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A via de sinalização Wnt no desenvolvimento pulmonar de galinha

Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Bioquímica, realizada sob a orientação científica da Doutora Rute Carina Silva Moura, Investigadora da Escola de Ciências da Saúde, da Universidade do Minho e da Doutora Rita Maria Pinho Ferreira, Professora Auxiliar Convidada do Departamento de Química, da Universidade de Aveiro

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Palavras chave Embrião de galinha, desenvolvimento pulmonar, via de sinalização Wnt, genes *hairy*, relógio molecular

Resumo

O estudo da biologia do pulmão é um campo de grande interesse, uma vez que a compreensão dos mecanismos subjacentes ao desenvolvimento pulmonar pode ajudar, não só a tratar/prevenir anomalias congénitas mas também ajudar na reparação, remodelação e regeneração do pulmão adulto. A morfogénese do pulmão fetal depende das interações epiteliais-mesenquimatosas controladas por diversos fatores de crescimento e transcrição, que regulam a proliferação, migração e diferenciação celular. Até à data, a regulação da morfogénese da ramificação pulmonar está bem documentada, no entanto o conhecimento dos mecanismos moleculares envolvidos no controlo espaço-temporal do desenvolvimento continua ainda desconhecido. Por outro lado, o controlo espaço-temporal do desenvolvimento embrionário e dos seus processos de padronização, na somitogénese e desenvolvimento do membro, é bem conhecido.

A via de sinalização Wnt é essencial durante o desenvolvimento dos vertebrados, tendo sido associado a diversos processos tais como a gastrulação, formação do eixo corporal e morfogénese de inúmeros órgãos, nomeadamente o pulmão. As proteínas Wnt atuam através de recetores transmembranares específicos, que ativam vias intracelulares que regulam processos celulares tais como, proliferação, diferenciação e morte. A via de sinalização Wnt é reconhecida como um elemento fundamental no desenvolvimento pulmonar mamífero mas pouco se sabe sobre a sua função no desenvolvimento pulmonar das aves. Além disso, é sabido o seu envolvimento nos mecanismos do relógio molecular descritos até ao momento.

O presente trabalho caracteriza, pela primeira vez, o padrão

de expressão de diversos membros da via de sinalização Wnt, tais como *wnt-2b*, *wnt-5a*, *wnt-7b*, *wnt-9a*, *β-catenin* e *axin2*, em estadios precoces do desenvolvimento pulmonar de galinha. Em geral, a sua expressão é similar à dos seus homólogos nos mamíferos. Estudos de inibição *in vitro* foram realizados de forma a avaliar a importância desta via de sinalização na ramificação pulmonar. Os explantes pulmonares, tratados com um inibidor da via canónica Wnt (FH535), apresentaram um comprometimento na formação de novas ramificações secundárias após 48 horas de cultura, para a maior dose testada. A análise morfométrica da ramificação confirmou a inibição. Quando incubados com as sondas *hairy1* e *hairy2* (dois genes descritos como intervenientes no mecanismo do relógio molecular), os pulmões tratados apresentaram uma redução na sua expressão, indicando um potencial papel da via Wnt como regulador destes genes.

Este estudo demonstra que a via de sinalização Wnt é crucial para uma correta ramificação pulmonar na galinha, e que pode ser um dos reguladores da expressão de genes *hairy* no pulmão.

Keywords Chick embryo, lung development, Wnt signaling pathway, *hairy* genes, molecular clock

Abstract

Lung biology is a field of great interest since the understanding of the mechanisms underlying pulmonary development may help not only to treat/prevent congenital anomalies but also aid adult lung repair, remodeling and regeneration. Morphogenesis of the fetal lung depends on epithelial-mesenchymal interactions that are governed by several growth and transcription factors that regulate cell proliferation, fate, migration and differentiation. So far, much is known about the regulation of lung branching morphogenesis, but the knowledge of the molecular mechanisms involved in the temporal-spatial control of lung development remains unknown. On the other hand, significant advances have been made regarding the comprehension of the temporal-spatial control of embryonic development, and its patterning processes, in somitogenesis and limb development.

Wnt signaling pathway is an essential player during vertebrate's embryonic development that has been associated with several developmental process such as gastrulation, body axis formation, and morphogenesis of numerous organs namely the lung. Wnt proteins act through specific transmembrane receptors, which activate intracellular pathways that regulate cellular processes such as cell proliferation, differentiation and death. Wnt signaling is recognized as a key molecular player in mammalian pulmonary development but little is known about its function in avian lung development. Moreover, it is accepted that it is involved in the molecular clock mechanisms described so far.

The present work characterizes, for the first time, the expression pattern of several Wnt signaling members, such as *wnt-2b*, *wnt-5a*, *wnt-7b*, *wnt-9a*, *β-catenin* and *axin2*, at early stages of

chick lung development. In general, their expression is similar to their mammalian counterparts. *In vitro* inhibition studies were performed in order to evaluate the importance of Wnt signaling pathway in lung branching. Lung explants treated with a Wnt canonical signaling inhibitor (FH535) presented an impairment of secondary branch formation after 48h of culture, in the highest dose tested. Branching analysis confirmed this inhibition. When probed with *hairy1* and *hairy2* genes (that are described as players in the molecular clock mechanism), treated lungs presented a reduction in their expression indicating a potential role of Wnt pathway as regulator of this genes. This study demonstrates that Wnt signaling is crucial for precise chick lung branching and that might be one of the regulators of *hairy* expression in the lung.

Contents

List of Figures xiii

List of Tables xv

Abbreviations xvii

1. Introduction 1

1.1 Embryonic development 3

1.1.1 Chicken embryology 3

1.1.1.1 Chick model 4

1.1.1.2 Stages of development 5

1.1.2 Lung development 5

1.1.2.1 Avian vs. mammalian 5

1.1.2.2 FGF signaling pathway 8

1.2 Wnt signaling pathway 9

1.2.1 Wnt protein family 10

1.2.1.1 Wnt signal transduction 10

1.2.1.2 Wnt canonical pathway 11

1.2.1.3 Wnt non-canonical pathway 13

1.2.2 Wnt in the embryo 14

1.2.3 Wnt signaling and lung development 15

1.2.3.1 Wnt expression pattern 16

1.2.3.2 Wnt role in lung morphogenesis 16

1.3 Segmentation clock 18

1.3.1 Signaling pathways involved in the molecular clock 20

1.3.2 Wnt and the molecular clock 22

1.3.3 *hairy* genes and lung development 23

1.4 Aims 25

2. Materials and methods 27

2.1 Eggs storage and embryo dissection 29

2.2 Lung explants culture 29

2.2.1 *In vitro* inhibition studies 29

2.2.2 Statistical analysis 30

2.3 *In situ* Hybridization 30

2.3.1 RNA probes 30

2.3.1.1 *E. coli* transformation 30
2.3.1.2 DNA extraction 31
2.3.1.3 Probe synthesis 32
2.3.2 *In situ* Hybridization 34
3. Results 37
3.1 Characterization of Wnt signaling members expression pattern, during chick lung development 39
3.1.1 Expression pattern of *wnt-2b*, *wnt-5a*, *wnt-7b* and *wnt-9a* during chick lung development 39
3.1.2 Expression pattern of *β-catenin* and *axin2* during chick lung development 41
3.2 *In vitro* inhibition studies 41
3.2.1 Wnt inhibition and morphometric analysis 42
3.2.2 Wnt inhibition and *hairy* genes 44
4. Discussion 47
4.1 Expression pattern of Wnt signaling members 49
4.2 Wnt signaling inhibition 51
4.3 Wnt signaling pathway in regulation of hairy genes expression 53
5. Conclusion 57
6. References 61
7. Appendices ..... a
7.1 Appendix 1 c
7.2 Appendix 2 i

List of Figures

Figure 1 - Comparison between human and chick embryos. (A) Human embryo at stage 14 (Carnegie stages) [adapted from [1]]; (B) Chick embryo at the Hamburger and Hamilton (HH) stage 26.. 4

Figure 2 – Representation of the avian lung and air-sacs, at day 4 (on the left) **and in the adult** (on the right). [adapted from [2]].. 6

Figure 3 - Development of the foregut into larynx, trachea and primordial lung at the third and fourth weeks of human gestation. (A) Ventral view of the origin of the partition of the foregut into the esophagus and the laryngotracheal groove (week three); (B) ventral view at week four. [adapted from [3]] 7

Figure 4 - Representation of the three different branches of the Wnt signaling pathway. In orange, PCP pathway; in blue Wnt/β-catenin pathway; in green the Wnt/Calcium pathway. [adapted from [4]]... 11

Figure 5 - Representation of the canonical Wnt pathway. (A) In the absence of Wnt, the canonical Wnt pathway is inactivated and β-catenin is degraded by the complex GSK-3/APC/Axin; (B) In the presence of Wnt, a signaling cascade is activated and results in the transcription of several target genes. [Adapted from [5]]. 12

Figure 6 - Wnt ligands expression pattern in early stages of chick lung development. Representative examples of whole mount *in situ* hybridization of stage b1 (A, F, K, P), b2 (B, G, L, Q) and b3 (C, H, M, R) - n=15 for each stage and probe. Lungs were probed with *wnt-2b* (A - E), *wnt-5a* (F - J), *wnt-7b* (K - O) and *wnt-9a* (P - T). Red arrow – medial mesenchyme, green arrow – proximal mesenchymal, orange arrow – secondary buds epithelium, asterisc – epithelial tip of the main bronchus, dagger - proximal epithelium. Magnification: A - C, F - H, K – M and P - R - 5x; D, I, N, S – 4x; E, J, O – 10x and T – 20x. 40

Figure 7 - *β-catenin* and *axin2* expression pattern in early stages of chick lung development. Representative examples of whole mount *in situ* hybridization of stage b1 (A, F), b2 (B, G) and b3 (C, H) - n=15 for each stage and probe. Lungs were probed with *β-catenin* (A - E) and *axin2* (F - J). Orange arrow - secondary buds epithelium, asterisc – epithelial tip of the main bronchus, arrowhead – peri-epithelial mesenchyme, black arrow - peri-epithelial mesenchyme around the secondary bronchi. Magnification: A – C and F – H – 5x; D, I – 4x and E, J – 10x. 41

Figure 8 - *In vitro* Wnt signaling inhibition. Representative examples of stage b2 lung explant culture, at D0:0h (A, D, G) and D2:48h (B, E, H) treated with DMSO (A, B), 20µM (D, E) and 40µM FH535 (G, H) and probed with *axin2* (C, F, I) – n=5 for each stage. Magnification: A, B, D, E, G, H – 4x; C, F, I – 5x. 43

Figure 9 - Branching analysis of lung explants. Branching analysis of stage b1 (n=20 for each condition), b2 (n=30 for each different condition) and b3 (n=20 for each different condition) explants treated with DMSO and FH535 (20µM and 40µM). Results are expressed as D2/D0 ratio. Data is represented as mean ± SE. $p<0.05$ * *vs* DMSO, § *vs* 20µM of FH535............................... 43

Figure 10 - *In vitro* Wnt signaling inhibition. Representative examples of stage b2 lung explant culture, at D0:0h (A, D, G) and D2:48h (B, E, H) treated with DMSO (A, B), 20µM (D, E) and 40µM FH535 (G, H) and probed with *hairy1* (C, F, I) – n=5 for each stage. Magnification: A, B, D, E, G, H – 4x; C, F, I – 5x. 44

Figure 11 - *In vitro* Wnt signaling inhibition. Representative examples of stage b2 lung explant culture, at D0:0h (A, D, G) and D2:48h (B, E, H) treated with DMSO (A, B), 20µM (D, E) and 40µM FH535 (G, H) and probed with *hairy2* (C, F, I) – n=5 for each stage. Magnification: A, B, D, E, G, H – 4x; C, F, I – 5x. 45

List of Tables

Table 1 – Wnt knock-out mice with a respiratory phenotype. ... 18

Table 2 – Molecular oscillators expressed on different species (adapted from [80]) ... 21

Table 3 – Information concerning the probe synthesis. In all cases DNA digestions were performed at 37ºC. (*) sequence confirmed by sequencing. ... 32

Abbreviations

AP	Anterior-Posterior
APC	Adenomatous polyposis Coli
BMP	Bone Morphogenetic Protein
CamKII	Calmodulin-dependent protein kinase II
CKI	Casein Kinase I
Dact	Dapper Homolog
Dkk	Dickkopf
Dsh	Dishevelled
DMSO	Dimethyl Sufoxide
DV	Dorso-Ventral
E	Embryonic Day
FGF	Fibroblast Growth Factor
FN	Fibronectin
FZD	Frizzled
β-Gal	β-Galactosidase
GRIP	Glucocorticoid Receptor Interacting Protein
GSK-3	Glycogen Synthase Kinase-3
Hh	Hedgehog
HH	Hamburger and Hamilton
Int-1	*Integration*-1
***IS*H**	*In Situ* Hybridization
LB	Luria Broth

Lef	Lymphoid enhancer-binding factor
LRP	Low-density lipoprotein (LDL) receptor-related protein
Lfng	Lunatic fringe
LR	Left-Right
NICD	Notch Intercellular Domain
Nkd	Naked
PCP	Planar-Cell Polarity
PKC	Protein Kinase C
PLC	Phospholipase C
PSM	Presomitic Mesoderm
RCAS	Replication-Competent Avian Retroviroses
Shh	Sonic Hedgehog
SOC	Super Optimal Broth with Catabolite Repression
Tcf	T-cell transcription Factor
TGF-β	Transforming Growth Factor-β
Wnt	Derived from Drosophila *Wingless* and the mouse *Int*

1. Introduction

1.1 Embryonic development

Embryonic development has always been a subject of interest for both the scientific and medical communities. Embryological studies are extremely important because serious birth defects may occur during development due to an alteration of a genetic event producing malformations that depending on the severity and the location may either be only unaesthetic or potentially lethal. It is important to understand the cause of these abnormalities in order to try to prevent and to treat them.[3]

The developmental process starts when a secondary oocyte is fertilized by a sperm. The stages between fertilization and birth are called *embryogenesis* and this period varies depending on the specie. In humans the gestation period takes around 9 months while, for example, in the chicken it takes about 21 days (of incubation).[1, 2]

At the beginning of the embryogenesis, cells migrate through the primitive streak, which starts as a posterior thickening of the epiblast, in order to lie in the appropriate position and ready to start the differentiation process. The migration of the cells at different rates determines their distinct fates: cells migrating earlier will be positioned more anteriorly than cells migrating later in development. This process is coordinated by the Hensen's node, an embryonic organizer. As a consequence of cell migration into a more anterior side of the embryo, a maturation gradient is created where anterior cells are more mature than posterior cells. This phenomenon is called *gastrulation* and is a key morphogenetic process that occurs in vertebrates, like chicken and mammals. This mass migration of cells defines all the embryo axes, namely, anterior-posterior (AP), dorsal-ventral (DV) and left-right (LR).[2, 6] Gastrulation results in the deposition of three main layers, the **ectoderm**, **mesoderm** and **endoderm**, which in turn will originate several different tissues and organs. The ectoderm originates the nervous system and the skin; the mesoderm will give rise to the urogenital system, the circulatory system and the somites, which are the precursors of the muscles, bones and cartilage; from the endoderm originates the lining of the gut, the liver and pancreas, and the respiratory system.

1.1.1 Chicken embryology

All vertebrate embryos (mammalians, birds, fishes, among others) share a similar and complex morphological structure, even though their adult forms are quite different. In

1828, when comparing chick embryos with other vertebrates, von Baer discovered that their general features are very alike at very early developmental stages, as seen in Figure 1. It's only in the later stages of development that specific features rise according to their class, order and specie.[3]

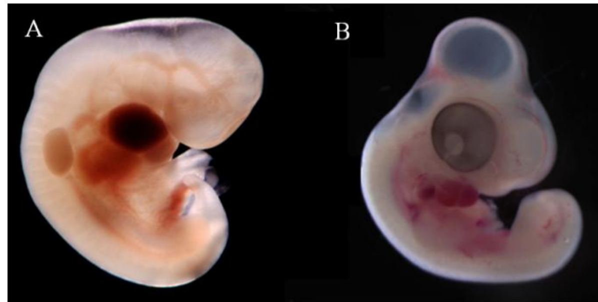

Figure 1 - Comparison between human and chick embryos. (A) Human embryo at stage 14 (Carnegie stages) [adapted from [1]]; (B) Chick embryo at the Hamburger and Hamilton (HH) stage 26.

Due to its similarity to the human embryo, the chick embryo is one of the best models for *in vivo* studies and, for this reason, investigators have dedicated their time to the comprehension of chick embryonic development.

1.1.1.1 Chick model

In order to be used in the laboratory, chicken eggs (*Gallus gallus variant Domesticus*) can be obtained from a commercial source and stored at 16ºC (for a maximum period of 7 days), prior to incubation at 37ºC, in a humid atmosphere (required to initiate the developmental process). When the egg is laid, the avian embryo contains a two-layered blastoderm, the upper (epiblast) and the lower one, throughout the area opaca and in the posterior part of the area pellucida. This blastoderm lies on the surface of the yolk, which is the main nutrient provider to the embryo. It's from the area pellucida that the main embryonic tissues rises, whereas the lateral borders of the area pellucida, along with the entire area opaca, forms the extra-embryonic tissues.[2] 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 (Figure 1). In the remaining time before hatching, the embryo grows in size, the internal organs fully 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.

For all its characteristics, the chick embryo is a longtime favorite in the study of vertebrate embryonic development. Furthermore, its accessibility, the fact that embryogenesis can be directly observed, since embryos are semi-transparent, and the possibility of surgical, pharmacological and genetic modifications make it an excellent study model.

1.1.1.2 Stages of development

In 1951, Hamburger and Hamilton described chick embryo's developmental stages according to their external characteristics [covering the entire incubation period (21 days)].[7] This illustration comprehends 46 stages of chick development starting from laying of the egg and ending with a newly hatched chick. Since chick embryos are one of the main sources of information when it comes to the study of not only embryonic development but also cancer and virus research,[8, 9] this developmental table has been a great help because its detailed information allowed standardization between researchers working with this animal model.

