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Final Project

Study of inner ear and lateral line hair cell regeneration

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Final project abstract
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Abstract

Death of sensory hair cells in the inner ear results in two global health problems that millions of people around the world suffer: hearing loss and balance disorders. Hair cells convert sound vibrations and head movements into electrical signals that are conveyed to the brain, and as a result of aging, exposure to noise, modern drugs or genetic predisposition, hair cells die.

In mammals, the great majority of hair cells are produced during embryogenesis, and hair cells that are lost after birth are not replaceable. However, in the last decades, researches have shown some model organisms that retain the ability to regenerate hair cells damaged after embryogenesis, such as Zebrafish and chicken, providing clues as to the cellular and molecular mechanisms that may block hair cell regeneration in mammals. This discovery initiated a search for methods to stimulate regeneration or replacement of hair cells in mammals, a search that, if fruitful, will revolutionize the treatment of hearing loss and balance disorders.

One aim of my project is to study the role of retinoic acid in adult Zebrafish and in mice, which is a metabolite of vitamin A known as an essential molecule to activate hair cell regeneration after cells damaged in Zebrafish embryo. We want to study important genes involved in retinoic acid pathway, such as Aldh1a3 and RARs genes, to check what their role is in the inner ear of adult Zebrafish and compare result obtained in the inner ear of mice.

On the other hand, Zebrafish lateral line contains neuromast, which are formed by the same structure than the inner ear: hair cells surrounded by supporting cells and neurons. The lateral line is a structure below the skin's surface that makes easier to damage hair cells to study their regeneration. For that reason, another aim of my project is to study how Sox2 and Atoh1, essential genes during the inner ear development, change their expression during hair cell regeneration in the lateral line.

In my project, the most important concepts related to Zebrafish world are explained in order to understand why we have studied this animal and these essential genes. Then, techniques that we used are explained, with their protocol attached in the annexes. Finally, results of my project are shown, but many of them were not expected and they would be needed to follow studying.

Summary

1-Introduction

Hearing loss and balance disorders are global health problems which affect millions of people around the world. These disorders normally have their origin due to genetic predisposition, ototoxic drugs, strong noises or a mix of them. These disorders result in the loss of the sensory hair cells which are responsible to convert sound vibration and head movements into electrical signals that are transmitted to the brain. In mammals, the majority of these hair cells are produced only during embryogenesis; probably that is why damaged hair cells after birth can't be regenerated. However there are some non-mammalian vertebrates, such as fish or amphibians, which do have the capacity of regenerating and self repairing these hair cells throughout life and recovering their sensory functions. Nowadays, this self-repairing capacity in non-mammalian vertebrates is being investigated. In this project we have assessed hair cell regeneration in Zebrafish. This fish has currently become one of most used animal model for these types of regeneration studies especially common in the study of genes involved in inner ear development, to try to understand their role in regeneration of hair cells. It is known that acid retinoic is essential for hair cell regeneration in the inner ear and neuromast of Zebrafish embryo, one of the aims of this project is to assess the role of retinoic acid in adult zebrafish hair cell regeneration and in adult mouse.

2-Background

Zebrafish, studied in order to understand how cells can regenerate in this organism, is a powerful model organism to understand and find solutions to this disease in humans. The main reasons are stated below:

2.1-Zebrafish as a cell regeneration model

The Zebrafish, Danio rerio, is a small tropical fresh-water fish which originally comes from south-eastern Himalaya region, but can also be found in rivers of northern India, northern Pakistan, Bangladesh, Nepal, Burma and in South Asia. Due to its regenerative skills, Zebrafish has become an excellent vertebrate model organism for biologists studying and scientific research. Zebrafish, known for its regenerative abilities, is nowadays considered a perfect animal model and used to identify gene functions in a vast range of developmental disorders and diseases.

2.1.1-Characteristics

A rapid development

The embryonic development of Zebrafish is very rapid: In the first 24 hours after fertilisation, all major organs appear and hatching takes place 12-36 hours later, depending on the embryo's internal condition and the external temperature, and then embryos start looking for food. The embryos develop from single cells to swimming fish in about two days.

After just 3 - 4 months, Zebrafish is sexually mature and capable of generating descendants. A single female can lay up to 200 eggs per week.

From: Kimmel et al. Stages of embryonic development of the zebrafish
Dev. Dyn. 203:253-310, 1995

Fig.2.1.1: Zebrafish development, from Kimmel et al. Stages of embryonic development of the Zebrafish

See-through embryos

The development of the Zebrafish is very similar to the embryogenesis in higher mammalian vertebrates but Zebrafish develop from fertilised transparent eggs to adult fish outside the female body. This embryos transparency is certainly another of the main reasons of their popularity in scientific research.   This allows observing the developing embryos easily inside during all the different steps of the process.

Moreover, the optical clarity of its embryo during the first few days of their lives allows researchers to observe the formation of internal organs "live" inside the living organism. This facilitates analysis of normal developmental processes and makes it much easier to detect and interpret changes caused by mutations or experimental manipulations.

This transparency also facilitates the detection of mutations; these can be identified quite easily just by using a fluorescence protein called GFP (Green Fluorescent Protein), which can be used to label individual cells, organs or even organelles.

Embryos can be genetically manipulated

Manipulating genes with chemicals is used to analyze what happens when gene's normal function is altered. It is probably considered one of the most effective ways for identifying new genes or discovering novel functions of known genes.

Moreover, Zebrafish mutations are generally less severe than human diseases, because the ancestral functions are typically subdivided amongst two or more homolog. This subdivision offer a great advantage to understand specific details of gene function and permits studying multiple functions. Due to the fact that mammalian mutations are more severe and often mask secondary functions of genes, this, it is better to use Zebrafish for functional genomics studies of human sequences.

Easy to breed

Zebrafishs are easy to breed and maintain in an aquarium, reducing the costs and making this animal model more economical than many other, making them accessible in large scale to laboratory researchers.

The Zebrafish genome

Zebrafish has 25 chromosomes and their genome contains about 1.5 x 10 billion base-pairs, half the amount if compared with the average mammalian genome size (aprox. 3 x 10 billion base-pairs). However, Zebrafish has many significant genes that have evolutionarily conserved across vertebrate species, including humans.

An ideal model organism

Zebrafish is one of the first non-mamalian vertebrates used in research. They show a much greater complexity than invertebrate models such as worms or Drosophila, when referring to organ systems. .

These important positive features such as: **rapid development**, **large number of offspring**, **transparency of the embryos**, **easiness in breeding** or its **genomic disposition**, make Zebrafish an ideal model organism to study the development of vertebrates using a genetic approach.

2.2-Definition of regeneration

The way how Zebrafish regenerates its cells, such as heart cells, nervous system cells or hair cells, has been studied and Scientifics have done an enormous progress during the past fifty years.

2.2.1-Regeneration

Regeneration is the process which some tissues, organs, genomes, cells and organisms renew and restoration themselves after an injury, a damage or organ part loss.

Humans can regenerate some organs, such as the liver or skin. Unfortunately, many other human tissues cannot regenerate as other animals can it, and this is one of the goals of regenerative investigations which try to understand why some animals can regenerate their tissues and humans cannot it.

2.2.2-How does regeneration work?

When damage has occurred, there are genetic induction factors that induce a cellular physiological response to regenerate from the damage, activating some specifics genes to work. Many of these genes, which are activated after an injury, are the same genes involve in the original development of tissues.

One of the main functions of this cellular physiological response is to produce signalling events to stimulate the production of additional cells that are capable of rebuilding lost structures. New cells coalesce near the site of injury, giving rise to a mass of undifferentiated cells called the regeneration blastema. Subsequent signals then regulate outgrowth and patterning of the newly formed tissue. To understand how early signalling events initiate regeneration and stimulate blastema formation,
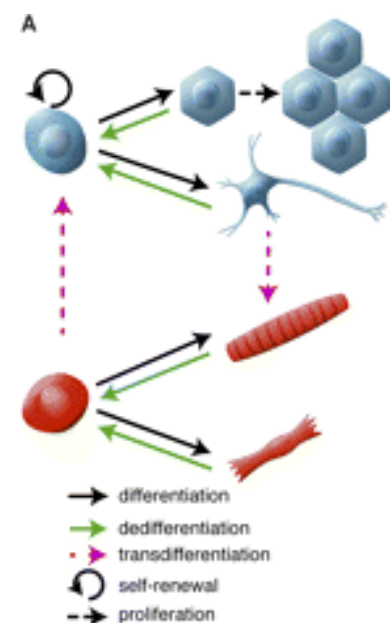

Fig.2.2.1 Cellular sources of regeneration (Ryan S. King and Phillip A. Newmark, 2012)**:** The ability to regenerate amputated structures often requires the production of new cells, and this production can be in different ways.

it is crucial to identify the cells upon which these signals act. New cells can be generated in a variety of ways, including proliferation of a resident stem cell population, division of terminally differentiated cells, or dedifferentiation/transdifferentiation of mature cells to a stem cell–like precursor or another cell type. The extent to which each mode is used varies between species and even across tissues within the same species (Ryan S. King and Phillip A. Newmark, 2012).

More research is needed to understand how differentiated cells can be made to divide and produce new tissue or cells, because the identification of genes and signalling pathways that direct progenitor cell behaviour is unknown and it is necessary to understand how cells regenerate and why humans have lost this capacity.

2.3-Inner ear structure

The inner ear is responsible for sound detection and balance and it comprises of two main functional parts: on one hand we have the lagena, responsible of hearing, which converts the sound into electrochemical impulses which are transmitted to the auditory nerve and then to the brain. On the other hand we have the vestibular system, which is responsible of balance.

Zebrafish inner ear develops during 13.5-14 hours postfertilisation (hpf) from and ectodermal thickening, a pair of surface sensory placodes, the otic placode, which is visible on either side of the hindbrain region. These cavities form a simple vesicle, a hollow ball of epithelium called otic vesicle, where all structures of the inner ear are thought to arise.

2.3.1-Vestibular system

In the vestibular embryonic system there are five sensory patches of epithelium: two macula and three cristae.

Three seimicircular canal

Two maculae

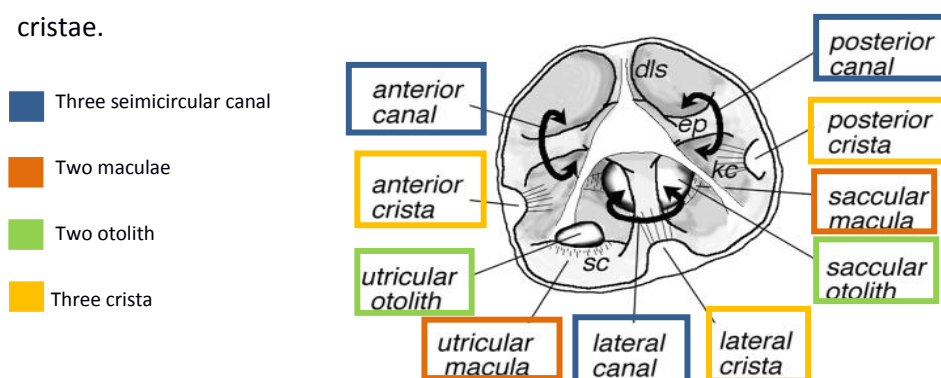**Two otolith**

Three crista

Fig.2.3.1 Wild-type Zebrafish ear at 4 days postfertilisation (TANYA T. WHITFIELD et al. 2002)**:** drawing of vesicle to give a three-dimensional impression of the structures within it. It shows the two macula, utricular and saccular macula, with their otolotih structures, and the three cristaes associated with three semicircular

Macula appears as the first sensory patch in the Zebrafish otic vesicle (Whitfield et al., 2002). There are two different maculae, posterior macula and anterior macula. Both contain receptor hair cells associated to otolith, which is a crystalline deposit of calcium carbonate and protein and are used as gravity, balance and movement indicators. Although maculae are formed for the same components, anterior macula just detects liner acceleration and has balance functions. On the other hand, posterior macula is the only organ that has hearing and balance functions, because detects linear acceleration and, moreover, sound vibration.

