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Characterization of *hairy1* and *hairy2* pattern expression during lung development of *Gallus gallus*

Tese de Mestrado

Mestrado de Genética Molecular

Trabalho efectuado sob a orientação de:

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Escola de Ciências da Saúde, da Universidade do Minho, Braga, Portugal

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DECLARAÇÃO

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Título da Tese de Mestrado:

Characterization of *hairy1* and *hairy2* expression pattern during lung development of *Gallus gallus*

Caracterização do padrão de expressão de *hairy1* e *hairy2* ao longo do desenvolvimento pulmonar de *Gallus gallus*

Trabalho efectuado sob a orientação de:

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Professora Doutora Paula Sampaio

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É AUTORIZADA A REPRODUÇÃO INTEGRAL DESTA TESE, APENAS PARA EFEITOS DE INVESTIGAÇÃO, MEDIANTE DECLARAÇÃO ESCRITA AO INTERESSADO, QUE A TAL SE COMPROMETE.

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Abstract

Although much is known about some aspects of lung development, the mechanisms that regulate the spatiotemporal control of this process are not well defined. The identification of a segmentation clock that ensures the correct spatiotemporal periodicity of somite generation, during chick somitogenesis, provided the first molecular evidence of how embryonic cells count time. The underlying mechanisms of this process involve the oscillation of "clock-genes" like *hairy1* and *hairy2* (members of hairy-enhancer-of-split –HES- family of transcriptional repressors), which are intimately related to the Notch signaling pathway. More recently, *hairy2* expression unveiled a molecular clock operating during limb development.

Lung development share some similarities with somitogenesis and limb development, namely: they all occur along the rostro-caudal axis, the signaling pathways implicated are the same, the formation of consecutive repetitive structures along the anterior-posterior axis is observed, and they occur with exact chronological precision. Considering this parallelism, the characterization of *hairy1* and *hairy2* during lung branching morphogenesis of *Gallus gallus* became relevant, since their expression might indicate a possible role in the development of this tissue, although their functions are not yet clarified. Moreover, taking into consideration that FGF and Notch signaling pathways are involved in the molecular clock mechanisms, the link between *hairy1* and *hairy2,* these pathways and branching morphogenesis was assessed by lung explant culture system.

The present work characterizes, for the first time, *hairy1* and *hairy2* expression pattern in early stages of chick lung development, by *in situ* hybridization. *hairy1* expression was evident mainly in the pulmonary epithelium of the respiratory tract, but also in the mesenchyme. *hairy2* expression was evident mainly in the chick pulmonary mesenchyme surrounding the trachea and the trachea bifurcation, and the most distal region of the main bronchus. *hairy1* and *hairy2* expression was not affected by FGF signaling inhibition; however, treated explants showed atypical lung branching when compared with control ones, which is consistent with the role of FGF signaling in branching morphogenesis. These results indicate that these genes are not downstream targets of FGF signaling pathway in the chick lung. Notch inhibition did not affect *hairy1* expression but, on the other hand, *hairy2* expression was dramatically reduced when compared with control explants. Moreover, treated explants showed not only an increased number of secondary branches but also an anomalous lung structure in the distal tip of the main bronchus. Notch signaling inhibition appears to affect only *hairy2* expression in the lung and seems to interfere with the correct process of lung development.

RESUMO

Hoje em dia muito se sabe sobre desenvolvimento pulmonar. No entanto, os mecanismos que regulam o controlo espacial e temporal deste processo não estão bem definidos. A identificação de um relógio molecular da segmentação responsável pela correcta periodicidade espacial e temporal da formação dos sómitos no modelo da galinha, forneceu a primeira evidência molecular de como de como é feita a contagem do tempo pelas células embrionárias. Os mecanismos subjacentes a este processo envolvem a oscilação de genes como *hairy1* e *hairy2* (membros da família de repressores de transcrição *hairy-enhancer-of-split*, HES), que estão intimamente relacionados com a via de sinalização Notch. Mais recentemente, foi descrita a existência de um relógio molecular no desenvolvimento do membro.

O desenvolvimento pulmonar partilha algumas semelhanças com a somitogénese e o desenvolvimento do membro, nomeadamente: ocorre ao longo de um eixo rostro-caudal, as vias de sinalização implicadas são as mesmas, observa-se a formação consecutiva de estruturas repetitivas ao longo de um eixo anterior-posterior, e ocorre com precisão cronológica. Considerando este paralelismo, tornou-se relevante caracterizar o padrão de expressão de *hairy1* e *hairy2* durante a ramificação pulmonar de *Gallus gallus*, uma vez que a sua expressão pode indicar um possível papel no desenvolvimento deste tecido. Além disso, tendo em conta que as vias de sinalização Notch e FGF estão envolvidas nos mecanismos do relógio molecular, estas vias conjuntamente com a relação entre *hairy1* e *hairy2* e a ramificação pulmonar foram avaliadas.

O presente trabalho caracteriza, pela primeira vez, o padrão de expressão de *hairy1* e *hairy2* em estadios iniciais do desenvolvimento pulmonar de galinha, por hibridização *in situ*. A expressão de *hairy1* foi observada principalmente no epitélio pulmonar do tracto respiratório, mas também no mesênquima. *hairy2* é expresso sobretudo no mesênquima pulmonar de galinha que rodeia a traqueia e a bifurcação da traqueia, e na região mais distal do brônquio principal. A expressão destes genes não foi afectada pela inibição da via FGF, indicando que estes genes não são alvos desta via de sinalização neste órgão. No entanto, estes explantes apresentaram uma ramificação pulmonar atípica, o que está de acordo com o papel da via FGF na ramificação pulmonar. A inibição da via Notch não afectou a expressão de *hairy1* mas, a expressão de *hairy2* foi dramaticamente reduzida. Além disso, os explantes tratados com DAPT apresentaram não só um aumento no número de ramificações secundárias mas também uma estrutura pulmonar anómala. A inibição da via Notch parece afectar a expressão de *hairy2* em regiões concretas do pulmão e parece interferir no correcto processo de desenvolvimento pulmonar.

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ABBREVIATIONS

ADAM desintegrin and metallopeptidase domain

AP alkaline phosphatase

BCIP bromo chloro indolyl phosphate

bHLH basic helix-loop-helix

BMP bone morphogenetic protein

CHAPS cholamidopropyl dimethylammonio propanesulfonate

CSL CBF1 suppressor of hairless Lag-1

DAPT difluorophenacetyl alanyl phenylglycine T-butyl ester

DMSO dimethylsulfoxide

DPBS dulbecco´s phosphate buffered saline

EDTA ethylen diamin tetra acid

EGF epidermal growth factor

EGTA ethylene glycol tetraacetic acid

FGF fibroblast growth factor

FGFR fibroblast growth factor receptor

HES *hairy* and enhancer of split

HGF hepatocyte growth factor

HH Hamburger and Hamilton

HSPG heparin sulfate proteoglycans

ISH *in situ* hybridization

LB Luria broth

LN Lin-12-Notch

NBT nitro blue tetrazolium chloride

NICD notch intracellular domain

NF nuclear factor

OR orange domain

PBS phosphate-buffered saline solution

PBT phosphate-buffered saline solution with Tween

PCR polymerase chain reaction

PM plasma membrane

PSM presomitic mesoderm

RAM RBP-Jk associate molecule

RBP-J recombination signal sequence-binding protein J

RT room temperature

RT-PCR real-time PCR

SHH sonic hedgehog

SSC standard saline citrate

St stage

SU5402 2-[(1,2-Dihydro-2-oxo-3H-indol-3-ylidene)methyl]-4-methyl-1H-pyrrole-3-propanoic acid

Su(H) supressor of hairless

TACE TNF-α−converting enzyme

Tbx T-box

TAD transactivation domain

TGF transforming growth factor

WNT wingless wint

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INTRODUCTION

1.1. CHICKEN EMBRYOLOGY

Avian and mammalian embryos are very similar both in its morphological complexity and in the general course of early embryonic development, despite the considerable differences in the adult stage. Avian embryos, however, are easier to obtain and observe: embryo observation and manipulation can be carried out simply by removing the egg's shell and embryos can be cultured in an *in vitro* system. The chicken is now one of the most versatile experimental systems available because of the advances in new technologies, including *in vivo* electroporation, isolation of embryonic stem cells, and sequencing of the chick genome (Wallis *et al.*, 2004).

1.1.1. EVENTS IN EMBRYONIC DEVELOPMENT

The large yolky egg is fertilized and begins to divide while still in the hen's oviduct. Initially, division is confined to a small patch of cytoplasm that contains the nucleus and lies on top of the yolk, leading to the formation of the blastoderm. A layer of cells, called the hypoblast, develops over the yolk to form the floor of the cavity and eventually gives rise to extra-embryonic structures. The embryo is formed from the remaining blastoderm, known as the epiblast. During the 20-hour passage down the oviduct, the egg becomes surrounded by albumen, the shell membranes and the shell. When the egg is laid, some embryonic development has already occurred and usually stops until proper cell environmental conditions are established for incubation to resume. At first all the cells are alike but, as the embryo develops, cell differentiation occurs.

Soon after incubation begins, gastrulation initiates in the epiblast and is marked by the development of a pointed thickened layer of cells visible in the caudal end of the embryo called the primitive streak. This structure is the forerunner of the main body axis of the embryo. Furthermore, a precursor of the digestive tract forms, blood islands appear and somites start to emerge at the anterior end of the embryo.

On the second day of incubation, the blood islands begin linking and form a vascular system, while the heart is being formed elsewhere. Flexion and arching of the embryo begins bringing the two heart rudiments together to form one organ lying ventral to the gut; the heart and vascular systems join and the heart begins to beat. At this stage, ears and lens begin to form.

At the end of the third day of incubation, the head is well developed, the heart is formed, blood vessels are forming and the limbs begin to develop. Blood vessels and blood islands, where blood cells are being formed, have developed in the extra-embryonic tissues. The vessels connect up with

those of the embryo to provide a circulation with a beating heart. At this stage the embryo lies down with its left side on the yolk, the head is strongly flexed so the embryo forms a "C" shape. The embryo gets its nourishment from the yolk through extra-embryonic membranes, which also provide protection. The amnion forms a fluid-filled amniotic sac that provides mechanical protection. A chorion surrounds the whole embryo and lies just beneath the shell, an allantois receives excretory products, and provides the site of oxygen and carbon dioxide exchange, and a yolk sac surrounds the yolk.

Torsion and flexion continue through the fourth day. The digestive and respiratory system develop and the heart continues to enlarge even though it has not been enclosed within the body. It is seen beating if the egg is opened carefully. By the end of the fourth day of incubation, the embryo has all organs needed to sustain life after hatching, and most of the embryo's parts can be identified. The chick embryo cannot, however, be distinguished from that of mammals. In the remaining time before hatching the embryo grows in size, the internal organs develop, wings, legs and beak are formed, and down feathers grow on the wings and body. The chick hatches 21 days after the egg is laid (Wolpert *et al.*, 2007; Gilbert, 2006).

1.1.2. STAGES IN CHICK EMBRYO DEVELOPMENT

The sequence of chick development has been described as an illustrated series of developmental stages by Eyal-Giladi and Kochav (1975) for the first seven hours of development and by Hamburger and Hamilton (1951) for development up to 20 days, allowing standardization between researchers working on chick development. Furthermore the detailed description of the developing anatomy of the chick embryo in '*The Atlas of Chick Development*' (Bellairs & Osmond, 2003), which builds on the Hamburger and Hamilton description of chick development, has allowed reliable reporting of manipulated chick embryonic anatomy.

The Hamburger-Hamilton stages (HH) are a series of 46 chronological stages in chick development, starting from laying of the egg and ending with a newly hatched chick. Chick embryos can be staged according to different morphological landmarks. Although most organ systems have a stereotypical appearance at each stage, there are a few which particularly lend themselves to use in staging chick development.

In the very early embryo, the primitive streak is the only visible landmark, and its shape and size is used to stage HH1-6 embryos. Stages 5-8 may be defined by the formation of a head fold, the neural folds, and their fusion to form the neural tube. The expansion of anterior neural tube to form

the brain may also be used to identify later stages. Somitogenesis, the progressive segmentation of the paraxial mesoderm, provides a convenient method for staging embryos between stage 7 and 14. Somites form with surprising regularity every 90 minutes. Stage 10 embryos have 10 somites, and as a rule of thumb, the embryo gains 3 somites during each stage. However, beyond stage 14 (HH14, 22 somites) it becomes increasingly difficult to determine the number of somites accurately. This is due in part to the dispersal of the mesoderm of the anterior most somites, and, in later stages, to the curvature of the tail. Total somite-counts given for the following stages are typical, but sufficiently variable so as not to be diagnostic. For these reasons, other markers (limb-buds, visceral arches) and other externally visible structures are used as identifying criteria from stage 15 onward. Formation of the brachial arches, which will give rise to the structures of the jaw, pharynx and larynx, begins at HH14 and is used as a marker throughout development. The morphology of the limbs, starting with the appearance of wing bud at stage 16, is a useful landmark for staging chick embryos until hatching. Between stages 15 and 35, the appearance of specific structures within the limbs (such as joints and digits), and at later stages the length of the toes are used. The formation and development of the eyelids, primordial feathers and beak are used in a similar way to stage later development (Hamburger & Hamilton, 1951; Bellairs & Osmond, 2003; Gilbert, 2006).

1.1.3. Chick Model

The chick embryo, including its extra-embryonic membranes, has long been used as a developmental model due to its accessibility for surgical manipulations and abundance of data elucidating cellular signaling and interactions during embryonic development (Coleman, 2008). It provides an excellent model system for studying the development of higher vertebrates wherein growth accompanies morphogenesis. This model has several advantages over mammalian systems for *in vivo* studies, as it is cost-effective, easily manipulated, easily accessible from early stages and throughout organogenesis, and can be used for transgenesis in conjunction with viral vectors or electroporation (Brown *et al.*, 2003). Furthermore, a large database exists on the descriptive aspects of normal and abnormal development of the early avian embryo, and a detailed fate map that shows the locations of progenitor cells prior to gastrulation as well as at later stages, when many different organ rudiments are forming, is available (Darnell & Schoenwolf, 2000).

Chicken eggs can be easily obtained from commercial sources. If eggs are acquired and used within a week, unincubated eggs can be stored in any cool place (16ºC), obviating the need for a special storage facility. At the time the egg is laid, the avian embryo consists of a flat, two-layered blastoderm that lies on the surface of the yolk and, therefore, is readily accessible. Subsequent

development occurs with incubation at 38°C and is rapid. During this period of early development, chick embryos can be easily removed from the shell for culture, or they can be cultured *in ovo* and follow subsequent development for up to several days. Embryos are semitransparent, allowing the visualization of internal tissues possible under the microscope, and they are big enough to make several types of micromanipulation practical at these early stages (Darnell & Schoenwolf, 2000). Chick embryology studies have demonstrated that it is possible to cleanly separate epithelial and mesenchymal tissues, recombine the two tissues in different orientations or tissues from embryos at different stages of development, and then graft the recombined tissues back into the embryo (Davey & Tickle, 2007).

