least one day the expression of Atoh1. This expression coincides with that of Bmp4 in the prosensory patches, and with some of the regions where Bmp7 is expressed (Wu and Oh, 1996). Remarkably, when in the presence of BMP4, otic vesicles exhibited a rapid and widespread induction of all Id1-3 genes, in a dose-dependent manner. This was accompanied with an increase in the levels of P-SMAD1-5-8 immunoreactivity, all across the otic epithelia that correlated with Id gene induction. Induction of Id1-3 was clearly observed after 4 hours post incubation. The initiation of the response was even faster judging from quantitative RT-PCR experiments that revealed that Id1-2 mRNA levels were already higher than control after 1 h of treatment with BMP4. The rapid response of Id to BMP exposure has been documemted in several other

systems, even in the absence of de novo protein synthesis (Norton 2000). This suggests strongly that Id1-3 are direct transcriptional targets of BMPs, and the results reported here indicate that this is the case in the otic epithelium.

As mentioned above, Id expression was induced in virtually the entire otic epithelium upon BMP4 incubation. This shows that the whole otocyst is competent to respond to BMP4, and is consistent with the reported ubiquitous expression of BMPRI-a in the inner ear (Chang et al., 2002). In chapter 2 we have shown that BMP4 addition was not able to generate ectopic sensory patches in the otic vesicle, and that cell death induced by BMP4 was restricted to the prosensory domains (see above). The fact that the entire epithelium is able to respond to BMP4 but functional effects are restricted, suggests that there must be additional factors that set the competence of the functional response to BMP4.

Perhaps the experiments with Noggin are the most relevant in relation to the understanding of the in vivo roles of BMP signaling in prosensory specification. As mentioned above, the effects observed with Noggin incubation reveal an endogenous Bmp activity that regulates the size of the sensory patch, as well as the generation of hair cells. It is remarkable that Id expression in the prosensory epithelium was completely abolished after 4 hours of incubation with Noggin, suggesting that this endogenous Bmp signaling is required to maintain Id expression at this stage, and may be important for regulating hair cell specification and cell cycle exit (see below). This also shows that Id mRNA turn-over is very fast, as it can be expected for its role as an immediate response gene (Norton 1998, 2000). Like most other proteins encoded by early response genes, Id proteins rapidly turnover in the cell, having a reported half-life of 20-60 min, depending on the cell type (Deed et al., 1996; Bounpheng et al., 1999a).

Furthermore, the pharmacological compound Dorsomorphin phenocopied the effects of Noggin. Dorsomorphin has been shown to specifically block the phosphorylation of R-Smad mediated by type I BMP receptors, withouth affecting other signaling pathways that can be activated by BMP (Anderson, Darshan. 2008; Cuny, et al. 2008; Hao, et al. 2008; Yu, et al. 2008). Therefore, this experiment shows that Id expression in the otocyst depends strictly on the steady activation of the Smad signaling pathway. In agreemet with that, overexpression of constitutively activated BMPRI-a (ca-ALK3) in the otic epithelia was able to induce Id1-3 expression and Smad phosphorylation within 6 hours post electroporation. P-SMAD activity was detected in electroporated cells and not in the neighbouring cells. Neither Id gene upregulation was present in other than those that overexpressed the activated receptor, showing that the effect of BMP activation is cell autonomous. This also suggests that activation of the BMP-Smad pathway induces Id, but it is unlikely that it reinforces BMP expression, since that would be reflected in a paracrine effect with P-SMAD and Id activation in neighboring cells. It would be interesting to test if ca-ALK3 overexpression actually reduces the expression of BMP-4 in an autoregularoty loop, as it was suggested by experiments with cultured otic vesicles.

Preliminary studies utilizing SU54202, a general inhibitor of receptor tyrosine kinases involved in FGF mediated signaling (Mohammadi et al 1997) suggest that Id expression does not depend on FGF signaling for its maintenance (not shown), suggesting that in spite of co-expression of various FGFs and BMP4 in the prosensory patches (Sanchez-Calderon, Martin-Partido, Hidalgo-Sanchez. 2002; Alsina, et al. 2004; Sanchez-Calderon, et al. 2007b), there is no cross talk between these two signaling pathways at the level of Id expression.

In summary, Id1-3 are expressed in the same areas than BMPs in the otocyst at E3. Id expression domains include the BMP4 prosensory patches, and their expression is correlated with higher levels of endogenous BMP activity, as evaluated by P-SMAD1-5-8 immunreactivity in vivo and in culture. Overactivation of the BMP pathway in two ways, either by addition of BMP4 to cultured otocyst, or by elecroporation of a constitutively active receptor, induces a rapid increase in P-SMAD1-5-8 and Id1-3 mRNA levels. In contrast, incubation with Noggin or Dorsomorphin rapidily abolished Id1-3 expression. Altogether, these results showed that Id1-3 expression in the E3 otocyst is dependent of BMP-induced P-SMAD1-5-8 activity and suggests that endogenous BMP4 in the presumptive sensory cristae is contributing to maintainence of the expression of Id1-3 genes.