In addition, macula and otolith are structures of saccule and utricle, which are the otolith organs and are parts of the balancing apparatus, being sensitive to gravity and linear acceleration. The saccule contains posterior macula giving sensitive to sound, and due to its orientation in the head, gives information about vertical acceleration. The utricule contains anterior macula and gives information about horizontal movement.

Fig.2.3.2 Utricular or saccular structure (Magnus Manske, 2008)**:** images shows structure of the otolith organs, which contains macula with a layer of hair cells and supporting cells, and covering this surface are otolith (otoconia).

The cristas act as a sensor of rotational acceleration and deceleration. Each crisata is associated with a semicircular canal, and are covered by hair cells.

2.3.2-Lagena: the hearing part

The lagena is an otolith organ comprised of the macula lagena and it is homologous to the cochlea of mammals, the auditory portion of the inner ear. Its functions are the detection of sounds and the transduction of sound vibration into electrical responses.

Fig. 2.3.3 Lateral view of the membranous labyrinth in a 2-year-old zebrafish. **(MICHELE MILLER BEVER, AND DONNA M. FEKETE):** In this image it is shown the utricle (u), the lagena (la), the saccule (s), and the three canals (posterior, pc, anterior, ac, horitzontal canal, hc).

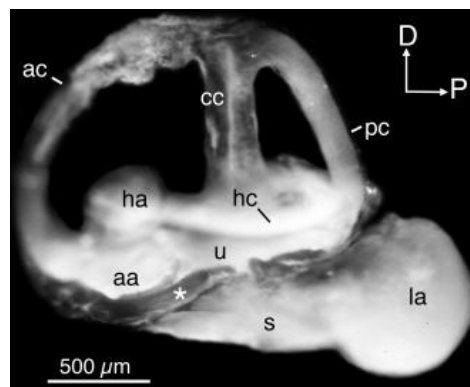These two types of sensory patches (lagena and vestibular system) differ in their function, and in the fine details of their cellular architecture, but they all conform to the same basic plan. They are relatively simple epithelia composed of arrays of two cell types, the sensory hair cells and their surrounding, non-sensory supporting cells, and each hair cell are associated with a neuron, which
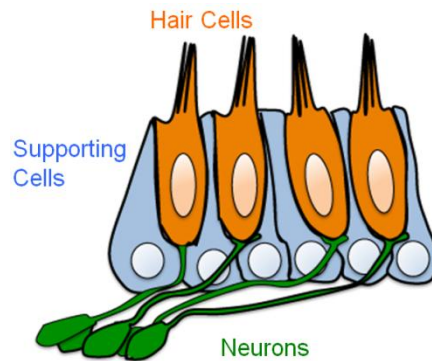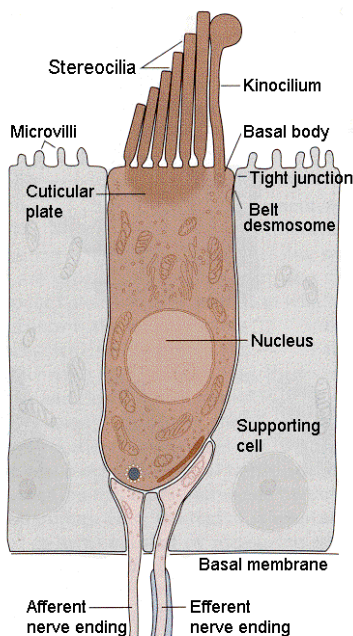

Fig. 2.3.4 Epithelia structure of sensory patches (BM Ryals and EW Rubel, Science, 1988): In this image it is shown the sensory hair cells and their surrounding, supporting cells and neurons.

2.3.3-Hair cells

Sensory hair cells are one of the components of the inner ear, and their function is to be the sensory receptor of both the auditory system and the vestibular system. They are responsible of hearing and balance capacity, and it is possible because of hair bundle on the apical surface of hair cells, the organelle of sensory transduction. Each hair bundles are formed by an elaborate microvillar arrays of stereocilia associated with a single microtubule-based kinocilium. This organelle is responsible for transducing sound energy or head movements into neural signal which is initial input to the auditory and vestibular nervous system.

The main functions of hair cells are to amplify low-level sound that enters the cochlea and to transform the sound vibrations into electrical signals. Hair cells are form synaptic connections from neurons at the basal ends, for that, electrical signals are sent via the auditory nerve to the auditory brainstem.

Fig. 2.3.5 Schematic diagram of a hair cell, showing the relationship of the cilia to the kinocilium and cell body as well as the innervations at the bottom of the cell.
Source: Kandel, Schwartz & Jessell, *Principles of Neural Science*, 4th Edn.

The loss of sensory hair cells causes hearing loss and balance disorders. In the mature mammalian organ of Corti, where there are hair cells and supporting cells, the hair cells are not replaced after ototoxic insults, and the deficit are permanent. In contrast, a replacement of this cells has been found in some kind of

vertebrates, such as chicken and Zebrafish. Hair cells regeneration seems to be originated from supporting cells that reenter the cell cycle when neighbouring hair cells are dying.

2.3.3.1-Atoh1 gene

Hair cells and supporting cells are produced from a prosensory equivalence group initially marked by expression of Atoh1. However, only cells that complete differentiation as hair cells continue to express Atoh1. The rest lose Atoh1 expression and become supporting cells. For that reason, Atoh1 expression is maintained only in hair cells.

It was demonstrated that Atoh1 gene is one of the first genes to be expressed in sensory hair cells, and it is necessary to direct hair cell formation. Zebrafish has two Atoh1 homologues, atoh1a and atoh1b, which are together necessary for hair cell development. These genes crossregulate each other but are differentially required during distinct developmental periods, first in the preocit placode and later in the otic vesicle (Bonny B. Millimaki, Elly M. Sweet, Mary S. Dhason and Bruce B. Riley). Otic expression of atoh1a began at 14 hpf in two domains in the otic placode and expression continued in the sensory maculae through 48 hpf. Expression of atoh1b began much earlier, at 10.5 hpf in the preotic placode and expression diminished at 22 hpf. So, both Atoh1 homologues are only expressed in cells that will become hair cells during the first hours of inner ear development. After this development happens, Atoh1 is not expressed anymore.

The principal regulator of hair cell differentiation, Atoh1 has receives great attention in recent years in research (Shailam et al., 1999; Lanford et al., 2002; Kelley,2006). Nowadays, it is believed that Atoh1 has an important role in zebrafish hair cell regeneration, for that reason we want to assess if during regeneration Atoh1 is expressed again to produce new hair cell.

2.3.4-Hair cell progenitors: Supporting cells

Supporting cells are non-sensory cells that surround hair cells, their main function in is structural and physiological. In 1988 it was published that to regenerate hair cells, a supporting cell division was necessary (Corwin and Cotanche, 1988; Jorgensen and Mathiesen, 1988; Ryals and Rubel, 1988).

Supporting cells can produce new hair cells following different methods. However, only two methods have really been used for this goal, the
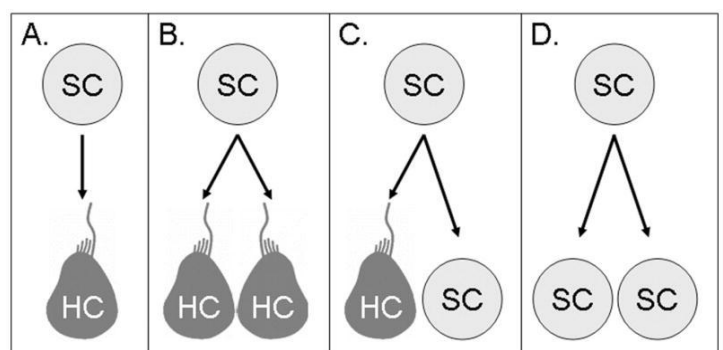

Fig.2.3.6 Methods of hair cell replacement(Heather R. Brignull, David W.Raible, Jennifer S. Stone). The production of hair cells may occur by several methods.

others are just non-proved hypothesis. . The most used method involves hair cells regeneration during its mitosis. Supporting cells divide asymmetrically during mitosis, generating a new hair cells and a supporting cell (Fig. 2.3.6-C). Moreover, with this method, it is thought that it could be possible that supporting cells divided symmetrically, generating a couple of hair cells (Fig. 2.3.6-B) or a couple of supporting cells (Fig. 2.3.6-D). These two possibilities are not yet tested, we still don't know if it occurs in regeneration of hair cells. At some point, it was shown that supporting cells may undergo a phenotypic conversion due to a set of morphological and molecular changes. This produces that supporting cells acquire all properties of sensory hair cells (Fig. 2.3.6-A).

Despite these known regeneration processes, it has been questioned if all the new hair cells come from supporting cells and some investigation have shown that not all of hair cells derived from active mitotic progenitor cells (Roberson et al., 1996, 2004). This is currently under study still.

Supporting cells are a heterogeneous population, there is a subpopulation of supporting cells which can be re-programmed to regenerate hair cells while other supporting cells cannot change their phenotype and do not contribute to hair cell regeneration. This depends on the expression of transcription factors, such as Sox2 or ATOH1, which define subpopulations of supporting cells that are able to regenerate hair cells.

Fig.2.3.7 Two tested methods of hair cell regeneration (Corwin and Cotanche, 1988): division and transdifferentiation, and direct transdifferentiation.

2.3.4.1-Sox2

Sox2 is known as a possible regulator for maintenance and regeneration of hair cells. This gene encodes a transcription factor well known for its role in maintaining pluripotent stem cell population, as well as differentiation during early development. However, the way Sox2 performs the mutually exclusive activities of maintaining pluripotency versus stimulating differentiation is not fully understood.

In the inner ear, Sox2 is initially expressed in progenitors of both hair cells and supporting cells, as Atoh1, but after hair cell differentiation, it is only maintained in supporting cells. It is known that nascent hair cells

at the periphery of the maculae still express sox2 but expression is lost as hair cells mature, but supporting cells constantly has sox2 expression.

Some studies show that knockdown of sox2 does not prevent the emergence of hair cells and support cells, but does lead to subsequent sporadic cell death of hair cells, and possibly support cells as well. Moreover, it was demonstrated that after hair cell damaged, with knockdown of sox2, supporting cells are transdifferentiated but not cell division, and this means that knockdown totally blocks the regeneration process.

These findings suggest that sox2 is required to maintain support cells in a pluripotent state or sox2 facilitates a discrete aspect of support cell differentiation that provides the facultative ability to transdifferentate under appropriate conditions. Finally, this indicate that sox2 is required for survival of a least some hair cells, either directly by regulating early stages of hair cell differentiation or indirectly by regulating essential non-autonomous functions of support cells. (Bonny B. Millimaki, Elly M.Sweet, Bruce B. Riley, 2010).