In the last few years, the classical approaches have been enormously enriched by three major technical advances: the introduction of new methods for gain- and loss-of-function and promoter analysis, the isolation of embryonic stem cells and development of new methods for transgenesis, and the sequencing of the chicken genome and establishment of numerous new electronic resources (Stern, 2005). The recent completion of the sequencing and assembly of the chicken genome represents a major leap forward in avian functional genomics research (Cogburn *et al.*, 2007). Its compact genome and its unique evolutionary position with respect to mammals have greatly facilitated the identification of putative regulatory gene regions, which show high sequence conservation to their mammalian counterparts (Groef *et al.*, 2008).

The special characteristic of chick embryos is that it is possible to bring together well-established embryological manipulations — the classical 'cut-and-paste' experiments, fate mapping and the application of biologically active molecules (by inserting small beads, which act as controlled release carriers, pre-loaded with growth factors, inhibitors, etc) — with more recently developed genetic manipulations, such as mis- and overexpression of genes and increasingly, gene inactivation. Importantly, such genetic manipulation can be precisely targeted in time and in space (Brown *et al.*, 2003).

1.2. SEGMENTATION CLOCK

Somitogenesis, the periodic formation of the vertebrae precursors, is a striking example of a dynamic embryonic process that relies on precise spatial and temporal control of gene expression (Aulehla & Pourquié, 2008). This process comprises the generation and translation of a temporal periodicity into the metameric pattern of somites (Leimeister *et al.*, 2000). Somites constitute the basis of the segmental pattern of the body and give rise to the axial skeleton, the dermis of the back,

and all striated muscles of the adult body. Individual pairs of somites, located symmetrically on either side of the neural tube, emerge from the rostral end of the presomitic mesoderm (PSM), while new mesenchymal cells enter the caudal paraxial mesoderm as a consequence of gastrulation. In the chick embryo, a somite pair is laid down every 90 min in a rostro-caudal progression, and a total of 50 somite pairs are formed during embryogenesis (Palmeirim *et al.*, 1997).

In 1997, Palmeirim and collaborators disclosed a molecular clock underlying vertebrate embryo somitogenesis when analyzing the expression pattern of the *hairy1* gene, an avian homolog of the *Drosophila hairy* segmentation gene, in chick embryos. They demonstrated that *hairy1* mRNA is expressed in a highly dynamic manner in the chick PSM appearing as a caudo-rostral wave, which is reiterated during the formation of every somite. The domain of *hairy1* expression has the appearance of a wavefront beginning in the broad, caudal PSM, progressing anteriorly and intensifying into the narrow anterior PSM. Finally, in each cycle, expression decays sharply throughout the PSM except for a thin stripe corresponding to the posterior part of the forming somite. This wavefront is not due to cell movements within the PSM, nor to the periodic production of an anterior-to-posterior diffusing signal, but is an autonomous property of the cells in this tissue: cells within the segmental plate cycle autonomously between *hairy1* "on" or "off" expression states until they segment and then sustain *hairy1* expression in the posterior somite half (Palmeirim *et al.*, 1997; Leimeister *et al.*, 2000).

Analysis of the *hairy1* sequence suggests that it belongs to the hairy-enhancer-of-split (Hairy E (Spl)/ HES) family of transcriptional repressor proteins. All HES members share conserved functional motifs: the basic domain (b) required for DNA-binding, a helix-loop-helix dimerization domain (HLH), an orange domain (OR) which confers specificity among family members and the C-terminal WRPW tetrapeptide required for co-factor binding. The evidence to date suggests that HES proteins can be involved in an array of repression mechanisms via the recruitment of different protein partners, forming complexes with different specificities, and regulate a wide variety of developmental processes such as negative control of differentiation, anterior-posterior segmentation in both invertebrates and vertebrates (probably by distinct mechanisms), and sex determination in flies. In many, but not all of these processes, HES proteins function as effectors of the Notch signaling pathway (Kageyama *et al.*, 2007).

Latter, a second chicken *hairy*-related gene, *hairy2*, has been identified and was shown to cycle in synchrony with *hairy1* across the PSM (Leimeister *et al.*, 2000). Like *hairy1*, *hairy2* is expressed as a wavefront, which sweeps across the PSM during the formation of each somite. Moreover, the

propagation of the *hairy2* wavefront is similar to that of *hairy1*. It does not rely on cell movement or on a propagatory signal traveling through the PSM, suggesting that this dynamic regulation of *hairy2* expression constitutes an intrinsic property of the PSM (Jouve *et al.*, 2000). When Jouve and collaborators compared the expression profiles of *hairy1* and *hairy2*, they realized that both genes exhibit similar anterior expression borders in the PSM but become expressed in complementary compartments in the rostral-most PSM. Furthermore, *hairy1* and *hairy2* expressions cycle together in the PSM, they share the same expression domain, except that *hairy2* extends more caudally in the PSM. At the end of one cycle, the expression domains diverge in the rostral PSM, where *hairy2* is located in the anterior part of the prospective somite, whereas *hairy1* is found in the posterior part (Jouve *et al.*, 2000).

The discovery of oscillating expression of *hairy1* in the PSM of chick embryos provided the first molecular evidence for the existence of a segmentation clock that ensures the correct spatiotemporal periodicity of somite generation. Meanwhile, several other genes have also been reported to have a dynamic expression at the level of the PSM in a caudal to rostral direction, such as *hairy2* in the chick, lunatic fringe *(Lfng)*, in the chick and mouse, *her1* and *her7* in zebrafish, *Hes1* and *Hes7* in mouse, *Hey2* both in chick and mouse, *esr9* and *esr10* in *Xenopus* and *her7* in medaka (Andrade *et al.*, 2007). Furthermore, Aulehla and colleagues (2003) have shown that a repressor of the Wnt signaling pathway, *axin2*, is also cycling in the mouse PSM (Aulehla *et al.*, 2003).

All studies concerning embryonic cyclic gene expression during development have focused exclusively on the somitogenesis process. However, time control is present during all embryonic processes, suggesting that the molecular clock may not be an exclusive property of the PSM cells but could also be operating in other developing tissues (Andrade *et al.*, 2005). Hirata and coworkers (2002) showed that serum treatment of a variety cultured cell lines induces cyclic expression of both mRNA and protein of Hes1 (mouse *hairy2* homologue), with 2-hour periodicity. Recently, *hairy2* expression was studied during chick limb bud development and it was observed that this gene is expressed from limb bud initiation until digit formation. This study provided for the first time evidence of a molecular clock working during chick forelimb autopod outgrowth and patterning. Pascoal *et al.* (2007) showed that the somitogenesis clock gene *hairy2* is expressed cyclically in the distal mesenchyme of chick embryo forelimbs, with a 6-hr periodicity and that this periodicity is correlated with the formation time of an autopod limb element. The results of this work support the hypothesis that the molecular clock is not an exclusive property of PSM tissue rather a more general way to count time during vertebrate development, providing positional information to different types of cells (Pascoal *et al.*, 2007).

Although the nature of the clock is still unknown, its existence is revealed by genes expressed in a cyclic fashion within the PSM and the majority of them is intimately related to the Notch signaling pathway (Aulehla *et al*., 2003; Andrade *et al*., 2005). These cyclic genes, as well as other components of the Notch signaling pathway, were shown to be required for the proper somite segmentation in mice and zebrafish by mutant analysis or morpholino oligo (MO)-mediated gene-knockdown experiments (Kawamura *et al.,* 2005). Somitogenesis is defective in animals in which this pathway is disrupted by either activating or inactivating mutations. Notch is linked to the segmentation clock although the exact nature of this relationship is not yet understood. Several observations suggest that Notch signaling is a central component of the oscillatory mechanism: (1) the cycling expression of mouse *Hes1* depends on the Notch ligand Delta1 (*Dll1*); (2) the activity of the Notch-dependent factors Her1, Her7, Hes1 and Hes7 is required for their own cyclic expression; (3) the oscillatory expression pattern of *lunatic fringe*, required for somite segmentation, is under the direct transcriptional control of Notch signaling and is lost in embryos lacking *Dll1* or *Hes7* (Pasini *et al.,* 2003). Alternatively, it has been proposed that the main function of Notch signaling is to maintain the synchronization of cyclic gene expression in the PSM (Jiang *et al.,* 2000, Aulehla & Pourquié, 2008).

The FGF signaling pathway has also been implicated in the somitogenesis clock mechanism. Kawamura *et al.* (2005) focused on a zebrafish *hairy/Esp1* gene, *her13.2* (Hes6-related hairy/Enhancer of split-related gene), which is expressed in the posterior PSM. They provided evidence that *her13.2* links FGF signaling to the Notch-regulated oscillation machinery in zebrafish. Expression of *her13.2* is induced by FGF-soaked beads and decreased by an FGF signaling inhibitor. *her13.2* is required for periodic repression of the Notch-regulated genes *her1* and *her7*, and for proper somite segmentation. Furthermore, Her13.2 augments autorepression of *her1* in association with Her1 protein. Therefore, FGF signaling appears to maintain the oscillation machinery by supplying a binding partner, Her13.2, for Her1. It is likely that FGF signaling is transmitted through several different molecular pathways in the posterior PSM, and that Her13.2 mediates specifically one of the roles of FGF signaling, the regulation of the cyclic genes.

1.2.1. NOTCH SIGNALING PATHWAY

Animal development involves many types of cell communication processes and the molecular bases of some of these processes have been unraveled in recent years. Curiously the same molecular pathways, such as Notch signaling pathway, are used to convey messages between cells in vertebrate and invertebrate animals (Bishop *et al.*, 1999). Notch signaling defines a conserved, pleiotropic cell-interaction pathway that controls cell fate and consequently differentiation, proliferation and apoptotic events throughout embryonic development and homeostasis of adult self-renewing organs (Lake *et al.*, 2009; Borggrefe & Oswald, 2009). The central element of this pathway is the transmembrane Notch receptor, which triggers signaling through interaction with membrane-bound ligands expressed on adjacent cells (Lake *et al.*, 2009).

The *Notch* gene was first identified in *Drosophila melanogaster* and encodes a large transmembrane protein that acts as a signaling receptor that is required throughout development to regulate the spatial patterning, timing and outcomes of many different cell fate decisions in both vertebrate and invertebrate species (Baron, 2003; Brennan & Gardner, 2002). In mammals, there are four Notch receptors (Notch1–4) and five transmembrane ligands from the Delta (Delta-like1, Dll1, Delta-like3, Dll3 and Delta-like4, Dll4) and Jagged families (Jag1, 2) (Benedito & Duarte, 2005; Borggrefe & Oswald, 2009). Avian have two Delta-like genes, Dll1 and Dll4, and two Jagged genes, called Serrate1 and Serrate2 in chicken. Both Notch and its ligands are integral membrane proteins and generally transmit signals only between cells in direct contact. Moreover, Notch activation has a direct and immediate effect on gene expression, mediated by the detached intracellular domain of Notch itself, acting as a transcriptional regulator in the nucleus (Borggrefe & Oswald, 2009). Thus Notch signaling can readily throw genetic switches that determine choices of cell fate. Furthermore, activation of Notch in a given cell frequently regulates the production of Notch ligands by that cell. Because the level of Notch activation in the cell depends on the level of ligand expression in its neighbors, and vice-versa, this gives rise to feedback loops that correlate the fates of adjacent cells and control spatial pattern of differentiation (Lewis, 1998).

1.2.1.1. Notch Structure

A prototypical Notch gene encodes a single transmembrane receptor composed in its extracellular region of a conserved array of up to 36 EGF-like repeats, mediating direct contact between ligand and receptor involved in interaction; three juxtamembrane repeats, known as Lin-12-Notch (LN) repeats, which modulate interactions between the extracellular and the membrane-

tethered intracellular domains. The intracellular region of Notch includes seven ankyrin repeats flanked by nuclear localization signals, a proline, glutamine, serine, threonine-rich (PEST) domain and a transactivation domain (figure 1.1) (Fiúza & Arias, 2007).
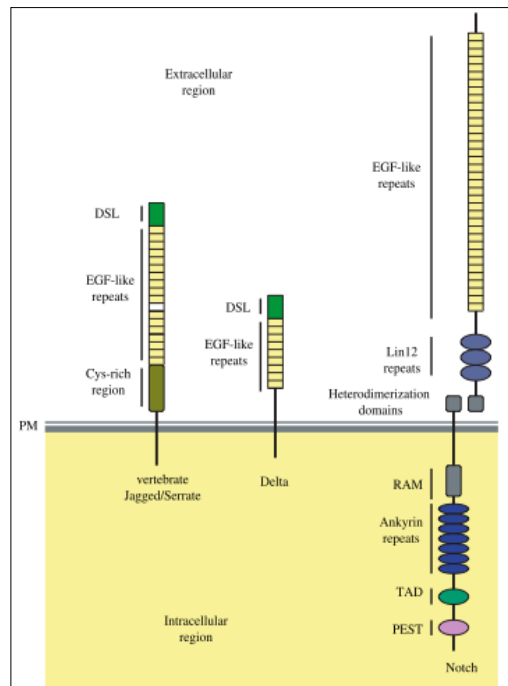

Figure 1.1. Structure of Notch and its ligands. Notch ligands, Delta and Jagged/Serrate, are composed of a cysteine-rich region, called DSL, responsible for the interaction with the Notch receptor and several EGF repeats. Jagged/Serrate also contains an extracellular cystein-rich region. Notch is composed by up to 36 EGF-like repeats. Notch also contains a cysteine-rich region known as Lin-12 repeats in close proximity with heterodimerization domains that bind non-covalently extracellular Notch with membrane-tethered intracellular Notch. In its intracellular part, Notch has a region called RAM followed by repeated structural motifs named Ankyrin repeats (mediate the interaction between Notch and CBF1/Su(H)), a transactivation domain (TAD) and a PEST domain. The PEST domain is involved in the degradation of Notch. PM, plasma membrane. (Adapted from Fiúza & Arias, 2007)

1.2.1.2. The Notch Cascade

The Notch signaling cascade appears remarkably simple with apparently no second messengers involved, although its role and the activation of downstream genes in a given tissue remain often complex and unpredictable (Borggrefe & Oswald, 2009). Notch signaling is activated upon cell-to-cell contact as a result of interactions between Notch receptors and their ligands (Delta or Jagged). Multiple studies focusing on both *Drosophila* and mammalian Notch receptors have led to a model for Notch signaling that involves a series of cleavages that eventually leads to the release of the intracellular domain, which carries nuclear localization signals, from the cell surface followed by its translocation to the nucleus where it participates directly in transcriptional events (Lake *et al*., 2009).

