Retinoic Acid signaling mediates hair cell regeneration by repressing *p27kip* and *sox2* in supporting cells

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TESI DOCTORAL UPF / 2016

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A Chiara,
Ai miei genitori

Acknowledgments/Agradecimientos/Ringraziamenti

En primer lugar me gustaría agradecer sinceramente a Berta Alsina, directora del proyecto, por su esfuerzo y dedicación. Gracias, no solo por tu guía y tus consejos científicos, si no también por tu confianza, tu paciencia (imprescindible), tu motivación y tu entusiasmo. Aprendí mucho durante estos años gracias a ti.

Un enorme gracias a los Alsinas, Laura y Esteban. Gracias por el apoyo y los abrazos en los momentos duros, las risas, los viajes, las discusiones, las comidas, las aventuras, y el pan cortado en los momentos buenos.

Gracias especialmente a Alex, que empezó como el duro y serio post-doc siempre demasiado ocupado, hasta convertirse en un gran amigo.

Quiero agradecer todos los miembros y ex miembros del grupo de Biología del Desarrollo, por las infinitas pero utilísimas discusiones y por las risas: Cristina, Fernando, Laura, Esteban, Simone, Sylvia, Javi, Alex, Hector, Jelena, Adria, Ivan, Andrea, Laura T, Marta y Miquel. Gracias a todos!

Gracias a toda la gente que ha entrado en mi vida en estos años y ha compartido conmigo un momento, una birra o una emoción: Simo, Davide, Valeria, Maria Aurelia, Luca, Matteo, Jose, Paula, Giulia, Sonia, Umberto, Armida, Egidia, Silvia, Luis, Izma, Marco.

Obviamente gracias al grupo del basket para las horas de diversión, las peleas y algún dolor de espalda!

Infine, un grazie gigante alla mia sorellina Chiara che per quanto lontani possiamo essere, siamo sempre insieme. E ai miei genitori, Silvia e Claudio, perché non potrei essere ciò che sono senza di loro e i loro insegnamenti.

Abstract

Hair cell damage, as a result of several causes such as aging, acoustic aggression, and ototoxic drug exposure, provokes/causes hearing loss in humans, one of the major health problems in the actual society. In mammals damaged hair cells cannot regenerate, whereas lower vertebrates have retained the ability to replace damaged hair cells by inducing cell proliferation of supporting cells and/or their transdifferentiation. Retinoic Acid (RA) has been implicated in limb, heart, spinal cord and peripheral nervous system regeneration. Although little is known about its role in the inner ear, a combination of gene expression profiling, functional assays and cell-lineage tracing experiments allows us to highlight the essential role of the RA pathway in hair cell regeneration in zebrafish. After hair cell death, regeneration is impaired upon blockade of the RA pathway in both the inner ear and lateral line systems. RA pathway blockade results in a severe reduction of supporting cell proliferation. Moreover, the expression of RA pathway components is induced during neuromast and lateral crista hair cell regeneration, confirming the activation of the pathway. Finally, we demonstrate that RA is critical for downregulating *p27^{kip}* and *sox2* in supporting cells, allowing them to re-enter the cell cycle. Altogether, we uncover a new role of RA in the regeneration of hair cells that hopefully could be relevant in the development of future therapeutic strategies.

Resum

La mort o lesió de les cèl·lules ciliades, com a conseqüència de diversos factors com l'envelliment, el soroll ambiental elevat o l´ús de fàrmacs ototòxics, provoca sordesa, un dels problemes més importants en la societat actual. Els mamífers no tenen la capacitat de regenerar les cèl·lules ciliades. No

obstant, els vertebrats inferiors han mantingut la capacitat de substituir les cèl·lules ciliades afectades mitjançant la proliferació de les cèl·lules de suport i/o la seva transdiferenciació. S'ha descrit la via de senyalització de l'àcid retinoic (AR) com a un factor important durant el procés de regeneració de les extremitats, el cor, la medul·la espinal i el sistema nerviós. Tanmateix, el paper que aquest pugui tenir a l'orella interna és poc conegut. L'estudi del patró d'expressió gènica, anàlisis funcionals i seguiment del llinatge cel·lular, ens ha permès determinar la importància de l'AR durant el procés de regeneració de les cèl·lules ciliades en peix zebra. Després de la mort de les cèl·lules ciliades, el bloqueig de la via de l'AR tant a l'orella interna com a la línia lateral impedeix la seva regeneració i redueix significativament la proliferació de les cèl·lules de suport. Altrament, durant el procés de regeneració dels neuromasts i la cresta lateral s'indueix l'expressió dels components de la via de l'AR, confirmant l'activació d'aquesta via. Finalment, podem demostrar que l'AR és essencial per reduir l'expressió de $p27^{kip}$ i *sox2* a les cèl·lules de suport permetent que aquestes entrin de nou al cicle cel·lular. Com a conclusió, el meu treball ha permès descobrir una nova funció de l'AR durant la regeneració de les cèl·lules ciliades que podria ser rellevant pel desenvolupament de futures estratègies terapèutiques.

Preface

Hearing loss, resulting from aging, genetic predisposition or environmental exposure to noise or ototoxic drugs, is one of the most prevalent chronic conditions affecting older adults, affecting more than 50% of individuals over the age of 60. Most of hearing and many balance deficiencies spring from irreversible damage or loss of sensory hair cells. In mammals these cells are generated only during embryonic development and must last through a lifetime. To treat hair cell loss, two broad strategies can be envisioned: prevention and/or replacement. Pharmacological approaches for preventing hair cell loss have been carried out in model systems and in human patients. However, to treat individuals already suffering from hair cell loss, new strategies must be investigated.

On the other hand, non-mammalian vertebrates such as bird, frog and fish have retained the ability to generate new hair cells also in the adulthood. Understanding the mechanisms used by low vertebrates to regenerate hair cells and how mammals lost this capacity is fundamental for the design of new therapeutic strategies to treat hearing loss.

This thesis identifies the retinoic acid, a derivative of the vitamin A, as an essential signal for hair cell regeneration in zebrafish. In addition to its role in inner ear development, we demonstrate the requirement of RA pathway in both inner ear and lateral line hair cell regeneration. Our results provide new insights into the molecular mechanisms involved in this process in non-mammalians organisms, hoping that this knowledge would be transferrable to induce generation of new hair cell in mammals.

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1. INTRODUCTION

1.1 The Vertebrate ear

Hearing loss is one of the major health problems of the actual society. Approximately 50% of elderly people, 60 years or older, suffer of partial or complete hearing loss that impairs their ability of social interaction. This disability can be classified in conductive hearing loss, as a result of the loss of function of the outer or inner ear, or neurosensory hearing loss, as a consequence of a damage of auditory nerve or of the loss of sensory hair cells of the cochlea. Neurosensory hearing loss can be congenital (affecting up to 1 in 500 newborns (Smith *et al.*, 2005)) or acquired, as a consequence of aging, loud noises, drugs toxic for the auditory system, viruses and tumors. More than half of congenital hearing loss are inherited, and can be classified into syndromic and non-syndromic (Kochhar *et al.*, 2007). While syndromic hearing loss are part of a more complex phenotype, genetists are committed to identify genes and mutations responsible for non-syndromic hearing loss (Venkatesh *et al.*, 2015 for review).

The ear is responsible for the sense of hearing, that allows us to interact with the external world and communicate, but also for the sense of balance. Although is not considered one of the main senses we dispose of, and probably underestimated, the sense of balance is the one that is essential for life. Balance disorders span from mild/moderate outcome as vertigo, characterized by the sensation of spinning or having the room spinning around you, normally accompanied by nausea, to more severe outcomes as disequilibrium, the sensation of being off balance resulting in recurring falls in one direction, or as the complete inability to stand (Sando *et al.*, 2001).

The organ that detects sounds and aids in balance and body position is the ear that is composed by three portions: the outer, the middle and the inner ear (Fig.1). The inner ear is the portion in which sound stimuli and head position are elaborated and converted into neural signals and is the subject of this thesis (Bever and Fekete, 2002).

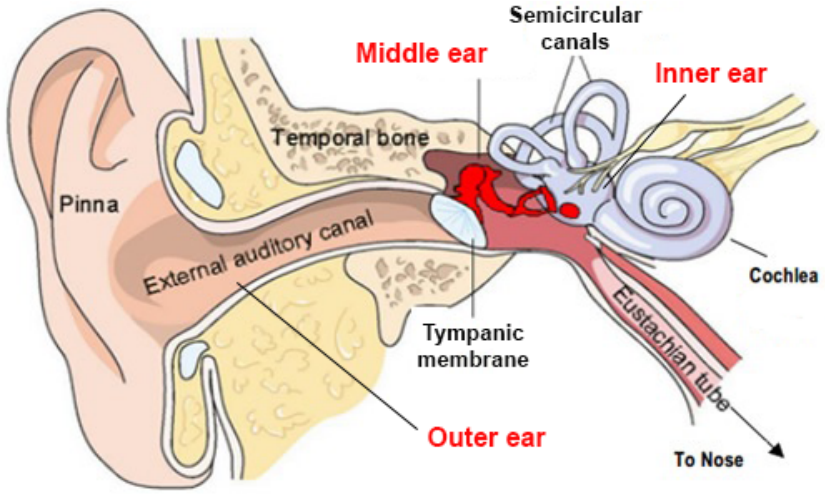

Figure 1: ***The vertebrate ear.*** *The vertebrate ear is composed by outer, middle and inner ear. Sound is collected by the outer ear through the auditory canal. The middle ear connects outer and inner ear and mediates the transformation of sound waves into mechanical vibrations of the fluid that fills the inner ear. The inner ear converts the mechanical stimuli into electrical signals that are conveyed through the acoustic nerve to the brain. The vestibular part collects information regarding angular and linear acceleration and sends them to the brain by vestibular nerve. Modified web image (http://www.news-medical.net/health/What-are-Balance-Disorders.aspx).*

The vertebrate inner ear is a complex 3D structure divided in two main parts called pars: the pars superior, placed dorsally, is highly conserved among vertebrates and consists of a central utricle and the semicircular canals. This pars is responsible for the sense of balance because contains a gravity-sensitive macula (placed in the central utricle), that detects linear acceleration in the horizontal axis, and three

rotation-sensitive cristae (placed in the ampullary connections between each semicircular canal and the utricle), that detect angular acceleration (Fig. 2). The ventral part of the inner ear, called pars inferior, is more specific to each class of vertebrate, but it typically consists of saccular or lagenear pouches, whose maculae have auditory functions, vestibular functions or both, and additional diverticula, such as the basilar papilla (in birds) or the organ of Corti (in mammals), specialized for hearing (Bever and Fekete, 2002) (Fig. 2).

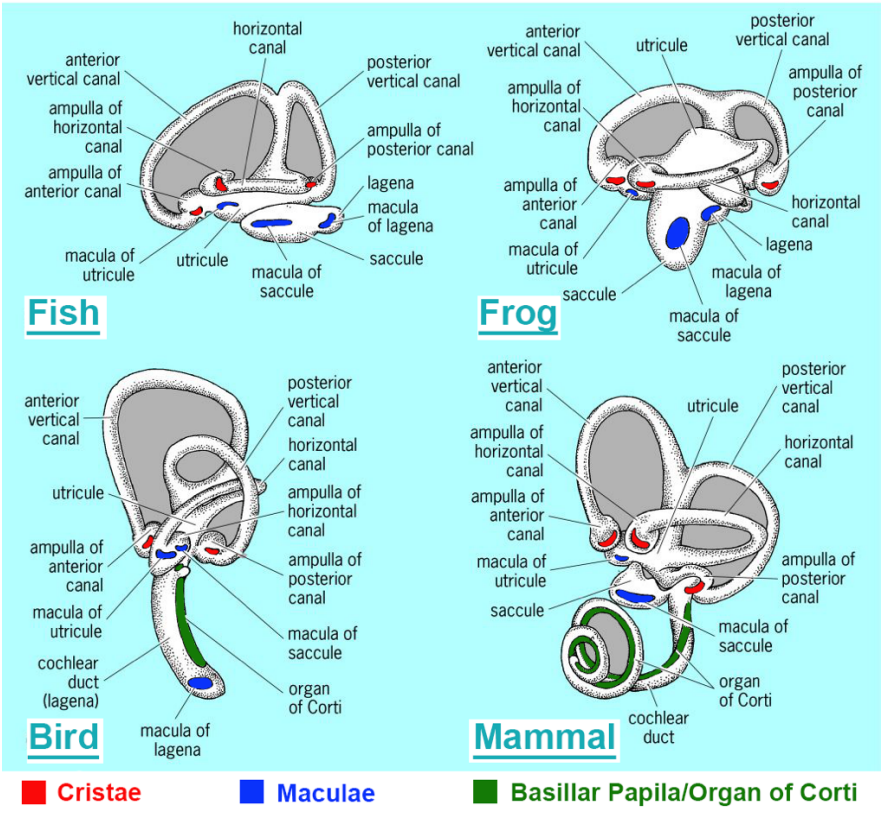

Figure 2: Schematic representation of adult inner ear of different vertebrates. *The pars superior, composed by a central utricule and the semicircular canals is highly conserved among species. The pars inferior, responsible for sound detection shows a prominent evolution. Modified web image (http://encyclopedia2.thefreedictionary.com).*

Sensory stimuli are captured by discrete and specialized structures of the inner ear called maculae and cristae. Although each patch has its own characteristic shape and polarity pattern, the core structure is highly conserved among species. These sensory domains are thickened, pseudostratified epitheliums, consisting of regular arrays of sensory hair cells (HCs) interspersed with non-sensory supporting cells (SCs) (Fig.3). Each patch is associated with sensory neurons whose cell bodies lie in a ganglion (the stato-acoustic ganglion – SAG) close beneath the ear epithelium. Mechanosensory HCs display a hair bundle protruding from the apical surface: this comprises a single kinocilium and a bundle of stereocilia that are immersed in a mobile gelatinous matrix that overlie each of the cristae (cupula) and maculae (otolithic membrane). The movement of these gelatinous structures provokes the deflection of the hair bundles that causes the opening mechanosensitive ion channels and the consequent generation of an electrical potential. The neurons from the SAG synapse with the HCs, sense the excitation and transmit the signal to the brainstem. Non-sensory SCs vary greatly in morphology and have several functions: they form the epithelial framework in which HCs are held, are involved in the maintenance and survival of the HCs and, more interestingly, in the last two decades SCs have been identified as essential for HC regeneration (Corwin and Cotanche, 1988; Ryals and Rubel, 1988; Haddon and Lewis, 1996; Presson *et al.*, 1996).