More recently, an extensive database of chicken embryo gene expression was created, gathering whole mount *in situ* hybridization (*IS*H) information and the corresponding metadata for genes expressed in the chicken embryos. This tool is extremely valuable for gene expression studies.[10]

1.1.2 Lung development

1.1.2.1 Avian vs. mammalian

Avian and mammalian lungs are quite different in their adult state, mainly due to the high metabolic rate of birds, which requires a special arrangement and architecture of the respiratory system. In the avian case, the lung air-sacs system presents an extremely complex structure without alveoli that resembles a sponge and allows an exceptional respiratory efficiency, letting the air to pass quickly through the lungs. In the mammalian case, the bronchial system exhibits interacting bifurcations that end in blind-ended conducts, originating the so-called "respiratory tree".[2, 3, 11, 12] With this structure, mammalian lungs have a large surface area allowing greater gas exchanges with the exterior.

During avian development, the larynx and the trachea originate from the gut and, at day 3, two primordial lungs arise from this laryngotracheal groove by tracheal bifurcation. These structures are built by a thin layer of endoderm and a thick covering of mesoderm. The two buds will grow in a posterior way forming the air passages and air-sacs, establishing a series of closed circular buds arising from the main airways branches. Each main bronchus may be further subdivided into two regions: the mesobronchus, which will become partly enclosed in the lung, and the extra-pulmonary bronchus that remains outside the lung (Figure 2). As it happens in the mammalian case, lung vascular patterning occurs in coordination with airway development. The pulmonary vasculature forms in a process named *vasculogenesis* and is guided by the budding process. The avian lungs are composed by a looping anastomotic network of air and vascular surfaces, the parabronchi (the gas exchange unit). This complex architecture produces a remarkably efficient respiratory system.[2, 13] Besides these transformation prior to the first contact with the world, considerable development takes place after hatching, since air-sacs develop their own bronchial tubes after hatching, and their dilation leads to the penetration of other regions of the body.[2]

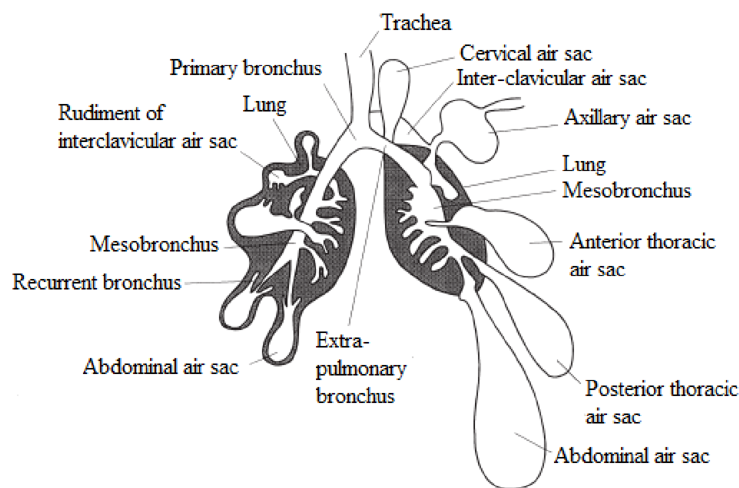

Figure 2 – Representation of the avian lung and air-sacs, at day 4 (on the left) **and in the adult** (on the right). [adapted from [2]]

During mammalian embryogenesis the ventral foregut endoderm differentiates into various epithelial cell types that contour the inner surface of the trachea and two primary buds, the primordial lung (Figure 3). The mesenchyme that constitutes the lungs is derived from the lateral plate mesoderm. This tissue is the main constitute of several components of the respiratory system like, the connective tissue, endothelial cell precursors, the smooth

muscle (that surrounds the airways and the blood vessels), the pleura (fine layer, of squamous mesothelial cells, that covers the lungs) and the cartilage of the trachea. The two main bronchi of the primordial lung branch successively into other finer buds and into even finer branches, the bronchioles, which are surrounded by parabronchial smooth muscle, originating the common respiratory tree. The bronchioles end in highly specialized structures named alveoli, where the gas exchanges occur. Besides this complex branch network of conducting airways, the mammalian lung is also constituted by a blood-vessels network that runs parallel to the bronchioles. It is the tight connection between the airways and the blood vessels that allow an effective gas exchange in these living organisms.[3, 13, 14]

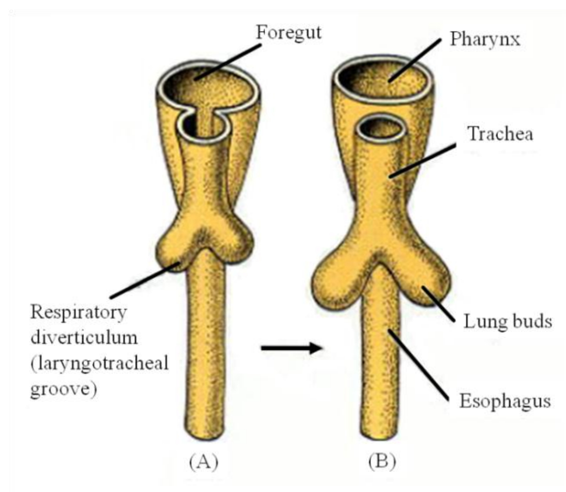

Figure 3 - Development of the foregut into larynx, trachea and primordial lung at the third and fourth weeks of human gestation. (A) Ventral view of the origin of the partition of the foregut into the esophagus and the laryngotracheal groove (week three); (B) ventral view at week four. [adapted from [3]]

A common feature to both avian and mammalian respiratory system is that, in order to assure the correct lung growth, there is an intricate crosstalk between the epithelium and the mesenchyme of the lung. It is this important, and well known, interaction that is the main impelling factor of pulmonary branching – sprouting of new pulmonary tubes from preexisting ones.[11, 15] *In vitro* studies showed that, when grafted adjacent to tracheal endoderm stripped of mesenchyme, distal mesenchyme induces ectopic budding but when the tracheal endoderm is grafted near the distal epithelium it inhibits the branching process.[16, 17] These experiments demonstrate the importance of the interaction between these two tissues in the lung's branching process.

The lung, the mammary gland and the kidney are only a few of the many organs where this elaborated process occurs, being tightly controlled by the interaction between several signaling pathways.[12, 18] In this sense, there have been a great number of studies focusing on the comprehension of the interaction between the major pathways that coordinate lung morphogenesis, namely FGF (Fibroblast Growth Factor), Notch, Shh (Sonic Hedgehog), Wnt, among others, using the mouse[19] and the chicken[18, 20] as models. These studies allowed a better understanding of the molecular mediators implicated in the reciprocal relation between epithelium and mesenchyme.

While the morphological characteristics of the adult respiratory system is well known,[20, 21] there isn't much information about the spatial-temporal dynamics of the mechanisms that ensure the maintenance of this complex and extraordinary respiratory system. It is possible that the positional information in the mesenchyme is sufficient to generate a fixed number of branches, at least until a certain number of branches, in a specific spatially manner and that the process subsequently becomes random instead of being a completely predetermined process. At this point, it could be determined by physiological conditions such as local oxygen and nutrient concentration. Consistent with this notion, individual variations in later branching patterns have been observed in humans.[11] Still, it is known that the early branching process does not vary between individuals, indicating that the early branching patterns are likely to be regulated by a hard-wired genetic program. Metzger and colleagues believe that the bronchial tree is composed by a complex and stereotyped scheme of branching modes, such as domain branching, planar bifurcation and orthogonal bifurcation that are genetically encoded. In their study, they propose that *Spry*-2 (a member of the Sprouty family) might regulate the site of initiation and number of branches in specific domains. They also suggest that all domains are controlled by a series of discrete patterning and morphogenesis events, which will vary according to the domain.[22]

1.1.2.2 FGF signaling pathway

FGF signaling pathway is involved in several processes like proliferation, survival, migration and differentiation, and is absolutely necessary for proper lung development. So far, 6 FGF ligands have been reported in the developing lung, such as FGF-1, 2, 7, 9, 10 and 18.[23] Through knock-out mice studies, the importance of FGF-10 in the respiratory

tract development was proven because these mice were incapable of developing lung buds and died soon after birth.[24] FGF-10 is considered a potential proliferative factor in lung development that stimulates distal epithelial growth.[25] A recent study established a connection between FGF signaling and chick lung development. This work showed that FGF receptors are essential for the epithelial-mesenchymal interactions that determine epithelial branching and mesenchymal growth in the chick lung. When FGF signaling was inhibited in this model no new buds were formed, as occurs in the mammalian lung.[18]

Several factors may lead to a compromised respiratory system, for example, premature birth, intrauterine infections or genetic mutations, which may disrupt the correct lung maturation. These factors may interfere with the required stimuli for lung development, resulting in deficient lungs in the newborn and, while an adult lung may undergo reparation, the pre-natal lung cannot fight these aggressions, resulting in disabilities like oesophageal atresia, tracheo-oesophageal fistula and life-long diseases, like chronic obstructive pulmonary disease.[26, 27]

1.2 Wnt signaling pathway

All developmental processes are ultimately controlled by a cooperative action between different signal transduction pathways. Among all the different pathways, Wnt signaling appears to be indispensable for orchestrating a complex cell behavior that occurs during development. Wnt signaling has been associated with cell proliferation, differentiation,[28] migration,[29] adhesion, survival and death. Moreover, it has been shown to be involved in a variety of early embryonic events such as gastrulation,[29, 30] somite patterning[31] and body axis formation,[32] and also in the morphogenesis of numerous organs, namely lungs[33] and kidneys.[34] Furthermore, it has been implicated in limb,[35, 36] nervous system,[37] craniofacial[38] development and also in the cardiovascular system.[39]

In the adult, Wnt controls the maintenance of the self-renewing tissue, for instance bone marrow, the gut and the skin.[40] An aberrant Wnt signaling pathway may result in serious malformations, including not only cancers but also osteoporosis[41] and neurodegenerative disorders,[42] which reveals the importance/relevance of this pathway.

1.2.1 Wnt protein family

Wnt was first identified, by Nusse,[43] as a proto-oncogene in mammary tumors that was activated by integration of the mouse mammary virus and, since then, it has been linked with many other tumors due to its deregulation in these cases.[44, 45, 46, 47]

The Wnt proteins owe its name to the *Drosophila* ***Wingless*** and the mouse ***Int**-1* (*Integration*-1) genes, and belong to a family of 19, cysteine rich, glycosylated and lipidated secreted proteins.[48, 49] So far, a vast number of target genes of Wnt signaling pathways were described in human, mouse, chicken and zebrafish.[39, 50, 51, 52] While in the human and mouse embryos, 19 proteins are encoded, in the chick embryo only 18 Wnt proteins exist. The chicken genome does not appear to have Wnt-2, 4b, 7c, or 10b.[38, 53]

Wnt proteins activate their intracellular pathways through the Frizzled (FZD) receptors, which are seven pass transmembrane receptors, like the smoothened receptors involved in transduction of the Hedgehog (Hh) signaling pathway. This family of Frizzled proteins contains a cysteine-rich N-terminal, which is responsible for ligand binding.

1.2.1.1 Wnt signal transduction

The Wnt family signals through an autocrine or paracrine fashion and is a high intricate pathway. The complexity of this pathway is due to the fact that both the receptors and ligands involved in the Wnt signal transduction belong to multi-gene families, allowing for a dazzling number of possible ligand-receptor interactions. The best known of these interactions results in the activation of β-catenin/Tcf transcriptional complexes,[54] leading to a variety of intracellular responses.

Depending of Wnt protein, the activation of the receptors may trigger several different responses, like the **Wnt/β-catenin pathway** (also known as the Wnt canonical pathway), the **planar cell polarity (PCP)** and **Calcium fluxes** (the non-canonical pathways) (Figure 4). The division in **canonical** or **non-canonical** pathway is due to the ability to induce, or not, an ectopic axis in *Xenopus* embryos respectively, and to cause the morphological transformation of mouse C57MG mammary cells.[54]

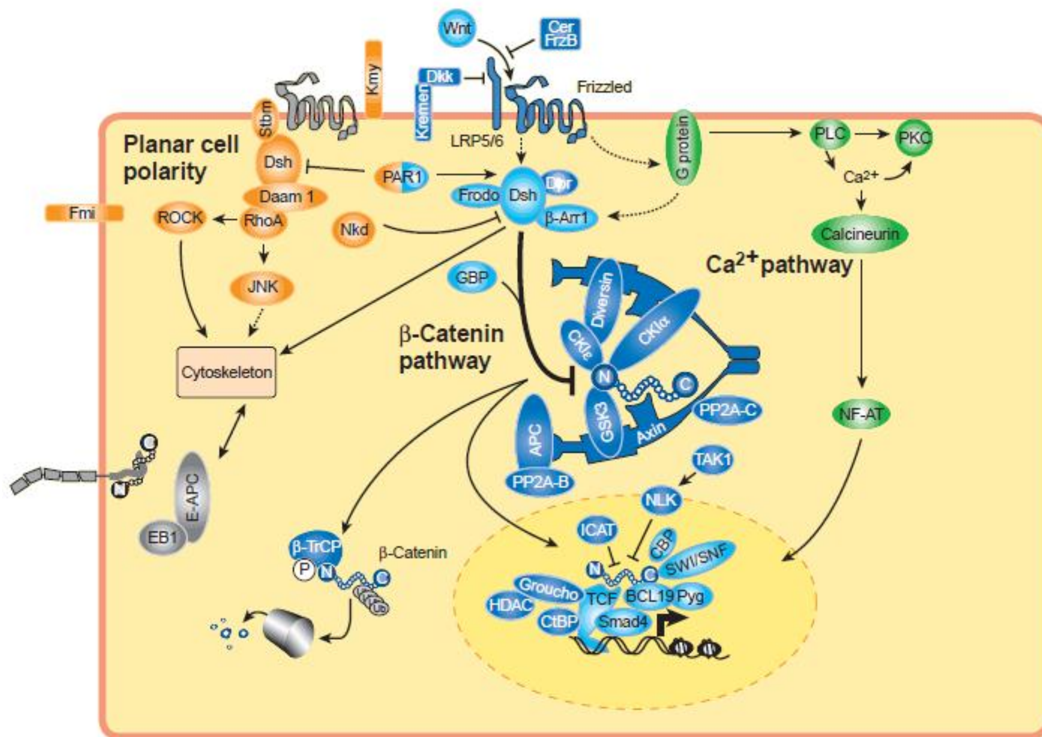

Figure 4 - Representation of the three different branches of the Wnt signaling pathway. In orange, PCP pathway; in blue Wnt/β-catenin pathway; in green the Wnt/Calcium pathway. [adapted from [4]]

1.2.1.2 Wnt canonical pathway

In the canonical pathway, Wnt binds to the FZD receptor and its co-receptors, **l**ow-density lipoprotein (LDL) **r**eceptor-related **p**rotein (LRP), LRP5 and LRP6, increasing both the cytoplasmic and, consequently, nuclear accumulation of β-catenin, a transcriptional regulator.

In the absence of Wnt proteins, β-catenin binds to scaffold proteins, such as Axin and Adenomatous Polyposis Coli (APC), and is phosphorylated at four N-terminal residues by Casein Kinase I (CKI) and Glycogen Synthase Kinase-3 (GSK-3). After this process, phosphorylated β-catenin becomes a target for ubiquitination and continuous proteosome-mediated degradation,[5] which is responsible for low levels of cytoplasmic β-catenin (Figure 5A).

As Figure 5B shows, when Wnt is present, the FZD receptors transduce a signal to several intracellular proteins, such as Dishevelled (Dsh), GSK-3, Axin, APC and β-catenin. Upon ligand binding, FZD and LRP 5/6 are activated and the co-receptor is phosphorylated by GSK-3 and CKγ in its cytoplasmic region. This phosphorylation is responsible for the recruitment of Dsh and Axin, inhibiting β-catenin's phosphorylation and allowing its binding to transcription factors, like T-cell transcription Factor/Lymphoid enhancer-binding factor (Tcf/Lef), that act as activators of target genes,[28] such as *c-Myc* and *cyclin D1* (potent cell proliferation activators). In the absence of β-catenin, Tcf forms a constitutive repressor complex with Groucho.[5, 54, 55] It is the orchestrated interplay between the activation and inhibition of this pathway that defines various patterning processes.[37, 56, 57]

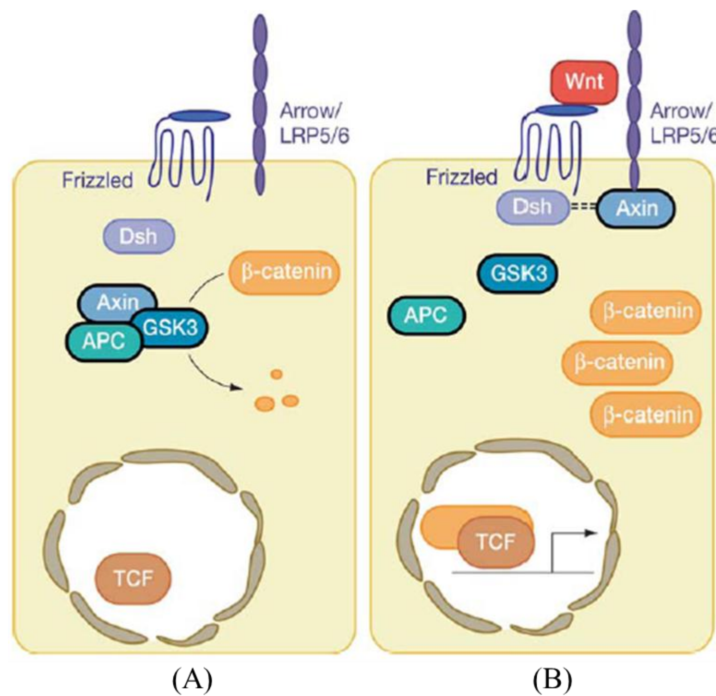

Figure 5 - Representation of the canonical Wnt pathway. (A) In the absence of Wnt, the canonical Wnt pathway is inactivated and β-catenin is degraded by the complex GSK-3/APC/Axin; (B) In the presence of Wnt, a signaling cascade is activated and results in the transcription of several target genes. [Adapted from [5]].