A most important feature of Notch is that it acts, at the same time as a transmembrane receptor and as a transcription factor. At the cell surface, extracellular domain of Notch can interact with the extracellular region of Delta and Jagged homologues expressed in a neighboring cell (Arias *et al*., 2002). This interaction results in the exposure of an extracellular metalloprotease site (S2 site) which thus becomes susceptible to cleavage by transmembrane proteases of the ADAM family (Mumm *et al*. 2000). Subsequently, two further intramembranous cleavages occur, named S3/S4, by γ-secretase activity of a membrane protein complex containing members of the Presenilin family and

Nicastrin as catalytic components (figure 1.2). γ-secretase activity can be inhibited with small cell-permeant molecules such as DAPT, leading to a blockade of Notch signaling (Daudet *et al.*, 2007). The intracellular domain of Notch (NICD) is thus finally released and translocates into the nucleus where it interacts with members of the RBP-J (also called CSL, CBF1, Su(H), Lag-1) family of transcription factors, regulating gene expression by acting as a transcriptional co-activator. NICD cannot bind directly to DNA but heterodimerizes with the DNA binding protein RBP-J and activates transcription of genes containing RBP-J binding sites. NICD binding to RBP-J is crucial for the switch from repressed to activated state. NICD first displaces corepressors from RBP-J, resulting in derepression of promoters containing RBP-J binding sites and subsequently recruits a coactivator complex to activate transcription of Notch target genes (Borggrefe & Oswald, 2009).
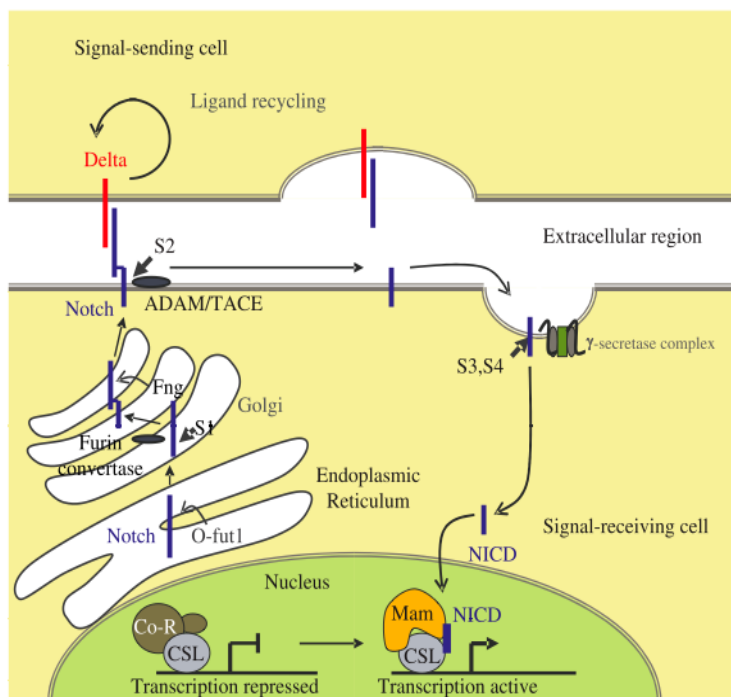

Figure 1.2. Notch signaling pathway. Notch binding to ligand elicits several steps of cleavage. The first one at the S2 site is mediated by the proteases ADAM10 or by TACE. This catalyzes the processing of Notch in the intramembranous S2 and S3 sites by the γ-secretase complex. Thus, Notch intracellular domain (NICD) is released and translocates into the nucleus where it dislodges repressors (co-R) associated with the DNA-binding CSL transcription factor. (Adapted from Fiúza & Arias, 2007)

Although signals mediated through Notch receptors have diverse outcomes, only a fairly limited set of Notch target genes have been identified in various cellular and developmental contexts. Hes is a highly conserved protein family that is regulated by Notch in multiple cell types. In mammals, the best-described Notch target genes are indeed the transcription factors Hes1, Hes5 and Hey1. Hes and Hey proteins are bHLH transcription factors that function as transcriptional repressors. Genetic studies in mice have shown that inactivation of many components of the Notch pathway results in dramatic segmentation defects and a severe impairment of the periodic expression of the cyclic genes (Dubrulle & Pourquié, 2002).

1.2.2. FGF SIGNALING PATHWAY

Fibroblast growth factors (FGF) and their specific cell surface receptors (FGFR) make up a large and complex family of signaling molecules that have been shown to play an important role in a variety of processes of embryonic development and tissue homeostasis (Dailey *et al*., 2005). The FGF family of ligands consists of 23 members in humans and mice, and 13 in chicken. These molecules signal by activating a smaller family of cell surface receptors encoded in four distinct genes (FGFR1-4) that, through alternative splicing in the extracellular immunoglobulin (Ig)-like domain adjacent to the membrane, can produce numerous FGFR isoforms. As the variable region involves the ligand-binding site, the FGFR isoforms differ in their binding affinity toward the FGF ligands. Experimental studies analyzing receptor specificity of the entire FGF family demonstrate a significant redundancy in FGF-FGFR interactions, with all the major FGFR variants being activated by at least five ligands. For instance, the prevalent isoform of FGFR3 (FGFR3c) appears to be strongly activated by FGF1, 2, 4, 8, 9, and 17-20 *in vitro*. However, its redundancy appears, in contrast, limited *in vivo* (Krejci *et al.*, 2009).

FGFRs are single-pass transmembrane proteins with tyrosine kinase activity. Ligand binding to the extracellular domain of the receptor initiates a signal transduction cascade (Ras-MAP kinase, PI3 kinase/Akt), that ultimately results in modification of gene expression (Dailey *et al*., 2005; Thisse & Thisse*,* 2005). The FGF-FGFR interaction requires the intervention of heparin or HSPG that bind both the ligand and the receptor at specific domains and stabilize the formation of a receptor dimmer bound to the FGF molecules (figure 1.3). FGF signaling can be blocked by chemical inhibitors such as SU5402, an FGF receptor antagonist, that inhibit the tyrosine kinase activity of all four FGFRs by interacting with the catalytic domain (Firnberg & Neubuser *et al.,* 2002).
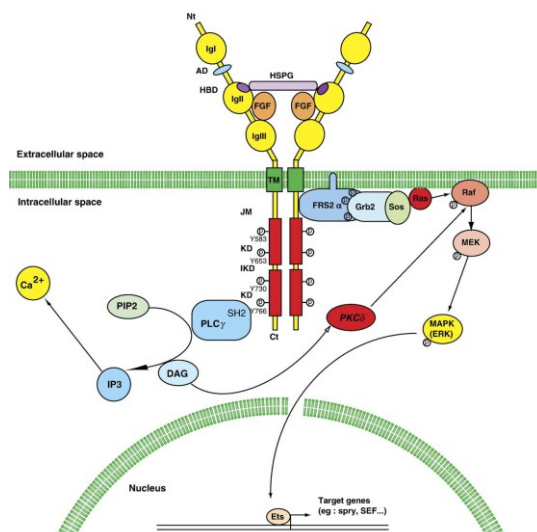
Figure 1.3. FGF receptors and FGF sinal transduction. FGFRs are modular proteins comprising 3 immunoglobulin domains (IgI, IgII and IgIII). FGF ligands linked to heparin sulfate proteoglycan (HSPG) bind to IgII and IgIII of FGFR. This results in the dimerization and the subsequent transactivation by phosphorylation of specific tyrosine residues. The main two transduction pathways involve the phospholipase C-g (PLCg) and the Ras/MAP kinase. (Adapted from Thisse & Thisse, 2005)

1.3. LUNG DEVELOPMENT

Lung development is a highly regulated and orchestrated process directed by mesenchymal-epithelial interactions that control and coordinate the temporal and spatial expression of multiple regulatory factors required for proper lung formation. It is now known that the molecular mechanisms involved in patterning, development and differentiation of the lung are orchestrated by finely integrated and mutually regulated networks of transcriptional factors, growth factors, matrix components and physical forces (Shi *et al.*, 2007).

1.3.1. BRANCHING MORPHOGENESIS

Early during mammalian embryonic life, the foregut endoderm is specified into domains that will give rise to organs, such as the thyroid, lung, liver, and pancreas. Once respiratory cell fate has been established, the tracheal and lung primordia form, and the lung subsequently develops into a tree-like system of epithelial tubules and vascular structures that ultimately becomes the airways and the alveoli (Cardoso & Whitsett, 2008; Muratore *et al.*, 2009). The foregut endoderm differentiates into various epithelial cell types, which line the inner surface of the developing lung and trachea. The lung mesenchyme originates from the lateral plate mesoderm and gives rise to multiple components of the lung, including its connective tissue, endothelial cell precursors, the smooth muscle that surrounds airways and blood vessels, the cartilage of the trachea, the lymphatics, and the mesothelial cells that cover the outer surface of the lung, the pleura (Cardoso & Lu, 2006).

Normal growth, morphogenetic patterning and cellular differentiation in the developing lung depend on interactive signaling between the endodermal epithelium and mesenchyme. For instance, distal mesenchyme induces ectopic budding and branching when grafted adjacent to the tracheal endoderm denuded of mesenchyme (Alescio & Cassini, 1962); conversely, tracheal mesenchyme inhibits branching when grafted next to distal epithelium (Wessels, 1970), showing that crosstalk between the epithelium and the mesenchyme drive the branching process. These intimate interactions are mandatory for formation and completion of lung development and ultimately reflect activation of local gene networks along the proximal-distal axis of the respiratory tract, which coordinates the temporal- spatial appearance of buds and clefts, resulting in the bronchial tree (Lebeche *et al.*, 1999). The presence of extracellular matrix molecules, including collagen, fibronectin, laminin, glycosaminoglycans, and proteoglycans, as well as cell membrane-bound integrins, play an important role in directing lung development by influencing the rates of cellular proliferation and differentiation (Shannon & Deterding, 1997). Mechanical distention exerted on the

lung as well as on specific cell types can also significantly affect gene expression and, ultimately, lung growth and development (Pinkerton & Joad, 2000). A myriad of diffusible factors such as FGF, TGF-β, BMP-4, retinoic acid, SHH, HGF hepatocyte growth factor and EGF, with their cognate receptors and intracellular signaling molecules modulate cellular proliferation/differentiation and branching (Muraoka *et al.*, 2000). Expression of each molecule occurs in a temporally, spatially, and cell type-specific manner. Also, the role of each molecule should be deemed not in terms of its isolated function in specific cells but, rather, in the context of epithelial-mesenchymal interactions, a process that is central to lung morphogenesis (Demayo *et al.*, 2002).

1.3.1.1. Notch signaling

Notch has been implicated in several aspects of lung biology, including a role in epithelial growth and differentiation. During development, Notch receptors and ligands have been identified in both epithelial and mesenchymal compartments of the lung, and there is *in vitro* evidence that Notch may act on epithelial differentiation and branching morphogenesis (Tsao *et al.,* 2008).

Aberrant Notch function is associated with several developmental disorders, neurodegenerative disease and cancers (Baron, 2003). Notch pathway activity in normal fetal lung development has been assessed primarily by *Hes1* mRNA and immunohistochemistry. *Hes1* mRNA has been detected in early pseudoglandular stage mouse lung starting at E12. In the mouse, *Hes1* mRNA expression progressively rises until birth and then remains detectable in adult lung as well. Hes1 immunoreactivity is readily detectable in fetal mouse lung in non-endocrine airway epithelial cells that express Notch1 and Notch3. Very limited data are available for other Notch effectors in lung development. *Hes5* has not been detected in whole fetal lung RT-PCR (Ito *et al.*, 2000). HeyL is apparently expressed in lung vasculature (Leimeister *et al.*, 2000a). *Hey1* mRNA is prominently expressed in adult lung, *Hey2* at lower levels (Steidl *et al.*, 2000). Early lethality of mice with homozygous deletions for *Notch1*, *Notch2*, *Dll1*, and *Jagged1* limits the assessment of these genetic alterations in lung development (Collins *et al.*, 2004).

Quantitative expression studies from the developing mouse lung demonstrate a progressive increase in *Notch1–Notch4*, *Dll1*, and *Jagged1* mRNAs from E11.5 into adulthood. *In situ* hybridization studies and immunohistochemistry suggest that Notch1 is expressed in the distal lung endoderm at least as early as E11.5 and persists through fetal development (Ito *et al.*, 2000). Notch1 expression in pseudoglandular lung clearly overlaps with areas of the epithelium undergoing active growth and branching morphogenesis. *Notch1* is not expressed at high levels in fetal lung

mesenchyme that surrounds the primitive epithelium (Post *et al.,* 2000). In contrast to *Notch1*, *Notch2* and *Notch3* are both expressed in lung mesenchyme. *Notch2* appears to be lacking in epithelial cells by *in situ* hybridization, whereas *Notch3* mRNA and immunoreactivity can be detected in epithelial cells as well as mesenchyme (Post *et al.*, 2000; Ito *et al.*, 2000).

1.3.1.2. FGF signaling

In the embryonic lung, FGF signaling is an essential component of the regulatory networks between epithelium and mesenchyme and is fundamental at several stages of mammalian lung development (Del Moral *et al.*, 2006). A restricted number of FGF family ligands and all FGFRs are present in the embryonic lung, and their expression is regulated in time and space. Perturbation of the FGF signaling pathway during lung development results in dramatic abnormalities of epithelial branching and differentiation (Warburton *et al.*, 1999).

FGF10 and its receptor, Fgfr2b*,* are required for epithelial branching. Targeted deletion of either genes prevents branching, causing the trachea to terminate as a blind sac (Colvin *et al.,* 2001). At early stages, FGF10 is expressed at high levels in the distal lung mesenchyme in a pattern that appears to correlate with sites of prospective bud formation, acting as a chemo-attractant of lung epithelium, being able to direct bud outgrowths to proper positions in lung organ cultures (Miura *et al.*, 2009; Lebeche *et al.*, 1999). FGF7 can be detected in the early embryonic lung and, together with other factors, plays a role in epithelial branching *in vitro*, acting as a proliferative factor for the lung epithelium (Lebeche *et al.*, 1999). FGF9 is expressed in the outermost layer of the lung, the mesothelium, and in the epithelium of the developing bronchi (Colvin *et al.*, 1999; Yin *et al.*, 2008). It has been identified as a key factor that signals to mesenchyme to regulate proliferation, differentiation and the expression of other factors that in turn regulate epithelial development. Classical inactivation of FGF9 leads to a reduction in proliferation of the lung mesenchyme resulting in severely reduced branching of the lung epithelium (Colvin *et al.*, 2001).

1.3.2. AVIAN LUNG DEVELOPMENT

Among the air-breathing vertebrates, the avian respiratory apparatus, the lung-air sac system, is the most structurally complex and functionally efficient. After intricate morphogenesis, elaborate pulmonary vascular and airway (bronchial) architectures are formed (Maina *et al*., 2006).

Although the anatomy of the avian lung differs from that of the mammalian lung, both develop similarly and have anatomical functional equivalents. The avian lung forms by a series of closed

circular buds arising from the main airway branches, which differs from the dichotomous branching morphogenesis in mammalian lung development. In contrast to the mammalian lung, which terminates in alveoli, the avian lung forms a looping anastomotic network of air-vascular surfaces (parabronchi) that end in terminal air buds and air capillaries (Loscertales *et al.*, 2008).