Nowadays, it is known that Sox2 expression is regulated by Atoh1a/b, because some studies indicate that supporting cells might have the ability to reduce the expression of Sox2 enough to allow Atoh1 activation during regeneration. This would be because cells that are differentiated into supporting cells and express Sox2 undergo dedifferentiation and become progenitor stem cells without expressing Sox2, and then, differentiate into hair cells expressing Atoh1. It is still being studied, and in this project we have been trying to discover what happens during hair cell regeneration.

2.3.5-Neurons

Research has shown that hair cells do not send neural signal to the brain, but that they only amplify low-level sound that enters the lagena. The inner hair cells transform the sound vibration into electrical signals that then relayed via the auditory nerve to the auditory brainstem.

To relay these signals, hair cells synapse with afferent neurons on their basal surfaces. Afferent neuron is a neuron which conveys sensory information centrally from the periphery to auditory nerve. Moreover, hair cells synapse with efferent neurons, which send impulses peripherally to activate muscles or secretory cells.

2.4-Lateral line

Moreover that there are hair cells in the inner ear, zebrafish has an array of hair cells in the lateral line neuromasts. The lateral line is located on the surface of the fish and relays information about both electrical currents and micro movements in the water about their bodies. These functions can be carried out thanks to a series of individual neuromasts below the skin's surface, that contain a set of hair cells which are innervated by afferent and efferent neurons and surrounded by supporting cells.
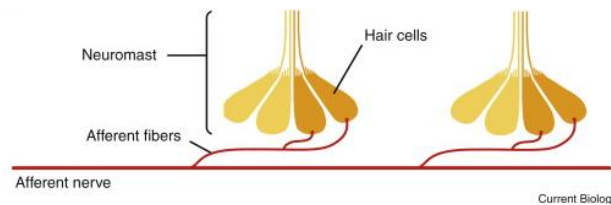

Fig. 2.4.1 Shematic lateral line (Eva Y. Ma and David W. Raible). The image shows an innervation of hair cells across multiple neuromasts by afferent neurons.

The lateral line of Zebrafish comprises two parts: an anterior lateral line, located around the head, and the posterior lateral line that runs down the length of the trunk and tail. The anterior lateral line consists of one ganglion neuromasts and posterior lateral line consist of five neuromasts with 2 or 3 terminal neuromasts.

The lateral line neuromast structure is quite different to the inner ear, nevertheless, it was demonstrated that zebrafish has the same regenerative ability in lateral line hair cells than inner ear hair cells. In addition, hair cell regeneration in the zebrafish lateral line is robust and rapid (Williams and Holder, 2000). Within 48 hours, hair cell have regenerated, re-established mechanotransduction, hair cell bundle polarity, and synapses with the auditory nervous system (Hernandez et al., 2006; Lopez-Schier and Hudspeth, 2006).

Lateral line hair cells also have supporting cells as progenitors and it was shown that supporting cells also regenerate hair cells by a mitotic proliferation (fig. 2.3.6 C) as in the inner ear. There is also a low level of direct transdifferentiation of supporting cells, but it is still being studied.

Besides, lateral line hair cells of zebrafish larvae are easily accessed making them ideal for experimental manipulation.
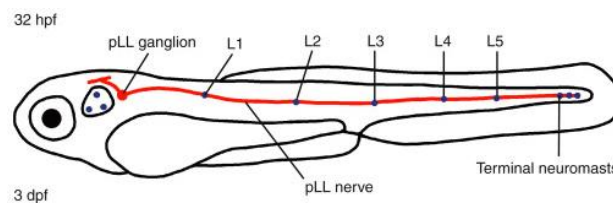

Fig. 2.4.2 Zebrafish lateral line(Eva Y. Ma and David W. Raible). The image shows the lateral line of a 3dpf zebrafish embryo. It is shown the anterior lateral line with its ganglion neuromasts, and the posterior lateral line with its five neuromasts and terminal neuromasts.

2.5-Retinoic Acid

Retinoic acid (RA) is a metabolite of vitamin A (retional) that is an important molecule signalling during vertebrate development and tissue differentiation. It has shown that most of the genes and proteins involved in retinoid signalling are expressed in specific regions of the Zebrafish otocit during development. For that reason, the role of RA in hair cell regeneration it has been studied and it is shown that is necessary to perform regeneration in embryos. However, it is not known if RA has the same role in adult Zebrafish than in embryo. Therefore, we wanted to study genes involved in RA pathway in adult Zebrafish and compare their expression with adult mice.

2.5.1-The retinoic acid pathway: generation of RA

Animals cannot synthesize vitamin A and they extract it from their diet in form of carotenoids (from plants) and retinyl esters (from animal products). These dietary components are stored as retinyl esters in specifics storage sites, and to transport them to cells that require them is performed by retinol, which circulates bound to retinol-binding protein 4 (RBP4)((Fig.2.5.1-A), Then, retinol is taken up by target cells through an interaction with a membrane receptor for RBP4, STRA6 (Fig.2.5.1-B); it then enters the cytoplasm, where it binds to retinol-binding protein 1 (RBP1) and is metabolized in a two-step process to acid retinoic (Fig.2.5.1-C), that, in embryos, first step is done by retinol dehydrogenase 10 (RDH10) which

Fig.2.5.1 Pathway that are involved in the generation of retinoic acid (Retinoic acid in the development, regeneration and maintenance of nervous system, Nature 2007).

metabolizes retinol to retinaldehyde (Ral), and then it is metabolized to all trans-RA by aldehyde dehydrogenases (RALDHs in mouse, ALDHs in Zebrafish).

Once RA is formed, it can be released from the cytoplasm and taken up by the receiving cell (paracrine signalling) (Fig.2.5.1-D), or can act back on its own nucleus (autocrine signalling) (Fig.2.5.1-E). (Retinoic acid in the development, regeneration and maintenance of nervous system, Nature 2007)

2.5.1.1-Aldehyde dehydrogenases (ALDHs)

The aldehyde dehydrogenase gene (*ALDH*) superfamily is an important family represented in all the three taxonomic domains (*Archaea*, *Eubacteria* and *Eukarya),* suggesting a vital role throughout evolutionary history (Update on the aldehyde dehydrogenase gene (ALDH) superfamily, 2011). ALDH activity is required

for the synthesis of vital biomolecules through the metabolism of aldehyde intermediates, such as retinoic acid, folate and betaine, to name a few.

ALDH superfamily is formed for different genes, but not all of them are in all the species. Concretely, in Zebrafish there are 25 ALDH genes, but only three are involved in the metabolism of retinoic acid: Aldh1a1, Aldh1a2 and Aldh1a3. In this project, we have focused the expression of Aldh1a3, because it is well-known that Aldh1a3 is expressed, during inner ear morphogenesis, in developing sensory epithelia of the cristae and utricular macula and is specifically up-regulated in epithelial projections throughout the formation of the walls of the semicircular canals and endolymphatic duct.

Latin name	Common name	# *ALDH* genes
Homo sapiens	Human	19
Pan troglodytes	Common chimpanzee	18
Callithrix jacchus	Common marmoset	16
Pongo abelii	Sumatran orangutan	18
Macaca mulatta	Rhesus macaque	20
Bos taurus	Cow	20
Rattus norvegicus	Norway rat	21
Mus musculus	House mouse	21
Taeniopygia guttata	Zebra finch	15
Gallus gallus	Chicken	14
Danio rerio	Zebrafish	25

Table 2.5.1. List of all species with their number of unique ALDH genes. (Update on the aldehyde dehydrogenase gene (ALDH) superfamily, 2011)

Nevertheless, it is not demonstrated if Aldh1a3 is still expressed in adult Zebrafish, for that reason, in this project we wanted to check it.

2.5.2-Paracrine signalling

When signalling in a paracrine fashion to active some specific gene, RA must be released from the cytoplasm and then up by receiving cells. RA binds to CRABP2 enters the nucleus (Fig.2.5.2-A), and binds to a transcription complex which includes a pair of ligand-activated transcription factors comprising the retinoic acid receptors (RAR) and retinoic X receptors (RXR) (Fig. 2.5.2-B), which themselves heterodimerize.
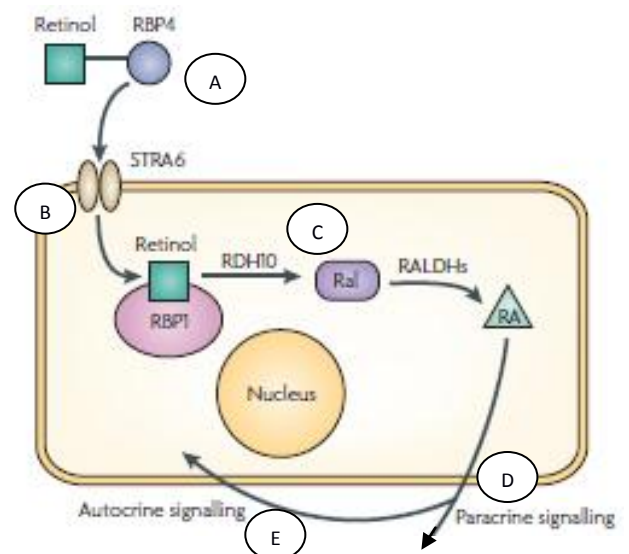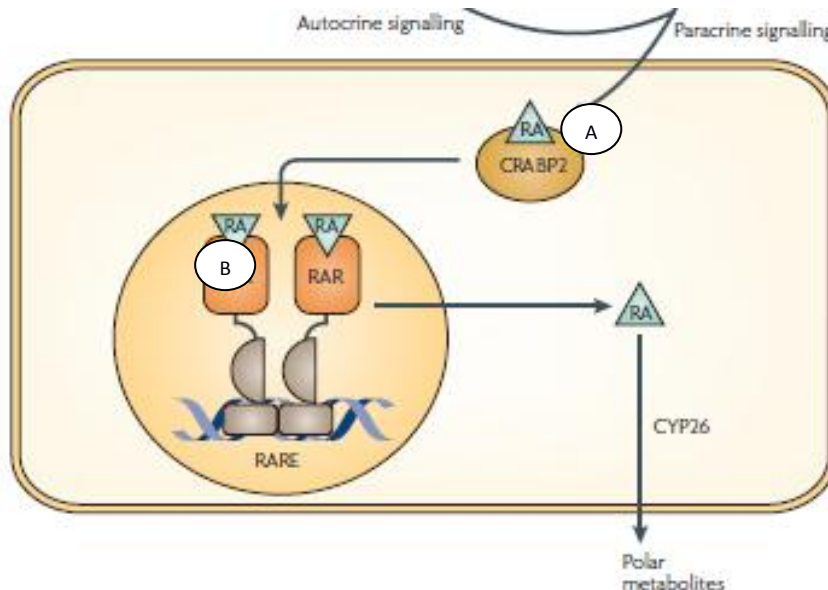

Fig.2.5.2 Pathway that are involved in the action and catabolism of retinoic acid (Retinoic acid in the development, regeneration and maintenance of nervous system, Nature 2007).

There are three RAR genes (RARα, RARβ and RARγ) and three RXR genes (RXRα, RXRβ and RXRγ) and together, the heterodimeric pair binds to a DNA sequence called a retinoic acid-response (RARE). This binding activates the transcription of target genes. After that, RA is catabolised in the cytoplasm by the CYP26 class of P450 enzymes.

2.5.2.1-Retinoic Acid Receptors (RAR)

The diverse actions of RA are mediated by a family of nuclear receptors, the RA receptors which, as it is explained above, heterodimerize with RXR receptors to form ligand-activated transcription factors complexes (Aranda and Pascual, 2001; Mark and Chambon, 2003). As we know, RA plays an important role in Zebrafish hair cell regeneration, as in embryo as in adult Zebrafish, and trying to understand why in mice hair cell regeneration is not allowed, we wanted to study the RARs genes expression in mice inner ear.