Wnt signaling doesn't only target effective proteins like β-catenin, but also molecules that can act as inhibitors, such is the case of Kramer, a single transmembrane-receptor and Dikkopf (Dkk), which is a soluble secreted protein. Together, these targets form a complex capable of preventing the co-receptor LRP from engaging the FZD

receptor, by promoting its endocytosis. Other Wnt signaling pathway inhibitors include Naked (Nkd), that involves the blocking of the Dsh complex, which in turn is responsible for stabilizing β-catenin through the hampering of the destruction complex, and the Frizzled-related protein (FrzB) that prevents the binding between Wnt proteins and the FZD receptor.[4]

Wnt canonical pathway was already related with other major pathways such as FGF, Shh and TGF-β (Transforming Growth Factor-β) during development.[58, 59, 60] When using a *Lac*Z transgene, behind the multiple Tcf/Lef complex binding site, a minimal promoter and β-Galactosidase (β-*Gal*) staining, Mohamed *et al* demonstrated that shortly before the onset of gastrulation and primitive streak formation, Wnt canonical signaling pathway was already activated.[56]

1.2.1.3 Wnt non-canonical pathway

Non-canonical pathway also comprises the activation of FZD receptors, but instead of cascading through the β-catenin pathway, it activates other intracellular pathways like planar cell polarity and the Wnt/Ca^{2+}.[61]

In the PCP pathway, Wnt activates Jun-N-terminal Kinase (JNK), leading to the asymmetric cytoskeletal organization and polarization of cell morphology within the plan of epithelial sheets.[61] This particular branch of Wnt non-canonical pathway branches at the level of Dsh and involves downstream components like the small guanosine triphosphatase Rho and a kinase cascade (Figure 4). PCP pathway has proven to have an important role in eye and wing development as well as in gastrulation movements, in both zebrafish and *Xenopus*.[52, 62]

Finally, in the Wnt/Ca^{2+} pathway, Wnt activation leads to the release of intracellular Calcium, possibly through mediators like G-proteins. During this pathway, there is activation of several enzymes, like Phospholipase C (PLC), Protein Kinase C (PKC). Besides activating PKC and PLC, Calcium also activates the Calcium/Calmodulin-dependent Protein Kinase II (CamKII) (Figure 4). This pathway has been linked to the ventralization of *Xenopus* and the regulation of convergent extension movements.[51]

Some theories favor that PCP and Wnt/Ca^{2+} pathways are overlapping because in studies conducted in *Xenopus* and zebrafish embryos, both Wnt/Ca^{2+} and the vertebrate

orthologues of the *Drosophila* PCP pathway are responsible for the convergent extension movements during gastrulation.[51]

Some investigators argue that rather than Wnt proteins being shared between pathways, it is the total net balance of signals that ultimately determine the response of the receiving cell.[54]

1.2.2 Wnt in the embryo

Members of *Wnt* family have distinct expression patterns in the embryo and adult organisms, and are critical for regulation of cell proliferation, establishment of tissue polarity, stem cell maintenance, differentiation and thereby organogenesis. A genetic screen showed that several of the Wnt signaling components were conserved across species and a significant repertoire of *Wnt* genes was already expressed in *Cnidarians*, an ancient metazoan phylum, which indicates that these genes diversity arose very soon in evolution and remain crucial for the correct development of multicellular organisms.[54]

This particular pathway was also linked to the neuroepithelial cells division, in *Drosophila*'s nervous system, where neuroepithelial cells divide symmetrically along the planar axis.[61]

Wnt/β-catenin pathway is probably the most studied branch of Wnt signaling and, in the embryo, β-catenin accumulation on the dorsal-anterior side is one of the signals of axis formation and precedes gastrulation. Besides its importance in species like zebrafish, *Xenopus* and *Drosophila*, β-catenin was also proven important in the mouse embryo for AP patterning during gastrulation: a posterior to anterior gradient of Wnt signaling occurs and while Wnts are being highly expressed posteriorly, Wnt inhibitors are more highly expressed anteriorly. Huelsken and Burchmeier showed that in mice lacking *β-catenin* gene, no mesoderm nor head structures were formed because *Cerebrus-like* and *Lim1* (anterior visceral endoderm markers) expressing cells were not reoriented into the anterior side.[61, 63] β-catenin is also implicated in the control of stem cell specification in the skin by cooperating with Lef-1 and Tcf-3 in order to control stem cells differentiation into the follicular lineage. But, in this case, β-catenin may not be enough to induce follicular differentiation and other mesenchymal signals may be needed.[28, 61]

Wnt-3 appears to be important in the formation of the primitive streak and anterior neuroectoderm but not in AP axis formation. Mouse embryos missing *Wnt-3a* present a

severe impairment in hippocampus development due to a lack of proliferative expansion in the caudal-medial cerebral cortex. But, the mouse embryos with a homozygotic condition for the dominant-negative *Lef-1* mutant allele, present a more severe phenotype, since severe defects in regional specification of the hippocampus primordium leads to the entire hippocampus loss. These facts corroborate the importance of *Wnt* genes, and also its transcription targets in brain development.[50, 61, 64]

During limb initiation and consequent development in vertebrates, several Wnt proteins play important roles, like for example Wnt-2b, which is expressed in the mesoderm of the presumptive forelimb region. Wnt-2b induces FGF10 expression, whereas in the mesoderm of the hindlimb region, FGF-10 expression is regulated by Wnt-8c instead. These interactions demonstrate the communication between Wnt and FGF signaling, through a regulatory loop, not only in limb development but also, for instance, in the induction of the inner ear.[36, 61, 65] As several studies reported, Wnt not only interacts with FGF signaling but also with other major pathways like Shh, and Bone Morphogenetic Protein-4 (BMP-4).[37, 49, 66, 67]

When studying the control of Wnt signaling pathway, inhibition seems to have a more important role than activation. At later stages of vertebrate development, suitable head formation relies on the involvement of some of Wnt signaling downstream targets and inhibitors (like Dkk-1 and FrzB-1), whereas ectopic activation of Wnt signaling blocks this process. Besides acting in a blocking fashion in head formation, Wnt signaling also plays an inhibitor role in cardiogenesis. Wnt signals from the dorsal neural tube as well as from the posterior lateral plate, blocking heart formation in the adjacent anterior paraxial mesendoderm. Dkk-1 also acts as an inducer in heart formation, by diminishing Wnt-3a and Wnt-8 activity.[39, 61]

Despite all the recent advances in the comprehension of Wnt signaling in the embryo, a lot is yet to be revealed and so, more studies need to be performed in order to fully understand this highly complex signaling pathway, in particular, its interactions with other pathways and also the deep connection between its own branches.

1.2.3 Wnt signaling and lung development

Over the last few years, significant progresses have been accomplished in the comprehension of the genetic and molecular mechanisms involved in lung organogenesis.

So far, several mediators of epithelium-mesenchyme interaction have been identified, including members of the Hh, FGF, TGF-β and Wnt families, among others.

As review by Horowitz and Simmons, one of the major functions of Wnt signaling in the developing lung is the regulation of the differentiation of precursor cells into several phenotypes along the growing lung.[12] A perturbation of this signaling pathway will disrupt the correct lung growth leading to an increased distal branching frequency. These findings are supported by the experiments concerning *Wnt-5a* null and transgenic mice, which present an abnormal distal lung morphogenesis.[68]

1.2.3.1 Wnt expression pattern

The expression of Wnts signaling components and β-catenin are precisely regulated during fetal lung development. For instance, *Wnt-2* mRNA is highly expressed in the rat fetal lung only in mesenchymal cells.[69] On the other hand, in humans *Wnt-11* is present in epithelial and mesenchymal cells,[70] while studies in mice revealed that *Wnt-7b* expression is restricted to airway epithelium (distal and proximal) during embryonic development.[71] During chick's respiratory system development, *wnt-11* is strongly expressed in the trachea's mesenchyme as well as in the mesenchyme surrounding the main bronchus.[72] Regarding *wnt-5a*, in mouse, its transcripts are present in both mesenchymal and epithelial compartments of the developing tissue, although more intense at the tip and around the branching epithelium. *wnt-5a* epithelial expression increases with gestation, in a proximal to distal fashion, while mesenchymal expression becomes progressively lower.[68] *β-catenin* is expressed in the airway and alveolar epithelial cells during human fetal lung development.[73] During lung development, other components of Wnt signaling like FZD receptors and Tcf/Lef transcription factors are also expressed.

1.2.3.2 Wnt role in lung morphogenesis

Studies on conventional knock-out mice of Wnt signaling pathway genes have revealed its essential roles in tissue morphogenesis; however, most of these knock-out mice die during embryogenesis or soon after birth and, for this reason, tissue-specific transgenic, conditional knock-out and knock-in mice needed to be performed.

A study carried out with *wnt-5a* knockout mice proved the importance of this protein in lung development because, on these mice, the trachea was truncated and there were serious abnormalities in the distal lung architecture, but not on pulmonary cellular differentiation. With the disruption of *wnt-5a* gene, these embryos presented overexpressed *fgf-10*, *Bmp-4* and *Shh* on their late stage, suggesting that Wnt-5a is crucial regulator of these mediators; consequently, the embryo presented attenuated pulmonary maturation and post-natal death due to apparent respiratory failure. Also, Wnt-5a appears to be important in capillary/alveolar apposition since it is not possible to have the correct apposition in mice lacking *wnt-5a* gene, hindering an appropriate gas exchange at birth.[68]

When studying *wnt-7b* in the mouse developing lung, Shu *et al* showed the importance of this gene in proliferation/growth of the lung mesenchyme and maturation of the airway epithelium. The result was perinatal death also due to respiratory failure. The lungs appeared not inflated, hypoplastic and also showed extensive hemorrhage. This phenotype was due to decreased cell proliferation in the distal mesenchyme adjacent to the growing tips of the distal airway epithelium, where *wnt-7b* is normally expressed. Also, the vascular smooth muscle appeared to be hypertrophic, which may also explain the hemorrhage.[33]

According to recent studies, Wnt-5a and Wnt-7a are not involved in early lung branching but instead in later phases of the embryo development.[33, 68]

Other important Wnt ligands in lung development are Wnt-2 and Wnt-2b, which are expressed in the mesenchyme surrounding the epithelial buds. Both *wnt-2* and *2b* are expressed later in developing lung mesenchyme but only *wnt-2* persists into adulthood. *wnt-2* null mice present small lungs and die soon after birth while the mice lacking *wnt-2b* gene are viable after birth.

On Table 1, are summarized different studies regarding knock-out mice that presented a phenotype in the respiratory system.

Table 1 – Wnt knock-out mice with a respiratory phenotype.

Approved symbol	Affected function/ Phenotype	Reference
Wnt-2	absent lung and absent trachea	74
Wnt-2b		
Wnt-5a	Abnormal lung development; Abnormal lung interstitium morphology; Decreased tracheal cartilage ring number; short trachea; Respiratory failure	75
Wnt-7b	Lung hemorrhage; Abnormal lung development – decreased mesenchymal cell proliferation and thin lung-associated mesenchyme; Abnormal right lung middle lobe morphology; Small lung – decreased lung weight; abnormal tracheal cartilage morphology; atelectasis	76

1.3 Segmentation clock

The vertebrate body is composed, along the AP axis, of a series of repeated structures called *segments*, that are periodically established in the embryo during *somitogenesis*.[77] Somitogenesis consists on the rhythmic formation, from the paraxial mesoderm, of epithelial cell blocks, called somites, which subsequently differentiate to give rise to the vertebrae and the skeletal muscles of the adult body. The number and rhythm of somite formation is characteristic of each species. For instance, in the chick, a new pair of somites is formed every 90 minutes, in a total of 52.[78, 79] In the zebrafish embryo it takes 30 minutes to form a new pair of somites whereas in mouse and human embryo it takes 2 and 8 hours, respectively.[80] This process relies on the translation of a temporal into a spatial periodicity.

The correct embryonic development depends on the timely proliferation and differentiation of progenitor cells into multiple cell types, which occurs in a scheduled time course. It is no coincidence that some structures are formed before others and that some organs are bigger than others. Several different types of biological clocks maintain the homeostasis of the organism; one example is the circadian clock, which regulates the sleep/wake cycle through molecular oscillators during, approximately, 24 hours. However, during embryo development, shorter cycles occur, in order to coordinate specific developmental events on a spatial and temporal fashion. This mechanism was reported, for

the first time, in somitogenesis by Palmeirim *et al*, who demonstrated the existence of a molecular oscillator (*hairy1*) and related it with well-timed somite formation. An intrinsically coordinated pulse of *hairy1* expression appears as a wave that sweeps across the presomitic mesoderm (PSM) during somite formation.[79] This event was called **segmentation clock**.

hairy1, the chick homologue of the *Drosophila* gene *hairy*, belongs to the hairy-enhancer-of-split (Hairy E (Spl)/ HES) family of transcriptional repressor proteins. All HES members share a few conserved functional motifs, such as the bHLH domain, composed by a basic region (b), necessary for DNA binding, and the helix-loop-helix (HLH) for dimerization; the Orange domain, which confers specificity of bHLH heterodimer partners; and finally the WRPW domain, which recruits transcriptional repressors, and a protein-protein interaction domain.[81, 82] These genes are present in mouse (*hes*), in zebrafish (*her*) and in chicken (*hairy*) and participate in functional biological clocks, measuring time during several embryonic events. In such developmental processes, Hes has been reported to act as a downstream target of Notch signaling repressing transcription. *hairy1* shares great homology with the mouse *hes* genes and is also a target of Notch signaling cascade.[79]

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. In the meantime, several other genes have been described as having a dynamic expression pattern in the PSM, namely *hairy2*, *hey2, Lfng, Delta and axin2*, just to name a few. *hairy2*, the chick homologue of mouse *hes1*, that encodes a transcription factor closely related to *hairy1,* is expressed throughout the PSM and is involved in the formation of one somite, every 90 minutes.[83]

Subsequent studies have showed that, besides somite formation, the molecular clock also controls the timing of other biological systems, like forelimb development in chick embryos. *hairy2* is expressed in limb chondrogenic precursor cells with a 6-hour periodicity and a limb skeletal element is formed by cells with “n” and “n+1” *hairy2* expression cycles.[84] 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.[84]

When trying to understand the nature of the timely somitogenesis, two important components were discovered. First, the molecular clock or oscillator, acts within each individual cell as a pacemaker, ensuring the periodicity of somitogenesis and second, part of this control mechanism is constituted by a gradient of developmental cell change - a morphogen gradient (wavefront).[85] This morphogen gradient may be responsible for the correct positioning of information inside the cell and for somite number and size.[86]

1.3.1 Signaling pathways involved in the molecular clock

Most of the knowledge concerning the molecular clock has been obtained through studies regarding somitogenesis,[6, 78, 79, 87] due to the fact that somites are the most obvious metameric structures and represent the basis of the segmental pattern of the body. The involvement of an oscillator during somitogenesis is responsible for the generation of a periodic pulse of Notch, FGF and Wnt signals.[86] A stripped pattern of gene expression, formed rhythmically in the PSM at a determined level of the gradients of Wnt and FGF – wavefront, in response to the segmentation clock, provides the blueprint from which the somite's structure will be formed.

The first pathway to be associated with oscillatory gene expression was the Notch signaling pathway.[80, 88] Notch is a transmembrane receptor and this receptor is able to recognize two sets of transmembrane ligands, Delta and Jagged/Serrate. Upon ligand binding, Notch undergoes a proteolytic cleavage at the membrane level, leading to the translocation of its intracytoplasmic domain (NICD) into the nucleus where, along with transcription factor CSL, activates the transcription of several genes, such as the *hes*1/*hes*5 in vertebrates.[89] Since Notch receptor and its ligands, are transmembrane proteins, Notch signaling takes place among neighboring cells.[77]

Several oscillator molecules have been linked to the Notch signaling pathway like *hairy*-related basic helix-loop-helix (bHLH) genes and include, among others, the genes mentioned on Table 2.

Table 2 – Molecular oscillators expressed on different species (adapted from [80]).