In the embryo of the domestic fowl, *Gallus gallus variant domesticus*, the development of the respiratory tract proceeds through three stages: the formation of the respiratory rudiments (between 2 and 4 days of incubation, fig. 1.4.A), the bronchial branching (5 days of incubation, fig. 1.4.B) and the formation of the air sacs (6 days of incubation, fig. 1.4.C and D) (Sakiyama *et al.*, 2000).

The lung buds become evident on day 3.5 of embryogenesis, i.e. about stage 23 of development (Hamburger and Hamilton, 1951). They appear as paired protuberances on the lateroventral aspect of the foregut (primitive pharynx) of the developing embryo. On day 5, after fusing on the ventral midline, the single bud divides into left and right primordial lungs that progressively elongate caudally while separating and shifting towards the dorsolateral aspects of the coelomic cavity.
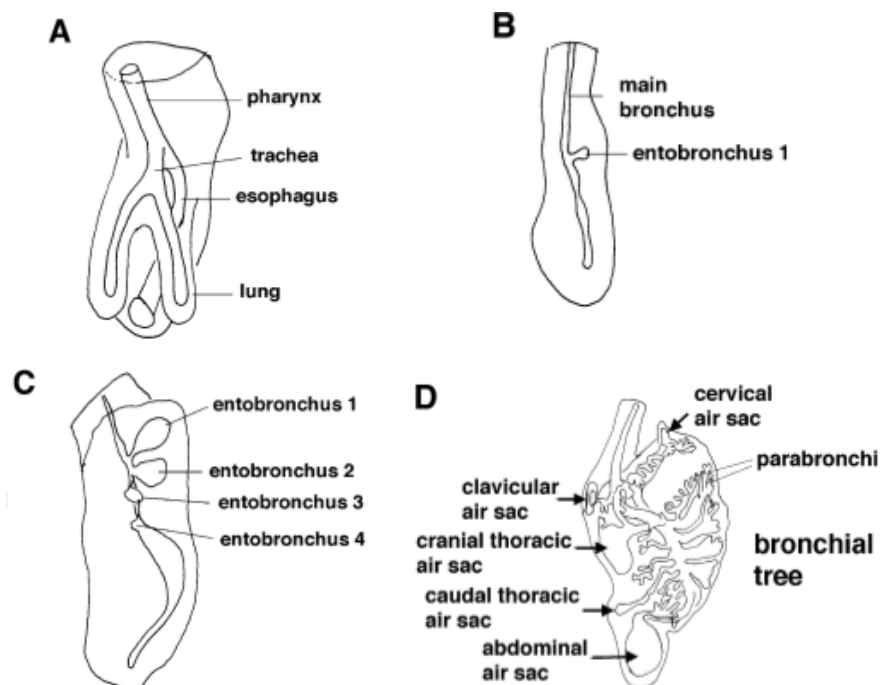
Figure 1.4. Chick lung development. (**A**) Drawing of the lung buds and surrounding organs after 3.5 days of incubation. Lateral view of the developing lung at day 5 (**B**), day 6 (**C**) and day 8 (**D**) of incubation. (Adapted from Sakiyama *et al.*, 2000).

On day 8, the lungs reach their definitive topographical locations in the coelomic cavity. As they develop and increase in size, they grossly changed from a saccular – to a wedge-shaped form.

After rotating through an angle of about 180º along the longitudinal axis, the lungs progressively insert and become firmly affixed to the ribs (Maina *et al.,* 2003; Maina *et al.*, 2006). Starting as a solid cord of epithelial cells that runs in a craniocaudal direction of the developing lung, progressively, the intrapulmonary primary bronchus begins to form. In a craniocaudal sequence, secondary bronchi sprout from the lumen of the intrapulmonary primary bronchus, radiating and extending outwards. On reaching the periphery of the lung, parabronchi (tertiary bronchi) bud from the secondary bronchi and project into the surrounding mesenchymal cell mass. The parabronchi proliferate, anastomose and connect the secondary bronchi. The parabronchial lung is capable of gas exchange by the end of the incubation period. On hatching no new structures appear, rather refinement of the existing structures occurs (Maina *et al.*, 2003a).

1.3.2.1. Molecular aspects of avian lung development

Although the morphogenesis of the mammalian lung is now well understood, there is a glaring dearth of data on the avian lung. To elucidate the molecular mechanism for regulating the region-specific morphogenesis of the chicken respiratory tract, Sakiyama and colleagues analyzed the spatiotemporal expression patterns of the *Hoxb* genes, *Bmp-2*, *Bmp-4, Wnt-5a,* and *Wnt-11* in the developing respiratory tract and found region-specific expression of these genes in the mesenchymal layer. By tissue recombination experiments, they found that the dorsal and the ventral pulmonary mesenchyme have different inductive capacities toward the tracheal epithelium. These observations suggested the possibility that *Hoxb* genes are involved in the system specifying regional differences in morphogenesis and cytodifferentiation of respiratory tract. In addition, they argue that it is possible that BMPs and WNTs mediate region-specific epithelial-mesenchymal interaction in this system (Sakiyama *et al.*, 2000).

Muraoka *et al.* (2000) suggested that nuclear transcription factor kB (NF-kB) may be required to mediate epithelial-mesenchymal interactions in the embryonic chick lung since NF-kB activity in the lung mesenchyme inhibits branching of underlying epithelium (Muraoka *et al.*, 2000). More recently Sakiyama *et al.* (2003) investigated the role of the Tbx4–Fgf10 system on the separation of the lung bud from the oesophagus in the chicken embryo showing that Tbx4 governs initial endodermal bud formation, respiratory endoderm formation, and septation of the respiratory tract and the esophagus. Moreover, the presence of a feedback loop between *Tbx4* and *Fgf10* in the regulation of lung development was demonstrated. In the mammalian developing lung, *fgf10* expression in the distal mesenchyme at sites where prospective epithelial buds will appear, and its ability to induce epithelial expansion and budding in organ cultures have led to the hypothesis that FGF10 governs the

directional outgrowth of lung buds during branching morphogenesis (Bellusci *et al.,* 1997).

In the chick lung, the branched structure is formed dorsally while the cyst structure (air sac) is formed ventrally during development. Sakiyama *et al.* (2000) carried out tissue recombination experiments, which showed that the cyst-branch difference in this system is caused by region-specific mesenchymal properties. The results of Miura and coworkers (2009) suggested that the regional cystic-branched difference within the developing chick lung results from a difference in the rate of diffusion of morphogen between the ventral and dorsal regions due to differential levels of HSPG and a different mesenchymal structure (Miura *et al.,* 2009).

1.4. AIMS

It is well known that *hairy1* and *hairy2* are involved in segmentation events that require tight spatial and temporal regulation, such as somitogenesis and limb development. These processes share some similarities with lung development, namely: they all occur along the rostro-caudal axis, the signaling pathways implicated are the same (FGF, Wnt, Notch), the formation of consecutive repetitive structures along the anterior-posterior axis is observed (somites, limb elements and secondary bronchi in somitogenesis, limb and lung development, respectively), and they occur with exact chronological precision. Considering this parallelism, the characterization of *hairy1* and *hairy2* during lung branching morphogenesis became relevant, since their expression might indicate a possible role in the development of this tissue, although their functions are not yet clarified.

The purpose of this work was to study the role of *hairy1* and *hairy2* during embryonic lung development of *Gallus gallus*.

Thus, the specific aims of this work were:

1. To characterize the expression pattern of *hairy1* and *hairy2* throughout different stages of lung development by *in situ* hybridization.

2. To evaluate the effect of Notch and FGF signaling pathway inhibition with DAPT and SU5402, respectively, using *in vitro* explant culture system:

   - In *hairy1* and *hairy2* expression pattern, assessed by *in situ* hybridization;

   - In lung branching morphology.

2

MATERIAL AND METHODS

2.1. EMBRYO DISSECTION

Fertilized chick (*Gallus gallus*) eggs, obtained from commercial sources, were incubated for 4-6 days in a humidified atmosphere at 37ºC. After incubation period, eggs shells were cracked open, and embryos removed and immersed in phosphate-buffered saline (PBS). The embryos were then transferred to dissection dishes immersed in PBS and staged according to the developmental table of Hamburger and Hamilton (1951).The attached yolk sac was cut and the embryos killed by decapitation. Embryonic chick lungs were carefully dissected under a dissection microscope (Olympus SZX16, Japan) and were then classified in b1, b2, b3, and so on, taking into account the number of secondary buds formed, 1, 2 or 3 respectively. Lungs were then processed either for ISH or for *in vitro* culture.

- o *PBS: 137mM NaCl, 10mM Phosphate, 2.7mM KCl, pH 7.4.*

2.2. *IN SITU* HYBRIDIZATION

In order to understand how gene expression is guiding development, it is essential to know exactly where and when particular genes are active. Genes are switched on and off during development and gene expression patterns are continuously changing (Wolpert *et al.*, 2007). One of the techniques that show where a gene is being expressed both within whole intact embryos and in sections is ISH.

In situ hybridization, as the name suggests, is a method of localizing and detecting specific RNA or DNA sequences in morphologically preserved tissues or cells preparations by hybridizing the complementary strand of a nucleotide probe to the sequence of interest. This technique involves: generation of a nucleic acid probe, labeled to enable subsequent detection; preparation of fixated tissues; pre-treatment of tissues to increase accessibility of target nucleic acid; hybridization of labeled probe to tissues; washing under conditions that remove non-hybridized probe; detection of the labeled probe, revealing the location of the target cellular nucleic acid (Wilkinson, 1999).

2.2.1. PROBES

2.2.1.1. DNA Extraction

DNA extraction was performed using the GenElute™ Plasmid Miniprep kit (Sigma, USA). This kit offers a simple, rapid, and cost-effective method for isolating plasmid DNA from recombinant *Escherichia coli* cultures. Glycerol stocks of plasmids containing the coding sequence of *Gallus*

gallus hairy1 and *hairy2* were kindly provided by Prof. Isabel Palmeirim, Life and Health Sciences Research Institute, School of Health Sciences, University of Minho. An overnight culture of recombinant *E. coli* containing *hairy1* and *hairy2* plasmid was harvested by centrifugation and subjected to a modified alkaline-SDS lysis procedure followed by adsorption of the DNA onto silica in the presence of high salts. Contaminants were then removed by a spin-wash step. Finally, the bound DNA was eluted in water and stored at -20ºC.

DNA extraction was performed according to the protocol described below.

1. An overnight culture grown in 5 ml of LB medium was harvested by centrifugation. Two ml of culture volume were transferred to a microcentrifuge tube, cells were centrifuged at 6000 rpm for 1 minute and the supernatant was discarded.

   ○ *LB medium: 1% w/v bacto-tryptone; 0.5% w/v bacto-yeast extract; 1% w/v NaCl*

2. The bacterial pellet was resuspended in 200 μL of Resuspension Solution, and then vigorously vortexed.

3. The resuspended cells were lysed by adding 200 μL of Lysis Solution and the contents were immediately mixed by gentle inversion.

4. Following cell lysis, the cell debris was precipitated with 350 μL of Neutralization Solution.

5. After gently inversion, the cell debris was centrifuged at 13000 rpm for 10 minutes.

6. Meanwhile, a GenElute Miniprep Binding Column was inserted into a microcentrifuge tube and 750 μL of Column Preparation Solution was added. After centrifugation at 13000rpm for 1 minute flow-trough was discarded.

7. The cleared lysate from step 6 was transferred to the prepared column and centrifuged for 1 minute at 13000 rpm.

8. Flow-through was discarded and 750 μL of the Wash Solution was added to the column to remove residual salt and other contaminants.

9. Column was centrifuged twice, 13000 rpm for 1 minute, to remove all the ethanol.

10. Finally, the column was transferred to a fresh collection tube and the purified plasmid DNA was eluted by the addition of 50 μL water and stored at –20ºC.

2.2.1.2. Plasmid Linearization

hairy1 plasmid, containing an 850 bp fragment of the *hairy1* coding sequence cloned in pBluescript KS-vector, was linearized using *Hind*III (Fermentas, Canada). *hairy2* plasmid containing a 350 bp of the *hairy2* cDNA cloned in pGEMT was linearized with *Nco*I (Fermentas) according to the manufacturer´s instructions. Both enzymes were selected to give a 5´overhang, since 3´overhangs are reported to lead to synthesis of abnormal long transcripts. This linearization step was controlled by agarose gel electrophoresis, since circular molecules that are left in the digestion mixture affect the transcription efficiency.

2.2.1.3. DNA Purification

Purification of enzymatic digestion was performed by the QIAquick® PCR purification kit (Qiagen, USA). This system combines the convenience of spin-column technology with the selective binding properties of a uniquely designed silica membrane. DNA adsorbs to the silica membrane in the presence of high concentrations of salt while contaminants pass through the column. Impurities are efficiently washed away, and the pure DNA is eluted with water. The DNA was stored at -20ºC.

DNA purification was performed according to the protocol described below.

1. Five volumes of Buffer PBI were added to 1 volume of the digestion and mixed.

2. The sample was applied to a QIAquick spin column, previously placed in a collection tube, and centrifuged for 1 minute at 13000 rpm.

3. The flow-through was discarded and 750 μL of Buffer PE was added, following a centrifugation (1 minute at 13000 rpm).

4. An additional centrifugation step, 1 minute at 13000 rpm, was performed so as to remove any residual Buffer PE, which may interfere with subsequent enzymatic reactions.

5. The QIAquick column was placed in a clean microcentrifuge tube and the DNA was eluted by the addition of 30 μL water to the center of the QIAquick membrane. After column was centrifuged (1 min, 13000 rpm). The DNA was stored at -20ºC.

2.2.1.4. Probe Synthesis

T7 RNA polymerase (Promega, USA) was used to synthesize the antisense digoxigenin-labeled *hairy1* and *hairy2*. The *in vitro* transcription reaction was performed using DIG RNA Labeling Mix (Roche Applied Sciences, Germany) according to the manufacturer´s instructions. The DIG labeling method is based on a steroid isolated from plants, which are the only natural sources of this molecule. Digoxigenin is linked to uridine nucleotides that are incorporated into the RNA probe by RNA polymerase. Hybridized DIG-labeled probes are then detected with high affinity anti-digoxigenin antibody conjugated with alkaline phosphatase (AP), in a colorimetric reaction with two AP substrates: NBT and BCIP (see ISH detection).

2.2.2. *In Situ* Hybridization

In situ hybridization was performed according to procedure described by Henrique *et al.* (1995) with minor modifications:

Pre-Hybridization

Prior to hybridization, samples were subjected to a series of pre-treatments that increase the efficiency of hybridization and/or decrease non-specific background.

1. After dissected, chick lungs were fixed overnight at 4ºC in 4% formaldehyde/2 mM EGTA, rinsed in PBS, dehydrated through a methanol series, and stored in 100% methanol at -20ºC.