3-Aims of project

Some research has shown that retinoic acid is essential for hair cell regeneration in larval stage of Zebrafish, and if it is performed an inhibition of genes involved in retinoic acid pathway, hair cells regeneration is not allowed in Zebrafish inner ear and lateral line. It is known in embryos but not in adult Zebrafish. For that reason, the principal aim of my project is to study the expression of different genes involved in retinoic acid pathway, such as ALDHs and RARs, in adult Zebrafish. Moreover, we want to study the same genes expression but in mice inner ear, to compare the level of expression between both species, to try to understand why Zebrafish hair cells can be regenerate and why mice hair cells cannot be.

Another aim is to study the expression of genes needed for hair cell regeneration, such as Sox2 and Atoh1, to try to understand what happens during hair cells regeneration in Zebrafish embryo and to check how gene expression changes. To study these variations, it's necessary to carry out a neomycin treatment in different times, to kill all hair cells and then to control the level of expression genes during hair cell regeneration, through a hybridization in situ.

4-Materials and Methods

4.1-Synthesis of RNA probes and sequencing

Labels are required to performed hybridization in situ, because it is the way to label cells which have expression of a particular gene. For instance, in my project, we wanted to know if Sox2 and ATOH1 had different level of expression in different hours of hair cells regeneration. To check these changes of expression, we had to prepare labels following the **protocol 7** (page 45).

First of all, we received plasmids with our gene sequence, and it was carried out linearization of them and then purification the product. Finally, it was performed a transcription and a precipitation, to get a DNA copy into RNA by the enzyme RNA polymerase. Then, we could use them for hybridization in situ, and if cell expressed the gene of interest, RNA copy would attach to RNA complementary chain, and we would know the level of gene expression.

After labels synthesis, it is necessary to check if RNA copy is the expected sequence. To test it, it is carried out a sequencing of the sequence, following **protocol 5** (page 44).

4.2-Neomycin treatment

One approach to understand how Zebrafish hair cells can regenerate is to identify molecules or genes needed for inducing hair cell regeneration. Our study is done to study lateral line hair cells death and their regeneration, because it is easier to kill hair cells from lateral line than from inner ear, because they are below the skin's surface.

First of all, to carry out this study, it is necessary damage cells with a neomycin treatment (**protocol 6, page 44**). Neomycin is a kind of aminoglycoside antibiotic which has been tested that has a relationship with lateral line hair cell damage, and results in hair cell replacement, the vast majority derived from proliferating precursors. For that, Zebrafish is exposed to neomycin for 1.5h, 3h, 5h and 12h, and then neuromast hair cell survival is analyzed, to check that all lateral line hair cells are damage.

To check if neuromast treatment has worked well, it is only necessary to check GFP fluorescent from hair cells, because we are working with Zebrafish Brn3c, which have been modified to have GFP in hair cells. If they are dead, there will not be fluorescent.

Then, these embryos are used to study genes or molecules needed for inducing hair cell regeneration, for instance, Sox2 and Atoh1 which are studied in our project.

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Tutors: Dr. Berta Alsina and Dr. Jordi Villà

4.3-Whole-mount in situ hybridization in zebrafish embryos

The in situ hybridization (ISH) technique allows the sites of expression of particular genes to be detected. Once labelled antisense RNA proves are prepared to use, carrying out the **protocol 1** attached in the annex (page 41), embryos may be fixed and permeabilized before to start to wet them in the labelled probe. After that, it is performed the hybridization using probes diluted in hybridization buffer. Embryos are incubated overnight at 70ºC, and then it is necessary to make a series of washes to remove traces of probes. Finally, hybrids are detected by immunochemistry using and alkaline phosphatase-conjugated antibody against digoxigenin (Anti-DIG-AP).
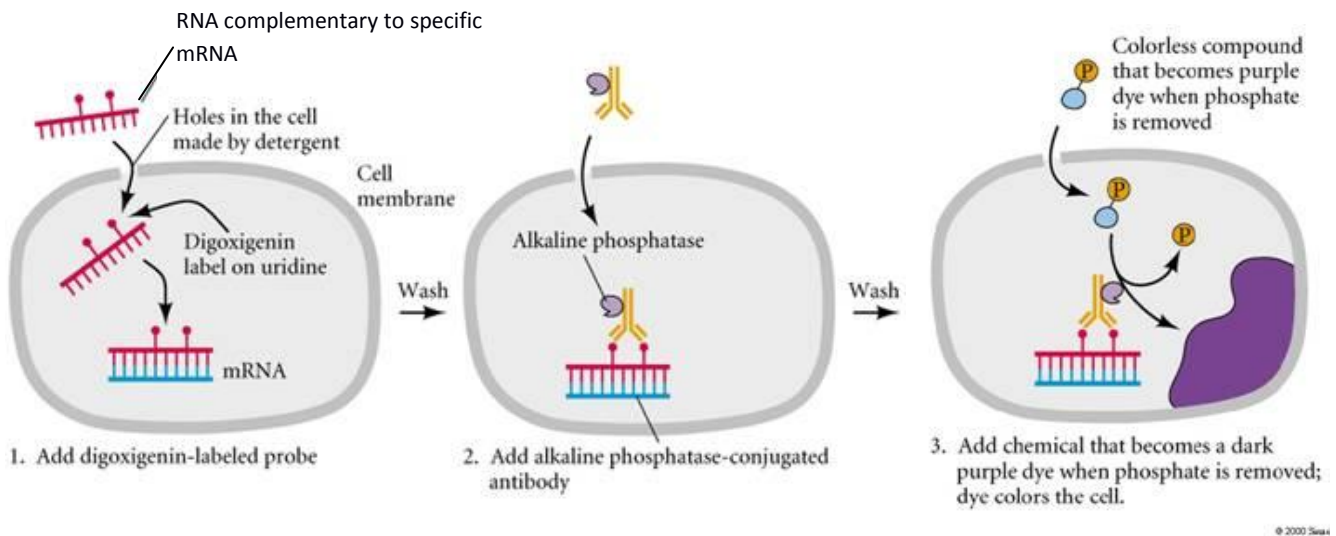

Fig. 4.3.1 In situ hybridization: The image shows how in situ hybridization is carried out until is detected the purple dye
(2000, Sinawer Associates, Inc.)

We studied genes which, if there was expression of them, were only expressed in progenitor supporting cells during hair cells regeneration. In order to also detect hair cells, it was performed an immunostaining, using a rabbit antibody anti-GFP and finally a monkey anti-rabbit with fluorescent. Anti-GFP was used because our Zebrafish embryos were transgenic and had hair cells labelled with GFP (green fluorescent protein). GFP exhibits bright green fluorescence when is exposed to light in the blue to ultraviolet range, but sometimes its fluorescence is lost during in situ hybridization process and it is necessary to make an immunostaining to recover hair cells fluorescence.
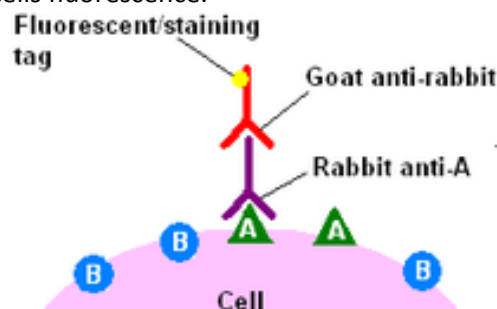

Fig. 4.3.2 Immunostaining (Victoria Male, 2005): The image shows how the method of immunostaining
uses one antibody against the antigen being probed for, and a second, label antibody against the first.
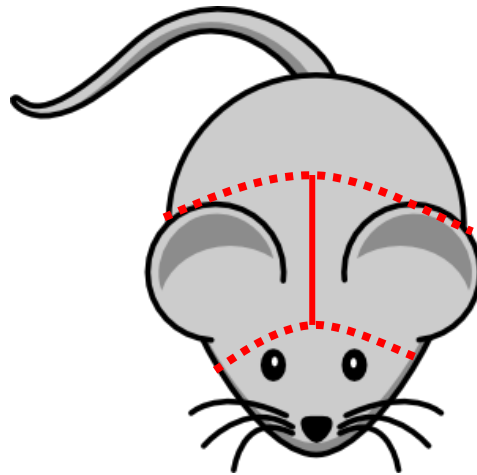
During my project, I have been carrying out in situ hybridization of Sox2, Aldh1a3, Atoh1 and RAR receptors, to check their expression in hair cell regeneration in addition to understand their function in the regeneration. However, to perform ISH for all of the genes that we wanted to study it was easy because ISH conditions were the same for each probe tested.

4.4-Generation of slices

4.4.1-Mices inner ear slices

Mice inner ear slices were needed to perform an immunochemistry of them, using RAR antibodies (αA,αB,γA,γB), and an in situ hybridization, using Aldh1a3 probes. The aim of that was to try to understand why mammals as mice cannot regenerate their hair cells. RAR labels were used as we wanted to check if in mice retinoic acid had the same role than in Zebrafish hair cell regeneration and Aldh1a3 probes to check if adult Zebrafish and mice had the same gene expression.

First of all, following **protocol 2 (page 43),** mice ears were slash by special scissors to discard nose and eyes which they were not necessary, and then, skin and skull were removed, keeping only the inner ear structure.

After getting a clearly inner ear structure, we followed three different protocols trying to cut the inner ear into slices:

1) Mousse inner ear was fixed with PFA and then it is cut by vibratome.

2) Mousse inner ear was fixed with PFA, incubated with agarose and then it is cut by vibratome.

Fig 4.4.1: Vibratome

3) After fixing the inner ear with PFA and washing a couple of times with PBS, we performed a decalcification of the inner ear bones and then it was incubated with agarose (**protocol 2, page 43)**

When we got the mousse inner ear slices, before to start the immunochemistry with RAR receptors, it was necessary a control of RAR labels. For that, it was needed some tissue where RAR receptors were always expressed, and we knew that chicken eyes had RARs expression. Therefore, we also prepare chicken eyes slices.

4.4.2-Chicken eyes slices

We had chicken embryos (5-6 days) dehydrated and, following **protocol 3 (page 43**), we rehydrated them gradually by MeOH (75%, 50% and 25%). After that, we also incubated them into agarose and then we could cut them successfully by vibratome (100-150 μm).

Fig 4.4.2 Chicken embryo 5 days (R. A. Ernst, F.A. Bradley, U.K. Abbott and R.M. Craig)**:** photo shows chicken embryo and red narrow shows the way we cut it.

4.4.3-Adult Zebrafish inner ear slices

Adult Zebrafish inner ear slices were needed to perform an in situ hybridization with Aldh1a3 labels. To make this slices, we followed a similar chicken slices protocol, and we cut slices of 90 μm by vibratome.

Fig 4.4.3 Adult Zebrafish: image shows an adult zebrafish and the red narrow shows the way we cut it.

4.5-Immunochemistry

4.5.1-Mice inner ear slices

One of my project's aims was to compare the differences of retinoic acid role between Zebrafish and mice. We knew that Zebrafish hair cell regeneration needed retinoic acid and its receptors to carry out the regeneration. Although it is known that during mice embryogenesis and tissue differentiation retinoic acid is an important signalling molecule, adult mice cannot regenerate damaged hair cells. That was the reason why we wanted to study the role of retinoic acid in adult mice, to try to understand which differences did not allow hair cell regeneration in mouse and in Zebrafish did.

