Universidade do Minho
Escola de Ciências

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Deciphering the role of Vitamin D in early lung development

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Deciphering the role of Vitamin D in early lung development

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Professora Doutora Rute Carina Silva Moura
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DECIPHERING THE ROLE OF VITAMIN D IN EARLY LUNG DEVELOPMENT - ABSTRACT

The high prevalence of vitamin D deficiency is currently considered a public health concern, affecting people of all ages worldwide, particularly childbearing women and children. There is now a large body of evidence linking maternal vitamin D deficiency and several maternal and fetal adverse health outcomes. Regarding the developing lung, it has been demonstrated that Vitamin D is crucial for lung maturation processes, and it influences the respiratory health of the offspring. Most observations point to a critical role of vitamin D during the later stages of lung development; however, little is known about its role in early branching stages, and nothing is known about its role in avian lung development. In this sense, this project aims to unravel the role of Vitamin D during early lung development, using the chicken embryo as a model. The present work unveils, for the first time, the expression pattern of Vitamin D signaling members (*rxr-α*, *rxr-γ*, *vdr*, *cyp24a1,* and *pdia3*) by *in situ* hybridization in embryonic chick lung. The similarity between the expression patterns and low expression levels of these genes with the mammalian lung point to a similar and, eventually, minor role during these early stages of development. Furthermore, in an attempt to unveil the role of Vitamin D in chick lung branching, *in vitro* lung explants were performed. Supplementation studies revealed that Vitamin D supplementation significantly reduced lung size and branching, independently of the dose. Additionally, *cyp24a1* increased expression in Vitamin D-treated explants, the main regulatory component of the pathway, confirmed Vitamin D signaling activation. Moreover, given the well-documented antiproliferative effect of vitamin D, we hypothesized that the significant reduction in lung size and branching could be explained by this event. EdU proliferation assay confirmed the presence of high proliferation levels in sites of active branching, distal and proximal lung, but further analysis demonstrated a significant reduction in proliferation levels in all Vitamin D-treated explants. This study unveils, for the first time, the potential role of vitamin D signaling in the early stages of chick pulmonary branching and contributes to better understand the intricate molecular events that ultimately regulate this complex process. Our results highlight the importance of regulating vitamin D levels during normal lung development and reinforce the need to establish appropriate doses and intervention windows to ensure an appropriate physiological response during embryonic development.

Keywords: Branching morphogenesis; Chicken embryo; Chick lung; Pulmonary development; Vitamin D.

DECIFRANDO O PAPEL DA VITAMINA D NO DESENVOLVIMENTO EMBRIONÁRIO DO PULMÃO - RESUMO

A elevada prevalência de deficiência de Vitamina D é considerada um problema de saúde pública, que afeta indivíduos de todas as idades em todo o mundo, principalmente mulheres grávidas e crianças. Existem evidências que apontam para uma associação entre a deficiência materna de Vitamina D e várias consequências adversas na saúde materno-fetal. No que diz respeito ao desenvolvimento pulmonar, sabe-se que a Vitamina D é crucial para os processos de maturação pulmonar, que ocorrem nos estadios finais do desenvolvimento, e que influencia a saúde respiratória da descendência. No entanto, pouco se sabe sobre seu papel nos estadios iniciais de ramificação, e nada se sabe sobre o seu papel no desenvolvimento pulmonar de galinha. Nesse sentido, este projeto tem como objetivo desvendar o papel da Vitamina D no desenvolvimento inicial do pulmão, utilizando o embrião de galinha como modelo. O presente trabalho caracteriza, pela primeira vez, o padrão de expressão de membros da via de sinalização da Vitamina D (*rxr-α*, *rxr-γ*, *vdr*, *cyp24a1 e pdia3*) por hibridização *in situ* no pulmão embrionário de galinha. A semelhança entre os padrões e os níveis de expressão destes genes com o mamífero sugere um papel idêntico, e porventura menos relevante, nestes estadios. Além disso, com o objetivo de elucidar o papel da vitamina D na ramificação, utilizaram-se explantes pulmonares *in vitro* suplementados com Vitamina D. Os estudos revelaram que a Vitamina D induziu uma redução significativa no tamanho e ramificação pulmonar, independentemente da dose. O aumento da expressão de *cyp24a1*, o principal componente regulador da via, em explantes tratados com Vitamina D confirmou a ativação desta via. Por outro lado, avaliámos se a redução do tamanho e da ramificação do pulmão poderia ser explicada pelo efeito anti proliferativo, já conhecido, da vitamina D. O ensaio de proliferação confirmou a presença proliferação ativa em locais de ramificação, no pulmão distal e proximal; no entanto, a análise quantitativa demonstrou uma redução significativa dos níveis de proliferação nos explantes tratados com Vitamina D. Este estudo revela, pela primeira vez, o potencial papel da sinalização da Vitamina D nos estadios iniciais da ramificação pulmonar e contribui para a melhor compreensão dos eventos moleculares envolvidos na regulação desse processo. Os nossos resultados destacam a importância da regulação dos níveis de Vitamina D durante o desenvolvimento pulmonar normal e reforçam a necessidade de estabelecer doses e intervenções adequadas para garantir uma resposta fisiológica apropriada durante o desenvolvimento.

Palavras-chave: Desenvolvimento Pulmonar; Embrião de galinha; Pulmão de galinha; Ramificação pulmonar; Vitamina D

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LIST OF ABBREVIATIONS

$1,25(OH)_2D$: 1,25-dihydroxyvitamin D

1,25D-MARRS/ PDIA3: 1,25D-membrane-associated rapid response steroid-binding protein