Id1, Id2, and Id3 are BMP-induced immediate early genes in ES cells, where they inhibit cell differentiation, and the main mechanism postulated for this effect is based on their interaction with bHLH proteins (Hollnagel, et al. 1999). Prosensory progenitors are highly proliferative at this stage and express genes typically associated with pluripotent stem cells like Sox2 (Neves et al., 2007). Therefore, BMP-mediated increase in Id expression within the prosensory patches, may function as a molecular switch for lineage specification by functionally blocking the activity of certain bHLH transcription factors, Atoh1 in the sensory patches. Such a role is supported by expression data that shows the withradwal of Id genes upon differentiation of hair cells at later stages of development and by the effect of Id blockade by Noggin or Dorsomoirphin.

Additionaly, BMP4 could be also stimulating cell cycle progression in sensory progenitors through Ids at this stage. Id proteins promote cell cycle progression either indirectly through the inhibition of proneural gene activity, or directly interacting with components of the cell cell cycle machinery as the cyclin-kinase inhibitors p21 and p27kip (Chassot, et al. 2007). Indeed, p27kip has been show to play a key role in the cell cycle exit of the mammalian cochlea (Chen, Segil. 1999), although its role in the chick inner ear has not been studied. In this context, we propose that BMP4 may function at this stage to maintain self-renewal and proliferation of prosensory progenitors, through the regulation of Id genes.

Id regulation of hair cell development

Downregulation of Id in the developing hair cells of the chick inner ear

Down-regulation of Id genes is necessary for terminal differentiation in many developmental processes, including myogenesis, myelopoiesis, lymphopoiesis,

bone morphogenesis, glomerular mesangial cell development, and trophoblast development (rev. in Norton 2000, Yokoda 2001, Ruzinova and Benezra 2003). Likewise, we found that Id1-3 are downregulated from the sensory organs as they differentitate.

The temporal sequence of sensory organ maturation in the chick inner ear has been analysed before, but based either on the histological differentiation or in the appearance of markers of hair cell differentiation (Knowlton. 1967)(Bartolami, Goodyear, Richardson. 1991; Katayama, Corwin. 1993). In this work we have also documented the sequence of Atoh1 expression, which shows that molecular differentiation of sensory patches follows a dorsal/cristae to ventral/auditory sequence. The experiments show also that Id genes follow a similar pattern, being sequentially down-regulated from the *Atoh1* postive regions. While Id2-3 withdrew from the vestibular system at E5, they were still expressed in the proliferative sensory epithelium of the basilar papilla where Atoh1 was not yet expressed. Later on, upon the onset of Atoh1 in the basilar papilla, Id genes were down-regulated. This regional and temporal pattern was further demonstrated by comparing Id expression with that of differentiation genes like MyoVIIa. All three Id genes explored Id1-3, showed a similar behaviour. Together, this data indicates that Id withdrawal from the sensory patches follows the dorsal to ventral sequence of maturation of the sensory organs (Bartolami, Goodyear, Richardson. 1991)

While this project was in progress, Jones et al (2006) reported a similar pattern of expression in the mouse cochlea. These authors performed in situ hybridizations in mouse embryos at the onset of hair cell differentiation in the mouse cochlea, and at the time point at which differentiated hair cells can be identified based on both morphology and *Atoh1* expression (Jones et al., 2006, Woods et al., 2004). They found that transcripts for Id1, Id2, and Id3 were expressed in very similar patterns in several different regions of the cochlea, including the developing cochlear duct, some mesenchymal cells, and the developing spiral ganglion. Expression of all three Ids was observed throughout all turns of the cochlear duct, indicating broad expression of Ids within the developing sensory epithelium. When individual cell types can be identified within the developing organ of Corti, and concomitantly with *Atoh1* expression, the basal turn of the cochlea showed down-regulation of all three Ids in developing hair cells (Jones et al., 2006). These same authors have shown that overexpression of Id3 in the prosensory progenitors of the mouse cochlea, by electroporation of isolated cochleas. produced a reduction in the number of hair cells generated in that system (Jones et al., 2006). This provides a strong argument for the notion that Id down-regulation is a requirement for hair cell differentiation.

There is no evidence about the mechanism of action of Id proteins in the inner ear. One possibility is that Ids are indirectly involved in the commitment of hair cells through the inhibition of Atoh1/E-protein heterodimers. This suggestion is supported by the fact that the inhibition of differentiation by the functional inactivation of bHLH transcription factors by Ids has been reported during different developmental events including myogenesis and neurogenesis (Benezra et al., 1990; Jan and Jan, 1993; Lee, 1997; Martinsen and Bronner-

Fraser, 1998). Additionaly, and not alternatively, Id proteins may negatively regulate the activity of Atoh1, by increasing the rate of degradation of this proneural protein, as it has been shown for Neurog1 and MyoD in other model systems (Vinals, Ventura. 2004; Vinals, et al. 2004).