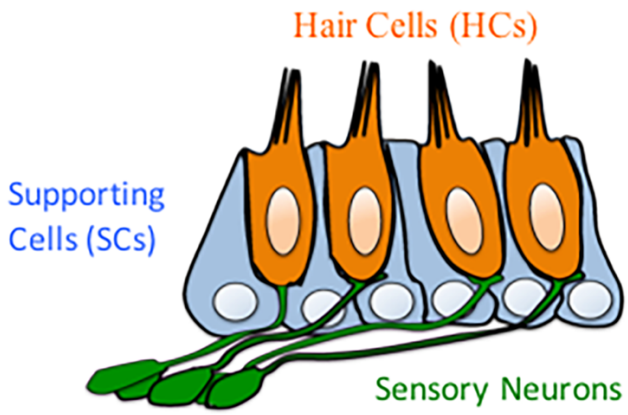

Figure 3: The Sensory Patch. *The inner ear sensory unit consists in hair cells (HCs, in orange) interspersed with supporting cells (SCs, in blue), and sensory neurons (in green) whose soma is located within the stato-acoustic ganglion (SAG).*

It is worth noting that most of hearing and many balance deficiencies spring from irreversible damage or loss of sensory HCs as a result of aging, genetic predisposition, viruses, tumors or environmental exposure to noise or ototoxic drugs (Beisel *et al.*, 2008). While in mammals these cells are generated only during embryonic development (Ruben, 1967) and must last thought a lifetime, other non-mammalian organisms such as bird, frog and fish have retained the ability generate new HCs also in the adulthood. For these reasons it became critical to study how those organisms regenerate sensory HCs and why mammals lost this ability.

1.2 The zebrafish as a model for studying hair cells

The auditory system of teleost fishes is composed only by two inner ears (no middle or external structures) each one consisting in three sensory otolithic end organs, the saccule, lagena and utricle (Bever and Fekete, 2002) and three cristae. Fishes do not have a specialized auditory organ like the mammalian cochlea or chick basilar papilla, but all the otolithic organs have both vestibular and auditory function. Despite the dual sensory capacity, studies in zebrafish suggest that the utricle is primarily a vestibular organ, the saccule is primarily responsible for sound detection, while lagena has roles in both orientation and hearing (Popper *et al.*, 2003; Kwak *et al.*, 2006; Khorevin, 2008). The utricle together with three semicircular canals form the pars superior and, as this anatomical structure is highly conserved among species, strongly resemble avian and mammalian counterparts. It is worth mentioning that during zebrafish development the basic configuration of the pars superiors, including the semicircular canals with ampullae and their sensory cristae, the utricle with sensory macula and overlying otolith, is already evident by the fifth day post-fertilization (Haddon and Lewis, 1996; Bever and Fekete, 2002). The homology with the mammalian structure and the early appearance in development positions the zebrafish inner ear pars superior as a good model for studying developmental processes and ototoxicity.

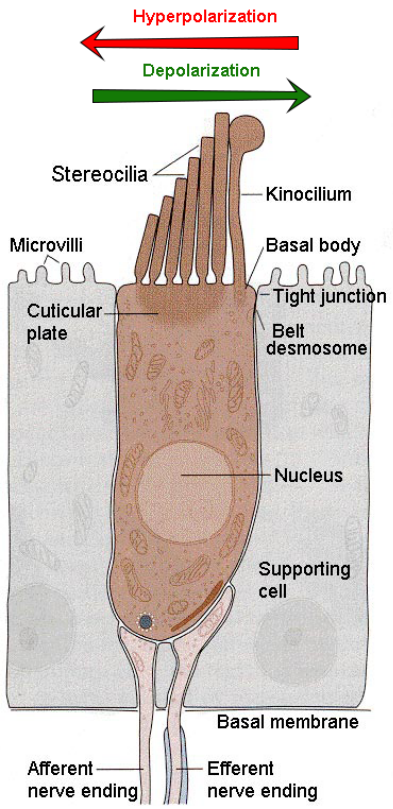

Figure 4: The Hair Cell. *Schematic drawing illustrating morphology and main anatomical features of the hair cell. Modified web image (http://www1.appstate.edu/~kms/classes/psy3203/Ear/Inner_Ear.htm)*

Teleost and mammalian HCs share many fundamental features: all are elongated epithelial cells that display a ciliary bundle on their apical surface composed by a single kinocilium and multiple stereocilia (Fig. 4). The kinocilium is placed at one side while stereocilia are positioned by size, with the longest ones closed to the kinocilium and the shorter one placed far away (Coffin *et al.*, 2004). The mechanotransduction ability is also conserved during evolution: is based on the deflection of the stereociliary bundle towards the kinocilium, leading to the opening of cation-selective channels and consequent cell

depolarization, or deflection away from the kinocilium that hyperpolarized the cell. Although the basic configuration and features are conserved, teleost and mammalian HCs present some morphological and physiological differences. For example, while teleost hair bundles are generally conical in shape, with a kinocilium and many rows of stereocilia, mammalian cochlear ciliary bundles lose the kinocilium upon maturation and fewer rows of stereocilia are arranged in a W-shape (reviewed Fettiplace and Hackney, 2006) (Fig. 5). Also, while HCs of teleost maculae are found in patches, equally-spaced and surrounded by SCs, mammalian cochlear HCs are arranged in precise rows (Corwin and Warchol, 1991) (Fig. 5). Moreover, a recent study described in detail the physiological properties of zebrafish HCs and compared them with mammalian counterparts (Olt *et al.*, 2014). Marcotti and colleagues concludes that teleost HCs physiologically resemble, to some extend, those from immature mammalian vestibular and auditory system.

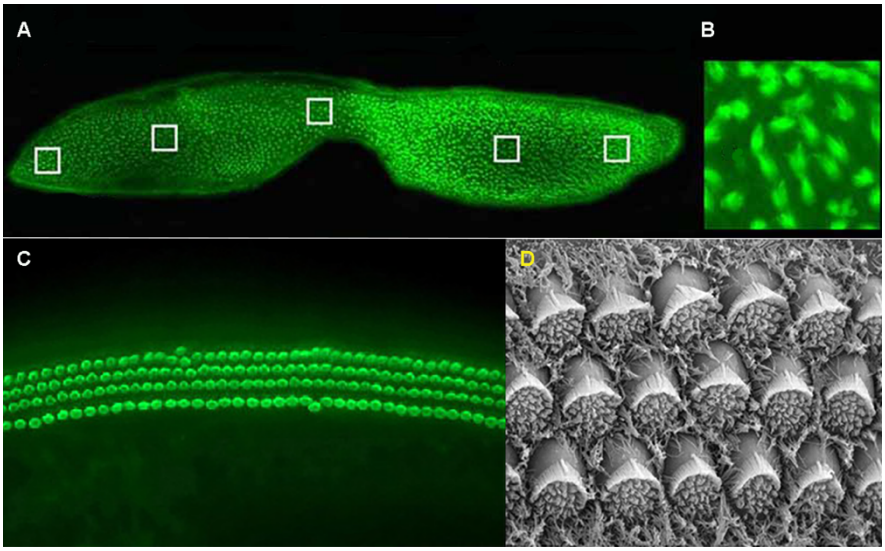

Figure 5: Fish and Mammalian HCs. *(A and B) Phalloidin-labeled saccular epithelia of zebrafish. HCs are dispersed and equally-spaced in the tissue and present conical hair bundle. (C) Lectin-staining and (D)*

scanning electron microscopy of mouse cochlea. HCs of mammalian cochlea are organized in defined rows and their hair bundle is arranged in a W-shape without a kinocilia. (A and B) modified from (Monroe et al., 2015), (C and E) modified from (Kiernan et al., 2005).

However, it is really important to underlie that, in addition to morphological and functional analogies, teleost and mammalian HCs are both damaged by similar chemical and acoustic insults (Ton and Parng, 2005; Hernandez *et al.*, 2006; Smith *et al.*, 2006; Olivari *et al.*, 2008; Schuck and Smith, 2009; Giari *et al.*, 2012; Casper *et al.*, 2013). Furthermore, zebrafish inner ear mutants' analysis and gene-expression studies unveil the existence of considerable genetic and organ system homology between zebrafish and humans (Malicki *et al.*, 1996; Coimbra *et al.*, 2002; Schibler and Malicki, 2007). In the last decades, these observations arise the researcher interest in zebrafish HCs as a model for studying mechanisms of HC death, otoprotection and regeneration.

During development, mechanosensory HCs and the other elements of the sensory patches, SCs and afferent neurons, arise from committed regions of the otic vesicle. First, at 10 somite stage (ss, 14 hpf) the otic placode appears as an ectodermal thickening adjacent to the developing caudal hindbrain (Jacobson, 1966; Streit, 2002). This simple embryonic anlage will generate the 3D structure of the inner ear and all the cellular components (reviewed in Torres and Giraldez, 1998). Then, the otic placode invaginates and pinches off the surface ectoderm to form the otic vesicle in amniotes. During these early stages, signals from surrounding tissues regionalized the newly formed structure, establishing two complementary neural and non-neural territories in the otic placode and otic vesicle (Fekete, 1999; Alsina *et al.*, 2004; Bell *et al.*, 2008; Vazquez-Echeverria *et al.*, 2008). Hedgehog (Hh),

Wnt, FGF and retinoic acid (RA) from adjacent tissues are the main signaling pathways involved in otic patterning (Riccomagno *et al.*, 2002; Hammond *et al.*, 2003; Riccomagno *et al.*, 2005; Schimmang, 2007; Radosevic *et al.*, 2011). The early proneural domain is characterized by the expression of the transcription factors *sox2* and *sox3* (Kiernan *et al.*, 2005; Abello and Alsina, 2007; Neves *et al.*, 2007; Abello *et al.*, 2010) (Fig. 6). Later in development, *sox2* expression is restricted to the prosensory patches and *sox2*-deficient mutants highlight the importance of this transcription factor for HCs and SCs generation (Kiernan *et al.*, 2005). The role of *sox2* is to commit the cell to a neurosensory fate, but, at the same time, maintaining the capacity of self-renewal. Specifically, *sox2* maintain this stem-cell state repressing the activity of proneural genes until cell cycle withdrawal (Bylund *et al.*, 2003). After extensive expansion, proneural progenitors start to express proneural differentiation genes, *neurog1* and *neuroD* for neurons (Alsina *et al.*, 2004) or *atoh1* for sensory cells (Pujades *et al.*, 2006) (Fig. 6). The basic helix-loop-helix (bHLH) proneural transcription factor atonal (*atoh1*) is critical during development and its loss leads to failed specification and/or differentiation of HC progenitors both in mammals and non-mammals (Bermingham *et al.*, 1999; Woods *et al.*, 2004; Pujades *et al.*, 2006; Millimaki *et al.*, 2007; Ma and Raible, 2009). As described by Riley and colleagues (Millimaki *et al.*, 2007), in zebrafish, *atoh1b* initially establishes a broad prosensory domain and subsequently activates Notch signaling to split the domain into separate utricular and saccular primordia. Immediately after the formation of the otic vesicle *atoh1b* activates the expression of *atoh1a* within the two maculae that is primarily responsible for specifying HCs and activating Delta-Notch-mediated lateral inhibition (Millimaki *et al.*, 2007; Radosevic *et al.*, 2011). In vertebrates, the Notch pathway plays several important roles during inner

ear development (Lewis, 1996; Kelley, 2006). In the early otocyst, Notch lateral induction of *Jagged1* (*Serrate1* in chick and *Drosophila*) is necessary for specifying regions of the otic epithelium as sensory. Later on, during sensory epithelia differentiation, Notch signaling is involved in cell fate specification, mainly by inhibiting the HC fate and promoting SC fate. Disrupting the Notch signaling at this stage results in a premature differentiation and overproduction of HCs (Haddon *et al.*, 1998; Lanford *et al.*, 1999; Riley *et al.*, 1999; Lanford *et al.*, 2000; Zhang *et al.*, 2000; Zheng and Gao, 2000; Kiernan *et al.*, 2001; Kiernan *et al.*, 2005; Li *et al.*, 2015). Interestingly, Notch1 indirectly repress, via *HES1/5*, *atoh1* transcription (Baker *et al.*, 1996) and, as expected, disruption of Notch pathway has the same result as the *atoh1* overexpression. Following commitment, developing HCs start to express a set of transcription factors required for HC survival and differentiation. One of the first gene to be expressed is the Pou-domain transcription factor *brn3c* (also known as Pou4f3). Deletion of *brn3c* leads to the ablation of the inner ear HCs in mammals (Erkman *et al.*, 1996; Xiang *et al.*, 1997).

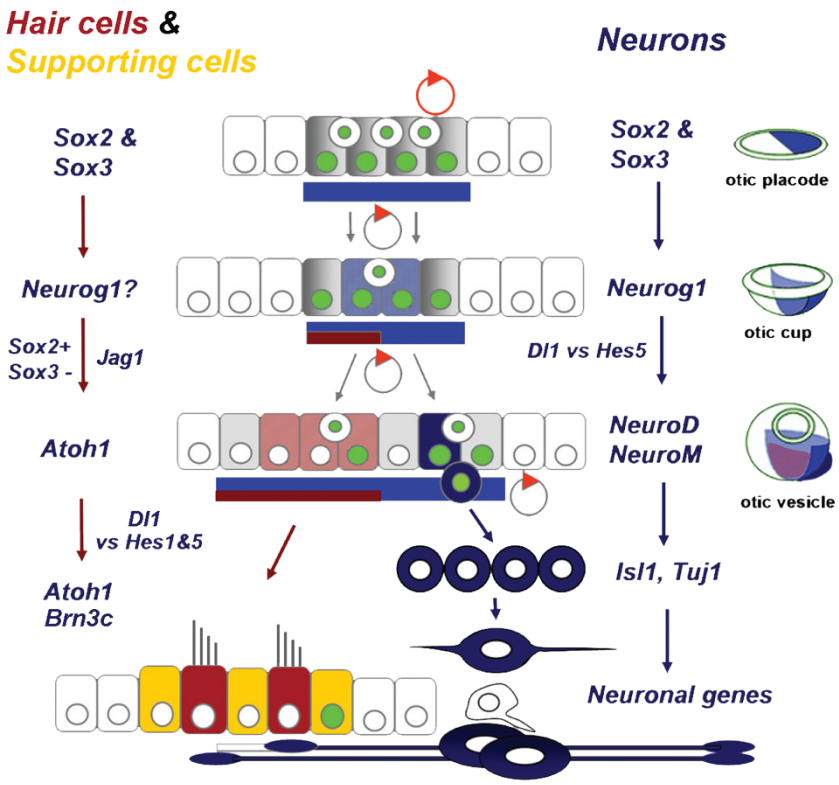

Figure 6: Inner ear cell fate specification in chick*. The diagram shows how the sensory patch component (HCs, SCs and Neurons) arise from committed proneural domain in amniotes. sox2 and sox3 transcription factors characterized this domain and maintain the cells in a renewal state. The domain is specified to give raise to the two main lineages: sensory and neuronal. Jag1 and activation of Notch lateral induction is necessary for sensory specification. Proneural genes are then activated and promote neuronal (neurog1 and neuroD) and hair cell (atoh1) fates. Delta-Notch lateral inhibition singles out HCs within atoh1 cluster. Modified from (Alsina et al., 2009)*

1.3 The Lateral Line

The majority of hair cell damage and regeneration research in fishes have primarily focused on another HC containing sensory organ, the lateral line. Placed on the surface of the fish, the lateral line is a sensory system that detects local water displacements and vibrations. This sensory information is crucial and is required for fundamental behavior including rheotaxis, shoaling, prey capture, predator and obstacle avoidance and sexual courtship (Liao, 2006; Suli *et al.*, 2012). The lateral line comprises a large number of sensory patches, called neuromasts, distributed over the body surface. Depending on the localization, they are classified as being part of either the anterior lateral line (aLL), which are positioned on the head, or the posterior lateral line (pLL), which are placed along the trunk and tail (Fig. 7A and B). In both cases the position of the neuromasts is highly stereotyped (Ghysen and Dambly-Chaudiere, 2004). Each neuromast is composed by 15-20 HCs and two SC types, the inner SCs that surround the HCs and the mantle cells located around the inner SCs. The HC ciliary bundles are embedded in a gelatinous compartment called cupula (secreted by the SCs) that directly contact the external water and drive the deflection of HC kinocilia allowing the mechanotransduction (McHenry and van Netten, 2007) (Fig. 7C and D). Due to the observation that neuromast cellular layers and nervous connection resemble those of the inner ear sensory patches and the superficial location of the neuromasts, that makes them easy accessible to visualization and manipulation, the lateral line has been mostly used as a model to study HC death, regeneration and ototoxicity.