	Zebrafish	Chick	Mouse
Molecular oscillators	*her*1	*hairy*1	*hes*1
	*her*7	*hairy*2	*hes*5
	-	*hey*2	*hes*7
	-	-	*hey*2

At first, Notch was believed to act as the segmentation clock pacemaker but some inconsistencies with the different findings led to the understanding that, on the zebrafish embryo, Notch signaling is important to maintain the synchronization of cell-autonomous oscillations, on the posterior PSM.[77] Since the initial oscillations of *her* genes in the zebrafish begin around the time of gastrulation, like in chick embryos, if the Notch signaling pathway had a role as the segmentation clock pacemaker, the treatment with an inhibitor of Notch signaling, DAPT, would disrupt the synchronization of the oscillation. Instead, the oscillations were still synchronized, and a somite boundary defect appeared only after a long delay, supporting the theory that Notch signaling operates only in the posterior PSM.[77, 90]

All these findings were major breakthroughs but unfortunately, most of them were only concerning the zebrafish. When it comes to the amniotes cyclic genes network, a more complex system is necessary that also involves the FGF and Wnt signaling pathways. In the case of amniotes, a specific glycotransferase, produced by the Notch target gene, *Lunatic fringe* (*Lfng*) has the ability to stop Notch signaling and in this way act as negative feedback inhibitor in the PSM.[91, 92] The disruption of Notch signaling in these embryos, with DAPT, proved that *Lfng* was completely downregulated, unlike in zebrafish embryos.[77, 92]

During somitogenesis, some of the oscillating genes, already linked to the segmentation clock, like *Snail1/2*, *Spry2*, *Dusp6* and *4*, were proved to be targets of the FGF pathway, allowing the connection of this signaling pathway to the segmentation clock. Many of the newly discovered oscillating genes in the FGF signaling pathway are negative regulators of the mentioned pathway, and have the potential to drive or pace the

oscillations. A few studies regarding the connection between FGF and Notch signaling pathways has proved that FGF signaling acts upstream of the Notch oscillations. The model proposed where FGF signaling initiated the *hes7* oscillations in the tail whereas Notch maintained the oscillations on the anterior PSM and the feedback loop identified between *hes7* and *Dusp4* supported this theory.[93, 94]

1.3.2 Wnt and the molecular clock

The first link between Wnt signaling pathway and the segmentation clock was documented due to oscillations of *axin2*, a target of Wnt canonical pathway, in the mouse embryo. As previously described, Wnt signaling is a very complex pathway that regulates the expression of its own components. Even though Wnt canonical pathway doesn't regulate the transcription of *axin*, it does regulate *axin2* (a homologue of *axin*). Since Axin and Axin2 are functionally equivalents, it is clear that Axin2 is able to act as a scaffolding protein in the destruction complex and as binding partner to Dsh and LRP5/6.[57]

Recent studies have identified several cyclic Wnt target genes, including some inhibitors of this pathway, such as *Dkk1* or *dapper homologue 1* (*Dact1*) and discovered that they exhibit oscillations in opposite phase to Notch and FGF target genes.[95] In recent trials with mouse embryos, *Lfng* was disrupted in Wnt pathway mutant *vestigial tail*, whereas *axin2* expression remains on RBP*jk* (a transcription factor of Notch signaling pathway) and *hes7* mutants. Even though Notch is one of the major components associated to the cyclic genes, it does not interfere with *axin2* oscillation. These data suggest that Wnt pathway acts independently and upstream from Notch signaling pathway and demonstrates an important role in the segmentation clock.[77, 96]

Wnt signaling was proven to establish a posterior-to-anterior signaling gradient along the PSM, since nuclear β-catenin exhibits a clear gradient running from the tail bud to the determination front level.[96, 97] Despite these findings, in a β-catenin gain-of-function experiment, *Dkk1* and *Lfng* cyclic genes were still oscillating in a dynamic fashion in the posterior PSM. These experiments demonstrated that, like FGF pathway, Wnt pathway is needed to determine the position and control the size of the oscillatory domain, but it doesn't control the rhythm at which *Lfng* is expressed.[77]

The truth is that so far, none of the pathways studied were successfully linked as the segmentation clock pacemaker, which leads to the understanding that the coordination of the spatial and temporal control of the periodic gene expression is managed by a complex interconnected signaling network that involves many subnetworks like Notch FGF and Wnt signaling pathways. These pathways appear to be responsible for regulating themselves and also coupling with each other, at least in amniotes. The challenge that remains is to understand how the different signaling pathways, that constitute the segmentation clock, interact with each other.

1.3.3 *hairy* genes and lung development

In 1992, Sasai and colleagues noticed a high expression level of *hes1* mRNA in both, the trachea and lungs of mouse embryos. Likewise, Ito demonstrated that Hes1 immunostaining was localized in non-neuroendocrine cells in the lung epithelium at an early stage (E14), reinforcing previous results. Furthermore, *hes1* knock-out mice exhibit smaller lungs than wild type, with reduced Notch1 immunostaining revealing that *Notch1* expression is tightly regulated by Hes1. Still, this dependence is not found in other Notch types. *Notch1* expression in the lung increases with fetal age and in the adulthood, but not in *hes1* deficient mice.[98, 99]

A recent study has described the expression pattern of *hairy* genes, *hairy1* and *hairy2*, in the chick lung. *hairy1* is present in the epithelial region of the main bronchus and the secondary bronchi, and also in lung mesenchyme. *hairy2* was found to be expressed in the tracheal mesenchyme and bifurcation, as well as in the distal part of the main bronchus and the distal epithelium of the primary and secondary bronchi. These results are in agreement with mouse expression pattern. Moreover, in lungs belonging to the same developmental stage, some differences in both *hairy1* and *2* expression pattern were detected, demonstrating their dynamic expression.[100]

Even though these transcriptional regulators have been described as Notch and FGF downstream targets, the inhibition of these pathways in the chick lung showed no influence in *hairy1* expression, even though lungs presented abnormal branching and growth. But, unlike *hairy1*, and besides not showing alteration after FGF signaling inhibition, *hairy2* expression was dramatically reduced in the lung, except in the most proximal and distal

secondary bronchi, and was vaguely diminished in the trachea and tracheal bifurcation of the lungs treated with DAPT (Notch signaling inhibitor). In these study conditions, *hairy* expression during chick lung development is not FGF signaling dependent. *hairy1* was also unaffected by Notch signaling inhibition, conversely *hairy2* showed a slight down-regulation after blocking Notch signaling.[100]

1.4 Aims

The molecular clock has been described in processes that require precise spatial and temporal regulation such is the case of 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. *hairy1* and *hairy2* expression pattern has already been well documented in the chick embryonic lung, however the signaling pathways involved in their regulation remains unknown.

The main aim of this dissertation is to understand the association between Wnt signaling pathway and *hairy* expression in the chick embryonic lung. For this purpose, the expression pattern of some members of Wnt family will be characterized, by *in situ* hybridization, in early stages of development. Moreover, *in vitro* inhibition studies will be performed using chick lung explants system and a specific Wnt signaling inhibitor. Explants will then be assessed for *hairy* expression and morphology alterations.

2. Materials and methods

2.1 Eggs storage and embryo dissection

Fertilized *Gallus Gallus* variant *domesticus* eggs were obtained from a commercial source (PintoBar, Portugal) and stored upon arrival at 16ºC, for no more than a week. Eggs were incubated for 4-5 days in a humidified incubator at 37ºC and then embryos were dissected in Dulbecco's Phosphate Buffered Saline without Calcium and Magnesium (DPBS; PAA, Austria) under a stereomicroscope (Olympus SZX 16, Japan). The lungs were staged according to the number of secondary buds formed, b1, b2 and b3.

The lungs obtained were processed either for *in vitro* explant cultures or for *in situ* hybridization.

2.2 Lung explants culture

2.2.1 *In vitro* inhibition studies

After dissection, lungs were transferred into Nucleopore polycarbonate membranes (8μm pore size) (Whatman, UK) in 24-well culture plates (Orange Scientific, Belgium). The membranes were previously stabilized with 400μL of Medium 199 (Sigma, USA) for about 1 hour. After stabilization, the Medium 199 was replaced with 200μL of culture medium, constituted by Medium 199 enriched with 5% heat inactivated Fetal Calf Serum (Invitrogen, UK), 10% Chicken Serum (Invitrogen), 1% L-Glutamine (Invitrogen), 1% Penicillin 5000IU/mL, Streptomycin 5000IU/mL (Invitrogen) and 0.25mg/mL Ascorbic Acid (Sigma). This entire procedure was performed in sterile conditions. The floating cultures were then incubated for 1 hour in a 5% CO_2 incubator, at 37ºC. Explants were kept in culture for 48 hours and the medium replaced daily. In order to monitor branching morphogenesis, explants were photographed at D0 (0h), D1 (24h) and D2 (48h) with a digital camera (Olympus U-LH100HG) coupled to the stereomicroscope. At D0 and D2 the total number of peripheral airway buds was determined. The results of branching were expressed as D2/D0 ratio.

For the inhibition of Wnt signaling pathway, lung explants were cultured with FH535 (Sigma), an antagonist of the canonical signaling pathway. FH535, dissolved in Dimethyl Sufoxide (DMSO), was added to the culture medium in order to obtain a final concentration of 20 and 40μM. Control explants were cultured with DMSO, at a final concentration of 1μL/mL.

After 48h in culture, lung explants were washed with DPBS and processed for *IS*H.

2.2.2 Statistical analysis

The branching results (D2/D0 ratio) were presented as mean ± SE. Statistical analysis was performed with SigmaStat 3.5 (Systat Software, Inc., USA) by One Way ANOVA on Ranks and the Turkey test was then used for post-test analysis. The statistical significance was set at $p<0.05$.

2.3 *In situ* Hybridization

In Situ Hybridization (ISH) is a semi-quantitative technique that allows for precise localization of a specific nucleic acid within a tissue or histologic section. A known target mRNA can be identified in a sample through the application/hybridization of a labeled RNA complementary strand (anti-sense RNA probe). This labeled RNA probe can then be detected by using an antibody that recognizes the label on the probe, and then visualized by a colorimetric method. The probes can therefore be used to detect expression and location of a gene of interest.

The most common approach that enables the detection of the hybridization is to label mRNA with a modified nucleotide, such as UTP conjugated with digoxigenin that is incorporated during *in vitro* transcription. Chromogenic visualization is achieved after incubation with an antidigoxigenin antibody conjugated with Alkaline Phosphatase (AP) and with two AP substrates (BCIP: 5-bromo-4-chloro-3-indolyl phosphate; NBT: 4-nitro blue tetrazolium). A dark blue precipitate will appear only in the areas where the target mRNA is present.

2.3.1 RNA probes

The chick probes used in this thesis were already described elsewhere: *hairy1* and *hairy2*;[79] *wnt-5a* and *wnt-7b*;[101] *β-catenin*;[102] *axin2*[103]. *wnt-2b* and *9a* were kindly provided by Dr Rodríguez-Leon (ICG, Portugal).

2.3.1.1 *E. coli* transformation

Plasmids provided in absorbent paper were soaked in about 30-40µL of ultrapure water and were left to elute overnight, at 4ºC. Then, 2µL of the plasmid were used to

transform 100µL of *Escherichia coli* DH5α competent cells. These cells were kept in ice for 30 minutes and then exposed to a heat shock at 42ºC, for 90 seconds, followed by 10 minutes on ice. 800µL of Super Optimal Broth with Catabolite Repression (SOC) (please see annex I for the reagents' composition) was added to the cells and these were incubated for 1 hour, at 37ºC, with agitation. After this incubation period, the mix was centrifuged for about 10 seconds, at 14000rpm. Most of the supernatant was removed and the transformed cells were plated in solid Luria Broth (LB) medium supplemented with Ampicilin (Sigma) (2mL/L), to select only the bacteria containing the desired plasmids, 5-bromo-4chloro-3-indolyl-B-D-galactosidase (X-Gal - Melford, U.K.) (1mL/L), a substrate of β-galactosidase and isopropyl-β-D-thiogalachopyranoside (IPTG - Melford) (1mL/L), an inducer of lacZ gene required for the production of galactosidase. These plates were incubated overnight at 37ºC. Colony plates were kept at 4ºC until further use.

2.3.1.2 DNA extraction

In order to extract DNA, the GenElute plasmid Miniprep kit (Sigma) was used. This kit is a rapid and cost-effective method for plasmid DNA isolation from recombinant *E. coli* cultures, by combining silica-binding technology and the convenience of a spin column format.

1. A colony or a patch of glycerol stock was transferred into 6mL liquid LB medium, supplemented with Amplicilin (2mL/L) and incubated overnight, at 37ºC with constant shaking.
2. When necessary, glycerol stocks were prepared from the cultured cells incubated overnight, 500µL, and 500µL of Glycerol 30%. These stocks were stored at -80ºC.
3. The remaining bacterial culture was centrifuged, in order to isolate cells (pellet) from the medium.
4. After ressuspension with 200µL Ressuspension Solution, 200µL of Lysis solution was used, to release cellular content. After pipetting up and down, it was allowed to clear for no more than 5 minutes.
5. Next, 350µL of Neutralization solution was added to the previous mix, allowing the precipitation of cell debris and so, after a new centrifugation,

the cell debris, proteins and chromosomal DNA were deposited in the bottom of the tube.

6. The cleared lisate was then added into a previously prepared binding column and, after spinning, the DNA bound into the column.
7. At this point, 750µL Washing solution was added and with it, the residuals and contaminants that were introduced during the column load, were washed away.
8. The final step involved the addition of 50µL of an Elution solution (a smaller volume in order to ensure a more concentrated plasmid DNA).

After DNA extraction, spectrophotometric analysis, through Nanodrop technology, was performed. This allowed the determination of DNA concentration (ng/µL) and purity through the ratio of absorbance at 260nm to 280nm (A_{260}/A_{280}). The size of the plasmid was determined by agarose gel electrophoresis. The DNA was stored at -20ºC.

2.3.1.3 Probe synthesis

Plasmids were processed as previously described and the information is summarized in table 3. Plasmids that were received during the course of this project were sequenced in the Molecular Biology service of ICVS, University of Minho, allowing the confirmation of the cloned cDNA fragment. (see Annex II for the sequence references). For amplification, a standard PCR program was used.

Table 3 – Information concerning the probe synthesis. In all cases DNA digestions were performed at 37ºC. (*) sequence confirmed by sequencing.

Probe	Linearization	Amplification	*In vitro* transcription	Reference
*wnt-2b**	-	M13 F and R	T3 RNA polymerase	104
wnt-5a	*Eco*RI	-	T7 RNA polymerase	105
wnt-7b	*Eco*RI	-	T7 RNA polymerase	
*wnt-9a**	-	M13 F and R	T7 RNA polymerase	104
*β-catenin**	-	M13 F and R	T7 RNA polymerase	102
*axin2**	*Not*I	-	T7 RNA polymerase	103
hairy1	*Hind*III	-	T7 RNA polymerase	79
hairy2	*Hind*III	-	T7 RNA polymerase	

Both PCR and linearization products were purified using GeneJet PCR Purification Kit (Fermentas, Germany). This kit is designed for rapid and efficient purification of DNA from PCR and other enzymatic reaction mixtures; it relies on a silica-based membrane technology in the form of a convenient spin column. This kit is commonly used to remove primers, dNTPs, unincorporated labeled nucleotides, enzymes and salts from PCR and other reaction mixtures.

1. Firstly, a Binding solution that contains a chaotropic agent that denatures proteins and promotes DNA binding to the silica membrane in the column, was added to the DNA (1:1).
2. After spinning for 1 minute at 14000 rpm and discarding flow-through, 700µL of Washing solution was added to remove all the remaining impurities and a new centrifugation was performed at 14000 rpm, for 1 minute. After discarding flow-through, the empty column was centrifuged, for 1 minute, to completely remove any residual wash buffer.
3. The final step involved the elution of the DNA with 50µL of Elution solution. The column was centrifuged in a new collection tube, for 1 minute. The purified products were then stored at -20ºC. 2µL were then analyzed by electrophoresis on an agarose gel.

During probe synthesis, an antisense RNA, complementary to the mRNA of interest, was synthesized through *in vitro* transcription. DNA fragments, obtained by digestion or PCR, were incubated with the appropriate RNA polymerase (Table 3) and Dig RNA labeling mix according to the manufacturer's instructions.

1. On a RNAse-free eppendorf, the reaction mixture was prepared with 1µg of linearized or amplified DNA, DTT (Promega), Dig-RNA labeling mix, RNAse out (Promega), the appropriated RNA polymerase and its respective transcription buffer and RNAse free ultrapure water, according to the manufacturer's instructions.
2. After vortex and spin down, the mixture was incubated at 37ºC, for 3 hours.
3. After this incubation period, 4µL DNAse (Promega) was added (to degrade the template) as well as 2µL of RNAse out. Once again the mixture was incubated at 37ºC, for 30 minutes.

4. On ice, the reaction was stopped and 600µL Ethanol 99,9%, 200µL of TE and 20µL of Lithium Chloride were added.
5. The mixture was incubated for 45 minutes, at -80ºC.
6. Afterwards, a centrifugation at 4ºC for 30 minutes, at 14000 rpm was applied and then, the supernatant was carefully discarded and 70% Ethanol was added for a new centrifugation, during 15 minutes.
7. The supernatant was discarded very carefully and the pellet was left on ice to evaporate the remaining ethanol.
8. The pellet was finally ressuspended in 10mM EDTA (Calbiochem, Germany) and analyzed on an agarose gel.
9. The probe was then stored at -20ºC.

2.3.2 *In situ* Hybridization

In situ hybridization was performed, as previously described by Henriques *et al*, with minor changes.[106] *IS*H protocol can be divided in 3 main steps: pretreatment and hybridization, post hybridization washes and post-antibody washes.

During **pretreatment and hybridization**, lung explants and embryos are rehydrated and treated with proteinase K, to favor the entrance of the probe into the cells. The hybridization process was done at 70ºC overnight, in order to ensure the optimal annealing of the Dig-labeled RNA probe to the target sequence.

1. After dissection, lungs were fixed overnight in fixation solution (Annex I), rinsed in PBS (1x), dehydrated through a methanol series and stored in 100% methanol, at -20ºC.
2. After a process of rehydration (with a decreasing amount of methanol), tissues were washed twice with PBT (1x) and treated with proteinase K. Incubation time varies depending on the tissue: whole embryos were incubated for 35 minutes while lungs were incubated for only 2 minutes.
3. After Proteinase K treatment, lungs were rinsed twice with PBT (1x) and then fixed for 20 minutes with post-fixation solution (see Annex I).
4. After rinsing twice with PBT (1x) for 10 minutes, tissues were incubated for one hour at 70ºC, with Hybridization solution (Hybmix, see Annex I), When used for

the first time, the Dig-labeled RNA probe is added to the Hybmix (1:200). This mix is also incubated at 70 ºC for 1 hour.

5. Afterwards, the Hybmix is replaced with the RNA dig-labeled probe diluted in Hybmix, and incubated overnight.
   After the hybridization step, the probe can be reused for 5 times.

During **post-hybridization washes**, tissues are washed in order to remove totally or partially unbound probe to any sequence other than the desired one.