Retinoic acid activates the retinoic acid receptor (RAR) nuclear transcription factor families. RAR isoforms are expressed in distinct patterns throughout development and in the mature organisms, and we wanted to check if adult mice still had expression of these receptors.

First of all, to understand why we performed an immunochemistry of mice inner ear tissue, it is necessary to know what it is. Immunochemistry is a process used to detect antigens, such as proteins, in cells of a tissue slices, using specific antibodies which bind to our protein of interest. Concretely, we performed an indirect immunochemistry (**protocol 4, page 43**).

Indirect immunochemistry is the same technique that we used to realize immunostaining after in situ hybridization, which involves an unlabeled primary antibody that binds to the target antigen in the tissue, and a labelled secondary antibody that binds to primary antibody due to it is raised against the IgG of the primary antibody animal species.

In our study of mice inner ear tissue, we used as a primary antibody a rabbit antibody anti Retinoic Acid Receptors. If we had found expression of them, it would have meant that retinoic acid still had some role in adult mice inner ear. However, it was completely expected that we would not find any expression because mice hair cell regeneration was known that it was not possible.

Nevertheless, we wanted to check the role of these receptors and to perform that, we used as a secondary antibody a donkey antibody anti-rabbit IgG, labelled with AlexaFluor 488.
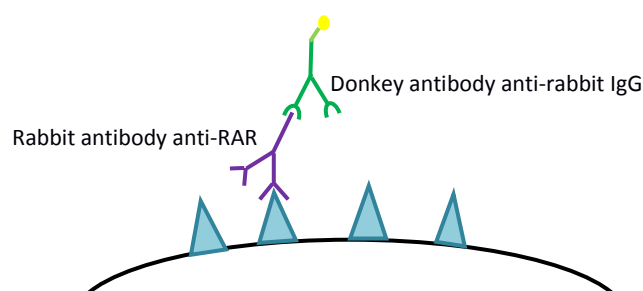

Fig 4.5.1 Immunochemistry of Retinoic acid receptors in mice inner ear tissue: this image shows the way how primary antibody binds to antigen and secondary antibody binds to primary antibody IgG.

Immunochemistry was carried out in four inner ear slices and we used a different concentration of anti-RAR for each: 1/50, 1/100, 1/200 and 1/400.

Such as we expected that we would obtain any signal, we had been sure that antibodies worked successful. To confirm that, it was necessary to realise a control positive of these antibodies. It was performed by using chicken embryo eyes, which always expressed retinoic acid receptors and we would obtain positive signal. We carried out another immunochemistry of chicken embryo eyes slices with the same condition than mice inner ear.

4.5.2-Chicken eyes slices

Chicken eyes slices were used as a control of antibodies α-RAR, and we performed the immunochemistry as in mice slices. We also used a different concentration of anti-RAR for each slices, to check which antibody concentration performed better.

4.6-In situ hybridization of adult Zebrafish and mice slices

As has been explained above (2.5-Retinoic Acid), during inner ear morphogenesis, it was known that Aldh1a3 was expressed in developing sensory epithelia of the cristae and utricular macula and was also involved in the formation of the walls of the semicircular canals. However, it had not been demonstrated if this member of Aldh1a-family was still expressed in adult Zebrafish. To check it, we performed an in situ hybridization of adult Zebrafish slices, following **protocol 8**(page 47), and using Aldh1a3 probes.

Moreover, following the same **protocol 8**(page 47), we performed an ISH of mice slices, to check the same gene expression. It was done to compare gene expression of both species trying to understand why mice cannot perform hair cell regeneration.

5-Results and discussion

5.1-Synthesis of probes and sequencing

We wanted to synthesize probes to check gene expression of Retinoic acid receptors (RARαA, RARαB, RARγA and RARγB) and Sox2 gene. To perform the linearization, it is needed a specific restriction enzyme for each label and a specific buffer for each restriction enzyme, and to perform the transcription of the sequence, it is also needed a specific RNA polymerase:

	Restriction enzyme	Buffer	RNA polymerase
RARαA	CLA I	Buffer H	T7
RARαB	EcoRI	Buffer H	T7
RARγA	BAMHI	Buffer B	T7
RARγB	BAMHI	Buffer B	T3
Sox 2	SAL I	Buffer H	Sp6

Table 5.1.1 Probes and their restriction enzyme, buffers and RNA polymerase.

During the process of synthesis, we performed controls using agarose gels to check if linearization and transcription were correct. But we had various problems with synthesis of probes. Some probes were not linearized or transcribed, and we had to repeat the process more than once, but here we only show some of them.

5.1.1-Linearization

In general, all of linearization which we performed was successful, only some was not it. We used 1 Kb Ladder DNA Marker (300-10.000bp) and we expected to get bands between 5.000 and 7.000 base pair, that it would be the vector and sequence length.

- **Unsuccessful linearization**

Some of the linearization that we performed was not successful. It was due to restriction enzyme did not work well and did not cut the vector, or maybe because the sequence expected was not the correct and did not have the right restriction sited. Others unsuccessful linearizations were because of a degradation of the vector during the process.
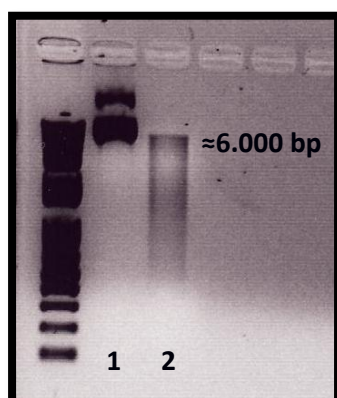
RARγA:

Fig 5.1.1 RARγA: During linearization, RARγA sequence suffered degradation

1: γA vector

2: γA was linearized ⊥

- **Successful linearization:**

The majority of performed linearizations were successful and we got a single band between 5.000 and 7.000 bp.
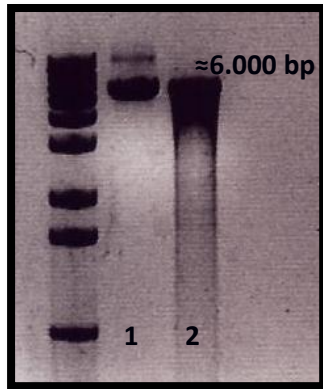
RARγA:

Fig. 5.1.2 RARγA: linearization was successful

1: γA vector

2: γA was linearized ⌐

RARαA, RARαB, RARγA, RARγB:

Fig. 5.1.3 RARαA, RARαB, RARγA, RARγB: linearization was successful, only RARαB linearization was not too clear.

1,3,5,7: αA, αB, γA, γB vectors
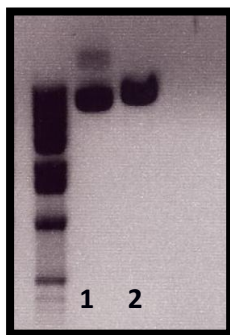
2,4,6,8: αA, αB, γA, γB were linearized ⌐

Sox2:

Fig. 5.1.4 Sox2: linearization was successful

1: sox2 vector

2: sox2 was linearized ⌐

5.1.2-Transcription

Transcription process was the most problematic. We performed a lot of transcription, using different concentration of vectors, different NTPs dilutions or increasing time of the transcription reaction incubation, trying to know why transcription did not work. Finally, it was necessary to carry out a vector sequencing, to check if the sequence of interest was the expected sequence.

We obtained results that we did not expect. The sequences of RARs and Sox2 labels were not right, for that reason, RNA polymerase could not transcript them.
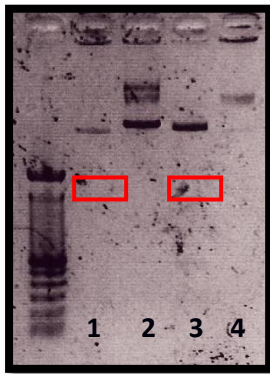
- **Unsuccessful Transcription:**

RARαA, RARαB:

Fig 5.1.5 RARαA, RARαB: an example of unsuccessful transcription.

2,4: αA, αB vectors

1,3: αA, αB were not transcripts

- **Successful transcription:**

After trying many times without success, we decided to prepare our own vectors with Sox2 labels, following protocol 9 (page 48), but we did not have enough time and only performed it once. In this case, the transcription was successful and we got a single band between 2.000 and 2.600 bp, using 100 bp DNA ladder.
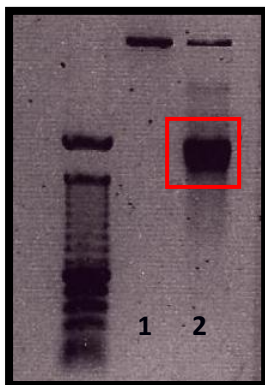
Sox2:

Fig 5.1.6 Sox 2: transcription was successful

1: sox2 was linearized

2: sox2 transcript

5.2-Neomycin treatment

Neomycin treatment worked successful and we obtained an increase of lateral line hair cells dead as we increased exposure time. In contrast, inner ear hair cell remained intact due to it was more difficult to access inside the inner ear structure than inside neuromast. Hair cell death was checked using fluorescent microscope, which allowed us to view GFP fluorescence of hair cells.
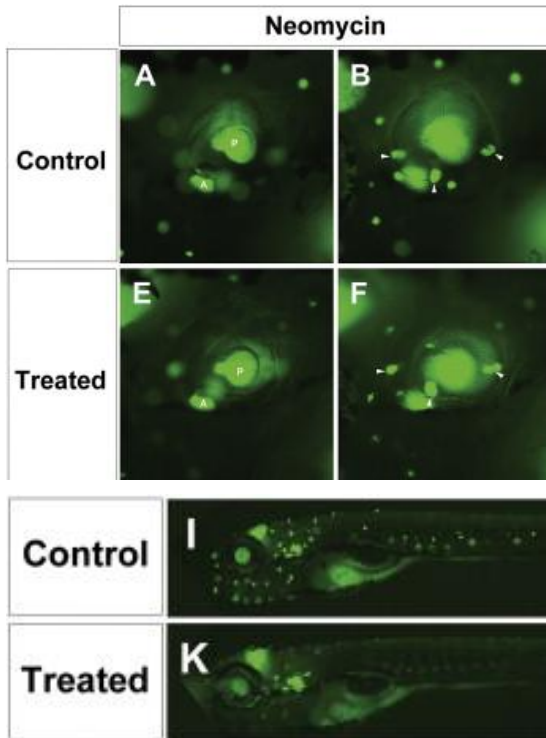

Fig. 5.2.1 The effects of neomycin exposure on the inner ear and lateral line larvae: Treatment with neomycin (E,F) did not affect GFP expression in the hair cells of the inner ear when compared to control treatment (A,B). These images were taken at both the anterior and posterior focal planes in live animals. A, anterior macula; P, posterior macula; arrowheads indicate the cristae of the semicircular canals. Treatment with neomycin (K) caused a strong decrease in GFP expression in hair cells of the lateral line, when compared to control treatment (I). (Ototoxin-induced cellular damage in neuromasts disrupts lateral line function in larval zebrafish, 2012)

5.3-Generation of cuts

5.3.1-Mice inner ear slices

As we have explained above, mice ears were slash by special scissors to discard nose and eyes which they were not necessary, and then, skin and skull were removed, keeping only the inner ear structure. The result was a similar structure to the picture below, which contains all the inner ear structure:
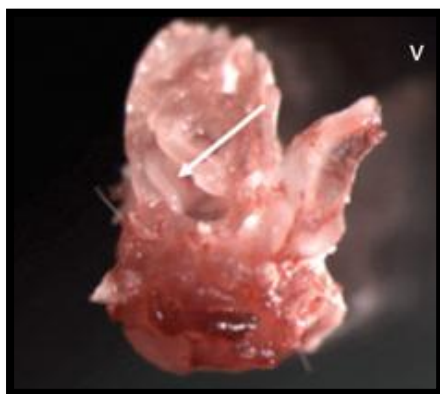

Fig. 5.3.1 Mouse inner ear (Rachel E Hardisty-Hughes, Andrew Parker & Steve D M Brown, 2010): After separation from the skull, the inner ear can be seen clearly. The photo shows a hole(white arrow) that is the apex of the cochlea.
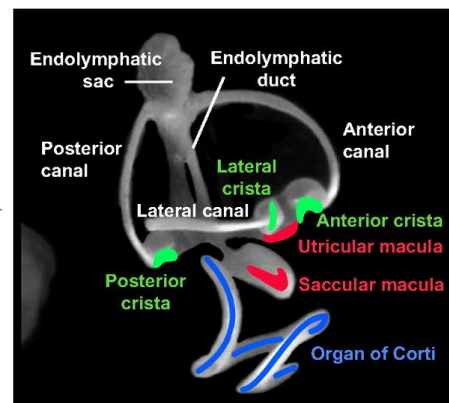
Fig. 5.3.2 Mouse inner ear (**Shaping sound in space: the regulation of inner ear patterning, 2012**): the image shows the mousse inner ear structure.