2. After a step of rehydration, and in order to increase the accessibility of the target RNA, tissues were treated with proteinase K (Promega) to partially digest cellular proteins. Proteinase K is an endopeptidase which is non-specific and attacks all peptide bonds, is active over wide pH range and not easily inactivated. It is used to remove protein that surrounds the target sequence (Polak & McGee, 1998). Incubation has to be carefully monitored because if the digestion proceeds to far you could end up destroying most of the tissue or cell integrity; in this case, and considering the small size of the tissues proteinase K incubation was only for 2 minutes.

   o *Proteinase K solution: 10μg/ml; 0.1% Tween20; PBS*

3. This step is followed by a refixation step, to avoid disintegration of the sample, after washing with PBT.

- o *PBT solution: 0.1% Tween20, PBS*

- o *Post-fix solution: 4% formaldehyde in PBT, 0.1% gluteraldehyde*

4. Pre-hybridization involves incubating the tissue/section with a solution that is composed of all the elements of the hybridization solution, minus the probe, in order to block non-specific binding sites. The hybridization solution is constituted by:

- o *Formamide (50%). Reduces the thermal stability of the bonds, allowing hybridization to be carried out at a lower temperature.*

- o *SSC (1.3x, pH 5). Decrease the electrostatic interaction between the two strands.*

- o *EDTA (5.0 mM, pH 8). Removes free divalent cations from the hybridization solution, which strongly stabilize duplex DNA.*

- o *Other components are added to decrease the chance of nonspecific binding of the probe and include: tRNA acts as a carrier RNA (50 µg/ml), heparin (100 µg/ml). 0.2% Tween20; 0.5% CHAPS.*

Hybridization

Following the pre-hybridization, hybridization was performed at 70ºC (the optimal conditions for the annealing of probe to the target nucleic acid in the sample). Hybridization depends on the ability of the probe to anneal to a complementary mRNA strand just below its melting point (Tm). The value of the Tm is the temperature at which half of the probe is present in a single stranded form (Polak & McGee, 1998).

5. The chick lungs were incubated in hybridization solution containing the digoxigenin-labeled probe (1%). The hybridization was carried out overnight at 70ºC. For *hairy1* probe, 167 lungs from b0 to b7 stages were hybridized; for *hairy2* probe, 169 lungs from b0 to b7 stages were hybridized.

Post Hybridization Washes

Following hybridization, the material was washed with hybridization solution in order to remove unbound probe or probe which has loosely bound to imperfectly matched sequences.

6. Washing should be carried out at or close to the stringency condition at which the hybridization takes place (70ºC) with a final low stringency wash (RT).

<u>Antibody Incubation</u>

7. After clear the tissue with washing solution, tissues were incubated with blocking solution in order to decrease background in the detection of transcripts.

    o   *Washing solution (MABT): 0.1 M Maleic acid; 0.15 M NaCl; pH 7.5*

    o   *Blocking solution: washing solution; 2% Goat Serum (Invitrogen, USA); 2% Blocking Reagent (Roche Applied Sciences)*

8. The chick lungs were then incubated with a high affinity anti-digoxigenin antibody conjugated (1/2000; Roche) with AP. This step was performed overnight with constant shaking (RT).

<u>Detection</u>

9. The samples were washed several times with washing solution at room temperature, with constant shaking, to remove all unbound enzyme conjugated antibody.

10. Next, lungs were incubated with two AP substrates: NBT and BCIP. This colorimetric reaction will form a dark blue precipitate, representing the presence of the target mRNA.

    o   *Developing solution: 0.1 M NaCl; 0.1 M Tris.HCl, pH 9.5; 0.4 M MgCl2; 1% Tween20; 0.35 mg/ml NBT; 0.175 mg/ml BCIP.*

<u>Imaging</u>

11. Lungs were photographed in PBT solution using an Olympus U-LH100HG camera coupled to Olympus SZX16 stereo microscope and stored in PBT/0.1% azide.

<u>Slide Sections</u>

Hybridized chick lungs, with *hairy1* and *hairy2*, were fixated in paraformaldehyed 4%, embedded in 2-hydroxyethyl methacrylate (Technovit 7100; Heraeus Kulzer, Germany) and processed for sectioning at 25 μm thickness using a rotary microtome (Leica RM 2155, Germany). Lung sections were photographed with an Olympus DP70 camera coupled to an Olympus BX61 microscope.

2.3. CHICK LUNG EXPLANT CULTURE

After dissection in DPBS (Lonza, Switzerland) lungs were transferred to Nucleopore membranes with an 8 μm pore size (Whatman, USA) and incubated in a 24-well culture plates (Orange Scientific, Belgium). The membranes were presoaked in 400 μL of Medium 199 (Sigma) for 1 h before the explants were placed on them. Floating cultures of the explants were incubated in 200 μL Medium

199 supplemented with 5% heat inactivated fetal calf serum (Invitrogen), 10% chicken serum (Invitrogen), 1% L-glutamine (Invitrogen), 1% penicillin 5000 IU/mL, streptomycin 5000 IU/mL (Invitrogen) and 0.25 mg/mL ascorbic acid (Sigma). Chick lung explants were incubated in a 5% CO_2 incubator at 37°C for 24h or 48 h (in this case the medium was replaced at 24 h). The branching morphogenesis was monitored daily by photographing the explants. At d0 (D0: 0 h), d1 (D1: 24 h) and d2 (D2: 48h) of culture, the total number of peripheral airway buds (branching) in all lung explants was determined. The results of branching were expressed as D2/D0 ratio.

2.3.1. *IN VITRO* INHIBITION STUDIES

2.3.1.1. FGF inhibition

In order to inhibit the FGF signaling pathway, lung explants (st b1 to b3) were cultured SU5402, an FGF receptor antagonist that has been shown to inhibit the tyrosine kinase activity of all four FGFRs by interacting with the catalytic domain. SU5402 (Calbiochem, UK) dissolved in DMSO was added to the medium to achieve a final concentration of 25 and 50 μM (n=13 and n=12, respectively) and 0.1% DMSO. Control explants consisted of medium containing DMSO at a final concentration of 1 μL/ mL (n= 13). After culture, lung explants were fixated overnight at 4ºC and processed for ISH.

2.3.1.2. Notch inhibition

In order to inhibit the Notch signaling pathway, lung explants (st b1 to b3) were cultured with DAPT, a γ-secretase inhibitor which prevents the release of the intracellular, active fragment of Notch and efficiently blocks the presenilin–γ-secretase complex and, as a consequence, efficiently prevents activation of the Notch response. DAPT (Calbiochem, UK) dissolved in DMSO was added to the medium to achieve a final concentration of 20 and 45 μM (n=67 and n=88, respectively) and 0.1% DMSO. Control explants consisted of medium containing DMSO at a final concentration of 1 μL/ mL (n=50). Additionally, lung explants were incubated with medium as culture and lung growth control. After culture, lung explants were fixated overnight at 4ºC and processed for ISH.

2.3.2. STATISTICAL ANALYSIS

The results were expressed as mean ± standard deviation. For statistical analysis SigmaStat 3.5 (Systat Software Inc., USA), was used and applied the One-way ANOVA test. When the

differences were statistically significant, the Holm-Sidak test for multiple pairwise comparisons was used. Statistical significance was set as $p < 0.05$.

3

RESULTS

3.1. CHARACTERIZATION OF *HAIRY1* AND *HAIRY2* EXPRESSION PATTERN DURING CHICK LUNG DEVELOPMENT

In order to characterize the expression pattern of *hairy1* and *hairy2* during chick lung development, *in situ* hybridization was performed. For this purpose, embryos with 4 to 6 days of incubation were collected, staged according to HH developmental table and lungs were carefully dissected (n= 336). Since HH developmental stages are based mainly in different external morphological landmarks, it is more accurate to group lungs according to some morphological features characteristic of this organ, namely the number of secondary bronchi formed. Therefore, lungs were staged according to the number of secondary bronchi: b0 stage correspond to lungs with no secondary bronchi, prior to bronchial branching; at b1 stage, the lung presents one secondary bronchus, at b2 stage it presents two secondary bronchi, and so on.

3.1.1. *HAIRY1* EXPRESSION PATTERN

The *hairy1* expression was evident mainly in the pulmonary epithelium of the respiratory tract, throughout all the stages studied (figure 3.1). *hairy1* is present not only in the epithelial region of the main bronchus (figure 3.1 B, E, green arrows) but also in the epithelial compartment of the secondary bronchi (figure 3.1 C, F, blue arrows). Moreover *hairy1* is also present in the mesenchyme: it appears as a "musky" expression pattern both in the medial region of the main bronchus (figure 3.1 B, C, purple arrows) and in the lateral region adjacent the secondary bronchi (Figure 3.1 C, D, black arrows).

Even though the expression pattern of *hairy1* remain constant throughout the different stages studied, it was possible to observe differences in lungs of the same stage both in the epithelial and the mesenchymal compartments. In some lungs *hairy1* expression in the epithelial tip of the main bronchus was absent (figure 3.2 C, D, blue arrows) contrasting with some examples where it was strongly expressed (figure 3.2 A, B, blue arrows). On the other hand, medial and lateral mesenchymal regions also presented distinct expression patterns: some lungs showed weak or undetectable expression (figure 3.1 H, orange arrow; figure 3.2 C, D) while others a strong expression (figure 3.2 A, B, white arrows).
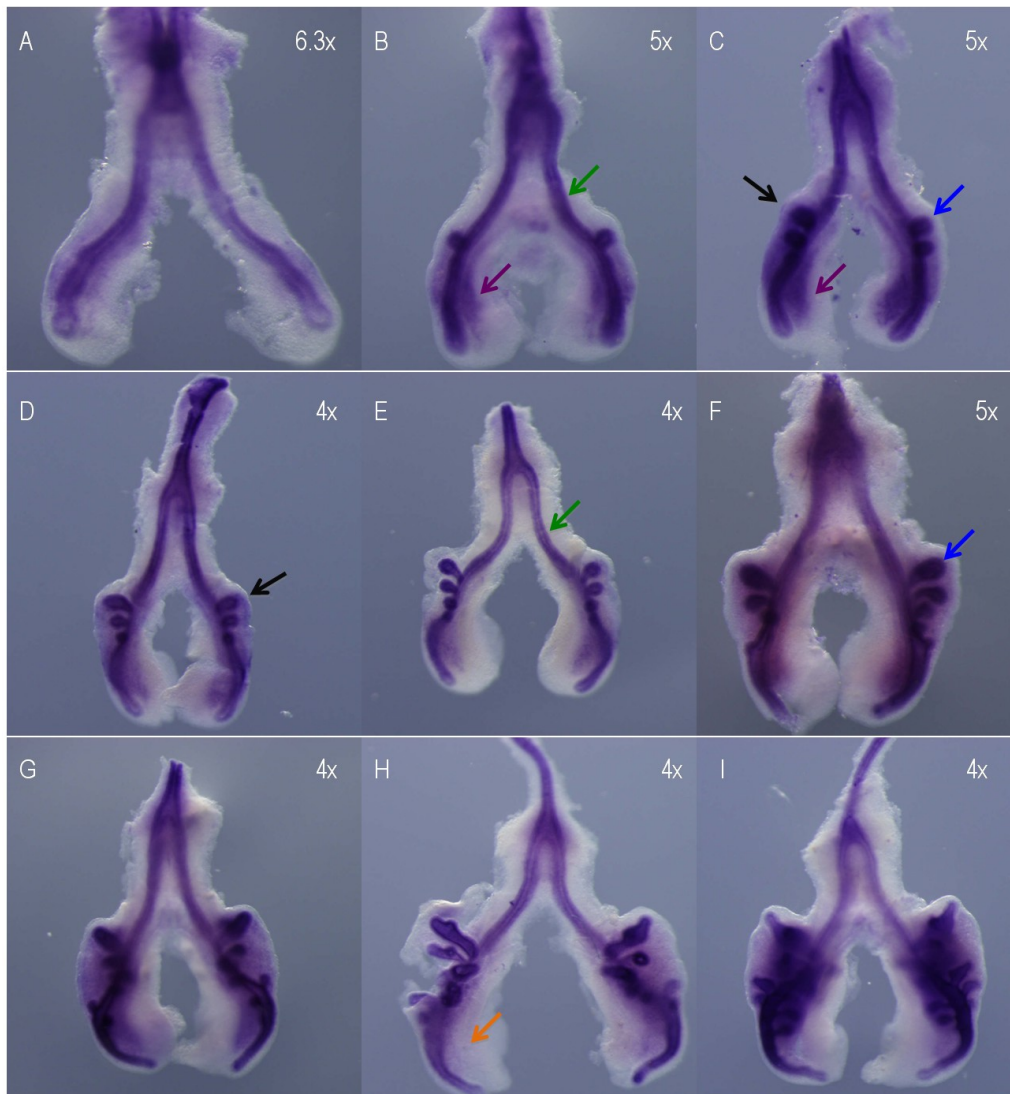
Figure 3.1. *hairy1* expression pattern during chick lung development, visualized by ISH, under precisely the same conditions. Representative examples of *hairy1* expression pattern of stage b0 (**A**), b1 (**B**), b1 (**C**), b3 (**D**), b4 (**E**), b5 (**F**), b6 (**G**), b7 (**H**), b8 (**I**). Green arrows (↑) - epithelium of the main bronchus; blue arrow (↑) - epithelium of secondary bronchi; purple and orange arrows (↑;↑) - medial mesenchyme of the main bronchus; black arrows (↑) - lateral mesenchyme of secondary bronchi.

Figure 3.2. *hairy1* expression pattern of st b2 lung. Positive (**A**, **B**) and negative (**C**, **D**) expression of *hairy1* in the epithelial tip of the main bronchus. White and orange arrows – distal medial mesenchyme of the main bronchus; blue arrows (↑) - distal epithelial bronchial tip; black arrows (↑), lateral mesenchyme.
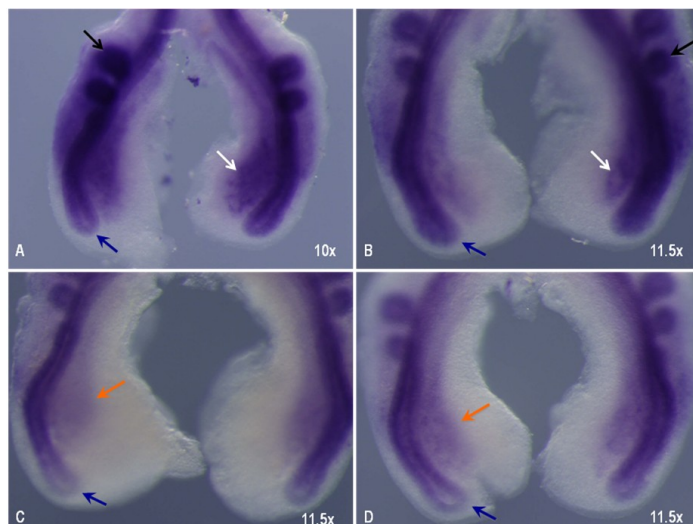
Representative examples of lungs from different stages of development, hybridized with *hairy1,* where processed for histological slide sectioning. Undoubtedly *hairy1* expression is observed not only in the epithelial compartment of the main bronchus (figure 3.3 A, black arrows) but also in the secondary bronchi (figure 3.3 A, C, blue arrow). On the other hand, medial mesenchymal expression is evident (figure 3.3 C, purple arrow); conversely *hairy1* is not present in the most distal mesenchyme (figure 3.3 B, green arrow).
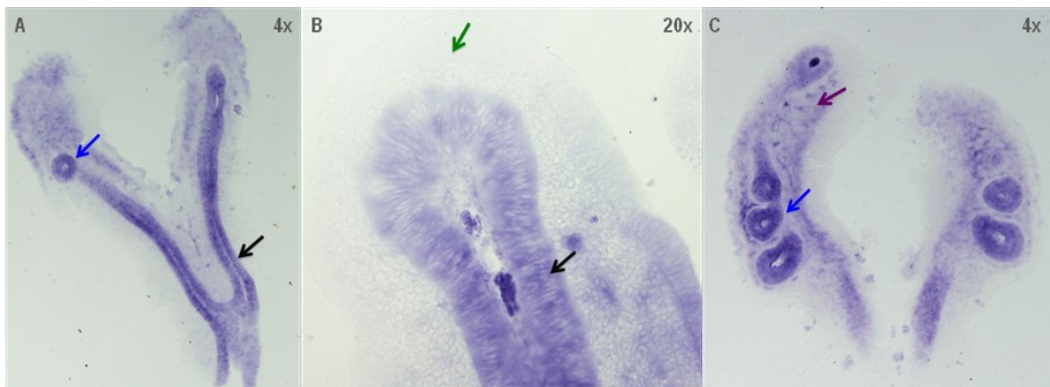

Figure 3.3. Representative examples of st b1 (**A**, **B**) and b3 lungs (**C**) probed with *hairy1*. Blue arrows (↑), secondary bronchi epithelium; black arrows (↑), main bronchus epithelium; green arrow (↑), distal mesenchyme; purple arrow (↑), distal lateral mesenchyme. 25μm Technovit sections.