BMP regulation of hair cell generation and Id genes

The results of the present report show that Id expression in the chick prosensory cristae is dependent on BMP-Smad signalling. This is illustrated by the effects of Noggin on hair cell development. Incubation of otic vesicles with Noggin increases the number of *Atoh1* positive hair cells even without expansion of the progenitor population. If Id genes are indeed repressing the activity of *Atoh1*, this would be a feasible mechanism to explain the increase in the number of hair cells upon Noggin incubation. Blocking BMP signalling would reduce Id expression, allowing Atoh1 activity to occur.

In summary, both expression profiles and the functional experiments in the mouse cochlea, and our experiments with cultured otic vesicles, are consistent with the hypothesis that downregulation of the Id1-3 genes is a key step in hair cell development

The differential regulation of the expression of Ids in sensory epithelia throughout development

It is worth noting that the BMP4 expression is maintained in sensory patches during hair cell differentiation (Wu and Oh, 1996). The downregulation of Id expression from the sensory patches at stages of hair cell differentiation, therefore, coexists with BMP4 expression in these domains. On the other hand, during hair cell differentiation, Id genes are expressed in some way complementary to Bmp4. We directly showed that exclusion by analyzing adjacent sections with Bmp4 and Id2-3 probes. Therefore, the early co-expression of Ids with Bmp4 and the fact that Id1-3 expression required BMP-SMAD activity, contrasts with their segregation from Bmp4 expression domains at later stages. And this raises interesting questions about the possible differential regulation of Id genes throughout development. The mechanisms that regulate the expression of Id genes at these late stages in the sensory organs of the inner ear are unknown. In particular, it is not clear how the expression of Id is downregulated in Atoh1-postive cells, or whether BMP-SMAD activity is still required for Id expression at these stages. Preliminary experiments with Noggin (not shown) indicate that in the auditory epithelium Id expression at E5 is not dependent on BMP, indicating that there must be other factors (intrinsic or extrinsic) that maintain Id expression. Experiments to address these questions have not been carried out yet, but we can speculate on several possibilities.

Within the otocyst, P-SMAD1-5-8 levels are higher in the prosensory patches, where BMP4 and Ids are co-expressed before hair cell generation. Assuming that this BMP-SMAD dependency is maintained at later stages, this suggests that the activity of BMP must be reduced, or inhibited in cells that downregulate Id expression. In this context, it would be interesting to determine whether or not

BMP activity is actually downregulated together with Id, for example by analysing the in vivo pattern of P-SMAD1-5-8 immunoreactivity at E5 and E7. In this context, it is interesting to note that Bmp4 expression is downregulated from differentiating hair cells in the chick vestibular organs (our results at E7, and Wu and Oh 1996).

Several mechanisms could account for such a reduction in BMP activity, such as extracellular antagonists of BMP. There are several BMP inhibitors that have been studied in this context and their gain or loss of function induced impairement of ear development. However, none of them has been shown to be expressed in the prosensory regions at these critical stages of hair cell differentiation. One exception is DAN, which has been shown to be expressed in the dorsal-lateral otic epithelium (Gerlach-Bank et al., 2004). However, siRNA inhibition of this gene in the chick otocyst mainly affected the endolymphatic apparatus suggesting that it does not play a key role in sensory development (Gerlach-Bank et al., 2004).

Several intracelullar inhibitors can modulate BMP activity, such as Smad6 or Smad7, or Smurfs (Massagué 2005). However, there is no data available about the expression of these molecules in the otic epithelia. Whereas Smad7 acts as a general inhibitor of TGF-b family member signalling pathway, Smad6 preferentially blocks BMP signalling (Ten Dijke et al 2003, Massague 2005). I-Smads are potently induced by TGF-b family members, and may thus participate in a negative feedback loop to control the intensity and duration of TGF-b signaling (Afrakhte et al., 1998; Ishisaki et al.1998). In this connection, Smad3 has been shown to promote differentiation in the neural tube, by repressing the expression of Id1 and Id2, promoting the parallel expression of proneural genes (Garcia-Campmany, Marti. 2007). Members of the TGFb family of ligands are also expressed in the mouse inner ear (Paradies et al., 1998), although their role in relation with sensory organ formation has not been addressed. Whether TGFb signalling can modulate regulation of Ids in hair cells remains to be determined.