25(OH)D: 25-hydroxyvitamin D

AP: Anteroposterior

AT2: Alveolar Type II Cells

BCIP: 5-Bromo-4-Chloro-3-Indolyl Phosphate

BMP: Bone Morphogenetic Protein

cDNA: Complementary DNA

D0: 0 Hours

D2: 48 Hours

DV: Dorsoventral

E: Embryonic Day

EDTA: Ethylenediamine Tetraacetic Acid

EGTA: Ethylene Glycol Tetraacetic Acid

FGF: Fibroblast Growth Factor

GLI: Glioblastoma Zink Finger Transcription Factor

ISH: *In Situ* hybridization

LF: Lipofibroblasts

MABT: Maleic Acid Buffer with Tween 20

mRNA: Messenger RNA

NBT: 4-Nitro Blue Tetrazolium Chloride

PBS: Phosphate Buffered Saline

PBT: Phosphate Buffered Saline with Tween 20

PBT: Phosphate Buffered Saline with Tween 20

PCR: Polymerase Chain Reaction

PTH: Parathyroid Hormone

RA: Retinoic Acid

RPM: Rotations Per Minute

RXR: Retinoid X Receptor

SD: Standard Deviation

SHH: Sonic Hedgehog

SP-A/B: Surfactant Protein A/B

TGFβ: Transforming Growth Factor β

VDD: Vitamin D Deficiency

VDR: Vitamin D Receptor

VDRE: Vitamin D Response Elements

WNT: Wingless-related Integration Site

CHAPTER I

INTRODUCTION

I. Introduction

I.1. Lung development

Lung development is a highly coordinated and conserved multistage process governed by a complex network of molecular, cellular, and physical events that ultimately control growth, differentiation, and patterning [1]. These rigorously regulated developmental routines culminate with the formation of a fully functional organ. Failure to correctly regulate these intricate series of events can lead to severe abnormalities that can compromise fetal development and postnatal survival or affect lung function throughout early life and adulthood.

Mammalian lung development is classically divided into five different stages: (1) embryonic; (2) pseudoglandular; (3) canalicular; (4) saccular; and (5) alveolar, mainly defined by molecular and morphological transitions (**Figure 1**) [2]. Due to non-synchronous pulmonary development, these stages overlap.

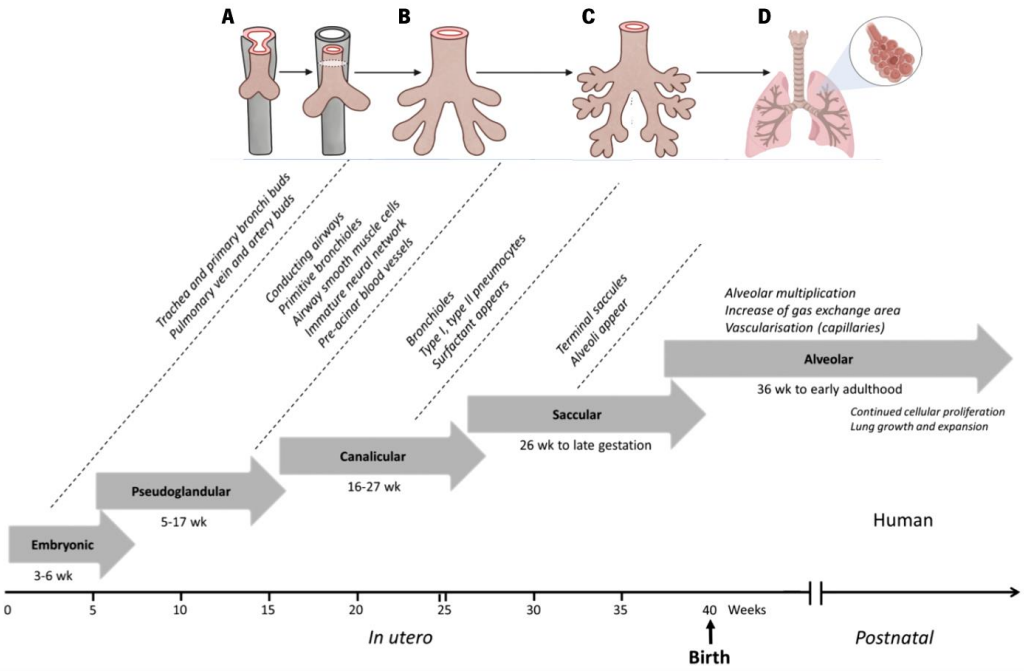


Figure 1 – Schematic representation of the main events underlying human lung development stages. **A** Embryonic phase; **B** Pseudoglandular phase; **C** Canalicular/Saccular stages and **D** Alveolar stage. Adapted from [3] and [4].

The respiratory system is organized into two main distinct tissues: the epithelial compartment, which derives from the endoderm layer, and the mesenchymal compartment that

arises from the mesoderm germ layer [5]. Proper lung morphogenesis relies upon elaborated interactions between the epithelium, the surrounding mesenchyme, and the extracellular matrix. This tissue crosstalk ultimately controls the spatial and temporal distribution of key signaling factors and diffusible signals. The activity of signaling pathways as, for instance, Fibroblast Growth Factor (FGF) [6], Retinoic Acid (RA) [7], Sonic Hedgehog (SHH) [8], Wingless-related Integration Site (WNT) [3], Transforming Growth Factor β (TGFβ), Bone Morphogenetic Protein (BMP) [9] and Hippo [10] play a fundamental role in this process.

In humans, these interactions start as early as the fourth post-conception week with the outgrowth of the primary buds from the ventral primitive foregut endoderm surrounded by splanchnic mesoderm (**Figure 1A**). Subsequently, during the pseudoglandular stage, the primordial lung buds elongate, and the prospective respiratory tree is generated by proliferation, reiterated budding, and branching of epithelial tubules (**Figure 1B**) [11]. During the canalicular stage, the existing epithelial airways continue to increase in size, and the epithelial terminal buds project into the distal airspaces, giving rise to the terminal sacs, the primitive alveoli. Additionally, epithelial differentiation occurs, allowing the morphological distinction between conduction and respiratory airways (**Figure 1C**). The saccular phase represents an intermediate phase between branching morphogenesis and alveolarization. This period is defined by the expansion and further division of the distal airspaces into numerous thin-walled terminal saccules, the alveoli precursors (**Figure 1C**) [4]. Finally, the alveolar phase comprises the process of alveolar formation to give rise to the functional gas exchange units (**Figure 1D**). In humans, this process starts before birth and continues postnatally until young adulthood. Overall, these events have been extensively studied in the mammalian lung; nonetheless, many controversies and questions about its molecular regulation remain unanswered.