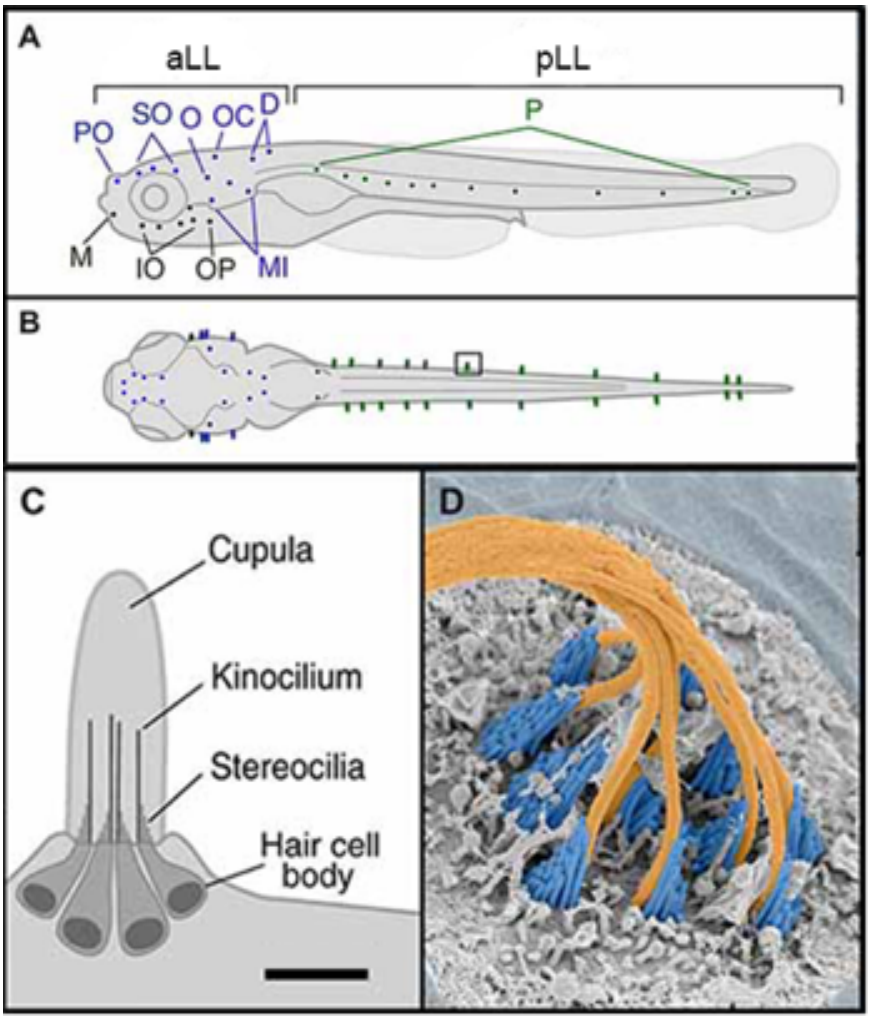

Figure 7: The Lateral Line. *Lateral (A) and dorsal (B) views of zebrafish larvae showing the stereotyped distribution of the neuromasts along the body. In the anterior lateral line (aLL), the supraorbital region (blue) includes the preoptic (PO) and supraorbital (SO) neuromasts. The infraorbital region (black) includes the mandibular (M), infraorbital (IO) and opercular (OP) neuromasts. The caudal-cranial region (purple) includes the otic (O), occipital (OC), dorsal (D) and middle (MI) neuromasts. The posterior lateral line (pLL) comprises the posterior (P) neuromasts located in the trunk region (green). (C) Schematic representation of a single neuromast and its major anatomical features. Scale bar: 10 μm. (D) False-colored SEM image of neuromast, where kinocilia (orange) and stereocilia (blue) are highlighted. Modified from (Monroe et al., 2015)*

The lateral line system has been widely used as a model to understand hearing loss leading to remarkable discoveries. Remarkably, much less is known about HC lost and regeneration in the inner ear. The two systems probably share the majority of the mechanisms and genes involved in the process, but there also could be some organ specific features. For this reason, some groups started to perform these studies in the inner ear (Smith *et al.*, 2006; Schuck and Smith, 2009; Uribe *et al.*, 2013).

The development of the posterior lateral line (pLL, the system used in this thesis) begins with the generation of a primordium from placodal cells adjacent to the ear. Prior to primordium migration, posterior lateral line pre-placodal cells are patterned via Notch signal: the most rostral region acquires proneural fate through the expression of proneural transcription factors *neurog1* and *neuroD*, while caudal cells are committed to sensory epithelial fate (Sarrazin *et al.*, 2010; Mizoguchi *et al.*, 2011). Inhibition of Notch signaling at this stage results in significant expansion of the *neuroD* expression domain and in an increase in the number of neurons in the posterior lateral line ganglion (pLLG), while overexpression of the Notch intracellular domain (NICD) leads to the opposite result (Mizoguchi *et al.*, 2011). At 20 hpf, the posterior lateral line primordium begins to migrate towards the tip of the tail and this migration is controlled by a set of chemokines and chemokines receptors (Metcalfe *et al.*, 1985; Dambly-Chaudiere *et al.*, 2007). Inside the migrating primordium, cells are patterned by the interplay between canonical Wnt and FGF signaling pathways. While canonical Wnt signaling is high in the leading zone and promotes cell proliferation, FGF signaling is strong in the trailing region and drive the generation of epithelial rosettes which finally form the neuromasts (Aman and Piotrowski, 2008;

Lecaudey *et al.*, 2008; Nechiporuk and Raible, 2008). Although Wnt signaling is required to establish FGF pathway in the trailing zone, both signaling pathways create a mutual local inhibition to maintain segregated the two cell populations (Aman and Piotrowski, 2008). In the trailing zone, cells begin to acquire an apicobasal polarity that is required for the consequent apical constriction and coinciding with the initiation of rosette formation (Hava *et al.*, 2009). Once mature, the epithelial rosettes detach from the primordium and are deposited in the right positions. Before detaching from the primordium, FGF signaling also promotes the expression of the proneural gene *atoh1a* in central cells of each rosette, committing these cells to HC fate (Lecaudey *et al.*, 2008; Nechiporuk and Raible, 2008). *atoh1a* mediates the expression of *deltaD* in the HC precursor, that is essential for the establishment of a Delta-Notch lateral inhibition which maintain *atoh1a* expression restricted to the protoneuromast center and allow the proper development of the neuromast (Matsuda 2010). As happens in the inner ear, blockade of Notch signaling results in supernumerary HCs at the expenses of the SC pool (Millimaki *et al.*, 2007; Matsuda and Chitnis, 2010).

1.4 Zebrafish as a model to study regeneration

The term regeneration means the restoration of organ mass, structure, and function after damage. The ability to regenerate is inversely proportional with the evolution, in fact while salamanders, newts, flatworms and lower vertebrate possess powerful regenerative capacity for nearly all the organs, mammals have only variable potential to regenerate depending on the organ: blood and skin are constantly highly regenerated, liver, skeletal muscles and bones can be well repaired after damage, but other organs such as heart, limb and neural tissues display almost no regenerative capacity (Fig. 8).

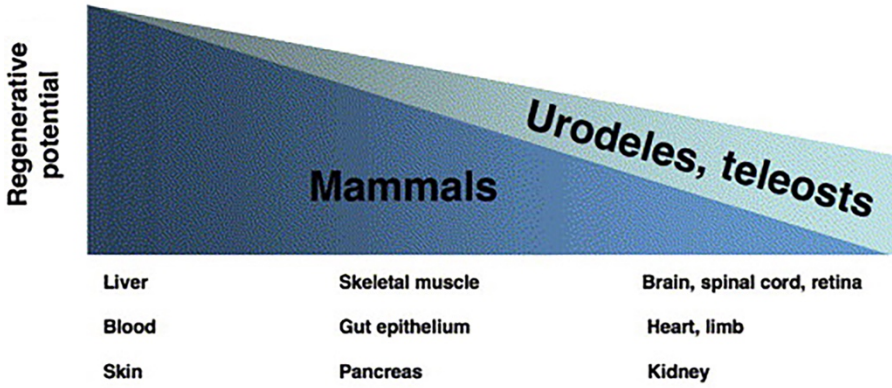

Figure 8: Regenerative potential spectrum. *Many mammalian tissues like liver, blood, skin, skeletal muscle, gut, and pancreas possess a significant capacity for regeneration. However, mammalian CNS structures like brain, spinal cord, and retina fail to regenerate, as do heart, kidney, and limb. Modified from (Poss, 2007)*

For its advantages, including ease and relatively low cost of maintenance, large number of offspring for pairing, easy genetics and transparent external development, zebrafish have been a popular model for developmental biologists over the past 20 years. During these years, researchers have

produced strains, reagents and tools that have been essential for the study of organ regeneration in adult zebrafish. Nearly all the organs of the adult fish are able to regenerate, extensively studied examples are the heart, fin, CNS, bone, pancreas, liver and kidney (reviewed in Poss, 2010; Gemberling *et al.*, 2013).

While cardiac injury in mammals and amphibians typically leads to the formation of scar tissue, adult zebrafish can regenerate heart muscle after damage (Poss *et al.*, 2002). Depending on the extension of the injury, two main kinds of regeneration have been observed. When the heart ventricle is partially injured, the resident *cmcl2*$^{+}$ cardiomyocytes served as the regeneration source. After damage these cardiomyocytes dedifferentiate, detach from one another and re-enter the cell cycle. The regenerating myocytes are produced by the wave of extensive proliferation and start to express *gata4*, a gene essential for cardiac development (Jopling *et al.*, 2010; Kikuchi *et al.*, 2010). On the other hand, when large parts of the larval heart are ablated, transdifferentiation was shown to be the key mechanism for complete regeneration (Zhang *et al.*, 2013). In this study, researchers killed the entire ventricular muscle and observed by cell-lineage tracing that *amhc*$^{+}$ atrial cardiomyocytes acquired ventricular muscle fate and migrated to the injured area.

Fin regeneration, particularly adult caudal fin, has been intensively studied in zebrafish. After a first phase of wound healing (immediately after injury), an essential element of the regeneration process, the blastema, is formed. The formation of this structure has been observed in many other organism regeneration events (Brown and Brockes, 1991; Brook *et al.*, 1993; Mescher, 1996). The blastema is a transient structure composed by a pool of proliferative pluripotent progenitor cells

that will give rise to a variety of cell types required for the reconstruction of the lost tissue (Nechiporuk and Keating, 2002). The appearance of the blastema is crucial and is the hallmark of fin regeneration because this structure is never formed in physiological conditions. Similar to heart regeneration, a dedifferentiation event is necessary: recent studies demonstrated that the cellular source for the formation of the blastema and the consequent fin reconstruction is the resident osteoblast (Singh *et al.*, 2012; Geurtzen *et al.*, 2014).

One of the most fascinating field in the study of zebrafish organ regeneration is the CNS regeneration because while in mammals this capacity is poor, adult teleost fishes maintain continual neurogenesis and regenerative capability (reviewed in Than-Trong and Bally-Cuif, 2015). It has been proposed that this characteristic of zebrafish CNS may lie on the ability to induce proliferation of a pool of stem/progenitor cells and on the maintenance of permissive environments, for example lacking overt scarring (Fleisch *et al.*, 2011; Kizil *et al.*, 2012). Well-described examples of zebrafish CNS regeneration are adult neurogenesis in the brain after physical injury, spinal cord or axon restoration after truncation and retina regeneration. Physical lesions of the telencephalon are widely used to study neuronal repair. It has been described that this kind of lesion induces ventricular radial glia progenitors to proliferate and generate neuroblasts that finally migrate to the damaged area (Kroehne *et al.*, 2011). Similarly, after resection of the spinal cord ependymo-radial glial cells proliferate and migrate to the lesion where form a bridge to re-connect the edges creating a permissive environment for axonal regeneration (Goldshmit *et al.*, 2012). Interestingly, Brand and colleagues observed that another gata factor, *gata3*, is strongly induced after injury and

is essential for proliferation and regenerative neurogenesis (Kizil *et al.*, 2012).

These are only few examples of organ and tissue regeneration studies in zebrafish. As mentioned before, experimental advantages of zebrafish for this use includes the ability to maintain and study zebrafish in large numbers, rapid external development, amenability to mutagenesis, a relatively small genome, and the fact that standard and new genetic approaches, including forward genetics and cell-lineage tracing, can potentially be applied to dissect the molecular mechanisms underlying regeneration. For all these reasons, zebrafish has become a great model to study organ and tissue regeneration. Teleost fishes, similar to avians, regenerate HCs and the state of art of this field (reviewed in Monroe *et al.*, 2015) will be discussed in the next sub-chapter.

1.5 Hair cell regeneration

A full complement of hair cells is required for normal hearing and balance functions. In humans, where these cells are generated only during development, the damage or loss of auditory and/or vestibular HCs results in permanent sensory deficits. Specifically, the organ of Corti of adult mammals shows no spontaneous formation of new HCs after acoustic trauma (Roberson and Rubel, 1994) or drug damage (Forge *et al.*, 1998; Hartman *et al.*, 2009) *in vivo*. In the organ of Corti, progenitor cell division ceased by embryonic day 14.5 (Ruben, 1967) and after trauma no increase in cell proliferation has been observed in adults (Roberson and Rubel, 1994; Forge *et al.*, 1998; Hartman *et al.*, 2009). On the other hand, the vestibular epithelium displays a small but significant induction of cell proliferation in response to HC damage *in vivo* (Rubel *et al.*, 1995; Kuntz and Oesterle, 1998). However, the differentiation of newly produced cells into HCs is rare or non-existent (Rubel *et al.*, 1995; Kuntz and Oesterle, 1998; Ogata *et al.*, 1999; Oesterle *et al.*, 2003). Other evidences show that mammalian HC regeneration is possible to some extent. In 2003, Heller and colleagues (Li *et al.*, 2003) purified a population of cells from mouse adult vestibular organs with the capacity to generate spheres and differentiate to HCs *in vitro* and *in vivo* when transplanted in chick (but not in mouse). Recently, Chen and colleagues determined that forced expression of *atoh1* can induce the generation of vestibular HCs *in vivo* until postnatal day P21 (Gao *et al.*, 2015). In accordance with that, a great effort was made to evaluate HCs regeneration potential in mammalian cochleae (Fujioka *et al.*, 2015 for review). Cells with otoshere-forming capacity were also purified from neonatal mouse cochleae (White *et al.*, 2006; Oshima *et al.*, 2007) but after several weeks of life those cells

lost their proliferative potential. However, recent data demonstrated that SCs expressing the Wnt signaling target *Lgr5* (leucine-rich repeat-containing G protein-coupled receptor 5) receptor, efficiently proliferate and differentiate into HCs in the newborn cochlea upon Wnt signaling activation (Shi *et al.*, 2013). The regeneration potential of the organ of Corti rapidly decreases and the capacities to divide and regenerate are lost by day P14 (White *et al.*, 2006; Oshima *et al.*, 2007). Recent studies showed this feature also *in vivo* (Bramhall *et al.*, 2014; Cox *et al.*, 2014; Mellado Lagarde *et al.*, 2014). Edge and colleagues using an elegant lineage-tracing approach demonstrated that *Lgr5*-expressing SCs from neonatal mouse cochlea are able to transdifferentiate to HCs after ototoxic damage, *in vivo* (Bramhall *et al.*, 2014) (Fig. 9). Moreover, Wang *et al.* showed that *Lgr5*-positive SCs could also generate new HCs via mitotic amplification *in vivo* (Wang *et al.*, 2015) (Fig. 9). The observation that *Lgr5*-expressing SCs act as HC progenitors was also confirmed *in vitro* (Lin *et al.*, 2015). The generation of new HCs is enhanced by Notch inhibition (Bramhall *et al.*, 2014), suggesting that SCs are maintained as SCs through Notch signaling (Mizutari *et al.*, 2013).