6. After overnight hybridization, lungs were rinsed with prewarmed Hybmix (70ºC), and then washed twice, for 30 minutes, with Hybmix at 70 ºC.
7. After this, lungs were washed with Hybmix:MABT (1:1) at, 70ºC (see MABT composition in Annex I), and then rinsed 3 times with MABT, at Room Temperature (RT).
8. After removing the previous solution, tissues were incubated for about 1 hour, with block solution 1 and then with block solution 2 (Annex 1) in order to remove any possible background of the probe.
9. Afterwards, tissues were incubated with anti-Digoxigenin antibody conjugated with Alkaline Phosphatase (AP; Roche) (see Annex 1), overnight, at RT, with constant shaking.
   To ensure the removal of unbound antibody, a series of washes are performed (post-antibody washes).
10. Firstly, lungs were rinsed several times with MABT at RT, and then washed for several hours with MABT.
11. Finally, lungs were incubated with a developing solution containing BCIP and NBT, two AP substrates. In this colorimetric reaction, a dark blue precipitate will form, indicating the presence of the target mRNA.

12 Lungs were photographed in PBT (1x) solution using a digital camera coupled to the stereomicroscope and stored in PBT/0,1% Azide, to prevent contamination.

Hybridized lungs were then processed for slide sectioning, in order to better characterize the mRNA location. For this purpose, lungs were fixated in paraformaldehyde (4%) (Panreac, Spain) overnight embedded in 2-hydroxyethyl-methacrylate and processed

for sectioning at 25μm thickness using a Rotary microtome (Leica, Germany). Lung sections were photographed with and Olympus DP70 camera coupled to an Olympus BX61 microscope.

3. Results

3.1 Characterization of Wnt signaling members expression pattern, during chick lung development

In order to characterize the expression pattern of several Wnt genes and other members of this signaling pathway, in early stages of chick lung development, *In Situ* Hybridization was performed. Moreover, representative examples of hybridized lungs from different stages of development were sectioned for histology. The lungs were harvested after an incubation period of 4,5 to 5 days and staged according to the number of secondary branches formed: b1 if only one secondary branch is present on each main bronchus, b2 if there are two secondary bronchi and b3 if there are three secondary bronchi in each main bronchus.

There are 18 Wnt ligands described in the chick embryo. The ones analyzed in this study were chosen considering their involvement in mammalian lung development: *wnt-2b*, *wnt-5a*, *wnt-7b* and *wnt-9a*. Wnt canonical signaling pathway has several target genes, including members of the protein activation complex such is the case of *β-catenin* and *axin2*.

3.1.1 Expression pattern of *wnt-2b*, *wnt-5a*, *wnt-7b* and *wnt-9a* during chick lung development

wnt-2b is present mainly in the mesenchyme surrounding the main bronchi particularly in the medial region (Figure 6B, red arrow), and almost absent from the most proximal area, the trachea, in the three stages studied (Figure 6A-E). Histological sectioning of hybridized lungs confirmed the mesenchymal expression, higher in the medial area (Figure 6E arrowhead). Moreover, it also showed that there is no expression in the pulmonary epithelium.

wnt-5a expression in the chick lung is confined to the most posterior area of the respiratory tract, namely the trachea (Figure 6G, green arrow), and no expression is observed in the main bronchi (Figure 6F-J). Slide sectioning confirmed that *wnt-5a* expression is only present in the mesenchymal compartment (Figure 6J, dagger). No differences were observed in the three stages studied.

wnt-7b is clearly expressed throughout all pulmonary epithelium not only in the main bronchus but also in secondary bronchi (Figure 6O, orange arrow) and in the tip of the main bronchus (Figure 6L, asterisk), in the three stages studied. *wnt-7b* transcript seems to be absent from the lung mesenchyme (Figure 6K - O). Histological sectioning of hybridized lungs confirmed this expression pattern (Figure 6N, O).

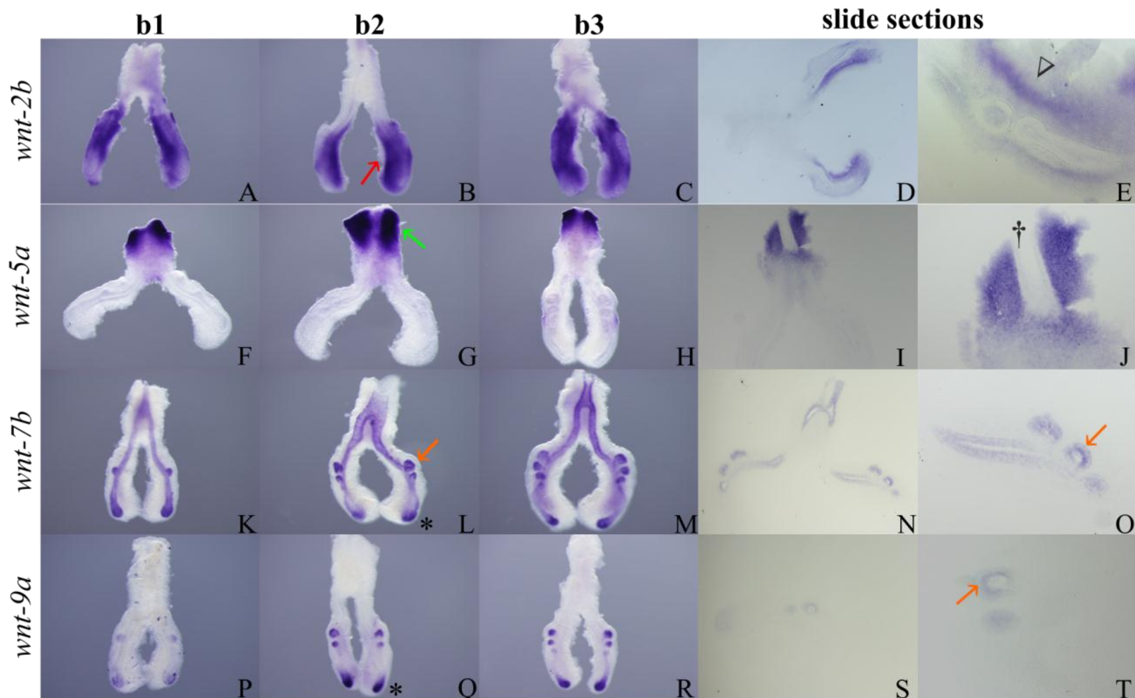

Figure 6 - Wnt ligands expression pattern in early stages of chick lung development. Representative examples of whole mount *in situ* hybridization of stage b1 (A, F, K, P), b2 (B, G, L, Q) and b3 (C, H, M, R) - n=15 for each stage and probe. Lungs were probed with *wnt-2b* (A - E), *wnt-5a* (F - J), *wnt-7b* (K - O) and *wnt-9a* (P - T). Red arrow – medial mesenchyme, green arrow – proximal mesenchymal, orange arrow – secondary buds epithelium, asterisc – epithelial tip of the main bronchus, dagger - proximal epithelium. Magnification: A - C, F - H, K – M and P - R - 5x; D, I, N, S – 4x; E, J, O – 10x and T – 20x.

wnt-9a is detected only in the growing tips of the lung, namely in the tip of the main bronchi and also in the secondary buds (Figure 6Q, asterisk and Figure 6T, orange arrow, respectively), and no expression is observed in the remaining epithelial compartment and the mesenchyme (Figure 6P-T). This expression pattern was confirmed in lung sections (Figure 6S, T).

3.1.2 Expression pattern of *β-catenin* and *axin2* during chick lung development

β-catenin is expressed throughout all the mesenchyme of the developing chick lung. This expression is more intense in the area adjacent to the secondary buds and in the tip of the main bronchus (Figure 7A - E). No differences were observed in *β-catenin* expression pattern in the three stages studied. Lung sections confirm this ubiquitous expression and also show an epithelial expression mainly in the tip of secondary buds and main bronchus (Figure 7E, asterisk and orange arrow, respectively).

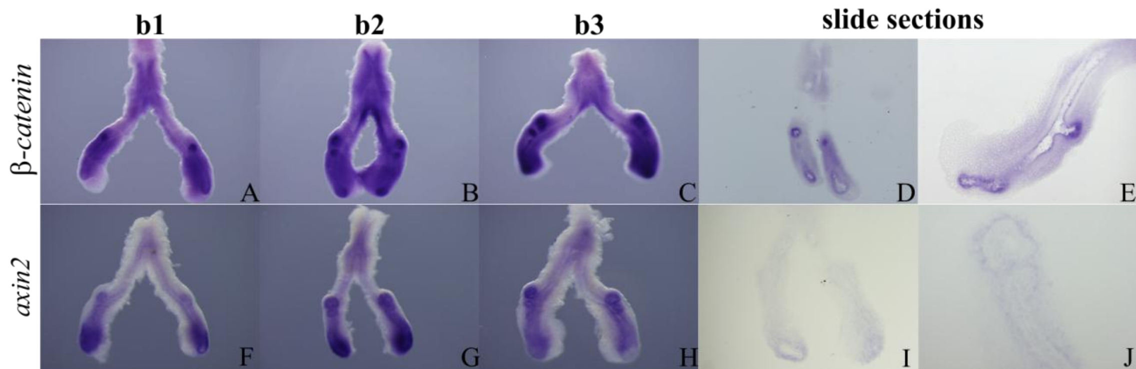

Figure 7 - *β-catenin* and *axin2* expression pattern in early stages of chick lung development. Representative examples of whole mount *in situ* hybridization of stage b1 (A, F), b2 (B, G) and b3 (C, H) - n=15 for each stage and probe. Lungs were probed with *β-catenin* (A - E) and *axin2* (F - J). Orange arrow - secondary buds epithelium, asterisc – epithelial tip of the main bronchus, arrowhead – peri-epithelial mesenchyme, black arrow - peri-epithelial mesenchyme around the secondary bronchi. Magnification: A – C and F – H – 5x; D, I – 4x and E, J – 10x.

axin2 mRNA is detected in the pulmonary mesenchyme of the chick respiratory tract (Figure 7F - J), in all stages studied, and expression is more intense in the peri-epithelial mesenchyme surrounding the secondary buds (Figure 7J, black arrow) and the tip of the main bronchus (Figure I, arrowhead). *axin2* transcript is absent from the epithelial compartment. Histological sectioning of hybridized lungs confirmed this expression pattern (Figure 7I, J).

3.2 *In vitro* inhibition studies

In order to understand the importance of Wnt signaling pathway during chick lung development *in vitro* lung explant cultures were performed. For this purpose, b1 to b3 lungs were processed for explant culture (as described in section 2.2.1) and incubated with

two different doses of FH535, a β-Catenin/Tcf inhibitor (deprives the complex β-catenin-Tcf/Lef from binding to Tcf/Lef DNA sites, possibly through the inhibition of the recruitment of β-catenin and Glucocorticoid Receptor Interacting Protein-1 (GRIP1), another coactivator, to this complex). The doses used, 20 and 40μM, were selected according to the literature and lung culture was maintained for 48 hours. Control explants were treated with DMSO, the dissolving agent of FH535. Also, lung explants were probed for *hairy* genes in order to try to understand if Wnt signaling pathway is involved in their regulation during chick lung development.

3.2.1 Wnt inhibition and morphometric analysis

Lung explants treated with FH535 show abnormal lung growth when compared with control explants treated only with DMSO (Figure 8). FH535 treated lungs present a decreased number of secondary when compared with controls, in a dose dependent manner. Branching analysis was performed and the results obtained are summarized in Figure 9. High FH535 doses lead to a decrease in the ratio of total number of peripheral airway buds (D2/D0) when compared to DMSO treated explants, in the three stages studied. No significant differences were found between control and 20μM treated explants. On the other hand, the highest dose of inhibitor induced a statistically significant decrease ($p<0.05$) when compared to both DMSO and 20μM treated explants.

To confirm Wnt signaling inhibition, b1 to b3 treated explants were probed with *axin2*, a direct readout of this signaling pathway. In DMSO treated explants *axin2* is present and displays an expression pattern that is in accordance with the previous results (Figure 7). On the other hand, FH535 treated explants present a reduction in *axin2* expression with increasing doses of inhibitor (Figure 8F, I). The explants treated with 20μM (Figure 8F) show a decrease in *axin2* expression levels when compared with controls, while 40μM explants exhibit almost no expression (Figure 8I). These results were consistent, independently of the stage.

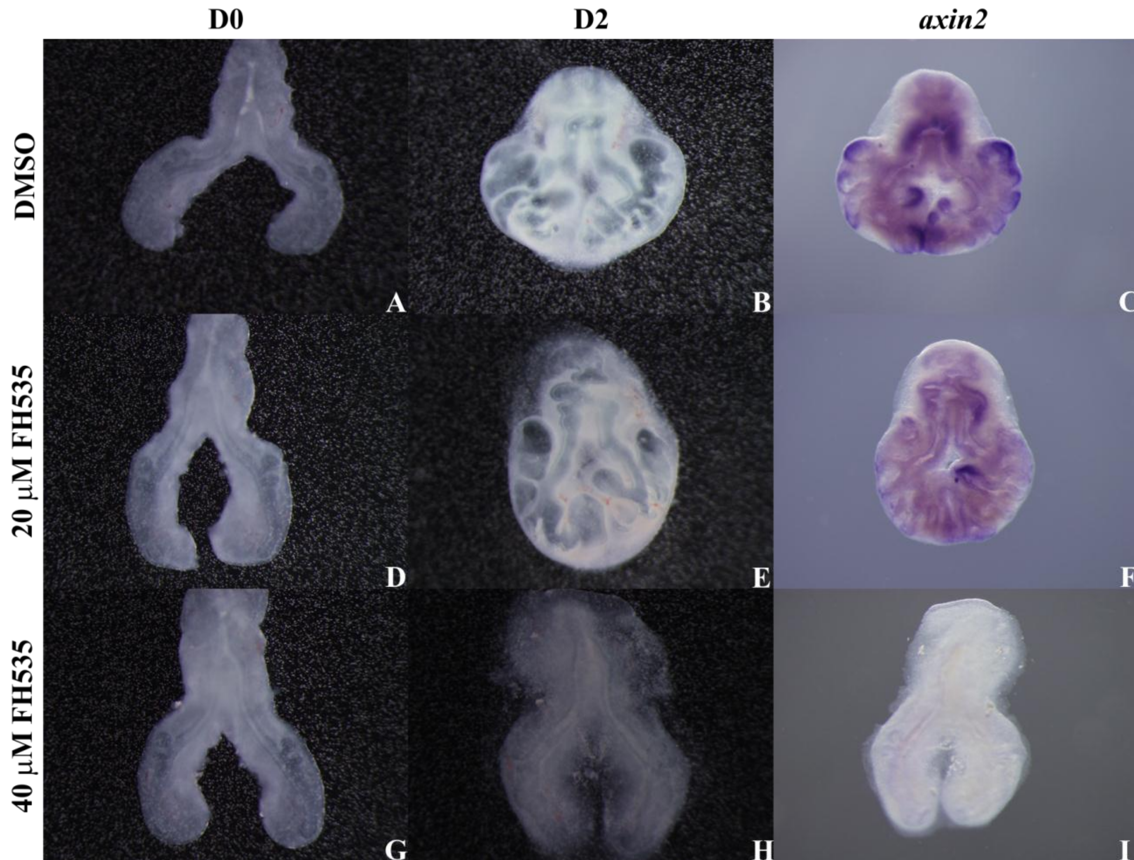

Figure 8 - *In vitro* Wnt signaling inhibition. Representative examples of stage b2 lung explant culture, at D0:0h (A, D, G) and D2:48h (B, E, H) treated with DMSO (A, B), 20μM (D, E) and 40μM FH535 (G, H) and probed with *axin2* (C, F, I) – n=5 for each stage. Magnification: A, B, D, E, G, H – 4x; C, F, I – 5x.

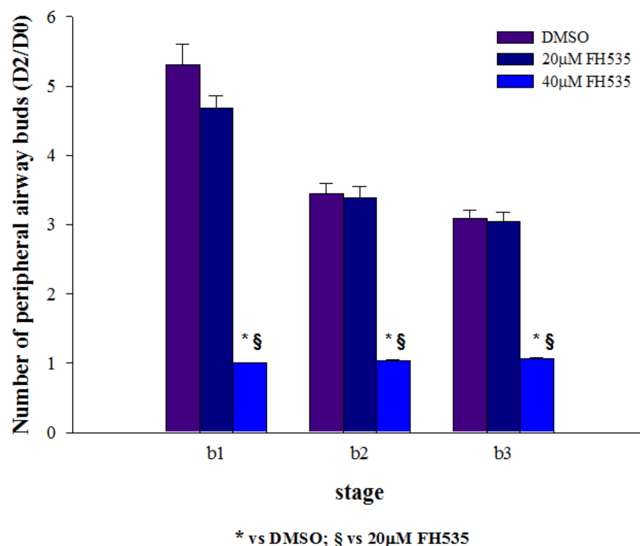

Figure 9 - Branching analysis of lung explants. Branching analysis of stage b1 (n=20 for each condition), b2 (n=30 for each different condition) and b3 (n=20 for each different condition) explants treated with DMSO and FH535 (20μM and 40μM). Results are expressed as D2/D0 ratio. Data is represented as mean ± SE. $p<0.05$ * *vs* DMSO, § *vs* 20μM of FH535.

3.2.2 Wnt inhibition and *hairy* genes

Lung explants treated with FH535 and DMSO were hybridized with *hairy1* and *hairy2* and the results are presented in Figure 10 and 11 respectively. *hairy1* expression is observed mainly in the pulmonary epithelium, which is in accordance with previous results. When compared with control, treated explants present a decrease of *hairy1* expression levels which is more evident in the highest dose studied.