After getting a clearly inner ear structure, we followed three different protocols trying to cut the inner ear into slices, but only one of them works:

1) Mousse inner ear was fixed with PFA and then it is cut by vibratome ➡️ it was unsuccessful, we could not cut the inner ear and it was broken.

2) Mousse inner ear was fixed with PFA, incubated with agarose and then it is cut by vibratome ➡️ it was unsuccessful, we could not cut the inner ear and it was broken.

3) After fixing the inner ear with PFA and washing a couple of times with PBS, we performed a decalcification of the inner ear bones and then it was incubated with agarose. Using this technique, we could cut inner ear into slices (100µm) by vibratome.

5.3.2-Chicken eyes slices

When we got the mousse inner ear slices, before to start the immunochemistry with RAR receptors, it was needed a control of RAR labels. For that, it was required some tissue where RAR receptors were always expressed, and we knew that chicken eyes always had RAR expression. Therefore, we also prepared chicken eyes slices and we obtained the following slices (130 µm).

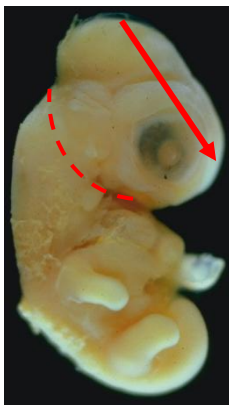**Fig 5.3.3 Chicken embryo 5 days (**R. A. Ernst, F.A. Bradley, U.K. Abbott and R.M. Craig)**:** photo shows chicken embryo and red narrow shows the way we cut it.
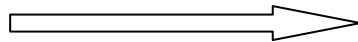
Fig 5.3.4 Slice of chicken embryo head that were obtained: photo shows chicken eyes (red narrow).

5.3.3-Adult Zebrafish inner ear slices

We also obtained successful slices of 90 µm, which showed the different parts of inner ear structure.

5.4- In situ hybridization (ISH)
5.4.1-ISH of Zebrafish embryos

5.4.1.1-Atoh1: Zebrafish Brn3c Neuromast (48hpf)

Fig 5.4.1 Zebrafish Lateral line 10x (48hpf)

Fig 5.4.2 Zebrafish Lateral line 40x (48hpf): images show Atoh1 gene expression in neuromast during hair cell regeneration (A-E). Images A and F it is a normal expression of Atoh1 and GFP hair cells. Atoh1 has a very low expression; instead GFP hair cell has a high expression.

It is demonstrated that Atoh1 expression increase in future new hair cells to carry out the hair cell regeneration. The image F to J shows how GFP expression of hair cells increase since first hours of neomycin treatment (B) that there is a few GFP expression, and then until 12 h after treatment there is not expression again (H-I).

It is means that Atoh1 genes are involved in hair cell regeneration.

It is known that Atoh1 only is expressed in future hair cells during Zebrafish development and after 48 hpf, it is not expressed. We wanted to know if Atoh1 is also involved in hair cell regeneration, for that reason, we performed an in situ hybridization using Zebrafish Brn3c (hair cells labelled with GFP) treats with neomycin to check what happens during hair cell regeneration.

The results that we obtained (Fig.5.4.2), show that in a Zebrafish without neomycin treatment (Fig.5.4.2-A and F), Atoh1 is not expressed in neuromast or there is a very slow expression, and GFP hair cells have a common expression. However, after neomycin treatment (Fig. 5.4.2 B-E) and hair cells death (Fig. 5.4.2 G-I), we observed an increase of Atoh1 gene expression into progenitors cells which would become new hair cells. After hair cell regeneration (Fig.5.4.2 J), Atoh1 gene expression decreases and supporting cells loss gene expression again.

Fig 5.4.3 Zebrafish neuromast 100x (48hpf): the images show Atoh1 gene expression in neuromast during hair cell regeneration (A-E). Normally, Atoh1 gene is not expressed (A), and after neomycin treatment, it is expressed during hair cell regeneration.

In figure 5.4.3 it is show how Atoh1 gene expression increases after neomycin treatment, and gets its highest expression between 3 and 5 hours after treatment, when hair cell regeneration is carried out. After hair cell regeneration, the expression decreases again.

These results mean that Atoh1, besides of have an important role in Zebrafish inner ear development, has an essential role in hair cell regeneration as well.

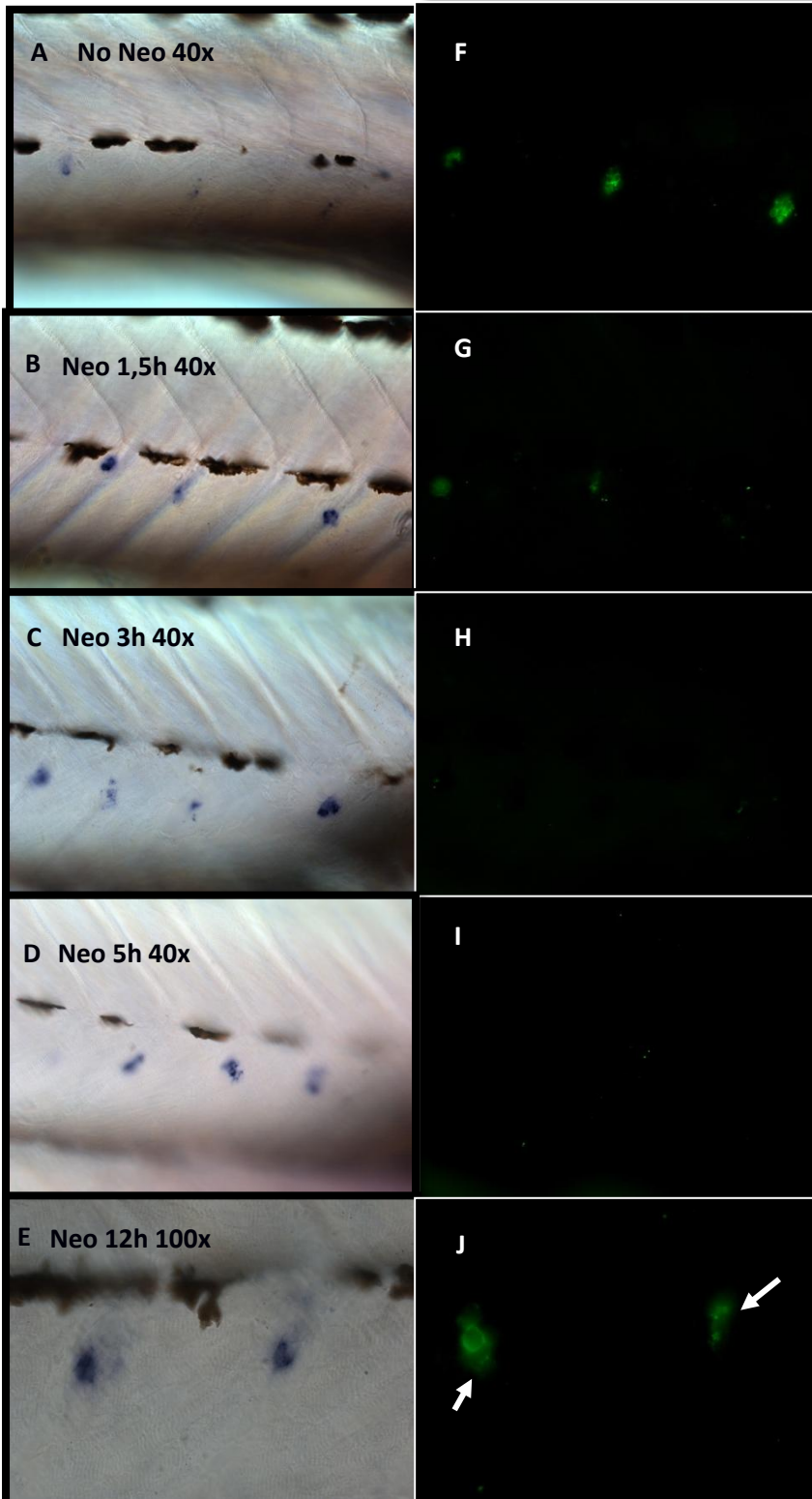**5.4.1.2-Sox2:** Zebrafish Brn3c Neuromast (48hpf)

Fig 5.4.4 Zebrafish Lateral line 40x (48hpf): images show Sox2 gene expression in neuromast during hair cell regeneration (A-E). This in situ hybridization does not show what was expected. Images A and F shows a normal Sox2 gene and GFP hair cell expression. Images B-E should show how Sox2 gene expression changes during hair cell regeneration, but ISH did not work. However, neomycin treatment worked well and images G-J show how hair cells are regenerated.

We performed many ISH using different Sox2 labels, but we obtained the same results in each ISH. Finally, we thought that there were some problems with the labels, and we prepared new Sox2 labels.

As we know, Sox2 is always expressed in supporting cells of inner ear and neuromast and we wanted to check its role in hair cell regeneration. However, we did not obtain the expected results. Some research shows that, during hair cell regeneration, supporting cells that express Sox2, lose expression, and then dedifferentiates into progenitor stem cells, to become new hair cells. For that reason, in our ISH of Sox2 we should have obtained a highly Sox2 expression in a normal neuromast (Fig.5.4.5 A), a decrease of expression after neomycin treatment (Fig.5.4.5 B-C), and an increase of expression after hair cell regeneration, but we could not check it.