3.1.2. *HAIRY2* EXPRESSION PATTERN

The *hairy2* expression was evident mainly in the chick pulmonary mesenchyme, throughout all the stages studied (figure 3.4). *hairy2* expression is present in the mesenchyme surrounding the trachea and the trachea bifurcation (figure 3.4 B, tr, trb), and tip of the main bronchus, in the most distal region (figure 3.4 D, E, green arrow) resembling a rostral-caudal wave; on the other hand in the proximal medial and lateral mesenchyme expression is absent (figure 3.4 D, blue arrow; figure 3.4 F, yellow arrow). Moreover, *hairy2* is also expressed in the distal epithelium of the primary and secondary bronchi (figure 3.4 E, purple arrow; figure 3.4 C, F, black arrows, respectively), quite the opposite of the remaining epithelial compartment which lacks its expression (figure 3.4 G, red arrow).

Differences in *hairy2* expression pattern were observed in lungs of the same stage primarily in the most distal mesenchyme, surrounding the main bronchus tip (figure 3.4 C, E). In some cases, the mesenchymal expression at the terminal region of the main bronchus is absent (figure 3.5 B, yellow arrow) and in others shows a strong expression (figure 3.5 A, red arrow). *hairy2* is always present in the medial distal mesenchyme and absent from main bronchus epithelium (figure 3.5 B, white arrow; 3.5 C, green arrows, respectively). The strong mesenchymal expression masks distal lung epithelium and, for this reason, epithelial variations in this region are difficult to detect.
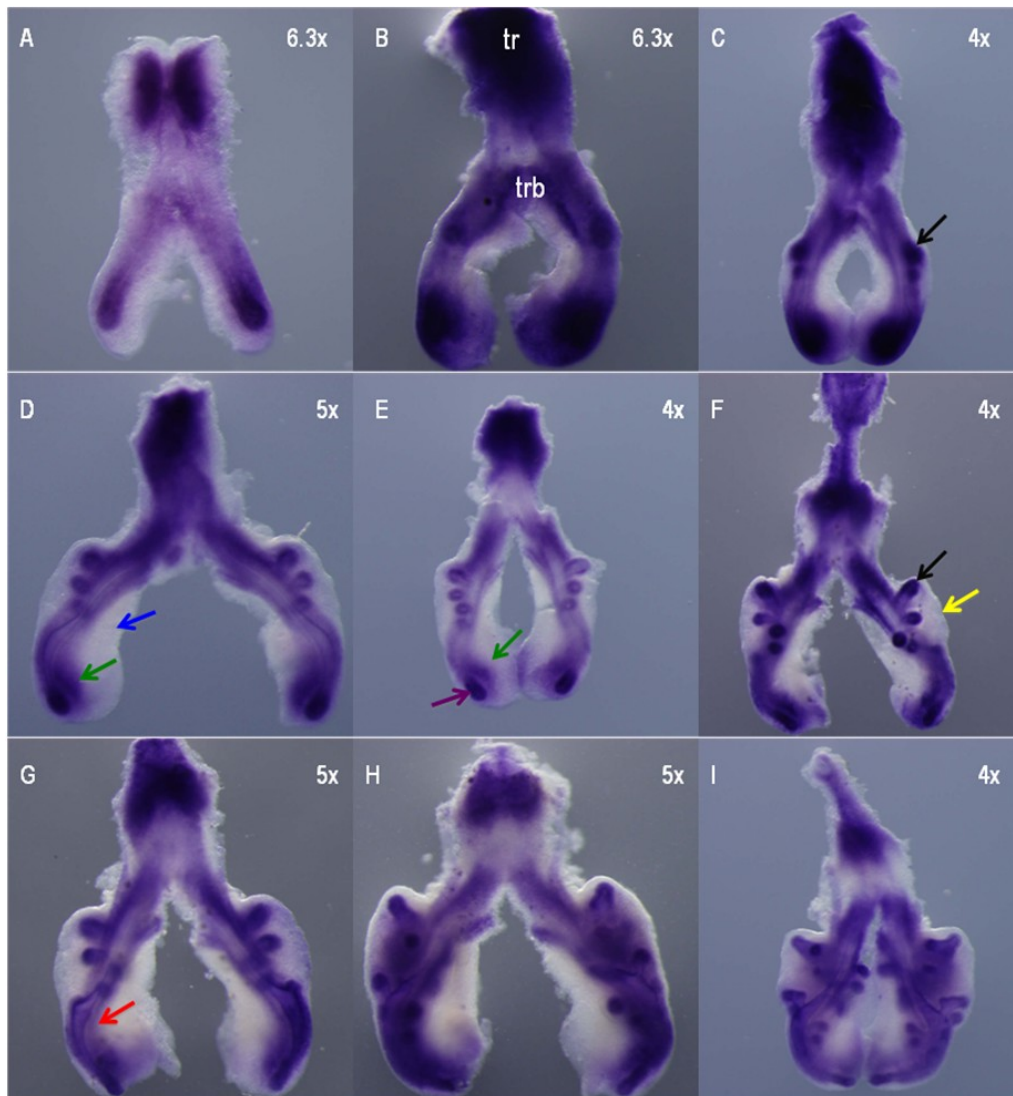
Figure 3.4. *hairy2* expression pattern during chick lung development, visualized by ISH, under precisely the same conditions. Representative examples of *hairy1* expression pattern of stage b0 (**A**), b1 (**B**), b1 (**C**), b3 (**D**), b4 (**E**), b5 (**F**), b6 (**G**), b7 (**H**), b8 (**I**). tr, trachea: trb, tracheal bifurcation; Black arrows (↑) - epithelium of secondary bronchi; blue arrow (↑) - proximal medial mesenchyme of the main bronchus; green arrows (↑)- distal medial mesenchyme of the main bronchus; purple arrow (↑) - distal epithelium of the main bronchus yellow arrow (↑) - lateral mesenchyme of the main bronchus; red arrow (↑) - epithelium of the main bronchus.
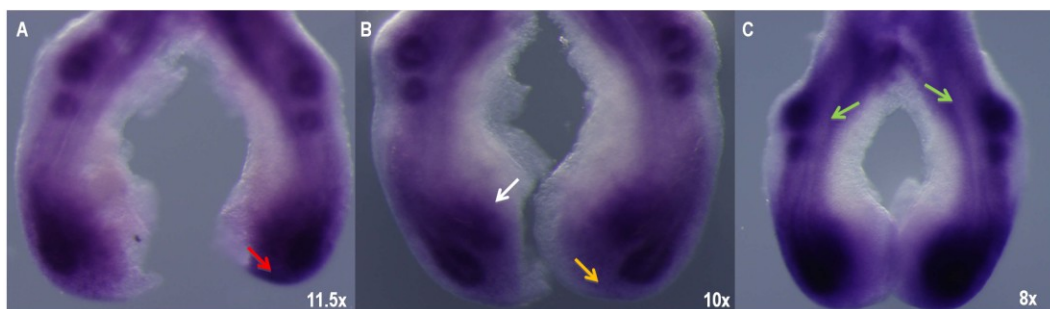

Figure 3.5. *hairy2* expression pattern of st b2 lung (**A**-**C**). Red and yellow arrows (↑;↑) - tip mesenchyme; white arrow - distal medial mesenchyme of the main bronchus; green arrows (↑) - main bronchus epithelium.

Representative examples of lungs from different stages of development, hybridized with *hairy2,* where processed for histological slide sectioning. *hairy2* is absent from the main bronchus epithelium (figure 3.6 A, B, black arrows) except in the most distal region of primary and secondary (figure 3.6 B, C, green arrows). Moreover, expression is also absent in the distal epithelium (figure 3.6 D, orange arrow): this observation could not be described previously since it was masked by the mesenchymal expression. Medial mesenchymal expression is observed in all stages studied (figure 3.6 A, B, C, purple arrow); on the other hand, tip mesenchyme presents presence *versus* lack of expression (figure 3.6 C, yellow arrow; figure 3.6 D, red arrow; respectively).
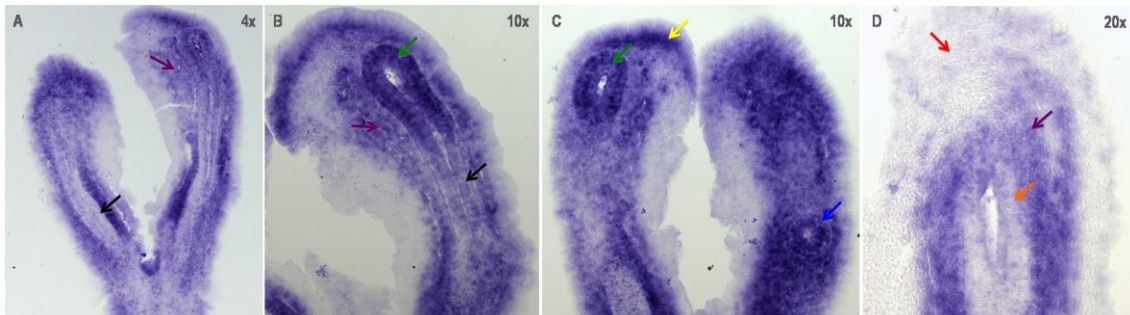

Figure 3.6. Representative examples of st b0 (**A**) and b1 (**B**, **C**, **D**) lungs probed with *hairy2*. Black arrows (↑), main bronchus epithelium; purple arrow (↑), distal medial mesenchyme; green and orange arrows (↑;↑), distal epithelium; yellow and red arrows (↑;↑), tip mesenchyme; blue arrow (↑), secondary bronchi epithelium. 25μm Technovit sections.

3.2. *In vitro* Inhibition Studies

In order to determine a possible role for *hairy1* and *hairy2* in chick lung development, *in vitro* inhibition studies were performed. The consequences of blocking FGF and Notch signaling in early stages of chick lung development were examined. For this purpose, chick lung explant culture system was optimized and explants were processed for ISH for both probes. Lung explants showed the same expression pattern as whole lungs, for both genes, as expected. After this optimization step, lung explants were incubated with SU5402 and DAPT so as to inhibit FGF and Notch signaling, respectively.

3.1.2. FGF Signaling Inhibition

Chick lungs from b1 to b3 stages were processed for explant culture as described in section 2.3.2 and incubated with an FGF receptor antagonist (SU5402), which has been shown to inhibit the tyrosine kinase activity all four FGFRs blocking FGF signaling. The doses used, 25 and 50 μM, were selected according to the literature and lung culture was maintained for 24 hours. In treated explants, *hairy1* and *hairy2* expression is not affected by FGF signaling inhibition (figure 3.7 and 3.8, F, I
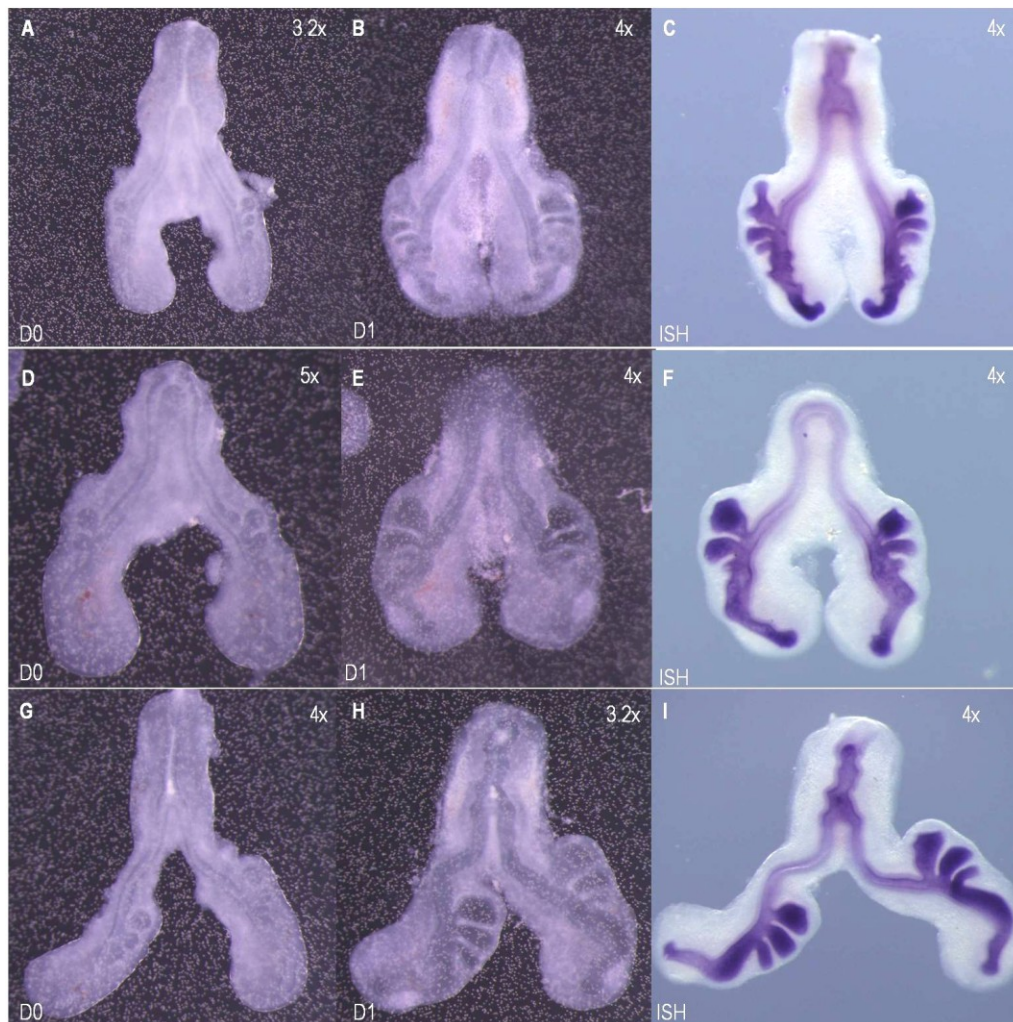
Figure 3.7. *In vitro* FGF inhibition studies. Representative examples of branching morphogenesis of st b3 lungs in the chick lung explant system (**A**-**B**; **D**-**E**; **G**-**H**), and ISH for *hairy1* (**C**, **F**, **I**). D0: 0 h, D1: 24 hours of culture. **A**-**C**, control explants. **D**-**F** and **G**-**I**, 25 and 50 μM SU5402, respectively.

respectively) presenting a pattern similar to control explants (figure 3.7 and 3.8, C, respectively). Moreover, *hairy1* and *hairy2* expression is not affected by increasing doses of inhibitor.