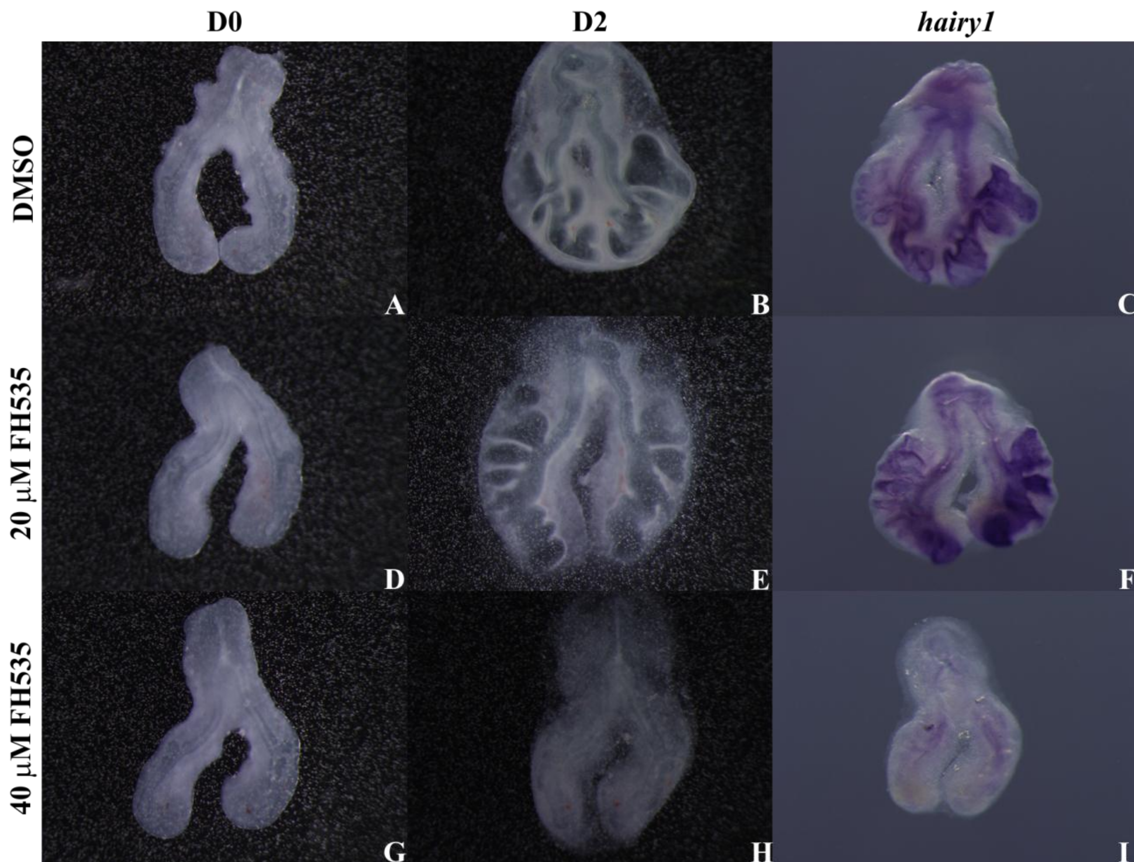

Figure 10 - *In vitro* Wnt signaling inhibition. Representative examples of stage b2 lung explant culture, at D0:0h (A, D, G) and D2:48h (B, E, H) treated with DMSO (A, B), 20μM (D, E) and 40μM FH535 (G, H) and probed with *hairy1* (C, F, I) – n=5 for each stage. Magnification: A, B, D, E, G, H – 4x; C, F, I – 5x.

In turn, *hairy2* expression is confined to lung mesenchyme which is in agreement with former studies. There is an obvious decrease in *hairy2* expression levels when compared to control explants, in both doses. This reduction is more patent in the 40μM FH535 treated explants (Figure 11).

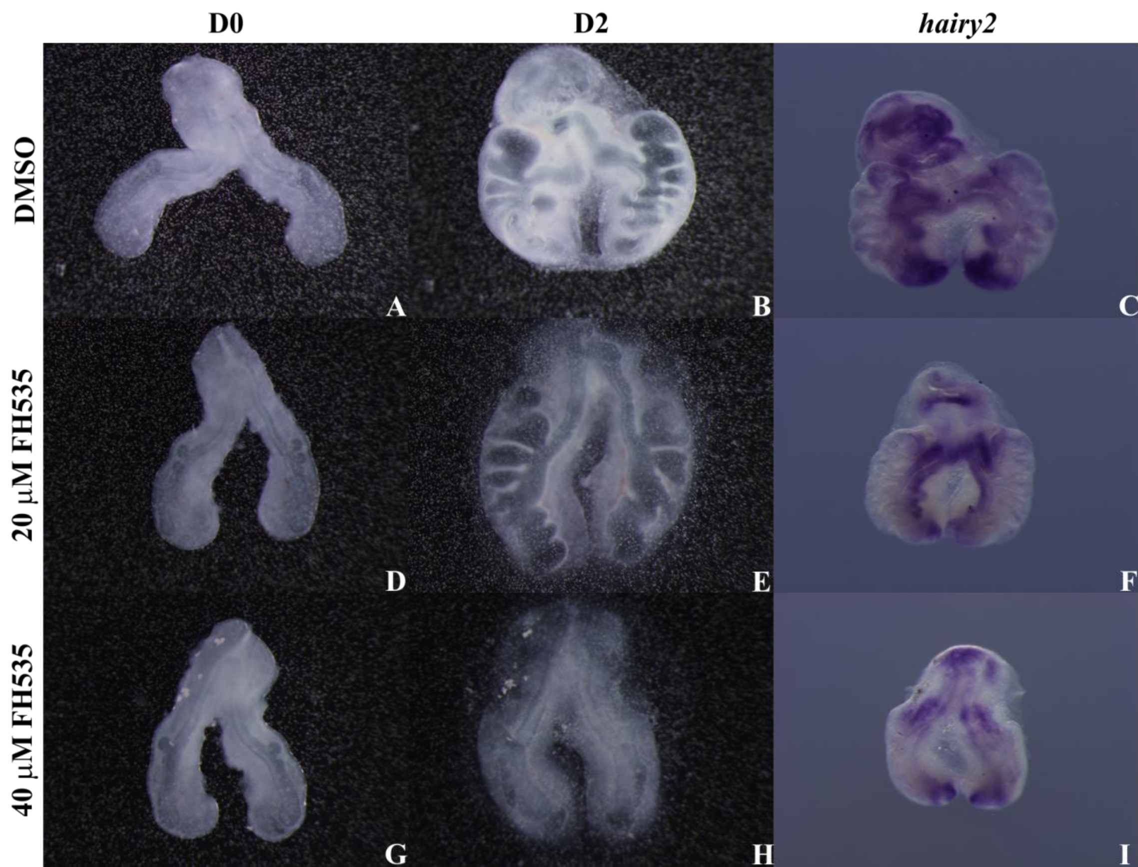

Figure 11 - *In vitro* Wnt signaling inhibition. Representative examples of stage b2 lung explant culture, at D0:0h (A, D, G) and D2:48h (B, E, H) treated with DMSO (A, B), 20µM (D, E) and 40µM FH535 (G, H) and probed with *hairy2* (C, F, I) – n=5 for each stage. Magnification: A, B, D, E, G, H – 4x; C, F, I – 5x.

4. Discussion

Wnt signaling pathway is of crucial importance during several embryonic developmental processes, namely the organogenesis of numerous organs, including the lung.[107] It has been established that lung development is coordinated by intrinsic epithelial-mesenchymal interactions involving numerous signaling pathways, such as FGF, BMP, HH and Wnt, which control several developing processes, like proliferation, differentiation and cell fate. However, there is little information regarding the control of the spatiotemporal mechanisms in this organ. Several animal models have been used in the study of pulmonary development, such is the case of the chick embryo, which has been recently validated for branching studies.[18]

The present work characterizes, for the first time, the expression pattern of some members of the Wnt signaling pathway, in early stages of chick lung development, such as *wnt-2b*, *wnt-5a*, *wnt-7b*, *wnt-9a, β-catenin* and *axin2*. Moreover, Wnt signaling pathway's role in the chick pulmonary branching process was accessed through *in vitro* inhibition studies. Considering that Wnt signaling pathway has already been linked to the molecular clock in other organs, the present work also analyzes the possible role of this signaling pathway in the regulation of two genes (*hairy1* and *hairy2*) that have already been associated with the molecular mechanisms of spatiotemporal development.[35, 96]

4.1 Expression pattern of Wnt signaling members

This study describes for the first time, the expression pattern of several Wnt family members, by *in situ* hybridization.

wnt-2b expression in chick lung is restricted to the mesenchymal tissue with higher expression in the medial region of the lung and no expression in trachea. These results are consistent with those of its homologue in mice where both *wnt-2* and *wnt-2b* are expressed in the mesenchyme, and that are known to have a role in lung specification of the foregut.[74]

wnt-5a expression in the chick respiratory tract is restricted to the trachea, in these stages of development. In embryonic mouse lungs (E12), this gene is greatly expressed in distal lung epithelium and surrounding mesenchyme; the highest level of *wnt-5a* expression however, is localized to the area of the pharynx,[68] which is consistent with the expression pattern in the chick lung.[108] De Langhe *et al* also described low levels of expression in the mesenchyme and epithelium, and high levels around the pharynx and

proximal trachea. A previous study carried out in chick embryos showed that by E7 *wnt-5a* appears to have no expression in the lung, but is already expressed in the esophagus. After embryonic day 11, *wnt-5a* expression is detected in both lung epithelium and adjacent mesenchyme, suffering a declination in the mesenchyme until hatching, becoming exclusively epithelial.[13]

While Wnt canonical pathway is known to regulate lung development early in branching morphogenesis, non-canonical pathway activators such as Wnt-5a seem to have an important role in mid-to-late gestational stages of airway and vascular patterning (during alveolarization), possibly through regulation of other major pathways (FGF and Shh).[107] In fact, Wnt mis/overexpression leads to abnormal chick lung phenotype from E10 onwards.[13] On the other hand, *wnt5a* (-/-) lungs present the correct number of lobes, nevertheless a truncation of the trachea occurs and abnormalities in distal lung architecture are detected without affecting pulmonary cellular differentiation. Chick *wnt-5a* expression is confined to the trachea region and may indicate a possible role of this gene in the morphogenesis of the proximal area of the lung at early stages of development.

In chick embryos, *wnt-7b* is clearly expressed in the entire epithelium compartment of the lung, mostly in the distal tips. In mouse embryonic lung *wnt-7b* is also expressed in the airway epithelium.[108, 109] It has been shown that epithelial Wnt-7b signaling is required for proper lung mesenchymal growth/differentiation and vascular development. Actually, *wnt7b* (-/-) mice die perinatally as a result of lung hypoplasia due a decrease in early mesenchymal proliferation, evident since E12.5, and severe defects in the smooth muscle of the major pulmonary vessels leading to an extensive hemorrhage at birth;[33] this event is a clear example of how inductive interactions between mesenchyme and epithelium are crucial to the generation and lung patterning. *wnt-7b* chick lung expression pattern is in agreement with its mammalian counterpart which might indicate a similar regulatory function for this gene in the chick.

wnt-9a presents a very characteristic pattern, since its expression is restricted to the epithelium of the peripheral airways and the tips of the main bronchi. It had already been described in *Xenopus* the presence of this gene in the lung primordia and in lung buds but without further description.[110] *wnt-9a* expression pattern was already described in mouse[111] chick limb development,[112] chick hepatic development[113] and in eye development[53].

However, this is the first time that is characterized, in detail, in early stages of lung development. The clear expression pattern of *wnt-9a* in the most distal areas of the lung, namely the peripheral airways and the tips of the main bronchi, may suggest a role in lung growth, as occurs with *wnt-7b* that, despite having a clear epithelial location, is also important for mesenchymal proliferation.

β-catenin is one of the most studied members of Wnt signaling pathway since it is responsible for the activation of transcription factors like Tcf/Lef, in the Wnt canonical pathway. In the chick lung, *β-catenin* is present in the mesenchyme and in the growing epithelial tips. In mouse, *β-catenin* is detected in epithelial cells lining the more peripheral lung tubules, and is not frequently observed in larger conducting airways.[114] Additionally, it was shown that β-catenin signaling is required for the formation of the distal, but not the proximal, airways since it's required for the determination of cell fate in the lung. In human lung it is highly expressed in the peripheral epithelium**.** Chick lung expression pattern is somehow consistent with the expression in mammalians, which might point to *β-catenin* as an important mediator in vertebrate lung development.

axin2, a direct readout of Wnt canonical signaling pathway, has been largely characterized during embryonic development.[115] In the chick lung, is present in the peri-epithelial mesenchyme around the secondary airways and the tip of the main bronchi. In human fetal lung *axin2* is expressed mainly in the peripheral epithelium of human fetal lung but also at low levels in the sub-adjacent mesenchyme.[115] The expression is observed in different compartments in these two models, but the localization is maintained: adjacent to the growing ends of the lung.

Considering the expression patterns described in this study, one might hypothesize that, in the chick lung, Wnt signaling might be involved in epithelium-mesenchyme interactions required for proper pulmonary development; also, that the overall mechanisms may perhaps be maintained across vertebrates, despite some minor expression differences between species.

4.2 Wnt signaling inhibition

Wnt signaling pathway is a major player during vertebrate's embryonic development that has been associated with several developmental process. As stated

previously, Wnt signaling is known to play an important role in some aspects of lung development such as: specification of the foregut,[74] mid-to-late gestational airway and vascular patterning,[107] regulation of distal lung morphogenesis[68] and lung mesenchymal growth/differentiation and vascular development.[33] So far, the effect on early branching morphogenesis has not been described in the chick embryonic lung and for that purpose, *in vitro* inhibition studies were performed.

Lung explants treated with 20µM of FH535 are similar to control explants. On the other hand, explants treated with the highest dose, 40µM, are quite different from controls and, consequently, from 20µM treated explant (Figure 8). 40µM treated explants don't form new secondary buds, after 48 hours in culture. To demonstrate that this morphological alteration was due to Wnt inhibition, lung explants were probed for *axin2* expression, a known readout of the canonical pathway. When compared to controls, 40µM treated explants lack *axin*2 mRNA (Figure 8I) supporting that Wnt signaling is clearly abolished in this condition. The observed phenotype most probably is the result of the Wnt pathway down-regulation. Conversely, 20µM treated explants display only a slight decrease in *axin2* expression level (Figure 8F), when compared with control explants (Figure 8C), which indicate that Wnt signaling is not fully repressed in this condition explaining the observed phenotype.

The morphometric analysis, assessed by the number of new secondary formed after 48 hours in culture (D2/D0 ratio), confirmed that Wnt signaling inhibition affects lung branching (Figure 9). Lung explants treated with the highest dose of FH535 (a chemical antagonist of this signaling pathway) don´t exhibit new secondary buds after 48 hours in culture, in the three stages studied. Only the higher dose of inhibitor induced a statistically significant decrease ($p<0.05$) when compared to both DMSO and lower doses treated explants. These results are consistent with those published by De Langhe *et al.*, who described a decrease in lung branching in DKK1 treated explants. Dickkopf-1 (DKK1) is a potent and specific inhibitor of Wnt action that is secreted by the distal lung epithelium. DKK1 treated explants are characterized by a defect in cleft formation due to a decrease in fibronectin (FN) deposition.[108] The extracellular matrix protein FN secreted by lung epithelium is known to be a Wnt target gene in *Xenopus*,[116] and is recognized as essential for cleft formation during the initiation of epithelial branching in several organ systems including the lung.[117] These results prove that Wnt signaling pathway is also important in

early stages of chick lung branching morphogenesis probably through the same molecular mechanisms described in mammalian, reinforcing that despite the differences in adult anatomies and functions, the molecular mechanisms of development are conserved.

4.3 Wnt signaling pathway in regulation of hairy genes expression

hairy1 and *hairy2* genes, that code for transcriptional repressor proteins, have been associated with the molecular clock mechanism described in segmentation processes such as somitogenesis[79] and limb skeletal elements formation[84]. The oscillatory expression of these genes has been correlated with the formation of repetitive elements, in both structures.

Branching morphogenesis and segmentation process share some resemblances: they all occur along the AP axis, the signaling pathways implicated are the same and 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). For this reason, the expression pattern of *hairy1* and *hairy2* had already been characterized in early stages of chick lung development.[100] Furthermore, it seems that these genes present a dynamic expression since lungs belonging to the same developmental stage exhibit differences in their expression pattern mainly in distal epithelium of the main bronchus. Moreover, previous studies have already determined the role of distinct signaling pathways (Notch, FGF and Shh) in the regulation of *hairy* expression, by *in vitro* inhibition studies. In the three cases, *hairy1* expression is not affected by the inhibition of these pathways in the conditions studied. On the other hand, only Notch inhibition induces a slight decrease in *hairy2* expression levels although not in all regions in the same way: tracheal area and the tracheal bifurcation showed only a minor decrease in *hairy2* expression.[100] Since the aforementioned experiments didn't undoubtedly show an association between Notch, FGF and Shh signaling pathways and *hairy* regulation, the role of Wnt signaling in the *hairy* gene expression was evaluated. For this purpose, *in vitro* inhibition studies were performed and explants were then analyzed for alterations in *hairy1* and *hairy2* expression levels.

hairy1 expression levels are decreased in FH353 treated explants, mainly in the highest dose tested (Figure 10); concerning *hairy2*, its expression in lung mesenchyme is also affected especially in explants treated with the highest dose (Figure 11).