I.1.1. The chicken embryo as a model to study lung development

Animal models play a crucial role in fundamental and biomedical research and are essential for deciphering the molecular and genetic circuits that control animal development and physiology. The avian embryonic lung has recently emerged as an alternative model system to study branching morphogenesis [12]. Several features make the chicken embryo an interesting animal model for developmental research: accessibility, availability, size, and developmental rate. This animal model is readily available all year round, easing the planning and scheduling of experimental protocols [13]. Moreover, the practical and economic advantages of this system

cannot be unnoticed: the low cost of the eggs and their housing conditions makes large-scale experiments more permissible. Eggs are nutritionally self-sufficient and do not require specialized facilities or equipment, ensuring consistent embryo viability without artificial support media or complex culture requirements [14]. Furthermore, embryo development is stationary and self-contained by nature, enabling its continuous monitoring and experimental manipulation through *in ovo* and *ex ovo* manipulations [15]. In addition, it has a rather convenient size and a short incubation period, completing its entire development by the time of hatching at 21 days. This process has been minutely documented by Hamburger and Hamilton [16], who provided a rigorous staging system for this animal model. Finally, it is phylogenetically closer to mammals than, for instance, zebrafish [14].

Despite the clear differences between avian and mammalian adult lung [17], the molecular events and signaling pathways underlying lung development at early stages are highly conserved in both phyla [18-21]. Additionally, there are remarkable morphological resemblances between mammalian and avian lung branching subroutines. Hence, as the comparative knowledge grows, there is an expanding potential for translation from the developing chicken lung to the human context [22].

I.1.2. Avian lung development

I.1.2.1. Overview

The avian respiratory system comprises the parabronchial lung, which is involved in gas exchange, and the air sacs that control air movements and serve as reservoirs for oxygen-rich air [23]. Together, this results in a remarkably efficient system that precisely responds to birds' high metabolic rate [17].

The mammalian and the avian lungs originate by the outpouching of a primordium from the foregut endoderm [17]. *nkx2.1* (also known as ttf1, thyroid transcription factor-1) expression in the endoderm distinguishes the respiratory progenitors in the chicken embryo [24]. The positioning and establishment of the mammalian lung is also associated with *nkx2.1* endodermal expression, which reflects the dorsoventral positional information in the gut endoderm [25].

In the chicken embryo, *Gallus gallus*, the embryonic lungs spring up from the laryngotracheal groove as a small ridge-like structure around embryonic day 3 (E3) [26] (**Figure 2A**). Initially, they are constituted by a thin layer of endoderm surrounded by a dense layer of

mesoderm. A series of complex and highly regulated molecular interactions coordinate growth and differentiation of these distinct cellular compartments to give rise to a fully functional organ. After fusing on the ventral midline, the single entity divides into left and right primordial lungs that progressively elongate caudally while deviating towards the coelomic cavity, resembling a pair of sacs lying on each side of the esophagus (**Figure 2B**) [26]. After the establishment of the lung primordium, the gross architecture of the avian airways is generated by lateral branching, a process wherein, by lateral sprouting, a new branch (secondary bronchi) emerges from the dorsal surface of the primary bronchus (mesobronchus) into the surrounding mesenchyme (**Figure 2C**) [26]. The lateral (or monopodial) branching observed in avian lung development is similar to the domain branching observed in the mammalian embryonic lung [27].

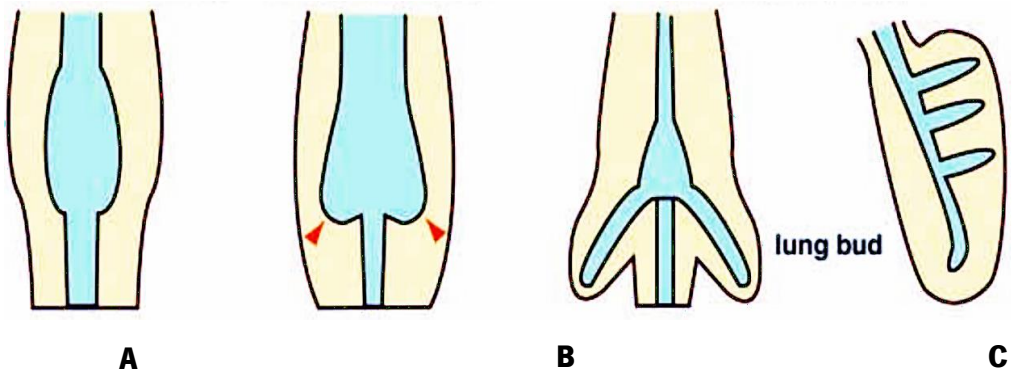


Figure 2 – Schematic representation of the chick respiratory tract development. **A** Laryngotracheal groove; **B** Primary bronchus formation; **C** Branching. Red arrowheads indicate primary lung buds evaginating laterally from the posterior edge of the endoderm. Adapted from [24].

The initial stages of chicken lung development are designated according to the number of secondary buds formed per mesobronchus. So being, the b1 stage corresponds to lungs with only one secondary bronchus; the b2 stage refers to lungs with two secondary bronchi, and the b3 stage presents three secondary bronchi, and so on (**Figure 3**).

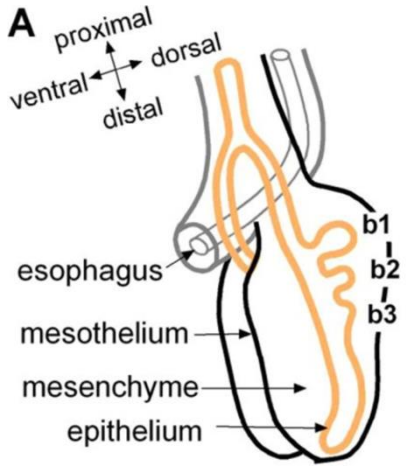


Figure 3 – Schematic representation of an E6 embryonic chicken lung. Lung epithelium (in orange) surrounded by mesenchyme undergoes monopodial branching, which occurs sequentially along the primary bronchus in a distal direction. The first lung bud (b1) and the subsequent buds (b2 and b3) emerge into the surrounding mesenchyme. b, bud. Adapted from [28].