SU5402 treated explants showed atypical lung branching when compared with explants treated only with DMSO (figure 3.7 and 3.8, A), presenting a reduced number of secondary branches (figure 3.7 and 3.8, B). The results of morphometric analysis on fetal lung explants are summarized in figure 3.9. The highest SU5402 dose (50 μM) induced a decrease in total number of peripheral airway buds when compared to control in stage b2 and b3 explants, whereas no statistical differences were found with the lower dose (25 μM). Curiously, no differences were found between the two doses in stage b2 explants (figure 3.9 A), but in stage b3 the differences have statistical significance (figure 3.9B). It was not possible to carry out statistical analysis in b1 lung explants due to the reduced *n* number.
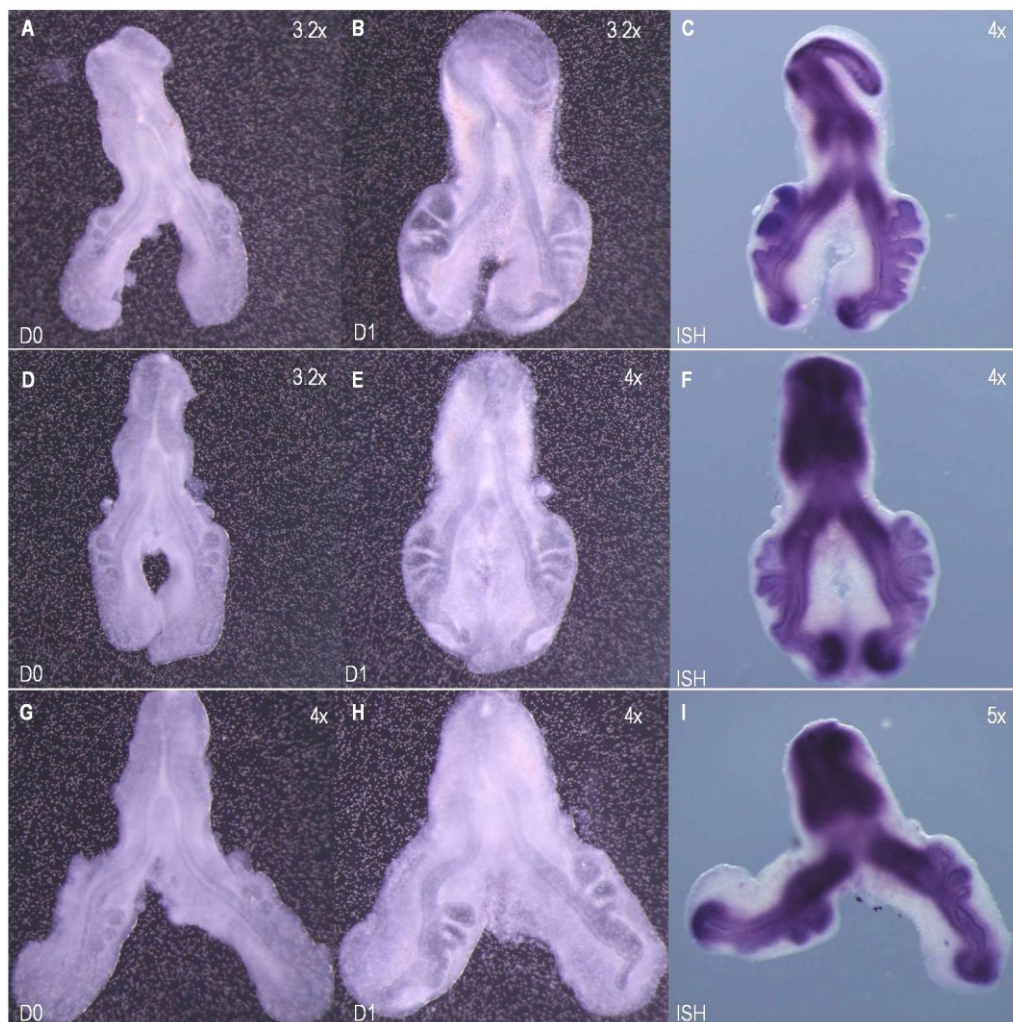
Figure 3.8. *In vitro* FGF inhibition studies. Representative examples of branching morphogenesis of st b3 lungs in the chick lung explant system (**A**-**B**; **D**-**E**; **G**-**H**), and ISH for *hairy2* (**C**, **F**, **I**). D0: 0 h, D1: 24 hours of culture. **A**-**C**, control explants. **D**-**F** and **G**-**I**, 25 and 50 μM SU5402, respectively.

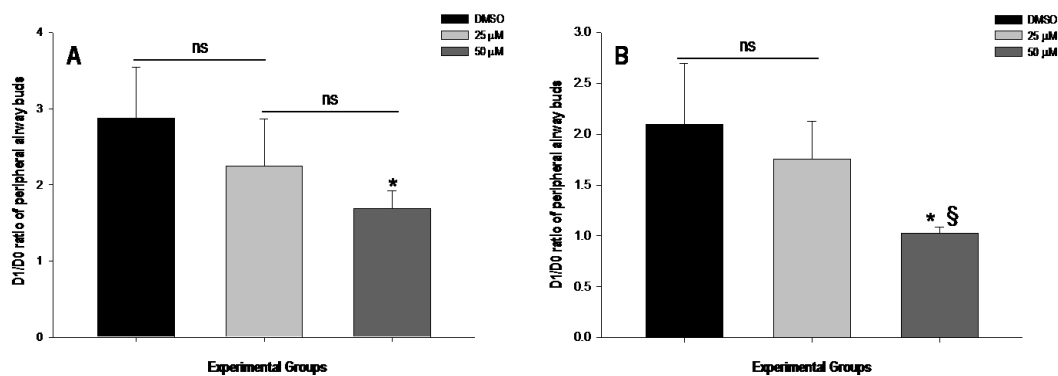**Figure 3.9.** Morphometric analysis of lung explant culture treated with SU5402. Results are expressed as D1/D0 ratio. (**A**) st b2 lung: DMSO (n= 6), 25 μM SU5402 (n= 6), 50 μM SU5402 (n= 4); (**B**) st b3 lung: DMSO (n= 5), 25 μM SU5402 (n= 7), 50 μM SU5402 (n= 7). Data is represented as mean $\pm$ SD. *p < 0.05 *vs* DMSO. § p< 0.05 *vs* 25 μM SU5402. ns, not statistically significant.

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3.1.2. NOTCH SIGNALING INHIBITION

Chick lungs from b1 to b3 stages were processed for explant culture as described in section 2.3.2 and incubated with a γ-secretase inhibitor (DAPT), which efficiently blocks the release of NICD and inhibits Notch signaling. The doses used, 20 and 45 μM, were selected according to the literature and, in this case, lung culture was prolonged for 48 hours.

Lung explants treated with DAPT show abnormal lung growth when compared with control explants treated only with DMSO (figure 3.10; 3.11). DAPT- treated lungs (figure 3.10 E-G, I-L; figure 3.11 E-G, I-L) show not only an increased number of secondary branches but also an anomalous lung structure in the distal tip of the main bronchus when compared with control lungs (figure 3.10 A-C; figure 3.11 A-C).
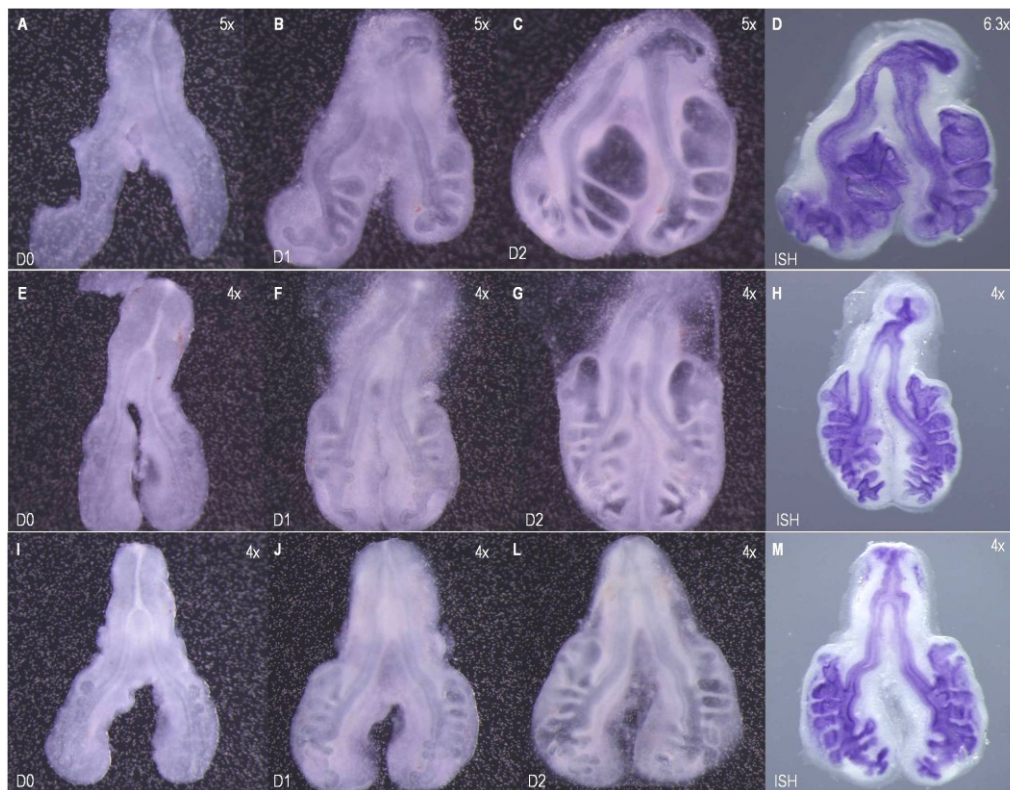

Figure 3.10. *In vitro* Notch inhibition studies. Representative examples of branching morphogenesis of st b3 lungs in the chick lung explant system (**A-C**; **E-G**; **I-L**), and ISH for *hairy1* (**D**, **H**, **M**). D0: 0 h, D1: 24 h, D2: 48h of culture. **A-C**, control explants. **E-G** and **I-L**, 20 and 45 μM DAPT, respectively.

In treated explants, epithelial *hairy1* expression is apparently not affected by Notch signaling inhibition (figure 3.10 H, M) presenting a pattern similar to control explants (figure 3.10 D). It seems that DAPT treated lungs might have a slight decrease in *hairy1* expression levels mainly in the mesenchyme; to confirm this observation additional RT-PCR studies are necessary. Moreover, branching and *hairy1* expression are not affected by increasing doses of inhibitor.

By contrast, *hairy2* expression was dramatically reduced in DAPT-treated lungs (figure 3.11 H, M) when compared with control explants (figure 3.11 D). In these DAPT-treated lungs, *hairy2* expression was selectively decreased in the tracheal area and in the tracheal bifurcation independently of the dose used (figure 3.11 H, M: tr, trb). On the other hand, *hairy2* expression was almost abolished in the mesenchymal and epithelial compartment of the lung, except for the proximal most and anterior most secondary bronchi which is only slightly diminished (figure 3.11 H, M, blue arrow and red arrows, respectively).
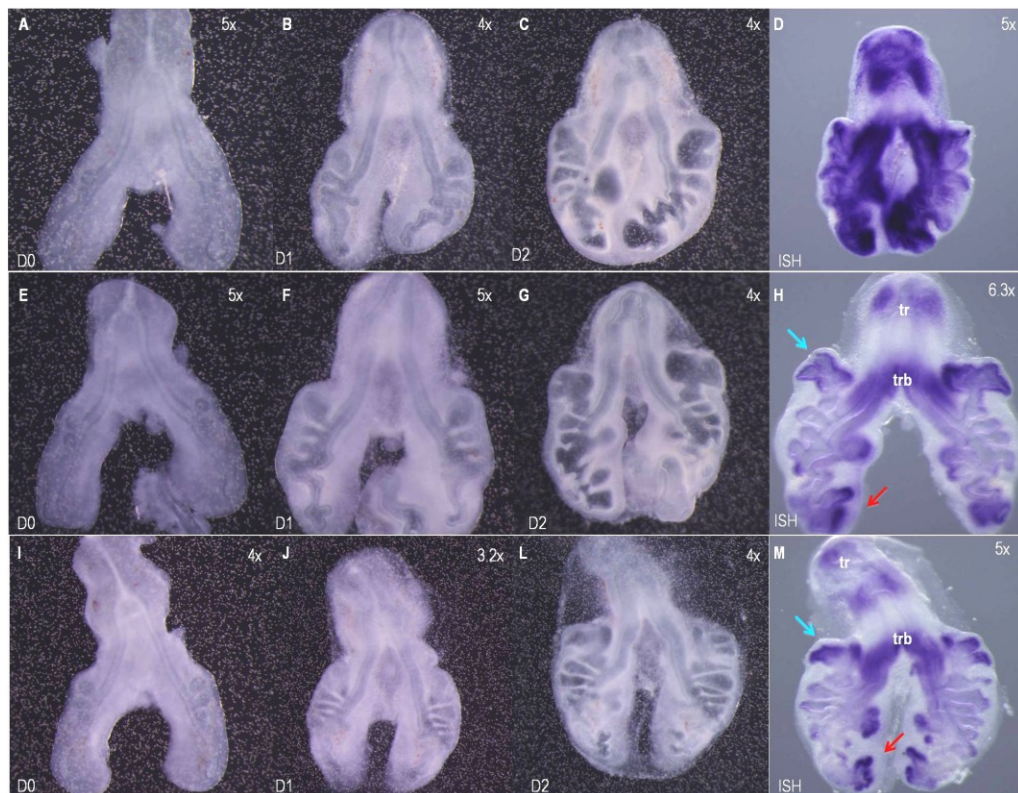

Figure 3.11. *In vitro* Notch inhibition studies. Representative examples of branching morphogenesis of st b3 lungs in the chick lung explant system (**A**-**C**; **E**-**G**; **I**-**L**), and ISH for *hairy2* (**D**, **H**, **M**). D0: 0 h, D1: 24 D2: 48h of culture. **A**-**C**, control explants. **E**-**G** and I-L, 20 and 45 μM DAPT, respectively. tr, trachea; trb tracheal bifurcation. Blue arrow (↑) -proximal secondary bronchi; red arrow (↑) - distal secondary bronchi.