The association between Wnt and Notch signaling has already been shown in the somitogenesis process. Aulehla *et al* demonstrated that the misexpression of *axin2*, a Wnt signaling target, affected the segmentation process since the somites were irregular in size and were not aligned on either side of the neural tube; in this study, it was also revealed that *axin2* expression in the PSM and the tail bud alternates with *Lfng* (a Notch signaling pathway cyclic gene) and continues even when Notch signaling is impaired. On the other hand, when *axin2* is misexpressed in the PSM, *Lfng* is upregulated, disrupting its cyclic expression pattern, and consequently disturbing the segmentation process. Also, when there was no Wnt-3a activity, *Lfng* was downregulated and didn't show a cyclic expression pattern.[103]

In another study, Li *et al* evaluated a possible interaction between Wnt and Notch signaling pathways, in early stages of cardiac differentiation: cells treated with LiCl or Wnt-3a exhibited an upregulation of *Notch-1* and *NICD-1* levels, whereas treatment with Dkk-1 (an inhibitor of Wnt signaling pathway) lead to a downregulation of *Notch-1* and *NICD-1* expression. Wnt-3a also upregulates the *hes1* promoter in undifferentiated cells. It was revealed that β-catenin binds with the NICD-1 and RBP-Jκ complex in the *hes1* promoter, indicating a direct regulation of canonical Wnt signaling on *hes1* transcription.[118]

A different study, performed in mice, relating to central nervous system development revealed that treatment with Wnt-3a caused a decrease in *hes1* and *hes5* expression levels. In this case, an increase of canonical Wnt signaling pathway interferes negatively with Notch signaling, enhancing neurogenesis.[119]

Taken together, these findings suggest that there is an interaction between canonical Wnt and Notch signaling pathways, even though with different outcomes, depending of the system/organ studied. *hairy1* is decreased when Wnt signaling is inhibited, however its expression in not Notch dependent in the chick lung. Wnt cascade might act directly in *hairy1* transcription by β-catenin/Tcf complex. *hairy2* expression is slightly diminished when Notch pathway is inhibited; in turn, Wnt inhibition also leads to a decrease in *hairy2*

expression levels. In this case, two scenarios are possible: *hairy2* might be regulated directly via β-catenin/Tcf complex or through Notch pathway by interacting with NICD-1 and RBP-Jκ complex as previously described in other systems.[96, 118, 119] This is the first evidence of *hairy* abrogation in the chick lung, however in order to establish a role for these genes in chick lung development it would be necessary to specifically target these genes instead of affecting the upstream signaling pathways. In cell culture, RNA interference is widely used however in whole tissues this method might not be the best approach due to the complex structure of the organ. An alternative method would be the use of virus (Replication-Competent Avian Retroviroses - RCAS or an adenovirus) injected directly in the tissue. Much work needs to be done in order to unveil the role of these genes in chick lung development.

5. Conclusion

The present work characterizes, for the first time, by *in situ* hybridization, the expression pattern of different Wnt signaling members in early stages of chick lung development, namely: *wnt-2b*, *wnt-5a*, *wnt-7b*, *wnt-9a*, *axin2* and *β-catenin*. Their expression patterns are in agreement with those of their mammalian counterparts, which may lead to the conclusion that the molecular mechanisms in early stages of lung development are conserved.

Moreover, in vitro inhibition studies were performed in order to determine the role of Wnt signaling in lung morphogenesis. Wnt inhibited lung explants presented branching impairment without formation of new secondary buds (decreased D2/D0 ratio), in the highest dose tested. In this condition, *axin2* expression was completely abolished indicating that Wnt signaling pathway was inhibited. These results show that Wnt signaling pathway is required for proper lung development.

Also, this study discloses an association between Wnt signaling and *hairy* genes in the lung, since their expression is downregulated when this pathway is inhibited. *hairy* transcription might be regulated directly via β-catenin/Tcf complex or indirectly by interaction of β-catenin with NICD-1 and RBP-Jκ complex.

6. References

1. Smith B. R., (2009). "The Multi-Dimensional Human Embryo." Retrieved 2011-12-15, from http://embryo.soad.umich.edu/.

2. Bellairs R. and Osmond M. (2005). The atlas of chick development. Amsterdam, Elsevier.

3. Gilbert S. F. (2006). Developmental biology. Sunderland, Massachusetts, Sinauer.

4. Huelsken J. and Behrens J. (2002). "The Wnt signalling pathway." *J Cell Sci* **115**(21): 3977-3978.

5. Logan C. Y. and Nusse R. (2004). "The Wnt signaling pathway in development and disease." *Annu Rev Cell & Dev Biol* **20**(1): 781-810.

6. Resende T. (2011). Temporal control of vertebrate embryo development: the role of Sonic Hedgehog in somite segmentation. Life and Health Sciences Research Institute. Braga, University of Minho. **PhD:** 172.

7. Hamburger V. and Hamilton H. L. (1951). "A Series of Normal Stages in the Development of the Chick Embryo." *J Morphol* **88**(1): 49-92.

8. Rubin H. (2011). "The early history of tumor virology: Rous, RIF, and RAV." *Proc Natl Acad Sci USA* **108**(35): 14389-14396.

9. Jing Y., Ma N., Fan T., Wang C., Bu X., Jiang G., Li R., Gao L., Li D., Wu M. and Wei L. (2011). "Tumor Necrosis Factor-alpha Promotes Tumor Growth by Inducing Vascular Endothelial Growth Factor." *Cancer Invest* **29**(7): 485-493.

10. Davey S., Darnell D. and Antin P., (2008). "Geisha database." Retrieved 2011-12-15, from http://geisha.arizona.edu/.

11. Chuang P.-T. and McMahon A. P. (2003). "Branching morphogenesis of the lung: new molecular insights into an old problem." *Trends Cell Biol* **13**(2): 86-91.

12. Horowitz A. and Simons M. (2009). "Branching Morphogenesis." *Circ Res* **104**(2): 784-795.

13. Loscertales M., Mikels A. J., Hu J. K. H., Donahoe P. K. and Roberts D. J. (2008). "Chick pulmonary Wnt5a directs airway and vascular tubulogenesis." *Development* **135**(7): 1365-1376.

14. Rawlins E. L. (2011). "The Building Blocks of Mammalian Lung Development." *Dev Dyn* **240**(3): 463-476.

15. Cardoso W. V. and Lü J. (2006). "Regulation of early lung morphogenesis: questions, facts and controversies." *Development* **133**(9): 1611-1624.

16. Alescio T. and Cassini A. (1962). "Induction in vitro of tracheal buds by pulmonary mesenchyme grafted on tracheal epithelium." *J Exp Zoo* **150**(2): 83-94.

17. Wessells N. K. (1970). "Mammalian lung development: Interactions in formation and morphogenesis of tracheal buds." *J Exp Zoo* **175**(4): 455-466.

18. Moura R. S., Coutinho-Borges J. P., Pacheco A. P., daMota P. O. and Correia-Pinto J. (2011). "FGF Signaling Pathway in the Developing Chick Lung: Expression and Inhibition Studies." *PLoS ONE* **6**(3): e17660.

19. Nogueira-Silva C., Santos M., Baptista M. J., Moura R. S. and Correia-Pinto J. (2006). "IL-6 is constitutively expressed during lung morphogenesis and enhances fetal lung explant branching." *Pediatr Res* **60**(5): 530.

20. Hans-Rainer D. (1974). "Structure of the avian respiratory tract." *Respir Physiol* **22**(1-2): 1-19.

21. Maina J. N. (2002). "Some recent advances on the study and understanding of the functional design of the avian lung: morphological and morphometric perspectives." *Biol Rev* **77**(1): 97-152.

22. Metzger R. J., Klein O. D., Martin G. R. and Krasnow M. A. (2008). "The branching programme of mouse lung development." *Nature* **453**(7196): 745-751.

23. Morrisey E. E. and Hogan B. L. M. (2010). "Preparing for the first breath: genetic and cellular mechanisms in lung development." *Dev Cell* **18**(1): 8-23.

24. Sekine K., Ohuchi H., Fujiwara M., Yamasaki M., Yoshizawa T., Sato T., Yagishita N., Matsui D., Koga Y., Itoh N. and Kato S. (1999). "Fgf10 is essential for limb and lung formation." *Nat Genet* **21**(1): 138-141.

25. Bellusci S., Grindley J., Emoto H., Itoh N. and Hogan B. L. (1997). "Fibroblast growth factor 10 (FGF10) and branching morphogenesis in the embryonic mouse lung." *Development* **124**(23): 4867-4878.

26. Harrison M. R., Keller R. L., Hawgood S. B., Kitterman J. A., Sandberg P. L., Farmer D. L., Lee H., Filly R. A., Farrell J. A. and Albanese C. T. (2003). "A Randomized Trial of Fetal Endoscopic Tracheal Occlusion for Severe Fetal Congenital Diaphragmatic Hernia." *New Eng J Med* **349**(20): 1916-1924.

27. Rottier R. and Tibboel D. (2005). "Fetal lung and diaphragm development in congenital diaphragmatic hernia." *Sem in Perinatol* **29**(2): 86-93.

28. Wang J. and Wynshaw-Boris A. (2004). "The canonical Wnt pathway in early mammalian embryogenesis and stem cell maintenance/differentiation." *Curr Op Gen & Dev* **14**(5): 533-539.

29. Hardy K. M., Garriock R. J., Yatskievych T. A., D'Agostino S. L., Antin P. B. and Krieg P. A. (2008). "Non-canonical Wnt signaling through Wnt5a/b and a novel Wnt11 gene, Wnt11b, regulates cell migration during avian gastrulation." *Dev Biol* **320**(2): 391-401.

30. Lickert H., Cox B., Wehrle C., Taketo M. M., Kemler R. and Rossant J. (2005). "Dissecting Wnt/β-catenin signaling during gastrulation using RNA interference in mouse embryos." *Development* **132**(11): 2599-2609.

31. Münsterberg A. E., Kitajewski J., Bumcrot D. A., McMahon A. P. and Lassar A. B. (1995). "Combinatorial signaling by Sonic hedgehog and Wnt family members induces myogenic bHLH gene expression in the somite." *Genes & Dev* **9**(23): 2911-2922.

32. Hobmayer B., Rentzsch F., Kuhn K., Happel C. M., von Laue C. C., Snyder P., Rothbächer U. and Holstein T. W. (2000). "WNT signalling molecules act in axis formation in the diploblastic metazoan Hydra." *Nature* **407**(6801): 186-189.

33. Shu W., Jiang Y. Q., Lu M. M. and Morrisey E. E. (2002). "Wnt7b regulates mesenchymal proliferation and vascular development in the lung." *Development* **129**(20): 4831-4842.

34. Pietilä I., Ellwanger K., Railo A., Jokela T., Barrantes I. d. B., Shan J., Niehrs C. and Vainio S. J. (2011). "Secreted Wnt antagonist Dickkopf-1 controls kidney papilla development coordinated by Wnt-7b signalling." *Dev Biol* **353**(1): 50-60.

35. Geetha-Loganathan P., Nimmagadda S. and Scaal M. (2008). "Wnt signaling in limb organogenesis." *Organogenesis* **4**(2): 109-115.

36. Church V. L. and Francis-West P. (2002). "Wnt signalling during limb development." *Int J Dev Biol* **46**(7): 927-936.

37. Quinlan R., Graf M., Mason I., Lumsden A. and Kiecker C. (2009). "Complex and dynamic patterns of Wnt pathway gene expression in the developing chick forebrain." *Neural Dev* **4**(1): 1-27.

38. Geetha-Loganathan P., Nimmagadda S., Antoni L., Fu K., Whiting C. J., Francis-West P. and Richman J. M. (2009). "Expression of WNT signalling pathway genes during chicken craniofacial development." *Dev Dyn* **238**(5): 1150-1165.

39. Person A. D., Garriock R. J., Krieg P. A., Runyan R. B. and Klewer S. E. (2005). "Frzb modulates Wnt-9a-mediated β-catenin signaling during avian atrioventricular cardiac cushion development." *Dev Biol* **278**(1): 35-48.

40. Hermle T., Petzoldt A. and Simons M. (2011). "The role of proton transporters in epithelial Wnt signaling pathways." *Pediatr Nephr* **26**(9): 1523-1527.

41. Rawadi G. and Roman-Roman S. (2005). "Wnt signalling pathway: a new target for the treatment of osteoporosis." *Exp Op Therap Targets* **9**(5): 1063-1077.

42. Vilariño-Güell C., Wider C., Ross O. A., Dachsel J. C., Kachergus J. M., Lincoln S J., Soto-Ortolaza A. I., Cobb S. A., Wilhoite G. J., Bacon J. A., Behrouz B., Melrose H. L., Hentati E., Puschmann A., Evans D. M., Conibear E., Wasserman W. W., Aasly J. O., Burkhard P. R., Djaldetti R., Ghika J., Hentati F., Krygowska-Wajs A., Lynch T., Melamed E., Rajput A., Rajput A. H., Solida A., Wu R.-M., Uitti R. J., Wszolek Z. K., Vingerhoets F. and Farrer M. J. (2011). "VPS35 Mutations in Parkinson Disease." *Am J Human Genet* **89**(1): 162-167.

43. Nusse R. and Varmus H. E. (1982). "Many tumors induced by the mouse mammary tumor virus contain a provirus integrated in the same region of the host genome." *Cell* **31**(1): 99-109.

44. He B. and Jablons D. M. (2007). Wnt Signaling in Stem Cells and Lung Cancer. Cancer Stem Cells. O. D. Wiestler, B. Haendler and D. Mumberg, Springer Berlin Heidelberg. **5:** 27-58.

45. Mazieres J., He B., You L., Xu Z. D. and Jablons D. A. (2005). "Wnt signaling in lung cancer." *Cancer Lett* **222**(1): 1-10.

46. van Es J. H. and Clevers H. (2005). "Notch and Wnt inhibitors as potential new drugs for intestinal neoplastic disease." *Trends Mol Med* **11**(11): 496-502.

47. Behrens J. and Lustig B. (2004). "The Wnt connection to tumorigenesis." *Int J Dev Biol* **48**(5-6): 477-487.

48. Verkaar F. and Zaman G. J. R. (2011). "New avenues to target Wnt/β-catenin signaling." *Drug Discov Today* **16**(1-2): 35-41.

49. Dodge M. E. and Lum L. (2011). "Drugging the Cancer Stem Cell Compartment: Lessons Learned from the Hedgehog and Wnt Signal Transduction Pathways." *Annu Rev Pharm Tox* **51**(1): 289-310.

50. Liu Y. G., Huang T. W., Zhao X. L. and Cheng L. P. (2011). "MicroRNAs modulate the Wnt signaling pathway through targeting its inhibitors." *Biochem Biophys Res Commun* **408**(2): 259-264.

51. Sheldahl L. C., Slusarski D. C., Pandur P., Miller J. R., Kühl M. and Moon R. T. (2003). "Dishevelled activates Ca^{2+} flux, PKC, and CamKII in vertebrate embryos." *J Cell Biol* **161**(4): 769-777.

52. Strutt D. I., Weber U. and Mlodzik M. (1997). "The role of RhoA in tissue polarity and Frizzled signalling." *Nature* **387**(6630): 292-295.

53. Fokina V. M. and Frolova E. I. (2006). "Expression patterns of Wnt genes during development of an anterior part of the chicken eye." *Dev Dyn* **235**(2): 496-505.

54. van Amerongen R. and Nusse R. (2009). "Towards an integrated view of Wnt signaling in development." *Development* **136**(19): 3205-3214.

55. Konigshoff M. and Eickelberg O. (2010). "WNT Signaling in Lung Disease A Failure or a Regeneration Signal?" *Am J Respir Cell Mol Biol* **42**(1): 21-31.

56. Mohamed O. A., Clarke H. J. and Dufort D. (2004). "β-catenin signaling marks the prospective site of primitive streak formation in the mouse embryo." *Dev Dyn* **231**(2): 416-424.

57. Jensen P. B., Pedersen L., Krishna S. and Jensen M. H. (2010). "A Wnt Oscillator Model for Somitogenesis." *Biophysic J* **98**(6): 943-950.

58. Shu W., Guttentag S., Wang Z., Andl T., Ballard P., Lu M. M., Piccolo S., Birchmeier W., Whitsett J. A., Millar S. E. and Morrisey E. E. (2005). "Wnt/β-catenin signaling acts upstream of N-myc, BMP4, and FGF signaling to regulate proximal–distal patterning in the lung." *Dev Biol* **283**(1): 226-239.

59. Skromne I. and Stern C. D. (2001). "Interactions between Wnt and Vg1 signalling pathways initiate primitive streak formation in the chick embryo." *Development* **128**(15): 2915-2927.

60. Ulloa F. and Martí E. (2010). "Wnt won the war: Antagonistic role of Wnt over Shh controls dorso-ventral patterning of the vertebrate neural tube." *Dev Dyn* **239**(1): 69-76.

61. Huelsken J. and Birchmeier W. (2001). "New aspects of Wnt signaling pathways in higher vertebrates." *Curr Op Gen & Dev* **11**(5): 547-553.

62. Qian D., Jones C., Rzadzinska A., Mark S., Zhang X., Steel K. P., Dai X. and Chen P. (2007). "Wnt5a functions in planar cell polarity regulation in mice." *Dev Biol* **306**(1): 121-133.

63. Huelsken J., Vogel R., Brinkmann V., Erdmann B., Birchmeier C. and Birchmeier W. (2000). "Requirement for β-Catenin in Anterior-Posterior Axis Formation in Mice." *J Cell Biol* **148**(3): 567-578.

64. Chapman S. C., Brown R., Lees L., Schoenwolf G. C. and Lumsden A. (2004). "Expression analysis of chick Wnt and frizzled genes and selected inhibitors in early chick patterning." *Dev Dyn* **229**(3): 668-676.

65. Monkley S. J., Delaney S. J., Pennisi D. J., Christiansen J. H. and Wainwright B. J. (1996). "Targeted disruption of the Wnt2 gene results in placentation defects." *Development* **122**(11): 3343-3353.

66. Yin A., Winata C. L., Korzh S., Korzh V. and Gong Z. (2010). "Expression of components of Wnt and Hedgehog pathways in different tissue layers during lung development in Xenopus laevis." *Gene Express Patt* **10**(7-8): 338-344.

67. Xu B., Chen C., Chen H., Zheng S. G., Bringas P., Xu M., Zhou X., Chen D., Umans L. and Zwijsen A. (2011). "Smad1 and its target gene Wif1 coordinate BMP and Wnt signaling activities to regulate fetal lung development." *Development* **138**(5): 925-935.