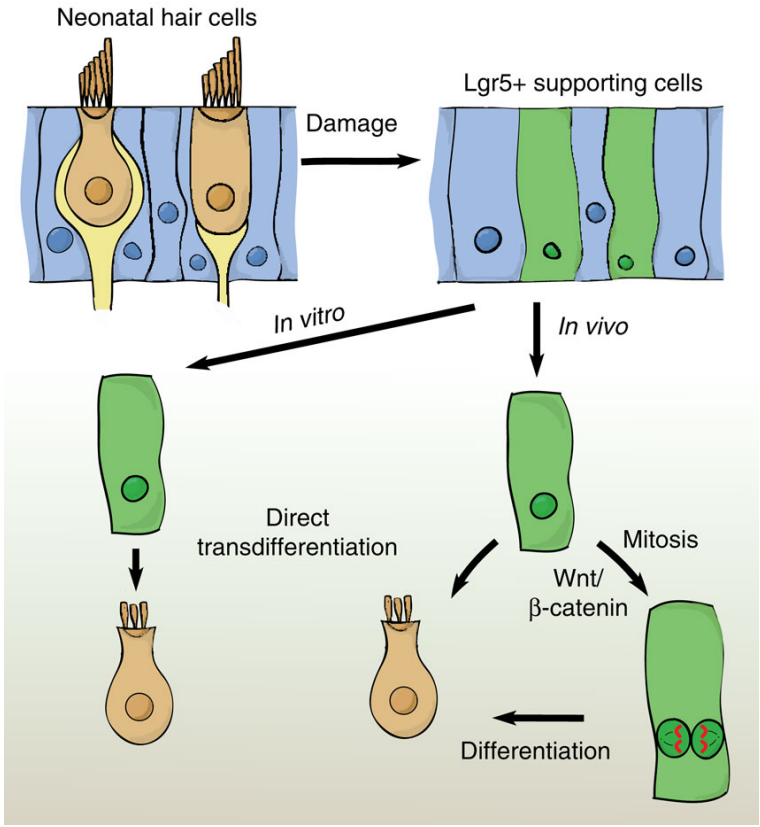

Figure 9: Mammalian HCs regenerative potential. *Several studies showed how a murine subpopulation of SCs (Lgr5-positive) could generate HCs after damage both in vitro and in vivo. Taken from (Wang et al., 2015)*

All these data highlight that mammals maintain a certain regenerative potential after birth but this capacity is lost after few weeks. Lessons from other organisms could unveil this mystery.

1.5.1 What we know from birds

The late 1980s were characterized by a few breakthroughs in the field, evidences of post-embryonic replacement of damaged HCs in non-mammalian vertebrates were presented.

In 1987, two studies demonstrated that birds retain the capacity to regenerate HCs after intense acoustic or ototoxic drug-induced trauma in the basilar papilla post-hatch (Cotanche, 1987; Cruz *et al.*, 1987). Cotanche showed that pure-tone noise exposure provokes restricted areas of HCs damage in the basilar papilla corresponding to the tonotopic regions. Two days after noise exposure cells morphologically similar to embryonic HCs appear in the damaged area and mature over the next two weeks to restore the normal cellular patterning. Similarly, Cruz and colleagues showed that injections of gentamicin, an ototoxic aminoglycoside antibiotic, result in complete HC loss in the total area of the basilar papilla. The number of HCs drops immediately after gentamicin treatment, but a partial restoration is already visible 3-4 weeks later. These exciting discoveries stimulated the field that only one year later produced another series of elegant papers identifying the precursors of the newly formed HCs. Although mitotic activity ceased by embryonic day 9 in the chicken basilar papilla, intensive noise exposure in post-hatch chickens or quails induce SCs in the damaged areas to leave the growth-arrest and re-enter the cell cycle. These divisions generate new precursors that then differentiate into HCs and SCs (Corwin and Cotanche, 1988; Ryals and Rubel, 1988). Researchers observed that SCs also serve as HCs progenitors during regeneration of avian vestibular epithelium (Jorgensen and Mathiesen, 1988; Roberson *et al.*, 1992). In addition to generate HCs precursors through dedifferentiation and divisions, SCs also have the capacity to transdifferentiate directly into HCs. This ability was first reported in chick basilar papilla in 1996 when Adler and Raphael blocked SC proliferation, using the inhibitor of DNA synthesis cytosine arabinoside (Ara-C), after acoustic overstimulation. Despite the SCs division inhibition, new HCs arose in the damaged areas confirming direct transdifferentiation (Adler and Raphael,

1996). In another study, researchers continually provided a traceable nucleoside analog and subsequently administered gentamicin to trigger HC loss. Only half of the newly generated HCs had incorporated the nucleoside analog, suggesting that the other half derived by a SC phenotypical conversion (Roberson *et al.*, 1996). Immediately after trauma SCs produce the earliest HCs via transdifferentiation, while HCs derived by mitosis appear later and eventually comprise a substantial proportion of the new sensory cells (Roberson *et al.*, 1996; Roberson *et al.*, 2004; Cafaro *et al.*, 2007).

1.5.2 What we know from fishes

In contrast to mammals, cartilaginous and bony fishes retain the ability to produce inner ear and lateral line HCs for a long time post-embryonically as part of their normal body growth (Popper and Hoxter, 1984; Corwin, 1985; Jørgensen, 1990) and homeostasis in adult (Williams and Holder, 2000; Cruz *et al.*, 2015; Pinto-Teixeira *et al.*, 2015). In addition, HCs of the lateral line system are rapidly regenerated following tail amputation (as part of the fin regeneration), after laser-ablation of individual HCs or after drug-induced HCs death (Balak *et al.*, 1990; Jones and Corwin, 1993; Jones and Corwin, 1996; Harris *et al.*, 2003). Likewise, fishes can also regenerate inner ear HCs to control levels within 1-3 weeks following acoustic traumas or ototoxic treatment, recovering normal inner ear functions (Smith *et al.*, 2006; Faucher *et al.*, 2009; Sun *et al.*, 2011). Similarly to avians, newly formed HCs derive from mitotic and proliferating SCs: in the inner ear saccule of goldfish, SCs can re-enter the cell cycle and generate HCs precursors (Presson *et al.*, 1995; Presson *et al.*, 1996). In the same manner, studies in the zebrafish neuromast showed that proliferation of SCs is required for HC normal turnover and

regeneration (Williams and Holder, 2000; Harris *et al.*, 2003): Rubel and colleagues describe the potential role of proliferation in regenerating HCs showing a dramatic increase in BrdU-positive cells after neomycin treatment (Harris *et al.*, 2003). Specifically in the neuromast, several evidences suggest the existence of (at least) two distinct populations of SCs, one population gives rise to HC progenitors following damage and is placed centrally, while the other is peripheral, less mitotically active and is required for maintaining the SC pool (Ma *et al.*, 2008; Cruz *et al.*, 2015; Romero-Carvajal *et al.*, 2015). To prove that, Raible and colleagues took advantage of a pulse-chase assay to differentiate mitotically distinct SCs populations: in detail, they used a transgenic line which express photoconvertible Eos protein in SCs. After Eos photoconvertion and five sequential neomycin treatments, peripheral SCs (at the anterior and posterior poles) retain Eos labeling, indicating low mitotic activity, while center SCs show no labeling, suggesting high proliferation (Cruz *et al.*, 2015). On the other hand, Piotrowski and colleagues tracked cell behavior of a regenerating neuromast *in vivo* and in real time. They identify two subpopulations of SCs, one located in the neuromast center that proliferate and differentiate into HCs, and one in the dorso-ventral poles less mitotically active and responsible for SCs self-renewal (Romero-Carvajal *et al.*, 2015). Chemical inhibitors of proliferation cause neuromast HCs regeneration failure (Lopez-Schier and Hudspeth, 2006; Ma *et al.*, 2008; Wibowo *et al.*, 2011; Mackenzie and Raible, 2012), suggesting that SCs proliferation is the main mechanism for generating new HCs in zebrafish. Increased rate of divisions was also observed after acoustic trauma in adult zebrafish (Schuck and Smith, 2009; Schuck *et al.*, 2011). However, one study report that in developing zebrafish larvae, laser-ablated inner ear HCs of macula were replaced by transdifferentiating SCs without mitotic events (Millimaki *et al.*,

2010). Briefly, researchers perform a lineage-tracing experiment in which few and disperse SCs were rhodamine-labeled. After HC laser ablation, only rhodamine-positive HCs were observed, with a corresponding disappearance of SCs rhodamine-labeled, suggesting SC direct transdifferentiation (Millimaki *et al.*, 2010). If this cell behavioral difference is due to the fish age or technical differences is unknown and further studies are needed to address it.

1.5.3 Regeneration signals

The focus of several studies in the field aimed at finding which signals are required for HC regeneration and which signals regulate SC quiescence and re-activation after HC loss. Recent gene expression studies uncover that some molecular pathways are activated specifically during regeneration (Hawkins *et al.*, 2007; Liang *et al.*, 2012). Liang and colleagues uses the digital gene expression, a powerful transcriptional profiling technique, to identify genes that modulate HC regeneration in adult zebrafish. They showed that the *stat3/soc3* pathway is strongly activated early after damage and promotes inner ear HC regeneration through SC activation, cell division and differentiation (Liang *et al.*, 2012). Moreover, other transcriptome analysis (Ku *et al.*, 2014) and RNA interference screens (Alvarado *et al.*, 2011) had the objective to unveil the whole transcriptome changes during regeneration. Interestingly, inflammatory modulators, chemokines and interleukins are robustly upregulated during HCs regeneration, suggesting a crucial role of injury-related inflammation in triggering regeneration process as in other systems (Ku *et al.*, 2014). In addition to regeneration-specific signals, many pathways involved in the development of HCs have also been implicated in their regeneration.

As described before, the basic helix-loop-helix (bHLH) proneural transcription factor atonal (*atoh1*) is critical during development. *atoh1* appears in the sensory patches (Bermingham et al,1999; Woods et al, 2004), becomes highly expressed in HCs after terminal mitosis (Chen and Corey, 2002; Lumpkin *et al.*, 2003) and is shut-down in mature HCs (Lanford *et al.*, 2000; Zheng *et al.*, 2000). Interestingly, during regeneration, *atoh1* is highly upregulated in the nuclei of SCs shortly after HC damage and is later highly expressed in the newly formed HCs both in chicken and zebrafish (Cafaro *et al.*, 2007; Jiang *et al.*, 2014). Based on these evidences, many laboratories tried to induce the production of new HCs in rodent models. Overexpression of *atoh1* is sufficient to induce a robust overproduction of HCs in postnatal mouse and rat cochlear cultures (Zheng and Gao, 2000; White *et al.*, 2006) and *atoh1* gene in utero transfer leads to supernumerary HC in postnatal mice (Gubbels *et al.*, 2008) (Fig. 10). Moreover, several other gene therapy studies conclude that *atoh1* is a master regulatory gene that is both necessary and sufficient for producing HCs in the mammalian cochlea (Izumikawa 2005, Atkinson 2014, Richardson 2015). However, these HC-like cells do not mature properly nor become fully functional.

As previously described, the Notch pathway plays several important roles during inner ear development. In normal conditions, Notch signaling is strongly active in order to maintain SCs in quiescence and the correct cellular pattern (Stone and Rubel, 1999), but after HC death Notch activity decreases allowing SCs to re-enter the cell cycle and/or transdifferentiate into HCs both in chick and zebrafish (Stone and Rubel, 1999; Jiang *et al.*, 2014; Ku *et al.*, 2014) (Fig. 10). Based on these results, blockade of the Notch pathway has been tested as possible strategy to induce HC regeneration in mammals. Inhibition of Notch induces the generation of new

HCs via SCs transdifferentiation with the consequence of depletion of SC population (Lin *et al.*, 2011; Mizutari *et al.*, 2013; Slowik and Bermingham-McDonogh, 2013).

Other pathways necessary for inner ear and HC development, such as Wnt and FGF, play an important role also in regeneration. Canonical Wnt signaling has been related to SC proliferation in both chick and zebrafish. Inhibition of Wnt/β-catenin signaling in zebrafish neuromasts results in a reduction of SC proliferation and HC differentiation, while activation of the pathway promotes SCs to re-enter the cell cycle, increasing HCs number (Head *et al.*, 2013; Jacques *et al.*, 2014) (Fig. 10). Transcriptome analysis and RNA interference-based screens highlighted the implication of Wnt in SC divisions also in chick (Hawkins *et al.*, 2007; Alvarado *et al.*, 2011). In addition, inhibition of Notch signaling causes proliferation of SCs in mouse cochlea by acting through the Wnt pathway, while transdifferentiation was Wnt-independent (Li *et al.*, 2015). However, Wnt signaling seems to be required for proliferation but not immediately after damage, precisely, Wnt signaling is downregulated during early time points but become upregulated later on (Jiang *et al.*, 2014). FGF signaling regulates also SC proliferation but in the opposite manner in respect to Wnt. In fact, FGF receptor 3 is abundant in quiescent SCs in chicken basilar papilla and is strongly downregulated in areas where numerous SCs are dividing, suggesting that the signaling must be attenuated to allow the re-enter in the cell cycle (Bermingham-McDonogh *et al.*, 2001) (Fig. 10). Consistent with this, FGF pathway is transiently inhibited in regenerating zebrafish neuromast (Jiang *et al.*, 2014).

sox2 is an essential transcription factor for inner ear development. As discussed above, its expression is strictly

controlled temporally and spatially to allow the correct patterning of the ear. Initially, expression of *sox2* defines the neurogenic region, subsequently is restricted to the prosensory patches and finally *sox2* is maintained only in SCs (Neves *et al.*, 2007; Millimaki *et al.*, 2010). Recent studies showed that *sox2* in transiently downregulated after neuromast HC loss and is necessary for inner ear HC regeneration in zebrafish (Millimaki *et al.*, 2010; Jiang *et al.*, 2014) (Fig. 10).