Other signalling pathways have been shown to modulate BMP activity at the level of Smad1-5-8, including IGF, FGF and Wnt (Rev. in Massagué 2003, Eivers et al 2008). This cross-talk occurs at the level of Smad1-5-8 proteins, and relies in different phosphorylation sites within the Smad proteins, in the so-called linker region (Massague 2003). In this way, FGF signals can reduce the intensity of the BMP activity, and this could contribute to silence BMP target genes (Massague 2003, Eivers et al 2008). Several FGF ligands have been shown to be expressed localized in the prosensory regions, including Fgf-10 and Fgf19 (Sanchez-Calderon, Martin-Partido, Hidalgo-Sanchez. 2002; Alsina, et al. 2004; Sanchez-Calderon, et al. 2007a), and could account for the inhibition of Bmp signalling, and downregulation of Id during hair cell development. In our hands, however, FGFs do not seem to interfere with Id expression at early stages, which does not imply that may be doing so during hair cell differentiation.

Some studies indicate that Ids are negatively regulated by cyclin-dependent kinase 2 (Cdk2) (Hara et al., 1997). Moreover, it has been reported that Cdk2 is

involved in the development of the organ of Corti (Malgrange et al., 2003), suggesting a possible link in the inner ear (Jones et al. 2006). However, there is no information about Cdk2 expression or function in the chick inner ear.

Interestingly, alternate longer splice variants have been identified for Id1 and Id3 (referred to as Id1L and Id3L). These splice variants are negative regulators of Id1 and Id3 function (Springhorn et al., 1994; Deed et al., 1996). Therefore, another possible mechanism for Id regulation in the cochlea would be a switch from expression of Id1/Id3 to Id1L/Id3L in developing hair cells. A study of the spatiotemporal pattern of Id splice variant expression could lead to valuable data regarding this hypothesis.

BMP4 target genes and apoptosis

BMPs are involved in proliferation and differentiation during development (Hogan, 1996; Mehler et al., 1997), as well as in programmed cell death, including the neural tube and the retina (Coucouvanis and Martin, 1999; Furuta et al., 1997; Golden et al., 1999; Liu et al., 2004; Trousse et al., 2001). Apoptosis is important for eliminating regressing tissue regions during embryonic development and several *Bmp* genes are involved in the regulation of apoptosis in the developing limb (Chen and Zhao, 1998; Merino et al., 1998; Pizette and Niswander, 1999). The effects of BMPs on precursor cell populations and its potential role in regulating the size of specific cell populations have not been discovered until very recently. BMP-signaling at early stages of chick neural tube development induces roof-plate cell fate, accompanied by an increase of programmed cell death and a repression of neuronal differentiation. At later stages, however, dorsal progenitor cells lose their competence to generate roof-plate cells in response to BMP-signaling and generate dorsal interneurons (Liu et al., 2004).

Normally occurring cell death during the development of the vertebrate inner ear has been known for a long time (reviewed by Leon et al., 2004). During otic placode and otic vesicle stages, cell death coincides with areas of cell proliferation, the closure of the otic pore, the formation of the endolymphatic duct and with the development of sensory organs (Alvarez and Navascues, 1990; Fekete et al., 1997; Lang et al., 2000; Sanz et al., 1999). Our results propose a causal connection between *Bmp4* expression and cell death that occur at the sensory patches (Alvarez and Navascues, 1990; Cole et al., 2000; Lang et al., 2000), and suggests that it is part of a mechanism required for establishing the appropriate size of the sensory patches and the number of hair-cells. BMP4 regulation of programmed cell death has been also documented in cranial neural crest development and limb development (Graham et al., 1994; Graham et al., 1996; Merino et al., 1999; Zuzarte-Luis and Hurle, 2002).

Very little is known about the mechanisms that couple BMPs with cell death. It has been postulated that different members of the *Msx* genes may mediate different aspects of BMP signaling during development of the neural tube, including dorsal cell fates and cell death, which in this case was associated with *Msx*1 (Liu et al., 2004). *Msx*1 promotes cell death in neural folds, neural crest

and animal cap assays (Tribulo et al., 2003). A study on ventricular zone progenitor cells has suggested that *Msx*2 and the cyclin-dependent kinase inhibitor p21 mediate cell death induced by BMP4 (Israsena and Kessler, 2002). $p21^{WAF1/Cip1}$ activity results in hypo-phosphorylation of Rb (retinoblastoma tumor suppressor protein) and arrest of the cells in the G0/G1 phase. The expression patterns of *Msx1* and *Msx2* coincide in the otocyst and are consistent with the local activation of BMP4, but they also suggest a differential role of *Msx*1 or *Msx2*. Exogenous BMP4 increased *Msx1* transcription in the *cristae* and the endolymphatic duct, but not *Msx2*, suggesting that *Msx1* could be one direct target of BMP4 involved with the effects described in the paper.

Id genes have been shown to promote apoptosis in a variety of experimental models..Transgenic mice with targeted Id1 expression in T cells showed a 96% reduction in the total number of thymocytes due to massive apoptosis (Kim et al., 1999). Moreover, cytotoxicity of Id proteins has been demonstrated by overexpression of Id proteins in cultured cells established from myeloid progenitors (Florio et al., 1998), osteosarcoma (Florio et al., 1998), myoblasts (Jen et al., 1992), primary cultures of embryonic fibrobroblasts (Norton and Atherton, 1998), neonatal cardiac myocytes (Tanaka et al., 1998), astrocytes (Andres-Barquin et al.1999), and cortical neural progenitors (Toma et al.,2000).