During mammalian branching morphogenesis, the lung also ramifies through terminal bifurcation, in which the main secondary branches subsequently bifurcate at their tips, forming the tertiary and later-generation branches [27]. In contrast, in the avian lung, secondary bronchi branch and invade the mesenchyme to give rise to the tertiary bronchi (parabronchi), that canalize, lengthen, anastomose, and ultimately connect the secondary bronchi, forming the gas-exchange unit of the lung [26]. Afterwards, the air sacs, cyst-like structures, are formed during development as ventral dilatations from the mesobronchi and are named based on their position along the craniocaudal axis of the primary bronchus (cervical, intra-clavicular, thoracic, or abdominal) [23]. The differences between the dorsal and ventral compartments of the lung are due to region-specific mesenchymal expression of *hoxb* genes (homeobox-containing genes, a family of transcription factors), *bmp-2*, *bmp-4*, *wnt-5a*, and *wnt-11* [29]. The dorsal-ventral patterning is crucial for defining branching *vs* air sac domains. Furthermore, the air sac/branch difference in the developing chick lung can also be explained by a variation in the diffusion rate of FGF10 between ventral and dorsal regions [30].

All these processes rely upon the crosstalk between the epithelial and mesenchymal compartments, which, in turn, depend on the activity of several conserved and tightly regulated intercellular signaling pathways that ultimately control proliferation, differentiation, and extracellular matrix remodeling in a temporal-spatial manner. The importance of reciprocal signaling is well documented in mammalian lung development [1]. While considerably less is known about the crosstalk during avian lung development, the molecular events that generate early branching

appear to be highly conserved with the mammalian, as similar expression patterns and similar functions have been uncovered. For instance, FGF [19], WNT [18], TGFβ, SHH [21], and RA [31] signaling pathways were described as critical players in chicken pulmonary branching morphogenesis.

I.1.2.2. Branching Morphogenesis

Branching morphogenesis sculpts the airway epithelium of the lung into a complex tree-like structure to conduct air and promote gas exchange after birth [28]. It has been shown, by tissue recombination, that the mesenchyme controls the branching pattern of the epithelium primordium of the chick lung, proving the importance of mesenchymal-epithelial interactions in chicken lung development [32]. Furthermore, each tissue compartment produces a unique set of growth factors and other signaling molecules, which operate in a paracrine manner between the epithelium and the mesenchyme [27]. This tissue crosstalk depends on the coordinated activation of conserved signaling pathways. For instance, FGF signaling, particularly FGF10, regulates normal lung development [33]. *fgf10* is expressed in the distal mesenchyme, at sites where epithelial buds will emerge, and acts in a paracrine fashion on the adjacent epithelium, where its cognate receptor *fgfr2* is expressed [34]. FGF10 signaling in lung epithelium is inhibited by its downstream target, *spry2*, which is expressed in the distal epithelium, proximal to sites of mesenchymal *fgf10* expression [35]. *fgf10*, *fgfr1-4,* and *spry2* are expressed at early stages of chicken lung branching and present a similar expression pattern as their mammalian counterparts, pointing a similar regulatory function in epithelial-mesenchymal interactions that determine epithelial branching and mesenchymal growth (**Figure 4A and 4B**) [19]. Furthermore, *in vitro* inhibition of FGF signaling lead to abnormal chick lung growth, with a cystic appearance of secondary bronchi, and reduction of the mesenchymal tissue [19]. Similarly, *fgf10*-knockout mice fail to correctly develop their lungs, which leads to neonatal death [36]. Altogether, this points to a similar role of FGF signaling in epithelial branching and mesenchyme growth of the developing chick lung.

SHH-GLI signaling is decisive for proper lung formation since it controls cell proliferation and differentiation in epithelial and mesenchymal compartments and regulates branching morphogenesis [37]. In the chicken embryo, *shh* is expressed in the trachea at E6 and in the proximal and distal epithelium at E10 [38,39]. Furthermore, as described by Moura *et al.* [21], all the canonical elements of SHH signaling pathway are expressed in the same cellular compartments as their mammalian counterparts; however, the proximodistal distribution is somewhat altered. *shh*

is expressed in the epithelial compartments of the main bronchus and secondary bronchi but absent from the most distal tip of the growing buds, whereas its receptor (*ptch1*) and effectors (*gli1*) mirror *shh* pattern, as it occurs in the mammalian fetal lung (**Figure 4C**). As so, it is expected that *shh* signaling is involved in the epithelial-mesenchyme interactions that govern avian branching morphogenesis. Together with FGF10, SPRY2, TGFβ, and BMP4, SHH signaling regulates lung bud outgrowth, branching, and subsequent growth arrest [40]. Moreover, the importance of this signaling pathway in chick lung branching has been proven in *talpid*^{3} chicken mutants that display an abnormal lung morphology due to defective SHH signaling [38]. Similarly, dysregulations of this pathway in the developing mammalian lung are implicated in abnormal tracheal and lung formation [41-43], including tracheoesophageal fistula [44]. Altogether, these observations point to a similar role for SHH signaling in chick and mammalian lung branching [45].

Canonical WNT/β-catenin signaling pathway also regulates numerous developmental processes, including cell fate and morphogenesis. Studies of the expression pattern of multiple WNT proteins suggest that this intricate pathway can originate from both the epithelium and mesenchyme tissues and works in an autocrine or paracrine fashion [46]. Several WNT ligands (*wnt-1, wnt-2b, wnt-3a, wnt-5a, wnt-7b, wnt-9a*) and other components of the WNT signaling pathway are differentially expressed during the early stages of chicken lung development [18]. Moreover, their expression is in agreement with their mammalian counterparts. *In vitro* inhibition of canonical WNT signaling leads to impairment of lung branching morphogenesis in both avian and mammalian lungs, indicating a similar regulatory function in the chicken pulmonary system [18]. The non-canonical WNT signaling appears to be involved in the mid-developmental stages of chick lung development. In the developing avian lung, WNT-5 acts non-canonically and is involved in the distal airway and vasculature development [39]. Disturbance in *wnt-5a* expression leads to abnormal vasculature pattern and lung hypoplasia, similar to what happens in the developing mammalian lung [47]. Furthermore, *wnt-5a* is also involved in tracheal development and in the inhibition of branching in this region since it is expressed in the mesenchyme of the trachea is detected at around E5 [18]. *wnt-5a* knockout mice display a decreased number of cartilage rings and reduced trachea size [47].