A critical step in the regeneration process is the re-entrance of SCs into the cell cycle. During embryogenesis, cells in the cochlear epithelium start to express the cycling-dependent kinase $p27^{kip}$ at the beginning of the terminal mitosis (Chen and Segil, 1999; Lowenheim *et al.*, 1999). $p27^{kip}$ levels remain high in differentiated SCs of the mature organ of Corti, preventing them from dividing. Unlike zebrafish (Jiang *et al.*, 2014), the high expression of the cycling-dependent kinase is maintained also after HC loss in mammals. However, deletion of $p27^{kip}$ in rodents leads to extended periods of progenitor proliferation and production of supernumerary SCs and HCs *in vivo* (Chen and Segil, 1999; Lowenheim *et al.*, 1999; Shi *et al.*, 2013; Walters *et al.*, 2014) and *in vitro* (White *et al.*, 2006) (Fig. 10). These results suggest that mammals lost the ability to repress $p27^{kip}$ in SCs postnatally, resulting in the impossibility to regenerate HCs after damage. The identification and characterization of the molecular pathways that regulates the expression of $p27^{kip}$ in non-mammalian model organisms could unveil mechanisms lost by mammals during evolution and could provide new therapeutic targets.

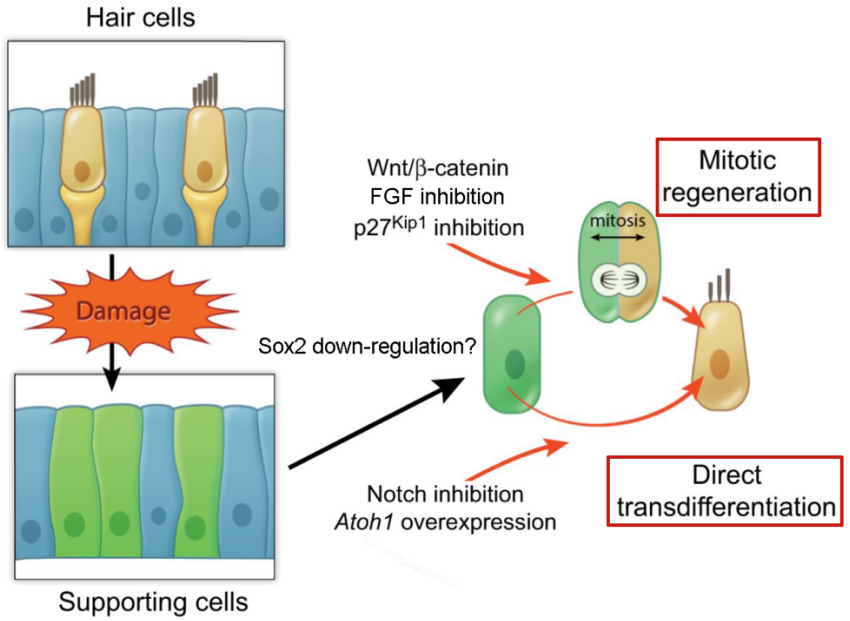

Figure 10: Hair cell regeneration signals. *Diverse signals promote SCs proliferation or transdifferentiation: while enhanced Wnt signaling, inhibition of FGF signaling and p27^{kip} promote mitotic regeneration, Notch inhibition and atoh1 overexpression enhance SC direct transdifferentiation. Modified from (Atkinson et al., 2015)*

These are only few examples of the signaling pathways and transcription factors known to be involved in HC regeneration, but the whole process is far from being understood. Further studies focusing on the identification of new players implicated in HC regeneration are necessary to comprehend the entire picture.

1.6 Retinoic acid signaling and functions

All-trans retinoic acid (RA) is a derivative of the liposoluble vitamin A (retinol). Vitamin A cannot be synthetized by animals but it must be extracted by their diet. Retinyl esters (the storage form of vitamin A) are converted by retinyl ester hydrolases to retinol that is released into the bloodstream bound to retinol-binding protein 4 (*RBP4*). When retinol enters into the target cells it binds to retinol-binding protein 1 (*RBP1*) in the cytoplasm and is metabolized. The last step of this process is carried out by retinaldehyde dehydrogenases (*aldhs*, also known as *raldhs*) that finally produce all-trans RA (Fig. 11). The newly synthetized RA can act in an autocrine or paracrine manner, hence is also a cell communication molecule. In both cases, RA is bound by the cellular retinoic-acid-binding protein 2 (*CRABP 2*) that translocates it to the nucleus. There, RA can exert its functions through a set of nuclear hormone receptors, the retinoic acid receptors (*RAR, alpha, beta* and *gamma*) that heterodimerizes with retinoic X receptors (*RXR, alpha, beta* and *gamma*) to form a transcription complex. The heterodimeric pair of receptors activated by RA can bind to a specific DNA motif, called retinoic acid-response element (RARE) (Fig. 11). Depending on the phosphorylation of these receptors and the recruitment of co-activators or co-repressors, the transcription complex is able to induce or repress gene transcription. Not all the RA-responsive targets show a RARE signal in their sequence but in these cases the mode of action of the transcription complex is poorly understood. Once all-trans RA has exerted its function, it goes back to the cytoplasm where is catabolized by the *CYP26* subfamily of P450 enzymes. The balance between synthesis and catabolism allows control of the level of RA in cells and tissues, frequently generating gradients of RA. In vertebrates, there are generally three *CYP26* enzymes, called *cyp26a1*,

cyp26b1 and *cyp26c1*. Those three enzymes present non-overlapping expression patterns suggesting specific roles for each enzyme in RA catabolism (reviewed in Maden, 2007; Rhinn and Dolle, 2012) (Fig. 11).

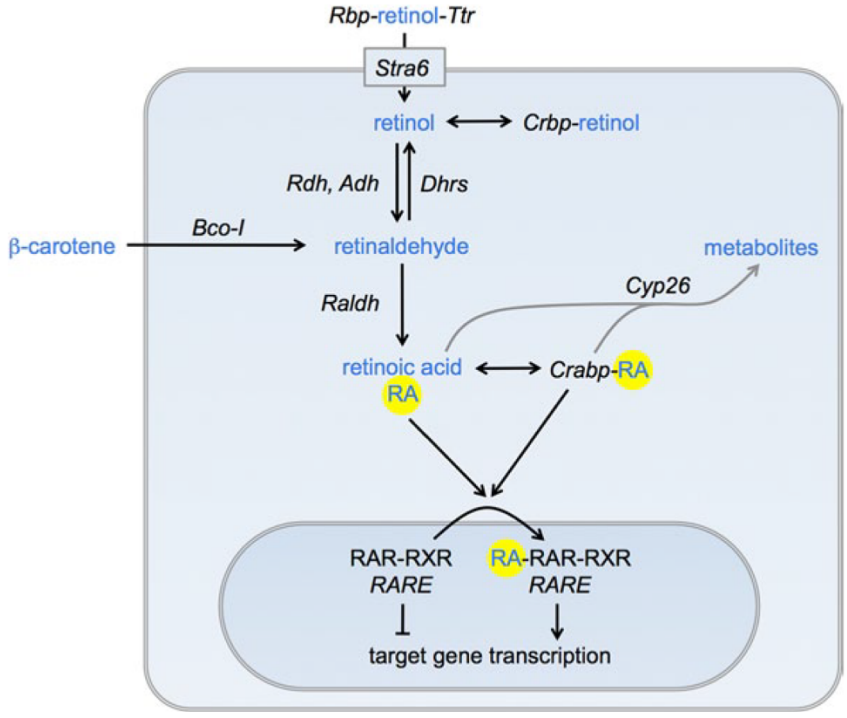

Figure 11: Retinoic Acid Pathway. *Taken from (Blum and Begemann, 2013)*

RA has been implicated in embryonic development, cell homeostasis and stem cell differentiation. During development, RA is produced by the somitic mesoderm that generate a posterior to anterior gradient of the molecule. This gradient is essential for the correct segmentation and patterning of the hindbrain. In fact, several studies described abnormal (enlarged, non-segmented and/or abnormally patterned) rhombomeres in case of endogenous deficiency in RA signaling (Gale *et al.*, 1999; Niederreither *et al.*, 2000; Wendling *et al.*, 2001). These alterations have dramatic

consequences on related developmental processes, such as branchial arches formation, neural/cranial nerve differentiation or inner ear patterning. Furthermore, RA plays another important role in limb development, in fact, through the inhibitory action on *fgf8* signaling, it creates a permissive environment in the forelimb field allowing limb bud induction (Zhao *et al.*, 2009). Similarly, the RA-*fgf8* mutual inhibition is also required for the correct somitogenesis (Sirbu and Duester, 2006) and neural tube neurogenesis (Diez del Corral *et al.*, 2003).

RA has been identified as a master differentiation factor in several tissues, such as the CNS (reviewed in McCaffery and Drager, 2000), pancreas (Tulachan *et al.*, 2003) and kidney (Rosselot *et al.*, 2010) among others. For this property, RA has for long been used in differentiation protocols for ES cells, suggesting that has the ability to begin transcriptional gene programs to commit stem cells to a particular lineage (Gudas and Wagner, 2011).

In the inner ear, RA has many roles at different stages. At early stages, it is required for correct size of the otic primordium (Hans and Westerfield, 2007). RA is crucial for proper embryonic development and RA depletion results in defects in the circulatory systems, limbs, trunk and hematopoietic system (Maden, 2002, for review). Hindbrain patterning is altered in embryos deficient in RA and, since hindbrain regulates otic development (reviewed by Romand, 2003), otic defects were considered mostly secondary consequences. Decrease levels of RA signaling generates supernumerary otic vesicles in amniotes, while the same phenotype has been observed in zebrafish embryos with an excess of RA (White *et al.*, 1998; Dupe *et al.*, 1999; White *et al.*, 2000). Hans and Westerfield proposed that precise levels of RA are crucial to determine the

extent of otic competence independently of hindbrain signaling (Hans and Westerfield, 2007). Later in development, RA signaling is required for correct positioning of the neurogenic domain (Bok *et al.*, 2011; Radosevic *et al.*, 2011) and control of *fgf3* expression (Cadot *et al.*, 2012). RA directly activates transcription factor *tbx1*, gene implicated in promoting posterior otic identity, conferring A-P identity to the otic placode and establishing the non-neurogenic domain (Bok *et al.*, 2011; Radosevic *et al.*, 2011). During otic placode generation and pattering, the main sources of RA are the somites and the posterior hindbrain mesenchyme which express *aldh1a2* (Niederreither *et al.*, 2000; Begemann *et al.*, 2001). Inactivation of this enzyme in mouse embryos results in a dramatic reduction in the size of the otocyst (Niederreither *et al.*, 1999; Niederreither *et al.*, 2000). Later on, *aldh1a3* is expressed in the developing vestibular organ (Romand *et al.*, 2006) and mouse null mutant of this enzyme showed several anatomical abnormalities resulting in an impairment of vestibular functions (Romand *et al.*, 2013). RA also affects otic patterning in zebrafish through *aldh1a3* in the anterior part of the otic vesicle. RA signaling in this region is required to restrict the anterior expansion of *otx1b*, regulating the position of the neural/non-neural boundary (Maier and Whitfield, 2014). Moreover, regarding HC development, it was shown to be a potent inducer of HC differentiation *in vitro* (Kelley *et al.*, 1993; Lin *et al.*, 2009) and to induce HC regeneration in 3 day old rat cochleae *in vitro* after ototoxic damage (Lefebvre *et al.*, 1993). To date, the cellular and molecular mechanisms of action of RA in HC regeneration need to be explored because surprisingly, no other clear report has linked RA with HC regeneration nor analyzed its implications in regeneration *in vivo.*

1.6.1 Role of RA in organ regeneration

Interestingly and linked to the developmental role, RA is emerging as a key player in regeneration. The first descriptions of the RA regeneration capacity were published three decades ago, when two studies described that the addition of retinoids to amputated amphibian limb generate duplications of proximal skeletal elements (Niazi and Saxena, 1978; Maden, 1982). The role of RA in amphibian limb regeneration has been extensively studied demonstrating the presence of RA, several *RARs* and *aldh1a3* in the blastema and epidermis of regenerating limbs (Giguere *et al.*, 1989; Hill *et al.*, 1993; Scadding and Maden, 1994; Monaghan *et al.*, 2012; Monaghan and Maden, 2012). In addition to development, RA has been identified as crucial component of the regeneration machinery for the peripheral and central nervous systems (reviewed in Maden, 2007). A representative example is the regeneration of the optic nerve in goldfish. Kato and colleagues found that *aldh1a2*, *CRABPs* and *RARs* were significantly upregulated after optic nerve injury, whereas *cyp26a1* was downregulated to about 50% of normal levels, suggesting the requirement of the RA pathway activation during optic nerve regeneration in goldfish (Nagashima *et al.*, 2009). In newts, also lens regeneration depends on RA: after lentectomy, *RARs* expression is strongly induced in pigmented epithelial cells and inhibitors of all *RARs* impair lens regeneration (Tsonis *et al.*, 2000; Tsonis *et al.*, 2002). Interestingly, RA has been involved in a particular example of tissue regeneration in mammals, the cervid antlers. In addition to promote osteoblast differentiation (Allen *et al.*, 2002), RA seems to control growth and other aspects of the earliest regenerative events. Although the process needs to be investigated, *rar*α and *aldh1a2* are expressed in the blastema (prechondrogenic mesenchyme)

suggesting a growth–promoting role (Kierdorf and Kierdorf, 1998; Kierdorf and Bartos, 1999).

Specifically in zebrafish, RA has been demonstrated to be essential in heart (Poss *et al.*, 2002; Kikuchi *et al.*, 2011) and fin (Blum and Begemann, 2012) regeneration. Zebrafish heart regeneration occurs through the activation of cardiomyocyte proliferation in areas of trauma. Within 3 hours of ventricular injury, expression of RA-synthetizing enzyme *aldh1a2* is highly induced and inhibition of RA receptors or expression of RA-degrading enzyme block regenerative cardiomyocyte proliferation (Kikuchi *et al.*, 2011). Following amputation, zebrafish lost appendage regrows through a proliferation-dependent process known as epimorphic regeneration that involves three successive stages: wound healing, blastema formation, and regenerative outgrowth and repatterning. RA has been described to be essential for the formation, survival and proliferation of the blastema, and overexpression of RA-degrading enzyme *cyp26a1* impairs fin regeneration (Blum and Begemann, 2012).

The results summarized here demonstrate the recurrent requirement of RA signaling for tissue regeneration in several organisms and suggest that RA might also be mediating regeneration of neuromast and inner ear HCs in zebrafish. The putative role of RA in HC regeneration might be useful to further understand the molecular mechanisms underlying non-mammalian vertebrate HC regeneration. Moreover, these new data could help us to identify mechanisms disappeared/silenced in mammals and, at longer term, this knowledge could be essential to develop new therapeutic strategies based on RA for treating hearing loss.

2. AIMS OF THE THESIS

Aims of the Thesis

The RA signaling pathway has been mainly implicated in cell differentiation in several tissues and it is critical for the development of a variety of organs and structures. For this property, RA is extensively used as a differentiation factor in cell culture protocols. Recent *in vivo* and *in vitro* studies suggest that RA might also have the ability to favor and/or promote cell proliferation. In zebrafish, RA has been demonstrated to be critical for the organ regeneration such as fin and heart after injury. Moreover, RA signaling has been reported to induce HC regeneration in postnatal mammalian cochlear explant. Surprisingly, no other clear report has linked RA with HC regeneration nor analyzed its implications in regeneration *in vivo.* If this putative role of RA is confirmed, the cellular and molecular mechanisms of action of RA in HC regeneration need to be explored.