It has generally been believed that an inappropriately strong signal for proliferation induces apoptosis (White, 1996), and in some of the above mentiones cases, the induction of apoptosis was invariably correlated with the ability of Id protein to promote the cell cycle S phase, highlighting the close coupling between the G1 progression and apoptosis functions of Id proteins (Norton, Atherton. 1998). Our results indicate that most of the apoptotic cells detected had incorporated BrDU, and therefore had undergone at least some period of S phase. Thus, over-expression of Id proteins upon excess BMP-4 addition may well contribute to this effect.

Btg2 and Btg1 expression during development of the inner ear

Little is known about the molecular mechanism of action of Btg genes in vivo, and most of the information comes from studies done in isolated cells in culture. To gain insights on the function of these genes during development, and since Btg1 and Btg2 are the more closely related genes in the family, we sought to analyse and compare their expression profile during early stages of chick development. We present here the first detailed description of Btg1 and Btg2 during early embryogenesis in the chick. Btg1 and Btg2 genes display differential expression patterns that suggest their connection with various events during early development. Overlapping pattern in particular places suggest that they can act redundantly in certain processes. However, distinctive, restricted and transient expression domains may offer insights into the specific in vivo regulation and function of these genes (Appendix II).

Moreover, we describe for the first time the expression of a Btg family member during the development of the inner ear, and speculate here about the possible role of Btg2 in this sensory organ.

Experiments *in vitro* and *in vivo* using different animal species have shown that Btg2 is expressed in differentiating neuronal cells that switch from proliferative to neuron-generating division (Iacopetti et al. 1994, Iacopetti et al. 1999), and that Btg2 overexpression results in negative regulation of the cell cycle and induction of neuronal differentiation (Malatesta et al. 2000, Corrente et al. 2002, el-Ghissassi et al. 2002, Canzoniere et al. 2004). Our results about Btg2 expression in the neural tube are consistent with those reported in mice (see Apendix II). In the inner ear, Btg2 expression was associated with areas where neural and sensory progenitos are located, and this expression appears to be restricted to a subpopulation of cells within those areas. Also, Btg2 was downregulated from the sensory neurons of the SAG as they differentiate, similar to the dowregulation of Btg2 in the TUJ1 positive cells of the neural tube (Iacopetti et al. 1999), suggesting that Btg2 could be playing a role in terminal divisions of neural progenitors of the inner ear as well. The mechanisms by which Btg2 may contribute to this process are unknown, but *in vivo* overexpression of Btg2 in mouse neuroepithelial cells affected cerebellar development by a mechanism that involves a double action of Btg2: 1) down-regulation of cyclin D1 promoting cell cycle exit, and 2) stimulation of Math1 promoter activity, stimulating differentiation of cerebellar progenitors located in the hindbrain rhombic lip (Canzoniere et al. 2004). Btg2 is also expressed in the vestibular prosensory progenitors, and seems to be dowregulated as hair cells are developed. It would be interesting to determine wheter Btg2 plays a similar role in the negative modulation of cell cycle in sensory progenitors, in promoting the initial expression of Atoh1 in the nascent hair cells, or both.

Btg1 expression has been associated with cells that have arrested cell cycle (Matsuda et al. 2001, Tirone. 2001). In this context, the role of Btg1 gene in myoblast differentiation has been well documented by *in vitro* studies, and seems to rely on its ability to interact with and stimulate the activity of several myogenic factors, including the bHLH transcription factor MyoD (Marchal et al. 1995, Rodier et al. 1999, Busson et al. 2005). Our results in the whole embryo reveal a high expression of Btg1 in the myotome, further suggesting this interaction *in vivo* (appendix II). Interestingly, Btg1 was strongly expressed in the differentiating neurons of the SAG at E6-5, when Btg2 has dowregulated. These cells are already postimtotic and characterized for the expression of proneural bHLH transcription factors (Alsina et al. 2004)

In the otic epithelia, Btg1 was expressed in the sensory patches, in regions where cell proliferation is reduced and not in the proliferating sensory progenitors. Moreover, Btg1 expression was absent in the developing hair cells, and seemed to be present in the supporting cell layer. It is worth mentioning that cell proliferation still occurs at this stage in the sensory organs (not shown), probably reflecting the presence of actively proliferating progenitors. The lack of markers for early supporting cell fate precludes an unequivocal identification of such cell type at this stage. However, given that Btg1 is absent from the proliferative Islet-1 positive progenitors in the basilar papilla, but present in the place where hair cells are being generated, it is tempting to suggest that Btg1 could be an early marker of supporting cell type.