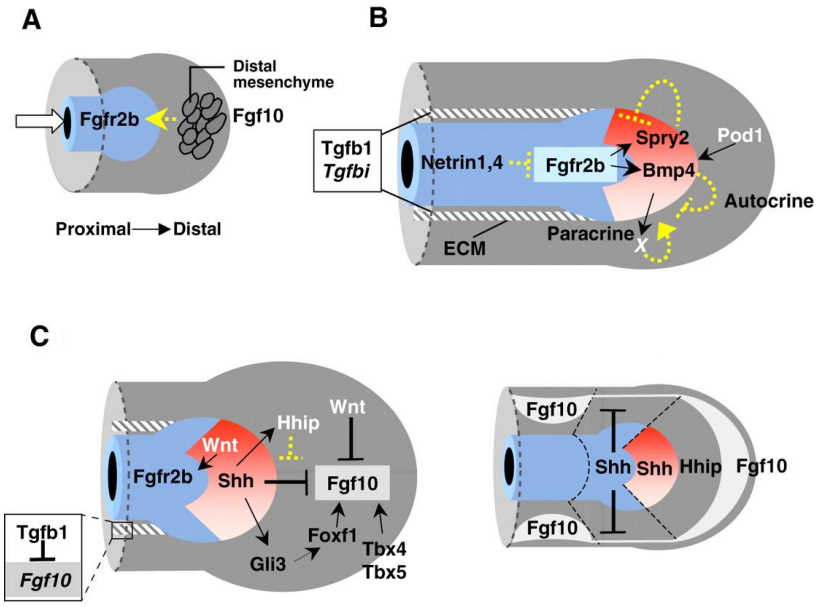


Figure 4 – Schematic representation of the signaling pathways involved in branching morphogenesis. Mesenchyme is depicted in gray and the epithelium in blue or red (distal bud). **A** Bud initiation and **B** elongation. **C** FGF-SHH-WNT signaling pathways crosstalk. Adapted from [33].

Retinoic acid (RA), the biologically active metabolite of Vitamin A, is involved in a series of cellular events underlying the morphogenesis of numerous organs, including the lung [48]. The RA signaling pathway has been characterized in the developing chick lung [31]. Whilst in the mammalian lung there is a combined expression in mesenchymal and epithelial compartments, in the chick lung RA signaling members are exclusively expressed in the mesenchymal compartment. Although this feature seems to be species-specific, it is reasonable to attribute RA signaling a similar regulatory function in the chicken pulmonary branching morphogenesis. In fact, RA exogenous supplementation stimulates lung branching, as it occurs in the mammalian lung [31]. In the mammalian lung, RA is essential for the process of primary lung bud formation in addition to distal bud outgrowth and alveologenesis [48,49]; deficiency/misexpression of RA signaling members is associated with severe lung abnormalities, including lung hypoplasia [50].

Furthermore, other molecular players, for instance, microRNAs (miRNAs/miR), also contribute to the lung developmental program. It has been recently described that miRNA processing machinery is expressed in the developing chick lung, uncovering a similar regulatory role of this previously described mechanism in epithelial and mesenchymal morphogenesis in the chick lung [20].

More recently, great attention has been given to the mechanics of branching and the physical forces that shape the developing airways. During branching morphogenesis, to generate a new secondary bud, the epithelium of the main bronchus must undergo a synchronized bending process, which requires the generation of conveyed forces from the adjacent cells. Kim *et al.* demonstrated that monopodial branching of the developing avian lung is initiated by the contraction of the apical/lumen side of epithelial cells [28]. In addition, recent data highlighted the importance of metabolic regulation during the early stages of chicken branching morphogenesis [51].

Overall, the molecular studies point to a strong resemblance between the mechanisms underlying avian and mammalian lung development, thus validating the use of the chicken lung for branching studies. In this sense, it is feasible to search for novel regulators of branching morphogenesis using the chicken embryo as a model. For instance, little is known about the role of vitamins, for instance, Vitamin D, in early branching stages. Broadening these insights will increase our understanding of the development of the lung and further allow more effective diagnostic and therapeutic interventions of developmental pathologies and abnormalities in pre- and postnatal lungs [17].

I.1.3. Influence of maternal nutrition and lung growth

In addition to the well-known molecular factors that determine and influence normal lung development, various genetic factors, environmental factors, and intrauterine factors encountered during critical points of prenatal development may disrupt the normal course of pulmonary morphogenesis and maturation, inducing changes in structure, function, and metabolism of the respiratory system.

Numerous experimental and epidemiological studies have demonstrated that nutrition plays a crucial role in fetal lung development, either by directly affecting the mechanisms involved in lung growth or by influencing developmental programming through epigenetic changes and can even have a lifelong impact on respiratory health [52-54]. During pregnancy, maternal nutritional status can influence the nutritional supply to the developing fetus. Due to their higher metabolic rates, pregnant women are commonly at risk of micronutrient deficiency [55]. Chronic restriction of some vitamins and minerals during the gestational period can result in structural alterations in the airways, causing abnormal lung function, depending on the type of nutritional deficit and the stage of lung development at which it occurs.