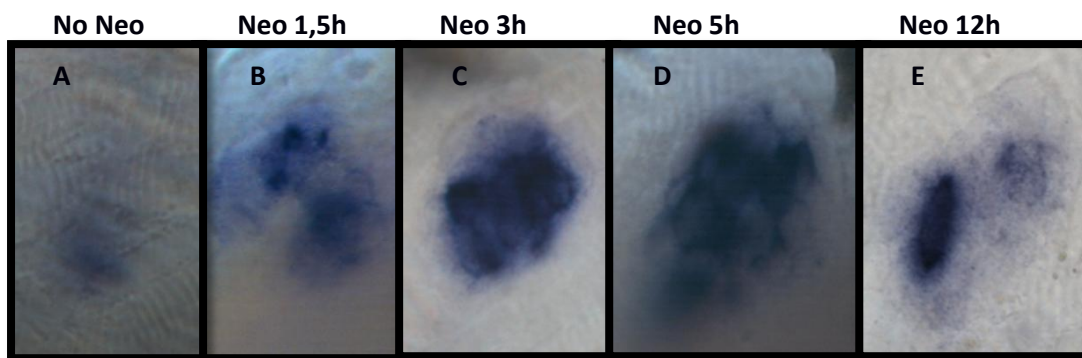**No Neo**   **Neo 1,5h**   **Neo 3h**   **Neo 5h**   **Neo 12h**

Fig 5.4.5 Zebrafish neuromast 100x (48hpf): the images show Sox2 gene expression in neuromast during hair cell regeneration (A-E). The results were not the expected, perhaps due to Sox2 label or due to a proteinase K treatment too strong.

The expected role of Sox2 in hair cell regeneration is that, when there is hair cell damage and Atoh1 genes are activated to perform hair cell regeneration, supporting cells that express Sox2 genes are dedifferentiates into progenitor stem cells to become new hair cells. It maybe means that Sox2 is regulated by Atoh1 and, when this increase after hair cell damage, Sox2 decreases. In other words, supporting cells lose Sox2 expression to become progenitor cells, and then, these express Atoh1 to become new hair cells.

5.4.1.3-Sox2: Zebrafish Brn3c inner ear(5 days post fertilization)

After getting wrong results in many sox2 ISH in lateral line, we wanted to check if the expression of Sox2 that we obtained in the inner ear, came from supporting cells or only was background and Sox2 really did not work well.

To check that, we performed slices of Zebrafish head (5 dpf) to see if Sox2 expression comes from supporting cells.

Fig 5.4.6 Zebrafish head slices 40x (5 dpf): the images show normal Sox2 gene expression in inner ear.

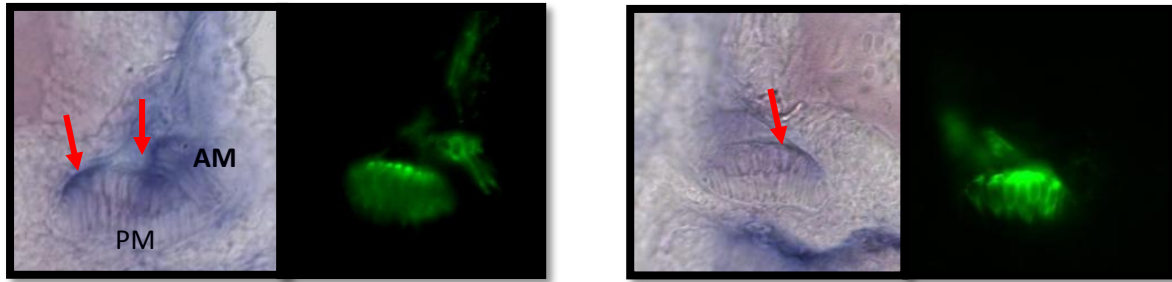**Fig 5.4.7 Zebrafish inner ear 40x (increased size images):** these two pictures show how Sox2 expression comes from supporting cells (red narrow), and after supporting cells there are hair cells (GFP fluorescents). AM: anterior macula, PM: posterior

These results mean that Sox2 labels work correctly because the expression is inside of supporting cells in the inner ear. In contrast, in neuromast we have not obtained incorrect results, and we think that it happened due to a too strong proteinase K treatment during ISH process.

5.4.1.4-Aldh1a3: Zebrafish Brn3c inner ear (5 days post fertilization)

We used Zebrafish Brn3c as a control to check that Aldh1a3 labels worked correctly, because we knew that Zebrafish embryo always has Aldh1a3 gene expression, but it is not yet investigated if this gene is still expressed in adult Zebrafish. For that reason, we wanted to perform ISH of Aldh1a3 in adult Zebrafish slices, but to ensure that labels worked correctly if we did not have expression in the slices, we did an ISH in embryos as a control.

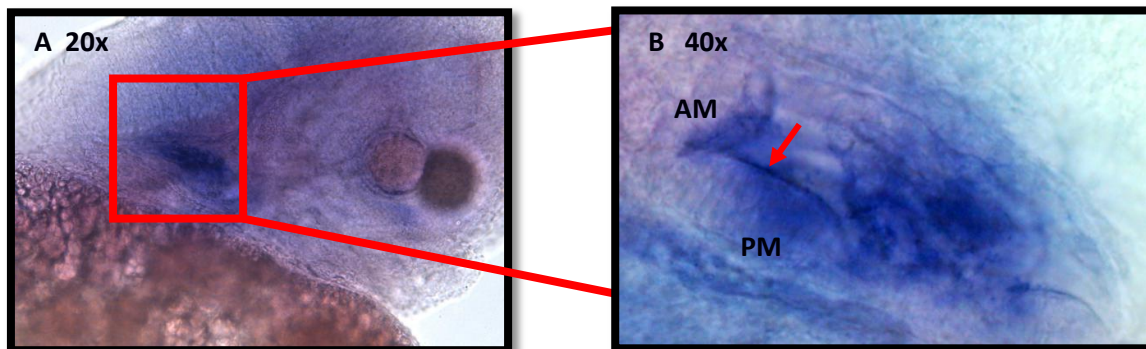**Fig 5.4.8 Zebrafish inner ear ISH Aldh1a3:** picture A shows a image of Zebrafish head and its inner ear, and picture B shows the inner ear (40x) with its posterior macula (PM) and anterior macula (AM), and Aldh1a3 gene expression in supporting cells (strong blue, red narrow)

After ISH we performed and immunostaining of GFP hair cells, to certify that expression comes from supporting cells and not from hair cells.
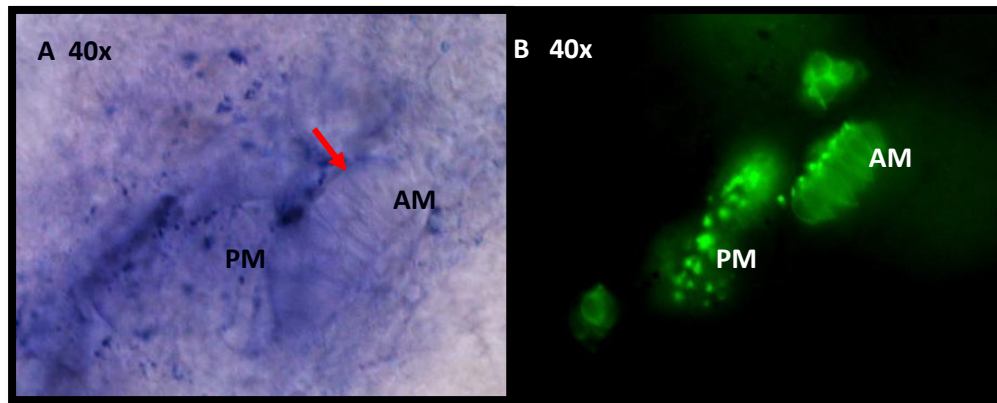
Fig 5.4.9 Zebrafish inner ear ISH Aldh1a3 and GFP immunostaining: picture A shows a image of Zebrafish inner ear with Aldh1a3 expression in supporting cells (red narrow), and picture B shows hair cells inner ear with its posterior macula (PM) and anterior macula (AM).

We obtained a correctly expression of Aldh1a3 in supporting cells of Zebrafish embryo inner ear. That means that Aldh1a3 probes work successful.

We also performed ISH of RARs genes, but they did not work due to RARs probes were not correct.

5.4.2-ISH of slices

5.4.2.1-Aldh1a3: adult Zebrafish Brn3c inner ear

While we were performing Alh1a3 ISH in adult Zebrafish slices, we had to change the protocol, because of after to carry out proteinase K treatment, slices were broken. It could be because this treatment was too strong for slices of 70 µm, and we decided to follow ISH without treatment.

Finally, we obtained unclear results, which did not allow us to distinguish where there was Aldh1a3 gene expression. Now, we are looking for a new protocol of ISH in slices to try to repeat the process.

5.4.2.2-Aldh1a3: mice inner ear slices

We performed the same ISH in mice inner ear slices, to test if ISH worked in mice slices, but we did not obtain any result. It could be possible due to labels could not go into the inner ear tissues.

5.5-Immunochemistry of mice and chicken embryo slices

We also have had problems to perform immunochemistry in these two species. Chicken embryo slices were used as a control of RARs antibody, because it is known that chicken eyes always express RAR genes. Even so, when we carried out immunochemistry of chicken eyes slices, we did not get any result and any expression. We are not sure why it did not work, maybe antibody were not suitable for an immunochemistry.

Despite this wrong result, we tried to perform an immunochemistry of RARs antibody in mice slices, but it did not work either. Our hypothesis is that decalcification process, which we performed to try to cut inner ear in slices, spoiled the inner ear tissue and for that reason, antibodies did not bind where they should.

Now, we have found a new protocol that it is not necessary to perform a decalcification process (Tadafumi Kawamoto, 2003), but we have not been able to prove it.

6-Conclusion

Nowadays, substantial researches have been made to try to understand the mechanisms of hair regeneration in the inner ear and lateral line of Zebrafish. It is known that retinoic acid has an essential role in Zebrafish embryo regeneration, because inhibiting some gene involved in its pathway; hair cell regeneration is not possible. The principal aim of my project was to study if retinoic acid had the same role in adult Zebrafish hair cell regeneration than in embryo, and if the essential genes involved in retinoic acid pathway had the same expression. Furthermore, we also wanted to study retinoic acid role in adult mice, and for both species we performed several in situ hybridization and immunochemistry to detect genes expression, such as Aldh1a3 and RARs genes, which are necessary to performed hair cell regeneration in Zebrafish embryo. However, we could not check if these two essential genes had the same expression or an important role to regenerate hair cells in adult Zebrafish or in mice. We generated right slices of both inner ear species, by vibratome, but ISH and immunochemistry did not work. We thought that it could be possible due to process of generate slices, perhaps for a strong process of decalcification in mice which damages tissue. Another possibility was that we did not performed a proteinase K treatment in adult Zebrafish because we thought that it damaged inner ear tissue, and if we do not performed it, it could not allow that probes entered to the tissue.

On the other hand, with our project we also wanted to study the expression of different genes of hair cells and supporting cells, to check how they change their expression during Zebrafish embryo hair cell regeneration in the lateral line. We focused with Sox2 gene and Atoh1, which the first one is always expressed in supporting cells and the second one in hair cells only during development. We also performed in situ hybridizations to check their expression during hair cell regeneration. In the case of Atoh1 genes, supporting cells progenitors of hair cells showed an increased expression, meaning that Atoh1 is essential to generate new hair cells. However, ISH of Sox2 did not show the expected results. The results of other researchers showed that Sox2 expression of supporting cells decreased when hair cell regeneration started, meaning that supporting cells lost sox2 expression and dedifferentiated into progenitor cells, to become new hair cells. But we did not obtained any expression in lateral line, only in the inner ear where there was not hair cell regeneration, because we had not killed hair cells in the inner ear. We checked if sox2 expression obtained came from supporting cells or it was only background and probes had not really worked, but it was verified that there was expression in supporting cells. We thought that ISH did not work in the lateral line due to the fact that we performed a too strong proteinase K during ISH process, and it damaged neuromasts, but it should be reviewed.