Chapter 6

CONCLUSIONS

1) Bmp4 anticipated the expression of Atoh1, and it was expressed in the otic vesicle at two distinct anterior and posterior patches. The expression of Bmp4 showed sharp boundaries and extended throughout most cells of the domain.

2) BMP4-inhibition increases the number of Atoh1-positive sensory cells, whereas the BMP4 excess inhibits hair-cell output in the otic vesicle. Otic vesicles incubated with the BMP antagonist Noggin, showed increased number of Atoh1-positive cells, the opposite effect being caused by BMP4. Neither BMP-4 nor Noggin interfered with the cellular or molecular mechanisms that generate the singling out and spacing of hair-cells.

3) Endogenous BMP signaling regulates the size of the sensory patches and the expression of Bmp4*.* Otic vesicles incubated with the BMP antagonist Noggin, showed larger prosensory regions than control, as evaluated by the expression domain size of LnFg, Fgf-10 and Bmp4. On the contrary, otic vesicles incubated with BMP4 showed smaller prosensory regions.

4) BMP4 induced cell death in proliferating progenitors within the prosensory domains. Otic vesicles incubated with BMP4 showed increased levels of picnotic nuclei, and apoptotic cells evaluated by the TUNEL method, respect to control condition. This increment was only present within LnFg positive domains, and most of the TUNEL positive cells were also positive fot BrDU. This effect is accompanied by an increase of Msx1 but not Msx2 in the sensory cristae

5) BMP4 induced cell death is dependent of S-phase progression. The density of TUNEL-positive cells induced by BMP4 was reduced by the presence of hydroxy-urea (HU), a compound that reduces DNA-synthesis by inhibiting the ribonucleoside diphosphate reductase.

6) Endogenous BMP signalling regulates hair cell specification. Incubation with Noggin produced and increased of the maximum diameter of Atoh1-positive patches above control levels after only 6 hours. This rapid effect suggests that expansion of the progenitor pool is not the only explanation for increased hair cell generation by Noggin.

7) Id1-4 genes are expressed in the prosensory region of the otic vesicle. Expression of all Id members included the prosensory regions, and persists until the onset of Atoh1 expression, up to E4. Id1-3 genes are also expressed in the periotic mesenchyme that originates the otic capsule of the dorsal otocyst.

8) Id1-3 genes are down-regulated from developing hair cells that that express Atoh1, and from differentiating hair cells expressing MyoVIIa. Expression remains high in the adjacent epithelia, and probably some supporting cells.

9) BMP signalling regulates Id expression in the inner ear. Otic vesicles incubated with the BMP antagonist Noggin, rapidly and strongly reduced Id1-3 gene expression in all regions of the otocyst, including prosensory patches and periotic mesenchyme. On the contrary, HH20-22 otic vesicles incubated with BMP-4 showed an induction of Id gene expression in all regions of the otic vesicle and periotic mesenchyme, indicating that the whole tissue is competent to receive the BMP signal.

10) Id gene regulation by BMP is dependent on SMAD signalling. Endogenous expression of Id1-3 at E3 correlates with higher levels of P-SMAD1-5-8 in vivo and in cultured otic vesicles. The inhibitor of SMAD1-5-8 phosphorylation Dorsomorphin, greatly reduced Id1-3 gene expression in all regions of the otocyst, including prosensory patches and periotic mesenchyme. Overexpression of a constitutively active form the the BMPRI-a in vivo, produced a cell autonomous increase in the levels of P-SMAD1-5-8, together with Id1-3 expression. Otic vesicles incubated with BMP4 increased the levels of P-SMAD1-5-8 in parallel with Id1-3 induction.

11) Btg1 and Btg2 are differentially expressed during early chick development. Btg2 was expressed associated with neurogenic regions during inner ear development. Btg2 was downregulated from differentiated cells in the SAG.

12) Btg2 expression during inner ear development was restricted and associated with vestibular prosensory regions and Btg1 is expressed in the developing inner ear associated with differentiating neurons of the SAG, supporting cells, and differentiating cells in the otic capsule surrounding the basilar papilla.

13) Taken together, these results show that the BMP signalling pathway regulates hair cell generation by controlling the activity and survival of prosensory progenitors. Id genes may play a role in this process as they are targets of the BMP-Smad activation. The restricted expression of Btg genes suggests that they may also contruibute to sensory organ development.

Chapter 7

MATERIALS AND METHODS

Embryos

Fertilized hens' eggs (Granja Gibert, Tarragona, Spain) were incubated at 38ºC for designated times and embryos were staged according to Hamburger and Hamilton, 1951. Embryos were dissected from the yolk and fixed by immersion in 4% paraformaldehyde in phospate-buffered saline (4%PFA/PBS) overnight at 4ºC.

Embryo collection

-Dissect the embryos in cold 1x PBS and fix them 4h for 0 to 15ss embryos and overnight for more than 15ss at 4°C in 4% Paraformaldehyde/PBS.