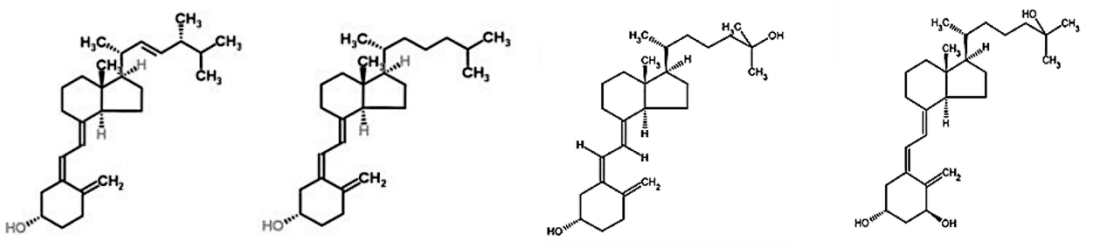
The influence in various aspects of the lung maturation of some micronutrients is generally accepted. For instance, the role of retinoic acid, the biologically active metabolite of Vitamin A, has long been recognized for its involvement in many aspects of lung development. From lung specification to the alveolar formation, RA finely tunes the reciprocal epithelial-mesenchymal interactions that give rise to the respiratory airway structure [7]. Vitamin A deficiency/impairment of the RA signaling pathway has detrimental effects on the development of the respiratory system; it is associated with chronic respiratory disorders, including pulmonary fibrosis, congenital diaphragmatic hernia (CDH), and chronic obstructive pulmonary disease COPD [56]. Regarding the effects of Vitamin A supplementation on lung development, retinoic acid treatment in animal models reversed some of the structural alterations induced by prenatal Vitamin A deficiency. In humans, Vitamin A supplementation in premature infants decreases the risk of bronchopulmonary dysplasia [57]. Nonetheless, RA levels must be tightly regulated, or instead of acting as a morphogen, RA has teratogenic effects. In fact, both deficiency and excess of this retinoid may lead to severe congenital lung malformations [58]. Other micronutrients with relevant effects on lung development are Vitamins C, E and D [59-61], selenium [62,63], and omega-3 docosahexaenoic acid (DHA) [64,65].

While the importance of a few micronutrients during pregnancy and lung development has long been recognized, for instance, Vitamin A as mentioned above, the role of many others is only recently becoming more appreciated, as is the case of Vitamin D.

I.2. Vitamin D

I.2.1. Vitamin D metabolism: overview

Vitamin D is unique among vitamins since it can act as a hormone and comes in two forms: vitamin D_2 (ergocalciferol) and vitamin D_3 (cholecalciferol). These two isoforms present similar biological activities and only differ in the side chain, where D_2 has a double bond between C22 and C23 and a methyl group at the C24 (**Figure 5A and 5B**) [66]. Vitamin D (both forms) can be synthesized in the skin from exposure to sunlight (ultraviolet B, UVB, 290-320 nm) or obtained through diet, from foods that naturally contain vitamin D, for instance, oily fish, cod liver oil, egg yolk, and vitamin D-fortified foods or supplements [67-69].



A Vitamin D2 **B** Vitamin D3 **C** 25-hydroxyvitamin D **D** 1,25-dihydroxyvitamin D

Figure 5 – Chemical structures of the main Vitamin D metabolites. A The physiologically inactive vitamin D_2 and **B** vitamin D_3; **C** the main circulating vitamin D_3 intermediate, 25-hydroxyvitamin D, 25(OH)D and **D** the bioactive vitamin D_3 metabolite 1,25-dihydroxyvitamin D, $1,25(OH)_2D$. Adapted from [70].

During exposure to sunlight, vitamin D_3 is produced in a two-step process under the influence of UVB: endogenous 7-dehydrocholesterol (7-DHC) in the skin is converted to pre-vitamin D_3, which then rapidly undergoes a thermal conversion to vitamin D_3 [68]. The amount of vitamin D_3 that results from cutaneous synthesis is determined by exposure of epidermal cells to UVB radiation that is highly dependent on a variety of factors, including UVB intensity, latitude, season and time of the day, exposed body surface, exposure duration, use of sunscreens, skin pigmentation level and age [67,71]. Independently of the source (skin or diet), for most of Vitamin D-mediated actions, Vitamin D must be firstly metabolized to 25-hydroxyvitamin D (or 25(OH)D; **Figure 5C**). This process occurs mainly, but not exclusively, in the liver [72]. 25(OH)D is the major circulating metabolite and thus is accepted as the biomarker for vitamin D status [73]. Numerous enzymes have 25-hydroxylation activity, but under physiological conditions, the major enzyme responsible for 25(OH)D production is cytochrome P450 family 2 subfamily CYP2R1 [74,75]. Knockout of *cyp2r1* in mice reduces 25(OH)D blood levels by more than 50% [76]. Moreover, in humans, mutations in *cyp2r1* are correlated with a form of hereditary rickets [77]. 25(OH)D is then transported to the kidneys, where it undergoes further hydroxylation by the CYP27B1 enzyme and is converted into the hormonally active form 1-α-25-dihydroxy vitamin D, or $1,25(OH)_2D$, also known as calcitriol (**Figure 5D**) [68,78]. CYP27B1 can also be found in extra renal sites, including the epithelial cells in the skin, the lungs, breast, intestines, prostate, placenta, immune cells, such as macrophages and monocytes, and bone cells [66,79-81]; these findings suggest that extra renal cells can produce $1,25(OH)_2D$ for their own needs, in a paracrine and/or autocrine fashion [82]. Inactivation of the *cyp27b1* gene in mice causes pseudo vitamin D-deficiency rickets due to inadequate $1,25(OH)_2D$ production [83]. Finally, $1,25(OH)_2D$ indirectly regulates its own levels by the action of CYP24A1, a 24-hydroxylase found in most tissues, that metabolizes both 25(OH)D

and $1,25(OH)_2D$ for degradation [73,84]. In addition to regulating the amount of calcitriol in circulation, this enzyme also regulates $1,25(OH)_2D$ levels within the cells to ensure the appropriate tissue response. Homozygous c*yp24a1* null mice died before 3 weeks of age and exhibited intramembranous bone lesion, resulting from defective mineralization [85]. Mutations in c*yp24a1* lead to elevated $1,25(OH)_2D$ levels, which causes idiopathic hypercalcemia in infants and nephrocalcinosis in adults [86,87]. Overall, vitamin D serum levels are tightly regulated through feedback loops resulting in changes in the expression of CYP24A1 and CYP27B1 enzymes. In this regard, the actions of $1,25(OH)_2D$, parathyroid hormone (PTH), and fibroblast growth factor 23 (FGF23) are intrinsically involved in the regulation of vitamin D metabolism [72].

I.2.2. Vitamin D signaling – mechanism of action

Vitamin D and its metabolites are generally carried in the blood, from their production sites to their target tissues, by Vitamin D binding protein (VDBP), and its uptake into cells occurs in both bound and unbound forms (**Figure 6A**). For most tissues, it is the unbound metabolite that is taken up into cells by diffusion across the membranes; however, at least for some tissues as, for instance, the kidney, placenta, and mammary gland, a fraction of 25(OH)D (and other vitamin D metabolites) are taken up into cells in the bound form, by endocytosis, via binding of VDBP to megalin30 and cubilin31 [88,89].