Using zebrafish larvae as a model system for inner ear and lateral line HC regeneration, the aims of this thesis are:

1. To analyse the requirement of RA in inner ear and lateral line HC regeneration.

2. To investigate the possible role of RA in promoting proliferation after HC death as occurs in other systems.

3. To confirm by lineage-traicing that SCs are the cellular sources for HC regeneration in the zebrafish.

4. To compare the expression pattern of the RA signaling pathway components in the inner ear lateral cristae and in lateral line neuromasts in homeostasis and after HC death.

5. To study possible organ-specific features of the inner ear and lateral line regarding HC regeneration.

6. To analyse the expression pattern of genes involved in HC development after injury.

7. To investigate putative molecular mechanisms of action of RA in HC regeneration.

8. To analyse the possible cross-regulation between RA signaling and FGF signaling.

3. RESULTS

Retinoic acid signaling mediates hair cell regeneration by repressing *p27^{kip}* and *sox2* in supporting cells

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Rubbini D, Robert-Moreno A, Hoijman E, Alsina B. Retinoic acid signaling mediates hair cell regeneration by repressing p27kip and sox2 in supporting cells. Journal of Neurosciece: 2015, 35(47): 15752-15766.

doi:10.1523/JNEUROSCI.1099-15.2015

4. DISCUSSION

Discussion

Regeneration intrigues countless biologists, biomedical engineers, and clinicians. How and why tissue regeneration occurs are the main questions in regeneration studies. A key goal of these studies is to gain knowledge that will promote the broad new field of regenerative medicine. This new branch comprises a variety of approaches from using exogenous stem cells or the strategy to stimulate endogenous stem cell activity, xenotransplantation, gene therapy to induce regenerative programs and the brand new field of 3D bioprinting of tissue and organs. In addition to regenerative capacity, also regenerative programs differ across organisms and organs. For that, although we already understand some forms of regeneration enough to manipulate them with therapeutic aims, for most examples of regeneration, we are far from comprehending the entire process and attempting to modify it. For each tissue or organ regeneration program, researchers try to recognize the main characteristics. First, it is crucial to find which are the cellular sources for regeneration and how the various cell types of the tissue are generated. Then, the focus goes into identifying which signals activate these cells and how these signals are generated and propagated from the injury site. Finally, it is essential to unveil which factors regulate proliferation and cell differentiation to recover the correct size and patterning and how the whole process is properly coordinated. Moreover, it is fascinating to investigate the evolutionary differences that have led the loss of regenerative potential in specific organs in humans compared to non-mammalian vertebrates. In my PhD, I analyzed deeply the HC regeneration process in zebrafish highlighting the importance of the RA in this process and its ability to allow supporting cell proliferation.

4.1 Supporting cells: the hair cell's supply

As previously mentioned, to understand any regenerating system, it is crucial to delineate the cellular origins of renewed tissues. In the case of HC regeneration, SCs have been proven to be the source of HC progenitors in several organisms *in vivo* and *in vitro* (Corwin and Cotanche, 1988; Ryals and Rubel, 1988; Adler and Raphael, 1996; Williams and Holder, 2000; Harris *et al.*, 2003; Bramhall *et al.*, 2014; Lin *et al.*, 2015). Under physiological conditions of HC death, SCs display stem-cell features, maintaining the capacity of self-renewal and the ability to generate HC precursors (Cruz *et al.*, 2015; Romero-Carvajal *et al.*, 2015). Specifically in zebrafish, activation of SCs proliferation is a key step of the regenerative process in saccule after acoustic trauma and in neomycin-treated lateral line (Harris *et al.*, 2003; Liang *et al.*, 2012). As discussed in the introduction, the group of Riley showed that, after HC laser-ablation in larval macula, SC transdifferentiation is the main mechanism to repair the sensory epithelia (Millimaki *et al.*, 2010). Studies in chick have shown that, in the first urgent phase after HC death, SC transdifferentiation is responsible for the production of the earliest HCs generated, while HCs derived by mitosis appear later (Roberson *et al.*, 1996; Roberson *et al.*, 2004; Cafaro *et al.*, 2007). In our work, we confirm by lineage-tracing experiments the central role of SCs in generating new HCs and we demonstrate the activation of SCs proliferation in both regenerating lateral line neuromasts and inner ear cristae. The methods used in this thesis do not allow us to detect and evaluate the contribution of transdifferentiation events during HC regeneration in the inner ear and further studies and different approaches are needed. In details, the aim of the cell-lineage tracing experiment performed here was to identify the cellular origin of the newly generated HCs. For that, after laser ablation and kaede

photoconversion we imaged the regenerated lateral cristae only at 48 hpa, making impossible evaluate the contribution of phenotypic cellular conversions. Time lapse *in vivo* imaging of the entire process would help us to detect possible transdifferentiation events. Moreover, *brn3c* (used in this study to identify the HCs) is a late marker of HC differentiation. Using *atoh1* instead, we would be able to identify earlier cells committed to HC fate before differentiation starts, and to determine if those cells arise from SC phenotypic conversions. Another possible approach would be to test the capacity for HC regeneration in the presence of chemical inhibitors of cell proliferation.

However, intrinsic differences between maculae and cristae might exist. As presented in the introduction, maculae are specialized to detect linear acceleration. In order to fulfil this function, HC hair bundles are immersed in a gelatinous matrix (otolithic membrane) containing denser structures made of protein and calcium carbonate called otoliths. The otoliths respond to gravitational force by moving the entire otolithic membrane, consequently deflecting HC stereocilia and kinocilia. On the other hand, cristae (responsible for angular acceleration detection) are covered by a gelatinous structure (cupula) that does not contain an otolith. The cupula is moved directly by a fluid, the endolymph, which fills the semicircular canals (Tavolga *et al.*, 2012). The crista structure closely resembles the one characterizing the neuromast. Moreover, each otolithic macula can be divided into a number of regions within which all the ciliary bundles are morphologically polarized in the same general direction. In other words, within macula HCs are oriented depending on the sensory patch area. This diversified orientation pattern is important to detect otolith movement in any direction. On the contrary, cristae

detect movement along the same axis, and in the crista of each semicircular canal all the sensory cells are oriented in the same direction (Tavolga *et al.*, 2012). Interestingly, in the lateral line, within an individual neuromast, all HCs have bundles oriented along the same axis (either anterio-posterior or dorso-ventral), making each neuromast only responsive to water movement on the same axis of their polarization, similarly to inner ear cristae (Thomas *et al.*, 2015). Finally, crista HCs are characterized by extremely taller ciliary bundle compared to macular HCs, with a kinocilium that is more than twice as long as the longest stereocilia, similarly to the HCs of the neuromast (Tavolga *et al.*, 2012). Apparently, ampullary cristae are morphologically and functionally closer to lateral line neuromast than to maculae. Hypothetically, this similarity might also be reflected during HC regeneration, where crista regeneration depends on SC proliferation as occurs in neuromast, while macula regeneration rely on SC phenotypic conversion.

4.2 Signals controlling HC regeneration

In tissues that are competent for regeneration, (extrinsic or intrinsic) signals are essential to initiate regenerative events and direct the cellular sources to activate regenerative transcriptional programs. In this thesis, we illustrate, for the first time, the role of RA signaling in HC regeneration *in vivo*. Our results indicate that RA instructs SCs to “dedifferentiate” and re-enter the cell cycle by repressing $p27^{kip}$ and *sox2*, and therefore generate new HCs. SC dedifferentiation has been proven to trigger significant levels of supporting cell S-phase entry in cultures of adult mouse utricles (Burns *et al.*, 2012). Infection of utricles with adenovirus vector encoding induced pluripotency transcription factors *c-Myc*, is both necessary and sufficient for the proliferative response (Burns *et al.*, 2012). It

is intriguing to propose that organisms competent for HC regeneration, retain the ability produce new HCs via SC dedifferentiation and consequent proliferation/transdifferentiation. Whether SCs in the zebrafish regress to a progenitor state and thus dedifferentiate, needs further investigation. Nevertheless, the fact that SCs re-enter the cell-cycle and induces *atoh1* would suggest that upon damage, SCs may be reverted to a progenitor state.

A number of developmental factors have been identified in chick and fish to be expressed very early after HC damage and whose activity is crucial for regeneration. After HC death, Notch signaling must be downregulated to allow SCs to transdifferentiate and/or proliferate in both chicken and zebrafish (Stone and Rubel, 1999; Ku *et al.*, 2014). Moreover, Wnt/β-catenin signaling pathway activation is required for SCs to re-enter the cell cycle (Head *et al.*, 2013; Jacques *et al.*, 2014), together with the attenuation of FGF signaling that regulates SC proliferation in the opposite manner (Bermingham-McDonogh *et al.*, 2001). Finally, activation of *atoh1* in HC progenitors is critical for the generation of new HCs in both chick and fish (Cafaro *et al.*, 2007). Interestingly, temporal mapping of the activation of these pathways in the regenerating lateral line indicates that while Wnt signaling is activated only 12 hours after neomycin treatment (which is consistent with its role in proliferation), Notch and FGF pathways are rapidly inhibited after HC death (Jiang *et al.*, 2014). Here we describe that another inner ear development-related signaling pathway, the RA signaling pathway, is also activated very early after damage in neuromasts as in lateral cristae. During development, RA signaling is active and has several functions. First, synthetized by the mesenchyme, RA is crucial for otic placode commitment, for the proper size of

the placode and its patterning (Hans and Westerfield, 2007; Bok *et al.*, 2011; Radosevic *et al.*, 2011). Later on, the RA, synthetized by *aldh1a3* within the otic vesicle, regulates morphogenesis and neuronal and sensory differentiation (Choo *et al.*, 1998; Romand *et al.*, 2002; Thompson *et al.*, 2003; Maier and Whitfield, 2014). *In vitro* studies have identified RA as a potent inducer of HC differentiation (Kelley *et al.*, 1993; Lin *et al.*, 2009). Addition of exogenous RA to embryonic cochlear explants results in production of supernumerary HCs and SCs, but these new cells appeared without signs of cell proliferation, implying that RA had changed the fate of postmitotic cell population towards the production of HCs and SCs (Kelley *et al.*, 1993). These data indicate that, during inner ear development, cell differentiation is the main effect of RA signaling. Different from Notch, which mode of action is common between development and regeneration, RA seems to have an additional role during HC regeneration, promoting cell proliferation instead or in top of differentiation. In our experiments, RA blockade impaired HCs production and therefore RA signaling, in addition to its role on SCs proliferation, could be required for HC differentiation. However, it is difficult to separate both events experimentally. To date, only one paper has linked RA to HC regeneration (Lefebvre *et al.*, 1993). In this study, researchers showed that RA favors the generation of new HCs in cultures of postnatal rat organ of Corti treated with ototoxic drugs. Interestingly, the regenerative potential of RA is blocked by cell cycle inhibitors, confirming the requirement of RA signaling to promote cell division (Lefebvre *et al.*, 1993).

Is worth notice that inner ear and lateral line systems display some differences: first, HC regeneration in the two organs follow different kinetics. While neuromasts completely recover the tissue functionality and HC number within 48 hours after

damage, HC regeneration in the lateral cristae last for 6 days. Second, although an intact RA pathway is required in both systems, different components of the signaling pathway are activated after HC death. In regenerating neuromasts *aldh1a3*, *rarαb*, *cyp26a1* and *cyp26c1* are transcriptionally activated by 1.5–3 hours post neomycin treatment, whereas in lateral cristae *aldh1a3*, *rarγa*, and *cyp26b1* are enhanced 24 hours after ablation. The meaning of this different expression is unclear. *rarα* receptors have widespread expression patterns, whereas the others (*rarβ*, *rarγ*) show more complex, tissue-specific expression (Dolle, 2009). At otic vesicle stage, three different *RARs* are expressed in the inner ear, *rarαb* and *rarγb* in nascent sensory patches and *rarγa* in a broader area coinciding with the non-neurogenic domain (Maier and Whitfield, 2014). On the other hand, during lateral crista regeneration, only *rarγa* is expressed and upregulated. This might suggest that while *rarαb* and *rarγb* are involved in HC development, *rarγa* is responsible for RA-mediated HC regeneration. Regarding the lateral line system, no reports have shown RA involvement in neuromast formation but we demonstrate the upregulation of *rarαb* after HC death and the presence of *rarγa*. However, gene knockout studies in mouse revealed a large degree of functional redundancy between *rar/rxr* heterodimers (Mark *et al.*, 2009). *CYPs* enzymes also display non-overlapping and tissue-specific expression patterns during development. Interestingly, *cyp26b1*$^{-/-}$ mouse mutants exhibit severe craniofacial abnormalities including defects in the inner ear structure (Maclean *et al.*, 2009). These findings may suggest that *cyp26b1* is the enzyme responsible for RA degradation in the inner ear which is consistent with the *cyp26b1* induction after HC laser ablation. Although little is known regarding transcriptional regulation of the *CYPs*, RA

treatments *in vivo* and in cultured cells showed rapid upregulation of the *cyp26a1* gene, which contains two functional RA-response elements (RAREs) (Loudig et al., 2005). Thus, *cyp26a1* might be induced by RA in cells that must be protected from RA-dependent transcriptional activation (further discussed later). Third, the expression of these components is specifically localized within the tissues: while *aldh1a3* is expressed closed to the center of the neuromasts and *CYPs* at the periphery, in the lateral cristae *aldh1a3* is enhanced laterally and *cyp26b1* medially. This localization suggests the generation of RA activity gradient within the sensory epithelia, latero-medial in lateral cristae and centro-peripheral in neuromasts. Along this gradient, different levels of RA might have different outcomes. While high levels promote cell proliferation (the mitotically active region coincides with the domain of RA synthesis, Fig. 18), lower levels might favor cell differentiation. Despite the common dependency on RA, our observations might indicate that inner ear and lateral line systems possess organ-specific regenerative features and further studies are required to elucidate divergences.

4.3 Retinoic acid role in hair cell regeneration

Our findings reveal that an intact RA pathway is necessary to permit SCs to reactivate the cell cycle, suggesting a direct regulation on proliferation, although other mechanisms of action on HC production are plausible. The RA capacity to promote cell proliferation has been previously described in zebrafish during heart and limb regeneration (Kikuchi *et al.*, 2011; Blum and Begemann, 2012), however no molecular mechanism has been proposed. In both systems *aldh1a2* expression is rapidly induced after injury. In heart regeneration, RA produced by endocardium and epicardium is required for

cardiomyocyte proliferation and appears to be a permissive, rather than instructive, signal of regeneration (Kikuchi *et al.*, 2011). On the other hand, during fin regeneration, RA is sufficient to confer mitogenic activity to the mesenchyme during blastema formation and maturation, since exogenous RA significantly increase mesenchymal proliferation (Blum and Begemann, 2012). Although we did not evaluate effects on cell division, treating larvae with all-trans RA does not alter the number of regenerated HCs, but slightly accelerates the process (data not shown). In fin regeneration, RA pathway is required for the activation of FGF and Wnt/β-catenin signaling, which both cooperate to blastema formation, maturation and survival (Blum and Begemann, 2012). This suggest a different mode of action of FGF in fin regeneration compared to HC, since previous studies report an inhibitory effect of FGF on SC proliferation (Bermingham-McDonogh *et al.*, 2001; Maier and Whitfield, 2014).