-Wash 3x5min in PBT (PBS-0.1% Tween20)

-Dehydrate in successive solutions of increasing Methanol concentrations (in PBT):

10min in 25% Methanol/PBT

10min in 50% Methanol/PBT

10min in 75% Methanol/PBT

10min in 100% Methanol/PBT

-Store in cold 100% Methanol at -20°C

Whole mount In situ Hybridization

-Rehydrate embryos:

10min in 75% Methanol/PBT

10min in 50% Methanol/PBT

10min in 25% Methanol/PBT

3x5min in PBT

-Treat with proteinase K 10μg/ml in PBT (stock: 10mg/ml)

The time of treatment depends on the size of the embryo:

-Post-fix with 4%Paraformaldehyde 0.2% Glutaraldehyde in PBT, 20min room temperature (RT)

-3x5min washes in PBT

-Prehybridize in hybridization buffer at 70°C for 1h

-Dilute the probe in hybridization buffer (2μl/300ml)

-Denature the probe in hybridization buffer for 10min at 80°C

-Replace the prehybridization buffer for the denatured probe in hybridization buffer

-Hybridize overnight at 70°C

(Pre-warm wash buffers at 70°C)

-Wash with: 3x30min wash buffer I at 70°C

2x30min wash buffer II at 65°C

2x30min TBST at RT (TBST-0.1% Tween20)

Immunostaining

-Block for 30min at RT with 10%NGS in TBST at RT

-Incubate for 2h to overnight with anti-DIG-AP or anti-FLUO-AP in blocking solution [1:2000] at RT or 4ºC

-2x10min TBST at RT and overnight at 4°C

Developing

(It is performed in the dark)

-Preincubate in NTMT 4x15min at RT

-Incubate in staining solution at RT in the dark until the staining is developed. Change the solution frequently, if the color of the solution turns to red change it and keep the embryos at 4°C in a fresh staining solution.

-Stop staining by several washes in PBT. It is recommended to wash for 1 or 2 days in PBT at 4°C.

-Embed in 50% Glycerol /PBT

-Store in 100% Glycerol at 4°C

Solutions

Hybridization buffer

50% Deionized Formamide (FAD)

5xSSC pH 4.5

1% SDS

50 µl/ml yeast RNA

0.05 mg/ml heparin

Wash I

50% FAD

5x SSC pH4.5

1% SDS

Wash II

50% FAD

2x SSC pH4.5

NTMT

100mM NaCl

100mM TRIS pH9.5

50mM $MgCl_2$

0.1% Tween20

Developing solution

0.3mg/ml NBT 0.175mg/ml BCIP /NTMT

For 10ml NTNT: 45µl NBT 75mg/ml in DMF

35µl BCIP 50mg/ml in DMF

Cryostat sectioning

-Fix the embryos in 4% Paraformaldehyde/PBS at 4℃

-Wash 3x10min PBS at RT

-Cryoprotect in 15% Sucrose at 4℃

-Embed in pre-warmed 30% gelatine/15% sucrose at 37℃ rocking

-Prepare the blocks with pre-warmed 30% gelatine/15% sucrose at 37℃

-Freeze the blocks in pre-cold 2-Methylbutane at -80℃

To improve tissue preservation, then section at 20µm thickness using Superfrost Plus Slides (Fisher, Pittsburg) and store at − 20℃. The cryostat Leica CM 1510-1 was used for sectioning. Sections were used either for *in situ* hybridization and/or anti-BrdU staining.

Organotypic cultures of otic vesicles

Otic vesicles were dissected from E3.5-4 embryos corresponding to stage HH20-23, transferred into four-well culture plates (NUNC, Roskilde, Denmark) and incubated in DMEM at 37°C in a water-saturated atmosphere containing 5% CO_2 as described (Leon et al., 1995) unless otherwise stated. Additions were 1% fetal bovine serum (Bio Whittaker Europe), recombinant human BMP4 and Noggin (R&D) were used in culture at concentrations between 1 and 100 ng/ml for BMP4 and 0.1-1µg/ml for Noggin.

***BrdU experiments and TUNEL assay* in cultures of otic vesicles**

Otic vesicles were incubated with 10 µg/µl 5-Bromo-2´-deoxyuridine (Aldrich) for 2 hours prior to fixation. Otic vesicles were incubated in 2N HCl for 30 minutes, three times washed in Sodium Borate pH 8.9 and processed for immunohistochemistry. BrdU mAb BMC9318 (Roche) was used in whole-mount at 1:200. Distribution of apoptotic cells in the otic vesicle was determined by TdT-mediated dUTP nick-end labeling (TUNEL) of the fragmented DNA. Briefly, cultured otic vesicles were fixed for 2 hours with 4% paraformaldehyde in PBS and dehydrated by a series of graded methanol steps. After rehydration, otic vesicles were incubated with 10 µg/ml proteinase K (Sigma) for 2 minutes at room temperature and post fixed with 4% paraformaldehyde and 0.1% glutaraldehyde in PBS. The otic vesicles were then incubated with the