The results of morphometric analysis on fetal lung explants are summarized in figure 3.12. DAPT induced an increase in total number of peripheral airway buds, independently of the dose used, in stage b1 and b3 explants (figure 3.12 A and C, respectively). However, in stage b2 explants only with the lower dose, this increase has statistical significance (figure 3.12 B). Curiously, no differences were found between the two doses in stage b1 and b2 explants, but in b3 lung explants the differences have statistical significance.
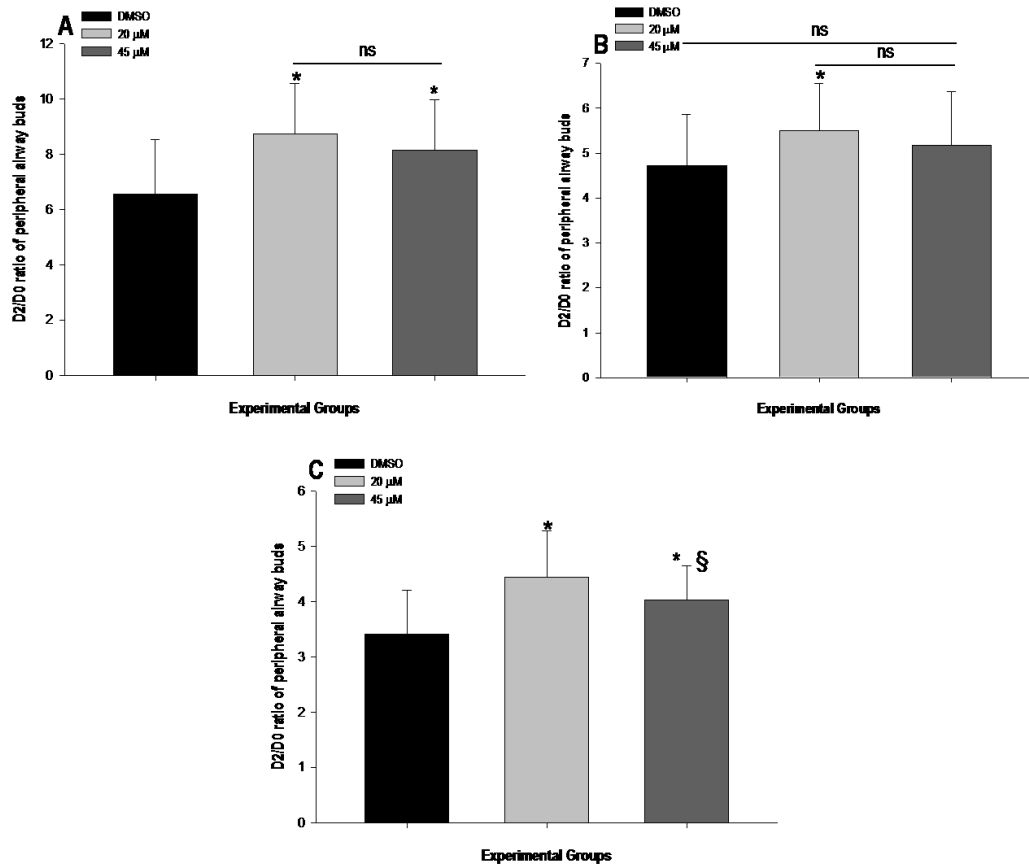
Figure 3.12. Morphometric analysis of lung explant culture treated with DAPT. Results are expressed as D2/D0 ratio. (**A**) st b1 lung: DMSO (n= 19), 20 μM DAPT (n= 24), 45 μM DAPT (n= 25); (**B**) st b2 lung: DMSO (n= 36), 20 μM DAPT (n= 46), 45 μM DAPT (n= 54); (**C**) st b3 lung: DMSO (n= 28), 20 μM DAPT (n= 37), 45 μM DAPT (n= 46). Data is represented as mean ± SD. *p < 0.05 *vs* DMSO. § p< 0.05 *vs* 20 μM DAPT. ns, not statistically significant.

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DISCUSSION

Although much is known about some aspects of lung development, the mechanisms that regulate the spatiotemporal control of this process are not well defined. The identification of a segmentation clock that ensures the correct spatiotemporal periodicity of somite generation, during chick somitogenesis, provided the first molecular evidence of how embryonic cells count time. The underlying mechanisms of this process involve the oscillation of "clock-genes" like *hairy1* and *hairy2*, which are intimately related to the Notch signaling pathway. Inactivation of this pathway results in dramatic segmentation defects and a severe impairment of the periodic expression of the cyclic genes (Dubrulle & Pourquié, 2002).

Given the importance of *hairy1* and *hairy2* in somitogenesis, and since this process share similarities with lung development, the main goal of this work was to describe the expression pattern of both genes during chick lung development. Moreover, taking into consideration that Notch and FGF signaling pathways are involved in the molecular clock mechanisms, the link between *hairy1* and *hairy2,* these pathways and branching morphogenesis was assessed by lung explant culture system.

hairy1 and *hairy2* expression pattern

The present work characterizes, for the first time, *hairy1* and *hairy2* expression pattern in early stages of chick lung development. The expression of *hairy1* is evident in the epithelial region of the main bronchus and secondary bronchi throughout all the stages studied. This general pattern expression in the pulmonary epithelium is, in part, consistent with the one described in the embryonic mouse lung. Sasai *et al.* (1992) has disclosed *Hes1* expression, a *hairy1* homolog in the mouse, in the mouse airway epithelium by *in situ* hybridization. More recently, Ito and coworkers (2000) demonstrated by immunohistochemistry that Hes1 is present in the nuclei of non-neuroendocrine airway epithelial cells in early stages of mouse lung development; the authors also showed, by northern blot analysis that *Hes1* mRNA expression progressively rises in the mouse lung until birth and then remains detectable in adult lung as well. Apparently, during early stages of chick lung development, *hairy1* expression levels remain overall constant, although in order to confirm this observation RT-PCR or northern blot analysis should be performed. Furthermore, *hairy1* is expressed also in chick lung mesenchyme. Collins *et al.* (2004) demonstrated by immunohistochemistry that Hes1 expression was negative in the surrounding lung mesenchyme and vessels (Collins *et al.*, 2004); conversely, recent work by Tsao *et al.* (2008) showed by *in situ* hybridization that E11–E12 mouse lung mesenchyme expresses *Hes1*, where it could potentially play a role in the development of the lung vasculature or other derivatives (smooth muscle or

cartilage), corroborating the results found in the chick model. The differences between the studies could be related to the fact that two different techniques have been used.

hairy2 is expressed in the mesenchyme surrounding the trachea and the trachea bifurcation and in the most distal region of the main bronchus resembling a rostral-caudal wave along the AP axis, and also in the distal epithelium of the primary and secondary bronchi *hairy2* expression pattern does not match completely with its mammalian counterpart, *Hes1*, since in the latter case no tracheal expression is observed. Very limited data is available for other Notch effectors in lung development. *Hes5* has not been detected in whole fetal lung by RT-PCR (Ito *et al.*, 2000). However, recent studies showed that *Hes1*, *Hes5* and *Hey2* were present in mouse airway epithelium predominantly in the distal buds, as it occurs with *hairy2* (Tsao *et al.*, 2008).

Our results suggest that *hairy1* and *hairy2* might have a dynamic expression since differences in the expression pattern of lungs belonging to the same developmental stage were detected. In the distal epithelium of the main bronchus it is possible to observe two different scenarios, either presence or absence of both genes, meaning that their expression is not constitutive. This type of expression had already been described in other developmental events such as somitogenesis (Palmeirim *et al.*, 1997; Jouve *et al.*, 2000) and limb bud outgrowth (Pascoal *et al.*, 2007), and associated with a mechanism responsible for translating temporal into spatial information. In order to confirm these results, additional experiments should be performed. Taking advantage of chick lung symmetry, both lungs will be dissected through the trachea axis and one half will be cultured for different time periods while the other will be fixed immediately; after hybridization of both halves, different expression patterns are expected in sibling lungs confirming the dynamic expression pattern of these genes. Moreover, we expect to find a relationship between the formation of a new secondary branch and the expression cycle, just as it occurs with the formation of a new somite and a limb element. Preliminary results in our lab show some evidence that these genes present a dynamic expression pattern during chick lung development.

Classical studies of lung structure raise the question of how the information required to generate a bronchial tree is biologically encoded. One possibility suggests that the process is not precisely controlled; branching occurs randomly to fill available space. Another is that control is precise but coding is simplified by repeated use of a branching mechanism. Lately, the complete three dimensional branching patterns and lineage of the mouse bronchial tree were described, and it was shown that it is generated using three geometrically distinct local modes of branching coupled in three different sequences throughout the lung. The authors found that Spry2 regulates the site of

initiation and number of branches in specific domains, and controls the proximal–distal register of entire domains (Metzger *et al.,* 2008). *Sprouty* genes (*Spry*) have a role in FGF signaling pathway regulation, functioning as inhibitors of intracellular signaling cascade of this pathway. They are part of a control mechanism of the size and number of lung buds, which may ultimately be responsible for the inhibition of branching. m*Spry2* is localized in the distal tips of the embryonic lung epithelial branches and is down regulated at sites of new bud formation (Sutherland *et al.,* 1996). Abrogation of m*Spry2* expression has a stimulatory effect on murine lung branching morphogenesis and differentiation in organ cultures (Tefft *et al*., 1999). Eventually, other genes might be involved in these control mechanisms, and *hairy* genes seem to be good candidates considering their role in other tightly regulated developmental process.

In vitro inhibition studies

In order to determine a possible role for *hairy1* and *hairy2* in chick lung development, *in vitro* inhibition studies were performed. For this purpose, the consequences of blocking FGF and Notch signaling pathway in early stages of chick lung development were examined.

hairy1 and *hairy2* expression was not affected by FGF signaling inhibition by SU5402, independently of the dose used, and lung explants presented a pattern of expression similar to the control group (figure 3.7, 3.8). These results seem to indicate that *hairy1* and *hairy2* are not downstream targets of this signaling pathway in the embryonic chick lung. In order to confirm these results, additional RT-PCR experiments would be necessary to quantify expression levels of both genes. In contrast, morphological differences were observed between treated *vs.* control explants: SU5402 treated explants presented a reduced number of secondary branches when compared to control explants. These results are in agreement with the role of FGF signaling in branching morphogenesis. In the developing mouse lung, a mechanism involving FGF-10 and FGFR-2 regulates airway branching. FGF-10 locally induces and guides bud outgrowths to proper positions during lung branching morphogenesis (Cardoso, 2001). In *Fgf10*-deficient mice tracheal development is normal but main-stem bronchial development as well as all subsequent pulmonary branching morphogenesis is completely absent (Chuang & McMahon, 2003). Therefore, when FGF signaling is impaired, as it occurs in SU5402 treated explants, FGF10 is not able to induce primary bud formation and for that reason no additional branches emerge.

hairy1 expression was not affected by Notch signaling inhibition by DAPT, independently of the dose used, and lung explants presented a pattern of expression similar to the control group (figure

3.10). These results point out that, in chick lung development, *hairy1* is not a downstream target of Notch signaling pathway. These findings are in agreement with a recent study that states that *Hes1* expression levels remain unaffected by DAPT treatment in mouse lung organ culture (Tsao *et al.,* 2008). On the other hand, *hairy2* expression was dramatically reduced (figure 3.11). In order to quantify these differences, additional RT-PCR analysis need to be performed to assess expression levels. Tsao and coworkers (2008) showed a down-regulation of *Hes5* mRNA, by RT-PCR, in DAPT-treated explants which is in accordance with the results obtained in this work. Despite the sequence homology between *hairy2* and *Hes1*, *hairy2* expression behaves like *Hes5* when Notch signaling is inhibited. Intriguingly, not all the regions showed the same expression abrogation, independently of the dose used. The tracheal area and the tracheal bifurcation showed only a minor decrease in *hairy2* expression, meaning that its expression might not be dependent of Notch signaling pathway in those regions; in the same exact areas, no variation of expression was observed in whole lungs which might indicate that *hairy2* expression is constitutive. These findings seem to indicate that dynamic *hairy2* expression is Notch-dependent whereas constitutive expression is not.

DAPT treated lungs showed not only an increased number of secondary branches but also an anomalous lung structure. These results are consistent with those described by Tsao *et al.* (2008) who described an overall increase in branching in DAPT-treated mouse lungs. Consistent with these findings, morphometric analysis of fetal lung explants (D2/D0) revealed, that DAPT induced an increase in total number of peripheral airway buds, independently of the dose used, in stage b1 and b3 explants.

Notch signaling is not necessary for lung bud initiation; however, Notch is required to maintain a balance of proximal-distal cell fates at early stages of lung development (Tsao *et al.*, 2008). The moderate hypotrophic phenotype of lungs from animals bearing a Hes1 mutation, and the expression of Notch components in the distal lung bud during branching morphogenesis, suggest that Notch may play a role in normal lung growth, especially in Clara cell precursors. Hes1 was detectable in fetal mouse lung in non-endocrine airway epithelial cells that are destined to become Clara cells (Ito *et al.*, 2000). This late-differentiating population of airway epithelial cells is the predominant, non-ciliated airway lining cell in the mouse (Collins *et al.,* 2004). In fetal lung development, Notch signaling appears to be essential for the lung to achieve its normal size. The Hes1−/− mouse exhibits a 20% decrease in lung length, compared to wild type littermates, airway pulmonary neuroendocrine cell hyperplasia and a decreased number of secretory cells (Ito *et al.*, 2000). In the chick lung, however, it was not possible to establish a similar role for *hairy1* since its expression was not abrogated. For this purpose, additional RNAi inhibition studies should be performed.

5

CONCLUSIONS

The present work characterizes, for the first time, *hairy1* and *hairy2* expression pattern in early stages of chick lung development, by *in situ* hybridization. The differences found in *hairy1* and *hairy2* expression pattern of lungs belonging to the same developmental stage suggest a dynamic expression for these genes in lung development, as it has already been described in somitogenesis and limb development.

hairy1 and *hairy2* are not downstream targets of FGF signaling pathway in the chick lung, since their expression was not affected by FGF signaling inhibition.

hairy1 expression is not affected by Notch inhibition, suggesting that this gene is not a downstream target of this signaling pathway. On the other hand *hairy2* expression is either dramatically reduced or maintained by Notch inhibition, suggesting two types of *hairy2* regulation in chick lung. Moreover, Notch signaling inhibition originated not only an increased number of secondary branches but also an anomalous lung structure in the distal tip of the main bronchus, which suggest that this inhibition interferes in the correct process of lung development.

6

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