As mention previously, Wnt/β-catenin signaling has been proven to be responsible SCs mitotic activation in neuromasts (Head *et al.*, 2013; Jacques *et al.*, 2014). While a low level of active Wnt/β-catenin signaling are detected in the homeostatic neuromasts, the induction of Wnt signaling coincides with the increase in proliferation observed in SCs several hours after HC damage (Jacques *et al.*, 2014; Jiang *et al.*, 2014). In line with this, in the regenerating chick cochlea and utricle, Wnt/β-catenin is downregulated immediately after ototoxic injury, but is significantly upregulated 48h after damage when the peak of proliferation occurs, similar to what has been described in the zebrafish neuromasts (Alvarado *et al.*, 2011). These studies suggest that pathways other than Wnt signaling may be responsible for initiating the regenerative response of SCs after HC loss but that active Wnt/β-catenin signaling is both

necessary and sufficient for proliferation in later stages of HC regeneration, both in bird and fish. Being activated earlier and regulating SC proliferation, RA could fit the role of signaling pathway upstream of Wnt. Moreover, as previously mentioned, RA activates Wnt/β-catenin signaling during fin regeneration. Would be fascinating to verify if Wnt activation in neuromasts is still be possible after the RA pathway blockade.

In neuromast, the cell cycle inhibitor *p27^{kip}* retains cells in a non-proliferative state but the expression levels drop dramatically after HC death. In mouse cochlea, SCs maintain sustained expression of *p27^{kip}* throughout life, and null mutants of this protein exhibit abnormal proliferation in the tissue in both newborn and adult mice (Chen and Segil, 1999; Lowenheim *et al.*, 1999; Oesterle *et al.*, 2011). Interestingly, *p27^{kip}* is characteristic of SCs, while other cyclin-dependent kinase inhibitors, such as *p19^{Ink4d}*, promotes mitotic activity of HCs, which then undergo apoptosis (Oesterle *et al.*, 2011). Here, we demonstrated that RA represses *p27^{kip}* transcription cell-autonomously, proposing for the first time a molecular mechanism through which the RA signaling pathway regulates proliferation. During embryogenesis, cells in the cochlear epithelium start to express *p27^{kip}* at the beginning of the terminal mitosis and its transcription is controlled by a gradient of an unknown factor (Chen and Segil, 1999). Recently, Corwin and colleagues reported that a RA gradient regulates differentiation in the chick basilar papilla (Thiede and Corwin, 2014). Is intriguing to propose that the RA gradient also controls *p27^{kip}* expression in developing cochlea as occurs in our system. Moreover, in zebrafish neuromasts, it has recently been reported that increased levels of NICD upregulate *p27^{kip}* (Romero-Carvajal *et al.*, 2015) suggesting an opposite effect of Notch on proliferation. An interesting scenario would be that, after HC damage, downregulation of Notch signaling and

activation of RA signaling are both necessary for SC proliferation. It remains to be tested if both signaling pathways regulate *p27^{kip}* in concert or independently. Transcriptional regulation of *p27^{kip}* is largely unknown and probably tissue specific. In vascular endothelial cells, *p27^{kip}* expression is induced by Rho family GTPase *Rac1* (Hirano *et al.*, 2007). Interestingly, Rac1 has been reported to be strongly activated after traumatic noise in mouse cochlea (Chen *et al.*, 2012). Thus, *Rac1* might regulate *p27^{kip}* also in mammalian cochlea, holding the SCs in a quiescent state and blocking regeneration. Would be fascinating to verify that interaction in the inner ear, and study the expression pattern of *Rac1* in organisms that can regenerate HC.

In addition to neuromast, we hypothesize that RA is also controlling *p27^{kip}* in the inner ear. Unfortunately, I was unable to detect clear expression pattern of *p27^{kip}* in lateral cristae since the *in situ* hybridization display high non-specific background signal in the head region. *In situ* hybridization on inner ear section will allow us to detect expression changes between homeostatic and regenerating cristae. In addition, in neuromast *p27^{kip}* downregulation is rapid and transient (1.5-3h after damage) and, since inner ear and lateral line HC regeneration follow different kinetics it will be challenging to find the exact timing of *p27^{kip}* repression after laser ablation. However, in the inner ear SC proliferation might not be controlled via *p27^{kip}* and further analysis will address this.

Together with *p27^{kip}*, the RA pathway also controls the expression of the transcription factor *sox2*. Essential for sensory development, *sox2* is maintained at highly levels in SCs in physiological conditions, but, during regeneration, *sox2* transcript disappeared rapidly after HC death both in lateral line

and inner ear. Although the meaning of this transient downregulation is unclear, our data demonstrate that depends on the RA pathway. Since *sox2* and *atoh1* display a mutual antagonistic interaction during HC development (Dabdoub *et al.*, 2008), *sox2* downregulation might be necessary to create permissive conditions for the expression of *atoh1* and the generation of new HCs. However, since *sox2* expression is controlled by Notch signaling (Dabdoub *et al.*, 2008; Millimaki *et al.*, 2010), *sox2* levels decrease could be only a result of the Notch pathway inhibition following HC damage. Would be interesting to investigate if the *sox2* downregulation during HC regeneration also takes place in other organisms, such as birds which inner ear SCs retain the expression *sox2* throughout life (Neves *et al.*, 2007). On the other hand, *sox2* is not involved in inner ear development or HC regeneration in frogs, suggesting intrinsic differential mechanisms between organisms. Similarly to bird and fish, mammal SCs are characterized by high levels of *sox2* from embryonic stages to adulthood, and this expression is not affected by drug-induced HC damage (Oesterle *et al.*, 2008).

The transcription factor *sox2* display a variety (and sometimes controversial) functions. *sox2* is a well-known key player in the maintenance of pluripotency and “stemness” and in fact is essential, for example, for osteoblast self-renewal inhibiting differentiation (Basu-Roy *et al.*, 2010). In recent years, *sox2* has been found to be aberrantly expressed in cancers, including those of the lungs, ovaries, bone, breasts, and others (Lengerke *et al.*, 2011; Basu-Roy *et al.*, 2012; Rudin *et al.*, 2012; Zhang *et al.*, 2012), where interferes with differentiation pathways such as the Hippo pathway and promotes uncontrolled proliferation (Basu-Roy *et al.*, 2015). For the ability to induce stem-cell feature, *sox2* is one of the four factors included in the widely used protocol to generate iPS

cells (Takahashi and Yamanaka, 2006). The expression of *sox2* in inner ear SCs in mammals, bird and fish might reflect the necessity of maintaining a pool of cells in a stem-like state with the goal to repair the tissue in case of damage. Why mammalian *sox2*-positive SCs are unable to self-renew nor to produce HC precursor in the adulthood like non-mammalian vertebrate counterparts is far from clear. On the contrary, a recent study has shown that $p27^{kip}$ expression is dependent on *sox2* in inner pillar cells (a subpopulation of SCs) in mouse cochlea, suggesting different modes of action of this transcription factor (Liu *et al.*, 2012).

Finally, we also examined the relationship between RA and FGF. During development, overexpression of FGF leads to an expansion of the *sox2* positive domain (Millimaki *et al.*, 2010). Moreover, *fgf3* is transiently inhibited in regenerating neuromast (Jiang *et al.*, 2014). Maier et al. have shown that RA signaling in the inner ear acts as a feedback inhibitor of FGF signaling (Maier and Whitfield, 2014). In detail, FGF signaling is initially necessary for the expression of *aldh1a3* in the otic vesicle, but then RA acts to downregulate FGF activity. Specifically, the blockade of RA signaling results in an upregulation of *fgf3* (Maier and Whitfield, 2014). In light of these observations during development, we speculated that upregulation of RA signaling during regeneration could derepress SCs, otherwise kept quiescent, by inhibiting FGF signaling. However, our results do not highlight a cross-talk between the two pathways suggesting that interactions during HC regeneration do not fully recapitulate development networks. However, further studies are needed to deeper investigate a putative interaction between the two signaling pathways.

Gene expression studies presented here show that RA synthesis and degradation are spatially coordinated across inner ear sensory epithelia and neuromasts, suggesting a source-and-sink regulatory mechanism. Recent publications support the existence of different SCs subpopulations in neuromasts, having specific features and roles during HCs regeneration (Ma *et al.*, 2008; Cruz *et al.*, 2015; Romero-Carvajal *et al.*, 2015). SCs located centrally show high levels of proliferation and are responsible for giving rise to the HC precursors, while another subpopulation, placed peripherally, is less mitotically active and is essential for maintaining the SC pool (Ma *et al.*, 2008; Cruz *et al.*, 2015; Romero-Carvajal *et al.*, 2015). One intriguing hypothesis is that the generation of a RA gradient from the center to the outside of the neuromast could instruct and contribute to define the separated SC populations. RA synthetized by *aldh1a3* enzyme in the center, enhances cell proliferation in this area by controlling $p27^{kip}$. On the other hand, *CYPs* expression at neuromast poles protects cells from RA signaling and maintain them in a quiescent state. Interestingly, *cyp26a1* display a peculiar expression pattern. *cyp26a1* was detected only in few regenerating neuromasts, and it was induced in two adjacent cells placed at neuromast poles. The expression of *cyp26a1* might define a specific SC subpopulation characterized by stem-cell features responsible for long term SC self-renewal as recently suggested by Raible's group (Cruz *et al.*, 2015).

4.4 Which signals may regulate RA pathway activation?

The rapid burst of RA signaling in SCs of both inner ear and lateral line suggest that RA is one of the first signaling pathway triggering HC regeneration. However, signals crucial for the initiation of the regeneration must exist and might regulate the

fast induction of the RA pathway. Studies of wound healing and wound epidermis revealed a number of regeneration-response genes during zebrafish caudal fin regeneration (Padhi *et al.*, 2004; Schebesta *et al.*, 2006). Moreover, recent findings have shown how apoptotic cells are associated with and can even stimulate nearby regenerative events (Tseng *et al.*, 2007; Jiang *et al.*, 2009; Pellettieri *et al.*, 2010). Jiang *et al.* found that *stat/jak* signaling plays a critical role in the regeneration of the epithelial cells in the *Drosophila* midgut by initiating cell division and inducing differentiation (Jiang *et al.*, 2009). Interestingly and in line with that, Burgess and colleagues observed a rapid induction of *stat3* transcription factor in SCs triggered by HC death, which activate the expression of *socs3a* in zebrafish (Liang *et al.*, 2012). The *stat3/socs3a* pathway is essential for both inner ear and lateral line HC regeneration in zebrafish and its activation occurs immediately after HC injury (0h after acoustic damage) (Liang *et al.*, 2012). These findings offer new insight and challenges for the HC regeneration field and may provide a link between HC death and activation of essential signaling pathways, including RA.

In conclusion, RA has been mostly associated with cell differentiation, and, due to its capacity to commit stem cells to a particular lineage, has been highly used in ES differentiation protocols (Gudas and Wagner, 2011). However, recent studies have proposed that RA is essential for reprogramming MEF cells into iPS cells (Wang *et al.*, 2011; Yang *et al.*, 2015). RA signaling acts in a highly dose-sensitive manner, precisely, low concentration favors MEF reprogramming while at high concentration, RA present its classical differentiation role (Wang *et al.*, 2011; Yang *et al.*, 2015). In line with these findings, we demonstrate for the first time an *in vivo* example of RA capacity to induce transcriptional changes aimed to

reprogram SCs. Although the molecular mechanisms of RA action have not been fully characterized and high-throughput analysis might be necessary, we show in the present study how the RA signaling is important for cell-autonomous inhibition of *p27^{kip}* and *sox2* transcription and SCs reactivation of cell proliferation, generating new HCs in both zebrafish inner ear and lateral line systems. These new data might be useful to further understand the basis of non-mammalian vertebrate HC regeneration, hoping that these results would be transferrable to inducing HC regeneration in mammals.

5. CONCLUSIONS

1. Lateral crista hair cell regeneration in zebrafish larvae is significantly impaired when the retinoic acid signaling pathway is blocked by the overexpression of a dominant negative form of the retinoic acid receptor alpha after hair cell ablation by two-photon microscopy.

2. Regeneration of lateral crista hair cells is also impaired after chemical blockade of the activity of RA synthetizing enzymes. Neither dnRAR overexpression nor DEAB treatment affects inner ear homeostasis or hair cell survival.

3. dnRAR overexpression inhibits supporting cell proliferation after hair cell damage in the lateral crista. This effect seems to be cell-autonomous, providing a mechanism by which retinoic acid signaling might regulate hair cell regeneration.

4. The retinoic acid pathway blockade via dnRAR overexpression also impairs hair cell regeneration and supporting cell proliferation in lateral line neuromasts after neomycin treatment.

5. Inner ear and lateral line hair cell regeneration are characterized by different kinetics. Neuromast fully

recovers within 48h after injury, while hair cell regeneration in lateral crista last for 6 days.

6. Newly regenerated hair cells derive from FGF-positive supporting cells in both inner ear and lateral line systems.

7. In the regenerating lateral cristae, the retinoic acid pathway components *aldh1a3*, *cyp26b1* and *rarγa* are upregulated 24h after hair cell laser ablation.

8. During hair cell regeneration, *aldh1a3*, *rarαb*, and *cyp26a1* are transcriptionally activated by 1.5h after neomycin treatment, while *cyp26c1* at 3 h.

9. The transcription factor *sox2* expressed in supporting cells, is rapidly and transiently downregulated in neuromast (1.5h after neomycin treatment) and in lateral crista (4h after laser ablation). Retinoic acid signaling blockade impairs the *sox2* downregulation in regenerating neuromasts in a cell-autonomous manner.

10. As previously described, the cell cycle inhibitor $p27^{kip}$ is repress in neuromast 1.5h after hair cell damage. Overexpression of dnRAR in neuromast cell-autonomously impairs the downregulation of $p27^{kip}$,

suggesting a molecular mechanism by which retinoic acid controls supporting cell proliferation.

11. *fgf3* transcription is inhibited 1.5h after neomycin treatment in regenerating neuromast, as previously reported, but retinoic acid signaling blockade does not alter this downregulation.

12. *fgf3* overexpression during hair cell regeneration in neuromast has no effect on the activation of *aldh1a3*, suggesting the absence of a cross-regulation between retinoic acid and FGF signaling.

6. MATERIALS AND METHODS

Zebrafish strains and maintenance

All the experiment performed for this thesis were done using zebrafish embryos and larvae obtained by pair mating of adult fish in the PRBB zebrafish facility by standard methods. Strains were maintained individually as inbred lines. In addition to wild-type (AB), the following zebrafish transgenic lines of either sex were used:

tg(brn3c:mGFP) is a stable reporter line where a GFP tagged to the membrane is expressed under the control of the *brn3c* promoter (Xiao *et al.*, 2005).

tg(hsp70:dnrarαa-GFP) is a transgenic line where a heat-shock promoter drive the expression of a dominant negative form of the Retinoic Acid Receptor (RAR) alpha a (Kikuchi *et al.*, 2011). We chose the dominant negative form of this particular receptor to block RA pathway since is the most potent of these inhibitory receptor mutants (Damm *et al.*, 1993).

tg(erm:gal4;UAS:kaede) is a stable reporter line that by GAL4-UAS system express the photoconvertible protein Kaede under the control of the FGF downstream target *erm* promoter (Esain *et al.*, 2010).

tg(hsp70:fgf3) is a transgenic line where *fgf3* expression can be induced by a heat-shock (Lecaudey *et al.*, 2008).

tg(claudinb:GFP) is a stable reporter line where *claudinB* promoter drives the expression of a membrane-tethered version of GFP (Haas and Gilmour, 2006).

tg(Xla.Eef1a1:H2B-Venus) is a transgenic line that ubiquitously expresses *H2B* histone protein fused to the Venus reporter, allowing nuclei staining. (Recher *et al.*, 2013).