terminal deoxynucleotidyl-transferase labeling mix for 30 minutes at 37℃ (Roche) and subsequently with the reaction enzyme terminal deoxynucleotidyl-transferase (Roche) for 2 hours at 37℃. The reaction was stopped by incubation with 2 mM EDTA in PBS for 1 hour at 65℃. Fluorescein-labelled deoxynucleotides incorporated in apoptotic cells were visualized in a DMR Leica fluorescence microscope. Fluorescent pictures were converted to black and white images for better analysis.

TUNEL assay in sections

-Hydrate the cryostat sections with PBS 5min

-Incubate with 10µg/ml Proteinase K for 2min at RT

-Post-fix with 4% Paraformaldehyde 0.25% Glutaraldehyde in PBT

-Block endogenous peroxidase with 0.3% H2O2/PBS for 2min at RT

-Incubate the sections with TUNEL reaction mixture (Label Solution and Enzyme Solution, Roche-Applied Science) for 60min at 37℃ in a humidified atmosphere in the dark

-Wash 4x15min with PBT at RT

-Block the sections with 10% Horse Serum/PBT for 1h at RT in a humid atmosphere

-Incubate with anti-Fluorescein-POD (Roche) [1:300] for 2h at RT in a humid atmosphere

-Wash 4x15min with PBT at RT

-The signal can be amplified with Tyramide using Cyanine 5 as fluorophore (TSA™ PLUS, Perkin-Elmer). Dilute Cy5-Tyramide 1:50 in Amplification Reagent (TSATM kit). Apply 100µl of working solution per slide, add a coverslip to keep a minimum volume, incubate for 10 min at RT in a humidified atmosphere in the dark

-Wash 8x15min with PBT at RT

-Mount the sections with Mowiol

Keep the sections in the dark.

Apoptotic cells were analyzed under a confocal microscope Leica TCS SP2 or under a fluorescence microscope Leica MZFLIII.

Immunostaining in sections

-Hydrate the cryostat sections with PBS 5min

-Incubate with 10µg/ml proteinase K for 2min at RT

-Post-fix with 4% Paraformaldehyde 0.25% Glutaraldehyde in PBT

-Block the sections with 10% Horse Serum in PBT for 1h at RT. Use a humid atmosphere.

-Incubate with the primary antibody [1:400] overnight at 4℃ in a humid atmosphere

-Wash 3x15min with PBT

-Incubate with secondary antibody [1:800] in 10% Horse Serum/PBT at 4℃ in a humid atmosphere for 2h.

-Wash 3x15min with PBT

-Post-fix with 4% Paraformaldehyde

- Wash 2x5min with PBT

-Mount the sections with Mowiol

Keep the sections in the dark.

Sections were analyzed under a confocal microscope Leica TCS SP2 or under a fluorescence microscope Leica MZFLIII.

Quantitation of results

Quantitation of the size of sensory patches was done from digitalized photomicrographs that were processed in Adobe Photoshop. Whole-mounted otic vesicles were flat mounted and photographed like shown in Figure 3. Patches were drawn in Adobe Photoshop and the surface area expressed in pixels (arbitrary units). In some experiments, the size of the patches was estimated from sections and approximated by measuring the maximum length of the patch from defined sections of the sample (ImageJ, NIH free software). Lengths were measured in pixels, calibrated and converted into microns. Quantitation of cell proliferation was done by counting BrdU-positive cells from photomicrographs of identical magnification. A surface of epithelial section was selected in Photoshop and analyzed for the number of BrdU-positive nuclei. Total nuclear density was measured and the fraction of BrdU-positive nuclei calculated. Quantitation of cell death was done as follows: images were converted to grayscale and inverted in Adobe Photoshop to enhance the apoptotic cells like shown in Figure 6. Domains of cell death were identified and drawn first in BMP4-treated otic vesicles and equivalent domains in control or Noggin-treated otic vesicles were also drawn and analyzed. Density of apoptotic cells was estimated as the surface occupied by dark-fluorescent spots against background. Signal and background was set equal for all samples by setting identical backgrounds and setting the threshold for signal at fifty per-cent of the signal amplitude. This gave values for the fraction of surface area of otic vesicle that was fluorescent and expressed in %.

Values are expressed as means ± SE and Student´s t-test was used for statistics when appropriate.

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Appendix I

Pujades C, Kamaid A, Alsina B, Giraldez F.
BMP-signaling regulates the generation of hair-cells.
Dev Biol. 2006 Apr 1;292(1):55-67. Epub 2006 Feb 3.

Appendix II

Kamaid A, Giráldez F.
Btg1 and Btg2 gene expression during early chick development.
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