The majority, but not all, of the physiological actions of $1,25(OH)_2D$ are mediated by its binding to a nuclear high-affinity vitamin D receptor (VDR), a ligand-activated transcription factor, member of the nuclear hormone superfamily [90]. The VDR is found in most cells, not just those involved in bone and mineral homeostasis, resulting in widespread physiologic actions of vitamin D. Once activated by $1,25(OH)_2D$, VDR interacts with any of the three retinoid X receptors (RXR) isoforms (RXRα, RXRβ, and RXRγ) to form a heterodimeric complex [91]. Subsequently, this heterodimeric complex is translocated to the nucleus and recruited to the vitamin D response elements (VDRE) within the promoter region of target genes [92] (**Figure 6B**). The impact on gene expression depends on VDR-RXR heterodimer's ability to recruit nuclear coactivators or corepressors hence activating or suppressing transcription of target genes, respectively [93]. *cyp24a1,* which encodes the enzyme that degrades the excess $1,25(OH)_2D_3$, is the most common target gene induced by vitamin D [94]. Conversely, $1,25(OH)_2D$-VDR-RXR may also down-regulate the transcription of the specific genes, such as *cyp27b1* and *pth* [95,96].

Vitamin D can also elicit rapid non-genomic responses, which are partly mediated by calcitriol binding to 1,25D-membrane-associated rapid response steroid-binding protein (1,25D-MARRS, also known as ERp57/PDIA3) [97]. This interaction then results in acute changes in cell signaling pathways, including the activation of protein kinase C (PKC), protein kinase A (PKA), and ultimately mitogen-activated protein kinase (MAPK), differing from actions of the classic vitamin D receptor [98] (**Figure 6B**). Evidence for a receptor that is VDR-independent includes studies performed in mice with a targeted deletion of PDIA3 receptor in the intestines, showing suppression of the rapid uptake of calcium and phosphate [99]. Deregulation of PDIA3 has been implicated in multiple pathologies, including airway inflammation [100] and cancer [101].

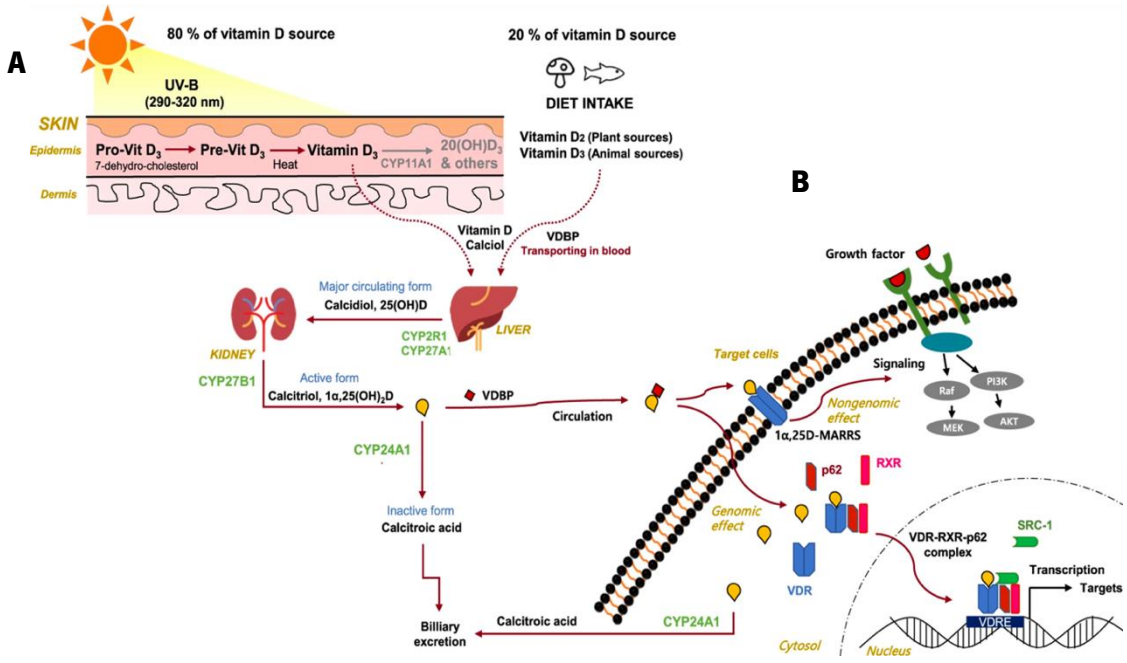


Figure 6 – Overview of Vitamin D metabolism (A) and mechanism of action (B). Retrieved from [97].

I.2.3. The classic and non-classic roles of vitamin D

Vitamin D has long been acknowledged for its classic role in calcium and phosphate homeostasis and skeleton maintenance [102]. Over the last years, the interest in vitamin D and its impact on various biologic processes has increased tremendously due to the finding of VDR presence in nearly every tissue and cell. More recently, the discovery of thousands of VDR binding sites throughout the genome unveiled a role for Vitamin D in controlling the expression of hundreds of genes [66]. The non-classic functions of vitamin D involve regulation of cellular proliferation, differentiation, apoptosis, and immune modulation [78]. Several signaling pathways and molecular

targets contribute to these effects in normal and malignant cells. Moreover, increasing evidence has recognized important roles of Vitamin D in many biological functions and several complex human diseases such as cancer, diabetes, hypertension, autoimmune and cardiovascular disorders [97,103].

For instance, multiple *in vitro* and *in vivo* studies have suggested that the active metabolite of vitamin D, 1,25$(OH)_2$D, presents an antitumoral effect by inhibiting the proliferation of cancer cells, inducing apoptosis, cell cycle arrest and differentiation, and inhibition of angiogenesis, metastasis and invasion [104]. One of the major mechanisms of Vitamin D antitumor effect is the inhibition of proliferation. Calcitriol induces G_0/G_1 cell cycle arrest in prostate, colorectal, and breast cancer cells. Additionally, most tumors express VDR and CYP27A1, but both decline with progressive tumor dedifferentiation [105]. On the other hand, CYP24A1 expression is often increased in tumors and is associated with 1,25$(OH)_2$D resistance.