68. Li C., Xiao J., Hormi K., Borok Z. and Minoo P. (2002). "Wnt5a Participates in Distal Lung Morphogenesis." *Dev Biol* **248**(1): 68-81.

69. Levay-Young B. K. and Navre M. (1992). "Growth and developmental regulation of wnt-2 (irp) gene in mesenchymal cells of fetal lung." *Am J Physiol* **262**(6): L672-L683.

70. Lako M., Strachan T., Bullen P., Wilson D. I., Robson S. C. and Lindsay S. (1998). "Isolation, characterisation and embryonic expression of WNT11, a gene which maps to 11q13.5 and has possible roles in the development of skeleton, kidney and lung." *Gene* **219**(1–2): 101-110.

71. Weidenfeld J., Shu W., Zhang L., Millar S. E. and Morrisey E. E. (2002). "The WNT7b Promoter Is Regulated by TTF-1, GATA6, and Foxa2 in Lung Epithelium." *J Biol Chem* **277**(23): 21061-21070.

72. Sakiyama J.-i., Yokouchi Y. and Kuroiwa A. (2000). "Coordinated Expression of Hoxb Genes and Signaling Molecules during Development of the Chick Respiratory Tract." *Dev Biol* **227**(1): 12-27.

73. Eberhart C. G. and Argani P. (2001). "Wnt signaling in human development: β-catenin nuclear translocation in fetal lung, kidney, placenta, capillaries, adrenal, and cartilage." *Pediatr Dev Pathol* **4**(4): 351-357.

74. Goss A. M., Tian Y., Tsukiyama T., Cohen E. D., Zhou D., Lu M. M., Yamaguchi T. P. and Morrisey E. E. (2009). "Wnt2/2b and β-Catenin Signaling Are Necessary and Sufficient to Specify Lung Progenitors in the Foregut." *Dev Cell* **17**(2): 290-298.

75. Yamaguchi T. P., Bradley A., McMahon A. P. and Jones S. (1999). "A Wnt5a pathway underlies outgrowth of multiple structures in the vertebrate embryo." *Development* **126**(6): 1211-1223.

76. Rajagopal J., Carroll T. J., Guseh J. S., Bores S. A., Blank L. J., Anderson W. J., Yu J., Zhou Q., McMahon A. P. and Melton D. A. (2008). "Wnt7b stimulates embryonic lung growth by coordinately increasing the replication of epithelium and mesenchyme." *Development* **135**(9): 1625-1634.

77. Özbudak E. M. and Pourquié O. (2008). "The vertebrate segmentation clock: the tip of the iceberg." *Curr Op Gen & Dev* **18**(4): 317-323.

78. Pourquié O. (2004). "The chick embryo: a leading model in somitogenesis studies." *Mech Dev* **121**(9): 1069-1079.

79. Palmeirim I., Henrique D., Ish-Horowicz D. and Pourquié O. (1997). "Avian hairy Gene Expression Identifies a Molecular Clock Linked to Vertebrate Segmentation and Somitogenesis." *Cell* **91**(5): 639-648.

80. Bessho Y. and Kageyama R. (2003). "Oscillations, clocks and segmentation." *Curr Op Gen & Dev* **13**(4): 379-384.

81. Kageyama R., Ohtsuka T. and Kobayashi T. (2007). "The Hes gene family: repressors and oscillators that orchestrate embryogenesis." *Development* **134**(7): 1243-1251.

82. Fisher A. L., Ohsako S. and Caudy M. (1996). "The WRPW motif of the hairy-related basic helix-loop-helix repressor proteins acts as a 4-amino-acid transcription repression and protein-protein interaction domain." *Mol Cell Biol* **16**(6): 2670-2677.

83. Jouve C., Palmeirim I., Henrique D., Beckers J., Gossler A., Ish-Horowicz D. and Pourquie O. (2000). "Notch signalling is required for cyclic expression of the hairy-like gene HES1 in the presomitic mesoderm." *Development* **127**(7): 1421-1429.

84. Pascoal S. (2006). New aspects in coordination of vertebrate limb bud development. Life and Health Research Institute. Braga, University of Minho**:** 220.

85. Aulehla A. and Herrmann B. G. (2004). "Segmentation in vertebrates: clock and gradient finally joined." *Genes & Dev* **18**(17): 2060-2067.

86. Aulehla A. and Pourquié O. (2008). "Oscillating signaling pathways during embryonic development." *Curr Op Cell Biol* **20**(6): 632-637.

87. Pourquié O. (2002). "Vertebrate Segmentation: Lunatic Transcriptional Regulation." *Curr Biol* **12**(20): R699-R701.

88. Jiang Y.-J., Smithers L. and Lewis J. (1998). "Vertebrate segmentation: The clock is linked to Notch signalling." *Curr Biol* **8**(24): R868-R871.

89. Pourquié O. (1999). "Notch around the clock." *Curr Op Gen & Dev* **9**(5): 559-565.

90. Jouve C., Iimura T. and Pourquie O. (2002). "Onset of the segmentation clock in the chick embryo: evidence for oscillations in the somite precursors in the primitive streak." *Development* **129**(5): 1107-1117.

91. Aulehla A. and Johnson R. L. (1999). "Dynamic Expression of *Lunatic fringe* Suggests a Link between *Notch* Signaling and an Autonomous Cellular Oscillator Driving Somite Segmentation." *Dev Biol* **207**(1): 49-61.

92. Dale J. K., Maroto M., Dequeant M. L., Malapert P., McGrew M. and Pourquie O. (2003). "Periodic Notch inhibition by Lunatic Fringe underlies the chick segmentation clock." *Nature* **421**(6920): 275-278.

93. Wahl M. B., Deng C., Lewandoski M. and Pourquié O. (2007). "FGF signaling acts upstream of the NOTCH and WNT signaling pathways to control segmentation clock oscillations in mouse somitogenesis." *Development* **134**(22): 4033-4041.

94. Niwa Y., Masamizu Y., Liu T., Nakayama R., Deng C.-X. and Kageyama R. (2007). "The Initiation and Propagation of Hes7 Oscillation Are Cooperatively Regulated by Fgf and Notch Signaling in the Somite Segmentation Clock." *Dev Cell* **13**(2): 298-304.

95. Dequéant M.-L., Glynn E., Gaudenz K., Wahl M., Chen J., Mushegian A. and Pourquié O. (2006). "A Complex Oscillating Network of Signaling Genes Underlies the Mouse Segmentation Clock." *Science* **314**(5805): 1595-1598.

96. Aulehla A., Wehrle C., Brand-Saberi B., Kemler R., Gossler A., Kanzler B. and Herrmann B. G. (2003). "Wnt3a Plays a Major Role in the Segmentation Clock Controlling Somitogenesis." *Dev cell* **4**(3): 395-406.

97. Aulehla A., Wiegraebe W., Baubet V., Wahl M. B., Deng C., Taketo M., Lewandoski M. and Pourquie O. (2008). "A β-catenin gradient links the clock and wavefront systems in mouse embryo segmentation." *Nat Cell Biol* **10**(2): 186-193.

98. Sasai Y., Kageyama R., Tagawa Y., Shigemoto R. and Nakanishi S. (1992). "Two mammalian helix-loop-helix factors structurally related to Drosophila hairy and Enhancer of split." *Genes and Dev* **6**(12b): 2620-2634.

99. Ito T., Udaka N., Yazawa T., Okudela K., Hayashi H., Sudo T., Guillemot F., Kageyama R. and Kitamura H. (2000). "Basic helix-loop-helix transcription factors regulate the neuroendocrine differentiation of fetal mouse pulmonary epithelium." *Development* **127**(18): 3913-3921.

100. Pacheco A. P. (2010). Characterization of *hairy1* and *hairy2* pattern expression during lung development of *Gallus gallus*. Life and Health Sciences Research Institute. Braga, University of Minho. **Master**.

101. Hollyday M., McMahon J. A. and McMahon A. P. (1995). "Wnt expression patterns in chick embryo nervous system." *Mech of Dev* **52**(1): 9-25.

102. Schmidt M., Tanaka M. and Munsterberg A. (2000). "Expression of β-catenin in the developing chick myotome is regulated by myogenic signals." *Development* **127**(19): 4105-4113.

103. Gibb S., Zagorska A., Melton K., Tenin G., Vacca I., Trainor P., Maroto M. and Dale J. K. (2009). "Interfering with Wnt signalling alters the periodicity of the segmentation clock." *Dev Biol* **330**(1): 21-31.

104. Ng J. K., Kawakami Y., Büscher D., Raya Á., Itoh T., Koth C. M., Esteban C. R., Rodríguez-León J., Garrity D. M., Fishman M. C. and Belmonte J. C. I. (2002). "The limb identity gene Tbx5 promotes limb initiation by interacting with Wnt2b and Fgf10." *Development* **129**(22): 5161-5170.

105. McBride H. J., Fatke B. and Fraser S. E. (2003). "Wnt signaling components in the chicken intestinal tract." *Dev Biol* **256**(1): 18-33.

106. Henrique D., Adam J., Myat A., Chitnis A., Lewis J. and Ish-Horowicz D. (1995). "Expression of a Delta homologue in prospective neurons in the chick." *Nature* **375**(6534): 787-790.

107. Pongracz J. E. and Stockley R. A. (2006). "Wnt signalling in lung development and diseases." *Resp Res* **7**.

108. De Langhe S. P., Sala F. G., Del Moral P. M., Fairbanks T. J., Yamada K. M., Warburton D., Burns R. C. and Bellusci S. (2005). "Dickkopf-1 (DKK1) reveals that fibronectin is a major target of Wnt signaling in branching morphogenesis of the mouse embryonic lung." *Dev Biol* **277**(2): 316-331.

109. Pepicelli C. V., Lewis P. M. and McMahon A. P. (1998). "Sonic hedgehog regulates branching morphogenesis in the mammalian lung." *Curr biol: CB* **8**(19): 1083-1086.

110. Garriock R. J., Warkman A. S., Meadows S. M., D'Agostino S. and Krieg P. A. (2007). "Census of vertebrate Wnt genes: Isolation and developmental expression of Xenopus Wnt2, Wnt3, Wnt9a, Wnt9b, Wnt10a, and Wnt16." *Dev Dyn* **236**(5): 1249-1258.

111. Witte F., Dokas J., Neuendorf F., Mundlos S. and Stricker S. (2009). "Comprehensive expression analysis of all Wnt genes and their major secreted antagonists during mouse limb development and cartilage differentiation." *Gene Expr Patt* **9**(4): 215-223.

112. Loganathan P. G., Nimmagadda S., Huang R., Scaal M. and Christ B. (2005). "Comparative analysis of the expression patterns of *Wnt*s during chick limb development." *Histochem and Cell Biol* **123**(2): 195-201.

113. Matsumoto K., Miki R., Nakayama M., Tatsumi N. and Yokouchi Y. (2008). "Wnt9a secreted from the walls of hepatic sinusoids is essential for morphogenesis, proliferation, and glycogen accumulation of chick hepatic epithelium." *Developmental Biology* **319**(2): 234-247.

114. Mucenski M. L., Wert S. E., Nation J. M., Loudy D. E., Huelsken J., Birchmeier W., Morrisey E. E. and Whitsett J. A. (2003). "β-Catenin Is Required for Specification of Proximal/Distal Cell Fate during Lung Morphogenesis." *J Biol Chem* **278**(41): 40231-40238.

115. Zhang M., Shi J., Huang Y. and Lai L. (2012). "Expression of canonical WNT/β-CATENIN signaling components in the developing human lung." *Bmc Dev Biol* **12**(1): 21.

116. Gradl D., Kühl M. and Wedlich D. (1999). "The Wnt/Wg Signal Transducer β-Catenin Controls Fibronectin Expression." *Molecular and Cellular Biology* **19**(8): 5576-5587.

117. Sakai T., AU - Larsen M. and AU - Yamada K. M. (2003). "Fibronectin requirement in branching morphogenesis." *Nature* **423**(6942): 876 - 881.

118. Li B., Jia Z., Wang T., Wang W., Zhang C., Chen P., Ma K. and Zhou C. (2012). "Interaction of Wnt/β-catenin and notch signaling in the early stage of cardiac differentiation of P19CL6 cells." *J Cell Biochem* **113**(2): 629-639.

119. Hirsch C., Campano L. M., Wöhrle S. and Hecht A. (2007). "Canonical Wnt signaling transiently stimulates proliferation and enhances neurogenesis in neonatal neural progenitor cultures." *Exp Cell Res* **313**(3): 572-587.

7. Appendices

7.1 Appendix 1

- **PBS "Phosphate Buffered Saline" 10x solution**

For 2L solution (RNase-free material):

160g NaCl

4g KH_2PO_4

23g $Na_2HPO_4.2H_2O$

Fill up the volume with ultrapure H_2O;

$7.2 \leq pH \leq 7.4$;

Sterilize;

Store at room temperature.

- **PBS 1x**

1% PBS 10x

Fill up the volume with ultrapure water;

Store at room temperature.

- **Formaldehyde fixation solution**

89.8% PBS 1x, without Ca^{2+} and Mg^{2+}, with EGTA;

10% Formaldehyde at 37%;

0.2% NaOH 1M.

- **PBT**

For 2L solution:

2L PBS 1x;

2mL Tween 20 (100%);

Store at room temperature.

- **Proteinase K** (20mg/mL)

Add 5mL of ultrapure H_2O to 100mg Proteinase K;

Prepare aliquots of 25µL;

Store at -20ºC.

- **Post-fixation solution**

89.6% PBT 1x;

10% Formaldehyde at 37%;

0.4% Glutaraldehyde at 25%.

- **Hybridization solution**

50% Formamide;

6.5% SSC buffer (20x);

1% EDTA (0.5M);

0.25% t-RNA (20mg/mL);

0.2% Tween 20 (100%);

0.5% CHAPS

0.2% Heparine (50mg/mL)

41.35% Ultrapure H_2O.

Store at -20ºC.

- **Heparine**

For each 25000 U add 3.1mL of ultrapure water and prepare aliquots of 400µL;

Store at -20ºC.

- **Probe**

Add 5µL of probe for each 1mL of Hybmix;

- **MAB 5x**

0.5M Maleic Acid ($C_4H_4O_4$);

744mM NaCl;

NaOH (adjust pH at 7.5);

Fill up the volume with ultrapure H_2O.

- **MABT (1x)**

20% MAB 5x;

1% Tween 20;

Fill with ultrapure water.

- **Blocking Reagent**

For 100mL

10g Blocking Reagent

100mL MAB 5x

- **NTMT**

0.1M NaCl;

0.1M Tris-HCl

50mM $MgCl_2$

1% Tween 20

- **Revelation Solution**

0.34% NBT

0.35% BCIP

99.31% NTMT

- **Azide (10x)**

For 50mL:

5g NaN_3

50mL H_2O ultrapure

- **PBT/Azide**

For every 50mL PBT add 500µL of Azide solution;

Store at 4ºC.

- **EGTA 0.5M pH 8**

For each 100mL:

19.02% EGTA (ethylene glycol)

4.34g NaOH

Fill up the volume with ultrapure H_2O

- **PBS, without Ca^{2+} and Mg^{2+}, with EGTA**

0.4% EGTA 0.5M

99.6% PBS 1x, without Ca^{2+} and Mg^{2+}

Store at 4ºC.

- **EDTA 0.5M pH 8**

19.01% EDTA tetrasodium salt;

Fill the volume with ultrapure H_2O;

Adjust pH with NaOH;

- **Tris-HCl 2M pH 9.5**

24.22% Tris-base (Trizma Base);

Fill the volume with ultrapure H_2O;

Adjust pH.

- **Sodium Chloride 5M**

For 1L:

29.25% NaCl;

Fill up the volume with ultrapure H_2O.

- **Magnesium Chloride ($MgCl_2$) 2M**

For 100mL:

40.66% $MgCl_2.6H_2O$

Fill up the volume with ultrapure H_2O.

- **Sodium Hidroxide (NaOH) 1M**

For 20mL:

4% NaOH

Fill up the volume with ultrapure water.

- **TE**

For 100mL:

1mL Tris-base 1M pH 8

0.2mL EDTA 0.5M pH 8

Fill up the volume with ultrapure water.

- **Tris-base 1M pH 8**

For 100mL:

12.11% Tris-base (Trizma base)

Fill up the volume with ultrapure water.

- **tRNA**

Add 5mL TE to 100mg tRNA;

Store at -20ºC (50µL aliquots).

- **Solid Luria-Broth (LB) medium**

For 1L:

10g Bactotriptone;

5g yeast extract;

10g NaCl;

20g agar;

Fill up the volume with ultrapure H_2O;

After sterilization, let cool down and then add Ampicilin (2mL/L), X-Gal (1mL/L) and IPTG (1mL/L); Plate.

- **Super Optimal Broth with Catabolite repression (SOC) medium**

For 500mL:

10g Tryptone;

2.5g Yeast extract;

0.0932g KCl

0.2922g NaCl;

1.2324g $MgSO_4.7H_2O$;

1.9817g Glucose. H_2O.

7.2 Appendix 2

Table 1 – Information regarding Wnt signaling members, after sequencing, obtained from Molecular Biology services at University of Minho.

Gene	Acession number	Description	Homology
β-catenin	NM_205081.1	*Gallus gallus* beta-catenin (chBcat) mRNA, complete cds: 3281 nts	98%
wnt-2b	NM_204336.1	*Gallus gallus* wingless-type MMTV integration site family, member 2B (WNT2B), mRNA > Gallus gallus wg/int-1 related gene product WNT-2B (Wnt-2b) mRNA, complete cds : 1158 nts	99%
wnt-9a	NM_204981.1	*Gallus gallus* wingless-type MMTV integration site family member 9a (WNT9a) mRNA, complete cd 1121nts	99%
axin2	NM_204491.1	*Gallus gallus* axin-related protein (AXIN2) mRNA, complete cds Length=2790	100%