Embryos were developed in an incubator at 28.5°C in system water containing methylene blue and staged according to standard protocols (Kimmel *et al.*, 1995).

Laser ablation of lateral crista hair cells and blockade of RA pathway

Double transgenic tg(brn3c:mGFP;hsp70:dnrarαa-GFP) embryos were obtained by pairwise mating of adult carriers. 4.5 day-old larvae were anaesthetised using Tricaine 20 µM (Sigma-Aldrich) and embedded on their sides in 1% low melting point agarose (Ecogen). Lateral crista hair cells were photoablated on SP5 upright Leica confocal microscope using a 25x water dipping objective and a two-photon laser beam (65-90% intensity for 3-6 seconds, depending on the experiment). To avoid damaging the entire sensory patch, only the upper row of hair cells was targeted, with 70-80% of hair cells ablated. The appearance of a transient air bubble confirms the ablation. After laser ablation of the lateral crista, transgenic larvae were heat-shocked at 39°C in pre-warmed system water for 45min-1h. The hair cells expressing membrane GFP (mGFP) were imaged using a SP2 Leica confocal microscope at different time points: 2, 48 hours post-ablation (hpa) and 6 days post-ablation (dpa). A second short 20-minute heat-shock was performed at 20 hpa. Z-stacks spanning the entire crista (ablated lateral crista and posterior non-ablated crista as an internal control) were taken at each time point (one z-plane imaged every 2-4 µm) using a SP2 Leica microscope. Raw data were analysed and hair cells were counted with FIJI software (Schindelin *et al.*, 2012).

DiAsp staining of mature hair cells

In another set of experiments, hair cell regeneration was analysed by counting the generation of matured hair cells in the inner ear. Prior to laser ablation, mature hair cells were labelled with 5mg/ml (1/6 of the stock solution in system water) of the fluorescent vital dye DiAsp (Collazo *et al.*, 1994) that exclusively labels cells with active mechanotransducing channels. 4.5 day-old wild-type or tg(hsp70:dnrarαa-GFP)

larvae were anesthetized, placed on their side in a injection chamber and DiAsp solution was injected into the right inner ear. One hour later, DiAsp stained lateral crista was laser ablated and heat-shocked only once to block the RA pathway (see above). Regeneration in ablated lateral crista was assessed at 48 hours and 6 days post-ablation by injecting DiAsp into the inner ear before SP2 Leica microscope confocal imaging.

Pharmacological loss-of-function studies

20 mM 4-(diethylamino)-benzaldehyde (DEAB, Sigma-Aldrich) stock solution was prepared and stored at -20ºC. Pharmacological blockade of RA activity was performed by incubating larvae with 100 µM DEAB in system water (1/200 of the stock solution) or DMSO as a control (diluted 1/200 in system water) just after laser ablation of hair cells. Larvae were incubated at 28.5ºC, in dark. DEAB solution was changed every 12 hours and maintained during the 6-day regeneration period. At each time-point, larvae were anesthetized using Tricaine 20 µM (Sigma-Aldrich) and embedded on their sides in 1% low melting point agarose (Ecogen) dissolved in 100 µM DEAB system water. The hair cells of lateral crista of tg(brn3c:mGFP) larvae were imaged at 4, 48 hpa and 6 dpa.

Neomycin induced hair cell damage to assess hair cell regeneration in the lateral line

Neomycin trisulphate salt hydrate (Sigma-Aldrich) was stored at 4ºC, protected by the light. Hair cell damage in the lateral line was induced by incubating 4.5 day-old tg(brn3c:mGFP) or tg(brn3c:mGFP;hsp70:dnrarαa-GFP) zebrafish larvae with 250-500 µm of neomycin in system water for 1 hour at 28.5ºC in dark, as previously reported (Harris *et al.*, 2003). Following neomycin treatment, larvae were washed 3 times with system

water and allowed to recover for 3 hours, at 28.5ºC. Washing extensively the larvae after neomycin treatment is crucial for the survival. Then, larvae were incubated with 5 mg/ml DiAsp solution (1/6 of the stock solution in system water) for 5 minutes shacking, and immediately anesthetized for live imaging. Some young and not fully mature Brn3c-positive hair cells that survived to antibiotic administration could be imaged at 4 hours post-treatment (hpt) and therefore, neuromasts were easily detected. Counting of brn3c-positive and DiAsp stained-cells was performed at 12, 24 and 48 hpt in every individual neuromast.

BrdU incubation and immunohistochemistry

Lateral crista: lateral crista hair cells of 4.5 day-old tg(brn3c:mGFP;hsp70:dnrarαa-GFP) and tg(brn3c:mGFP) larvae were laser ablated as previously described, and were heat-shocked at 39ºC for 1 hour in pre-warmed system water. 24 hpa larvae were treated with 10mM 5-bromo-2'-deoxyuridine (Sigma-Aldrich) dissolved in system water for 24 hours, washed two times and fixed in 4% paraformaldehyde. After several washes with 0.1% PBT, larvae were incubated 1h in 15% sucrose (in PBS) then in 15% sucrose/7.5% gelatin and placed in cryomold in the desired orientation. Blocks were frozen in 2-Methylbutane (Sigma-Aldrich) for tissue preservation and cryosectioned at 20 µm on a Leica CM 1510-1 cryostat. Sections were collected on Superfrost slides and mounted with mowiol.

Neuromast: 4.5 day-old tg(claudinb:GFP;hsp70:dnrarαa-GFP) and tg(claudinb:GFP) larvae were treated with 250-500µm of neomycin trisulphate salt hydrate, as previously described, and heat-shocked at 39ºC for 1 hour. After washing the larvae, they were allowed to recover and were treated with 10mM 5-bromo-2'-deoxyuridine (Sigma-Aldrich) dissolved in system water, for

6 hours at 9 hpt and 34 hpt. Larvae were fixed in 4% paraformaldehyde and washed several times with 0.1% PBT. Immunohistochemistry: after a DNA denaturation step in 2N HCl for 30 minutes at RT, larvae (to reveal BrdU-positive cells in the neuromast) or sections (for the inner ear) were washed three times in 0.1M sodium borate for 15 minutes, washed in 0.1% PBT and blocked in blocking solution (0.1%Tween-20 in PBS (PBT), 2% bovine serum albumin (BSA) and 10% goat serum) for 1.5 hour at RT. Mouse anti-BrdU (Sigma; 1:200) and rabbit anti-GFP (Torrey Pinnes; 1:400) were incubated overnight at 4ºC in blocking solution. After washing with PBT for the whole day, anti-rabbit Alexa488 and anti-mouse Alexa648 (Invitrogen; 1:400) were incubated overnight at 4ºC in blocking solution.

Neuromast hair cell regeneration time-lapse

4.5 day-old tg(Xla.Eef1a1:H2B-Venus;brn3c:mGFP;hsp70:dnrarαa-GFP) triple transgenic larvae were treated with 250-500µm of neomycin trisulphate salt hydrate and heat-shocked at 39ºC for 1 hour as described before. To perform the imaging we used a confocal sequential acquisition mode of 2 channels: 1) excitation at 488nm (good excitation wavelenght for GFP and poor one for venusFP) and detection window was optimized to detect GFP emission and minimize venusFP emission (green colour was assigned to this channel for presentation purposes); 2) excitation at 515nm (good excitation wavelenght for venusFP and also excites GFP) and detection window was optimized to maximize venusFP emission detection (red colour was assigned to this channel for presentation purposes). With these settings, in channel 1 we only detect the signal from the dnRAR-GFP fusion while in channel 2 signals both nuclear FP were detected (the membrane of hair cells coming from brn3c-

mGFP signal is observed in red in the video because the increased sensitivity settings for channel 2). The video displays an overlay of the two channels showing GFP- nuclei in red and GFP+ nuclei in yellow (expressing both FPs). z-stacks of 50 µm were collected for every time point and a z-projection of maximal intensity comprising about 10 µm depth (10 slices including all nuclei from the neuromast) was obtained to generate the video for presentation. The time lapse was performed during 9 hours. Divisions were identified by a detailed 4D analysis of every plane.

Cell lineage

Tg(brn3c:mGFP;erm:Gal4;UAS:Kaede) embryos were obtained by pairwise mating of adult carriers. Hair cells from lateral crista of 4.5 day-old larvae were ablated as described above and immediately afterwards the Kaede protein of that crista was photoconverted from green to red by exposure to UV light (12.5x intensity for 10 seconds). Larvae were imaged at 48 hpa using a Leica SP5 confocal microscope.

In the case of the neuromast, Kaede photoconversion was performed 3 hours after neomycin treatment. Immediately after photoconversion, Z-stacks spanning the entire neuromast were taken every 10 minutes (one z-plane imaged every 1 µm) using a Leica SP5 confocal microscope for 3 hours (from 3hpt to 6 hpt). The larvae were again imaged every 10 minutes between 12 and 21 hpt and at 30 and 50 hpt using a Leica STED confocal microscope. Raw data were analysed and hair cells were counted with FIJI software (Schindelin *et al.*, 2012).

Whole mount in situ hybridization and immunohistochemistry

Antisense RNA probe synthesis was done by *in vitro* transcription of linearized DNA vectors or of PCR amplification products. In the first case, vectors carried the sequence of interest, flanked by T3, T7 or SP6 polymerases sequence. The

following probes were used: *atoh1a* (Millimaki *et al.*, 2007), *aldh1a2* (Begemann *et al.*, 2001), *aldh1a3* (Canestro *et al.*, 2009), *cyp26a1*, *cyp26b1* and *cyp26c1* (White *et al.*, 2007), *sox2* (Marz *et al.*, 2010), *cdkn1b* (Geling *et al.*, 2003), *fgf3* (Maves *et al.*, 2002).

DNA linearization and purification:

1 µg of plasmid DNA were incubated at 37ºC with the specific restriction enzyme (see table 1) in the final volume of 20 µl. After 2h, the enzymatic reaction was stopped by adding 1 µl of proteinase K (10 mg/ml) and 1 µl of SDS 10%, and incubating the reaction at 37ºC 30 min. Then, the linearized plasmid was purified adding 80 µl of H2O, 11 µl of 3M Na Acetate, 278 µl of 100% ethanol, incubating at -20ºC for 1h and, finally, centrifuging 30 min at 13000 rpm at 4ºC. The pellet was next washed with 500 µl of 70% ethanol and centrifuged 10 min at 13000 at 4ºC. Once dry, the linearized plasmid was resuspended in 20 µl of H_2O.

The restriction enzymes and polymerases used to synthesize each probes are listed below:

Probe	*Restriction enzyme/RNA polymerase*
atoh1a	KpnI/T7
aldh1a2	NotI/T3
aldh1a3	HindIII/T7
cdkn1b	NotI/T3
cyp26a1	SalI/T7
cyp26b1	EcoRI/SP6
cyp26c1	XbaI/SP6
fgf3	SalI/T7
sox2	BamHI/T7

Table 1 *Restriction enzymes and RNA polymerases used for the generation of riboprobes for in situ hybridization*

On the other hand, *rarαb* and *rarγa* probes were generated by PCR amplification from 72 hpf embryos cDNA, adding T7 polymerase binding side at 5' of the reverse primers and following RNA transcription.

cDNA library generation specific PCR amplification

Total RNA isolation was done using Trizol (Invitrogen) extraction protocol. Reverse transcription of obtained RNA was performed using SuperScript III Reverse Trascriptase Kit from Invitrogen. To selectively amplify *rarαb* and *rarγa* genes the Expand High Fidelity PLUS PCR system (Roche) and the following primers were used:

rarαb-FW: 5'-GATGTGTGGTTTGTGTGGCCTTC-3'

rarαb-RV-T7: 5'-TAATACGACTCACTATAGGGATGCCTTCC CCTCGCTCTGTCAG-3'

rarγa-FW: 5'-CGAGGCTAGGAACAGCTCAC-3'

rarγa-RV-T7: 5'-TAATACGACTCACTATAGGGATGCAAGCAGGCAGATTT GAGAAGG-3'

Once prepared the mix as described in the manufacturer datasheet, the following cycler program was used:

94ºC	2 min	
94ºC	30 sec	
55ºC	30 sec	30x
68ºC	3 min	
68ºC	7 min	
4ºC	hold	

In order to verify the PCR product, 1 µl of the reaction was run on 1% agarose gel/1xTBE.

RNA probe transcription

For the generation of antisense probes, linearized DNA or PCR products were incubated 37ºC with the specific RNA polymerase (see table 1) and DIG labeled nucleotides (DIG RNA labeling mix (Roche)) in a final volume of 20 µl. After 2h, riboprobes were purified adding 30 µl of H_2O, 300 µl of cold 100 % ethanol, 10 µl of 4M LiCl, incubating the reaction 30 min at -20ºC and centrifuging for 30 min at 13000 rpm at 4ºC. The pellet was then washed with 500 µl of cold 70% ethanol and centrifuged 10 min at 13000 rpm at 4ºC. Once dry, the RNA probes were resuspended in 20 µl of H_2O and 1µl was run in a 1% agarose gel/1xTBE to verify the transcription.

Whole-mount in situ hybridization (ISH) in zebrafish larvae

Single whole-mount in situ hybridization was carried out with DIG-labeled riboprobes detected by alkaline-phosphatase coupled anti-DIG antibody (anti-DIG-AP), and developed with NBT/BCIP according to Thisse et al, 2004. After *in situ* hybridization, larvae were post-fixed overnight in 4% PFA and analysed in whole mount for neuromast imaging (larvae mounted in 100% glycerol) or in sections for inner ear expression analysis. In the second case, larvae were incubated 1h in 15% sucrose (in PBS) then in 15% sucrose/7.5% gelatin and placed in cryomold in the desired orientation. Blocks were frozen in 2-Methylbutane (Sigma-Aldrich) for tissue preservation and cryosectioned at 20 µm on a Leica CM 1510-1 cryostat. Sections were collected on Superfrost slides and mounted with mowiol.

Whole mount immunohistochemistry

Immunostaining with rabbit anti-GFP (Torrey Pines) and donkey anti-rabbit Alexa Fluor 488 (Life technologies) was performed to detect brn3c cells expressing mGFP and/or the

fusion protein dnRAR-GFP. Briefly, larvae were blocked 1.5h at room temperature with blocking solution (2% Bovine serum albumin (BSA), 10% heat inactivated goat serum, 0.1% PBT). Then, larvae were incubated overnight with rabbit anti-GFP primary antibody (1:400) in blocking solution. After washing 3 times for 15 min with PBT, larvae were incubated 2h with Alexa Fluor 488 secondary antibody (1:400) in blocking solution, and washed again extensively. As for in situ hybridizations, larvae can be prepared for whole-mount imaging or cryosectioned as described above.

Statistics

All statistical comparisons were performed by unpaired Student t-test. In figures Mean and SD values are shown. **$p<0.01$ and * $p<0.05$.

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ANNEX

During my phD I also participate in a project that result in the following publication:

Hoijman E, Rubbini D, Colombelli J, Alsina B. Mitotic cell rounding and epithelial thinning regulate lumen growth and shape. Nature Communication: 2015, Jun 16;6:7355
doi: 10.1038/ncomms8355