In the world of research, everything needs to take its time and sometimes we do not get the expected results in a short period. Although we have not obtained clearly results in this project, hair cell regeneration research will continue growing to develop therapies to treat, and perhaps to cure, hearing and balance disorders.

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8-Annexes

PROTOCOL 1: Whole-mount in situ hybridization (ISH) in zebrafish embryos

Single whole-mount in situ hybridization is carried out with DIG-labeled RNA probes and alkaline-phosphatase coupled anti-DIG antibody (anti-DIG-AP), and detection with NBT/BCIP according to Thisse et al., 2004, or with FastRed.

1) dechorionate embryos
2) fix the embryos at desired stages with 4% paraformaldehyde (PFA) in PBS overnight at 4ºC
3) wash the embryos 2x5min with PBS-0,1% Tween-20 solution (PBT)

Pre-Treatments

4) dehydrate embryos with 100%MeOH at least 1h at -20ºC
5) rehydrate embryos gradually by rising them at room temperature 10 min with 75% MeOH, 10 min 50% MeOH, 10 min 25% MeOH and 2x10 min PBT
6) incubate embryos with proteinase K (10mg/ml, 1/1000 in PBT) for 10 min if they are younger than 48 hpf, of for 20 min if they are 48 hpf or older
7) wash 5 min PBT
8) postfix with 4% PFA for 40 min
9) wash 2x5 min in PBT

Hybridization

10) prehybrize the embryos with hybridization buffer, 1h at 70º

        Hybridization Buffer (50ml):

- 25 ml 100% formaldehyde (FAD)
- 250 ul 10mg/ml heparin
- 1 ml 25mg/ml tRNA
- 500 ul 10% Tween-20
- 125 ul 1M citric acid pH 6.0
- 10,5 ml ddH$_2$O

11) hybridize the embryos with probes diluted in hybridization buffer 1/200, overnight at 70º

Washes

12) incubate the embryos with:
    a. Wash 1, 10 min at 70º
    b. Wash 2, 10 min at 70º
    c. Wash 3, 10 min at 70º
    d. Wash 4, 10 min at 70º
    e. Wash 5, 2x30 min at 70º
    f. PBT, 2x10 min at room temperature
       HYB-Buffer (50ml):

- 32,5 ml 100% formaldehyde (FAD)
- 12,5 ml 20x SSC
- 500 ul 10% Tween-20
- 4,5 ml ddH$_2$O

Wash 1: 75% HYB-Buffer / 25% 2xSSC

Wash 2: 50% HYB-Buffer / 50% 2xSSC

Wash 3: 25% HYB-Buffer / 75% 2xSSC

Wash 1: 2xSSC

Wash 1: 0,5xSSC

Immunohistochemistry

13) incubate embrys at least 90' at room temperature with blocking solution (2% Bovine serum albumin (BSA), 10% heat inactivated goat serum (NGS), 0,1% PBT)
14) incubate embryos overnight at 4ºC with anti-DIG-AP diluted in blocking solution 1:4000
15) wash 10x10 min with PBT
16) incubate the embryos 3x10 min with alkaline phosphatase buffer:

Alcaline phosphatase buffer (50ml total)

- 5 ml 1M Tris-HCl pH 9,5   (final conc. 100mM)
- 2,5 ml 1M $MgCl_2$ (final conc. 50mM)
- 1 ml 5M NaCl   (final conc. 100mM)
- 1 ml 10% Tween-20   (final conc. 0,1%)
- 100 ul Triton X-100   (final conc. 0,2%)

Develop with NBT

17) Develop the reaction by incubating embryos in the dark with the following solution:
- 45 ul NBT
- 35 ul BCIP
- 10 ml alkaline phosphatase buffer
18) stop reaction with PBT
19) dehydrate embryos with 100%MeOH at least 1h at -20ºC
20) rehydrate embryos gradually by rising them at room temperature 10 min with 75% MeOH, 10 min 50% MeOH, 10 min 25% MeOH and 2x10 min PBT
21) wash overnight with PBT and postfix with 4%PFA, 2h at room temperature

Anti-GFP and anti-pH3 immunostaining

- after ISH is developed, postfix embryos with 4%PFA for 30'
- incubate with blocking solution (2% Bovine serum albumin (BSA), 10% heat inactivated goat serum (NGS), 0,1% PBT) 1h at RT
- incubate overnight at 4ºC with primary antibody diluted 1/400in blocking solution

- wash 10x10 min with 0,1% PBT
- incubate 2h at RT or overnight at 4ºC with secondary antibody diluted 1/400 in blocking solution

- wash 10x10 min with 0,1% PBT
- detect fluorescence under the fluorecent microscope

PROTOCOL 2: Generation of mice inner ear slices

1) Slash mice ears to discard nose and eyes.

2) Remove skin and skull, keeping only the inner ear structure.

3) Fix inner ear structure with 4%PFA, 4h at room temperature.

4) Wash 3x10 min with PBS.

5) Decalcification. 1,5h-3h

6) Incubate mice inner ear in agarosa at least 30 min at 80ºC.

7) Cut inner ear with vibratome (100µm)

PROTOCOL 3: Generation of chicken and adult Zebrafish slices

1) Dehydrate sample with 100%MeOH at leat 1h at -20ºC

2) Rehydrate sample gradually by rising them at room temperature 10 min with 75% MeOH, 10 min 50% MeOH, 10 min 25% MeOH and 2x10 min PBT.

3)Incubate sample in agarosa at least 30 min at 80ºC

4)Cut sample with vibratome (100-150µm)

PROTOCOL 4: Immunochemistry of slices

1) Incubate slices with blocking solution (2% Bovine serum albumina (BSA), 10% heat inactivated goat serum (NGS), 0,1% PBT) 2h at room temperature.

2) Wash slices 3x5min with PBT (PBS-0,1% Tween-20 solution) at room temperature.

3) Incubate overnight at 4ºC with primary antibody diluted 1/50, 1/100, 1/200 and 1/400 in blocking solution.

4) Wash 10x10 min with 0,1% PBT

5) Incubate 2h at RT or overnight at 4ºC with secondary antibody diluted 1/200 in blocking solution.

6) Wash 10x10 min with 0,1% PBT

7) Detect fluorescence under the fluorescent microscope

PROTOCOL 5: Sequencing. Kit Big Dye 3.1 sequentiation and precipitation

PCR

7,32 µL ddH$_2$O

1 µL DNA

1 µL PREMIX Big dye 3.1

0,68 µL Primer (5 Mm)

Program: seq-auto

Precipitation

Add 10 µL ddH$_2$O

In a new epp:

10 µL sample

5 µL 125Mm edta

60 µL ETOH 100%

Vortex and Incubate 15' RT

Centrifugate 30' at max. 4ºC

Descart supernatant

Add 200 µL EtOH 70%

Centrifugate 5' at max. 4ºC

Descart super natant and dry the pellet 15-30' RT

Freeze samples

PROTOCOL 6: Neomycin treatment

1) Incubate embryo with neomycin and stop once at 1,5h, at 3h, at 5h and at 12h.

2) Fix the embryo with 4% PFA

3) Keep them at -20ºC with MeOH 100% to dehydrate embryos.

PROTOCOL 7: Synthesis of probes

1)Linearization

10 µL cDNA

2 µL Buffer (specific for each restriction enzyme)

1 µL Restriction enzyme

7 µL H_2O

Run gel: vector + linealized

2) Purification

50 µL EtOH 100%

1 µL 3M NaAc

1 µL EDTA

Incubate 30' at -20ºC

Centrifugate 30' at 4ºC, 13.000RPM

Discard supernadant

Add 500 µL EtOH 70%

Centrifugate 5' at 4ºC, 13.000RPM

Discard supernadant

Dry at 37ºC

20 µL H_2O

Run gel: cDNAs+linealized

3) Transcription

a) Prepare NTPs dilution

UTP dil 1:10 (1 µL UTP + 9 µL of H_2O)

-6,5 µL UTP (dil)

-3,5 µL DIG-UTP - 7 µL H_2O

-1 µL ATP   -1 µL GTP

-1 µL CTP

b) Transcription reaction (20 µL for each epp.)

-Prepare mix for each epp.

-4 µL NTPs

-2 µL transcription buffer 10x

-1 µL RNAsine

-1 µL RNA pol

-4 µL DNA linealized

-8 µL H_2O

c) Vortex and spin down

d) Incubate the transcription reaction, for 2 h at 37ºC

e) Control: run a 1% agarosa gel to confirm the transcription

4) Purification

a)Ethanol 100%

-Add 100 µL H_2O

-Add 300 µL Ethanol 100%

-Add 10 µL of 4M LiCl

Mix well

Incubate 30' at -20ºC

Centrifugate 30' at max. 4ºC

Descart supernatant

b)Ethanol 70%

-Add 500 µL EtOH 70%

Centrifugate 10' at max. 4ºC

Descart super natant and dry the pellet 15-30' at 37ºC

Add 20 µL H_2O

Store at -20ºC

Control: run a gel

PROTOCOL 8: In situ hybridization of slices

1) Incubate slices with 100% MeOH at least 1h at -20ºC to dehydrate

2) rehydrate slices gradually by rising them at room temperature 10 min with 75% MeOH, 10 min 50% MeOH, 10 min 25% MeOH and 2x10 min PBT.

Hybridization

3) Prehybrize slices with prehybridization buffer, 1h at 70ºC

4)Hybrize slice with probes diluted in hybridization buffer 1/200, overnight at 70ºC

Washes

1)Wash I 1h 70 ºC   SCC 5x pH 4.5 + 1% SDS + 50% formamida + H_2O

2)Wash II 1H 65ºc SCC 5x pH 4.5 + 50% formamida + H_2O

3)TBS-T 15minx3

Immunodetection

1)Incubate slices with block solution (TBS-t 10% GS) 60-90 min

2)Wash with TBS-T

3)Incubate with block solution + Anti-Dig-AP (1:2000), overnight 4ºC

4) wash 10x10 min with TBS-T
5) incubate the embryos 3x10 min with alkaline phosphatase buffer:
        Alcaline phosphatase buffer (50ml total)

- 5 ml 1M Tris-HCl pH 9,5   (final conc. 100mM)
- 2,5 ml 1M $MgCl_2$ (final conc. 50mM)
- 1 ml 5M NaCl   (final conc. 100mM)
- 1 ml 10% Tween-20   (final conc. 0,1%)
- 100 ul Triton X-100   (final conc. 0,2%)

  Develop with NBT

1) Develop the reaction by incubating embryos in the dark with the following solution:
- 45 ul NBT
- 35 ul BCIP
- 10 ml alkaline phosphatase buffer

2)stop reaction with PBT

PROTOCOL 9: Design probes

1) Transcription of Zebrafish genome

2) Retrotranscription of genome to get RNA into cDNA

3) Design specific primers to amplify the target sequence by PCR.

4) PCR product is bound to vector pGENT

       -5 ul 2x Rapid lig Buffer

       -1 ul vector (50 mg)

       -3 ul PCR product

       -2 ul t4 DNA ligase

1h RT

5)Prepare IPTG-XGAL (1:1) on plate + dry a bit (80 ul IPTG and 80 ul XGAL)

6)Transform all ligation 10 ul in 160 ul cells

       -10' on ice

       -5' on ice (add lig)

       -50'' 42ºC

       -5' on ice

7)Plate 50 ul and incubate ON at 37ºC

8)Select white colonies → have the vector.