Furthermore, VDR is expressed in most immune cells, which are also capable of synthesizing the active metabolite of Vitamin D [106]. Therefore, besides its endocrine functions, Vitamin D can also act in a paracrine or autocrine manner. Additionally, Vitamin D can modulate both the innate and the adaptative immune responses, and its deficiency has been associated with increased susceptibility to infection and is prevalent in autoimmune diseases [107].

Overall, interventional studies have pointed to a protective effect of calcitriol on the mortality of certain cancers and the prevention of metabolic, cardiovascular and autoimmune diseases. However, other studies have revealed contrasting or even contradictory evidence in other non-skeletal tissues. In this sense, the extra skeletal actions and effects of 1,25$(OH)_2$D remain to be further explored, and, consequently, pharmacological supplementation is still a matter of debate.

I.2.4. Maternal vitamin D status during pregnancy and child outcomes

It is now generally accepted that Vitamin D deficiency (VDD) is a worldwide health problem with concerning prevalence rates, requiring actions from a public health perspective [108]. Recent large observational data have suggested that nearly 40% of Europeans are vitamin D deficient, and 13% are severely deficient [108]. Although there is no consensus on the definition of VDD, most experts agree that a 25(OH)D concentration less than 20 ng per milliliter (50 nmol per liter) is an indication of VDD [109]. A level of 25(OH)D of 21-29 ng/mL (51-74 nmol/L) is indicative of relative insufficiency. If the concentration is 30 ng or higher (>75 nmol/L), it is considered adequate [110].

Pregnant women and newborns are at greater risk of vitamin D deficiency [111]. In fact, there is growing evidence that many women worldwide are Vitamin D deficient, both before and during pregnancy [112-114]. Significant alterations in Vitamin D metabolism occur during pregnancy, mostly to provide adequate calcium for fetal bone mineral accretion [115]. However, VDD not only affects musculoskeletal health but has also been implicated in a range of acute and chronic diseases. Considering recent literature, VDD is associated with maternal adverse health outcomes, including preeclampsia, gestational diabetes, and preterm delivery; in offspring, VDD is associated with type 1 diabetes, inflammatory and atopic disorders [113,114,116]. On this subject, several epidemiological studies have revealed an association between maternal VDD and an enlarged risk of chronic lung disease in offspring, including respiratory distress syndrome (RDS) and bronchopulmonary dysplasia (BPD), both major complications to preterm birth [117,118]. Furthermore, hypovitaminosis D during pregnancy has also been implicated in the pathogenesis of early childhood chronic respiratory infections, including COPD [119], and increases the risk of childhood wheezing and asthma later in life [120,121]. For instance, *in vitro* and *in vivo* studies have suggested that $1,25(OH)_2D$ is involved in the epithelial-mesenchymal transitions that occur in asthma and fibrotic diseases [122,123]. Moreover, it has been shown that low perinatal vitamin D levels are associated with deficits in lung function, pointing to a role for this vitamin in lung development [124].

I.2.5. The role of Vitamin D in lung development

The importance of Vitamin D for fetal lung development, particularly during the later stages of lung maturation, has been demonstrated in animal models. Vitamin D contributes to pneumocyte type II maturation [125], surfactant-related phospholipids and surfactant protein B biosynthesis [126], and enhances surfactant release in fetal rat lung explants [127]. Furthermore, it has been shown to promote epithelial-mesenchymal interactions during pulmonary maturation and modulate alveolar septal wall thinning by regulating lipofibroblasts proliferation/apoptosis [128]. Moreover, prenatal Vitamin D deficiency in mice has been linked to deficits in lung function and altered lung structure, with increased airway resistance and a significant decrease in lung volume [129]. In rats, maternal VDD was associated with increased airway hyper-responsiveness and increased airway smooth-muscle mass, reflected by alterations in surfactant phospholipids synthesis functional markers and tracheal and alveolar mesenchymal differentiation markers [130]. Genomic analysis of lung tissue from humans and mice identified numerous VDREs-containing genes involved in lung

development, pattern formation, and branching morphogenesis, revealing a wide range of potential roles for vitamin D in normal lung growth [131].

Other *in vivo* studies support the role of vitamin D in pulmonary function. Taylor *et al.* [132] showed promising results using a neonatal rat model, in which pups were randomized to receive one of three different doses of active hormone, $1,25(OH)_2D$ and its physiologic precursor 25(OH)D via nebulization, for 14 days. The nebulized vitamin D treated group showed an improvement in lung maturation, supported by increased surfactant production, increased expression of alveolar epithelial, mesenchymal, and endothelial differentiation markers, and increased alveolarization.

Moving to human studies, a combination of two randomized clinical trials showed that, by the age of three, children born to mothers who received a higher dose of Vitamin D_3 (4000 IU/day or 2400 IU/day) had a 25% reduced risk of recurrent wheezing than those whose mother received a smaller dose 400 IU/d (controls) [133-135]. Moreover, VDD during pregnancy and decreased 25-hydroxyvitamin D levels at birth were correlated with reduced lung function in offspring [121,136]. Nonetheless, whether Vitamin D supplementation in pregnant women is necessary to reduce the risk of chronic lung disease and improve lung development in the offspring is still uncertain. The outcomes of several recent randomized controlled trials have revealed no beneficial effects of maternal Vitamin D supplementation on lung health and development [133,137]. Figure 7 summarizes current epidemiological and experimental evidence supporting the relationship between maternal Vitamin D and lung development and health outcomes.

Collectively, these observations point to a critical role of vitamin D during the later stages of lung development (**Figure 7**). However, little is known about the impact of vitamin D in early branching stages.

Despite the pieces of evidence of the benefits of Vitamin D supplementation during antenatal, perinatal, and postnatal periods due to an improvement on lung maturation, structure, and function [138], the role of vitamin D supplementation, and the optimal vitamin D dosing and status is still a subject of great debate. In fact, the normal Vitamin D levels for term and preterm infants are still not well-established, and optimal daily requirements for correct lung maturation in neonates are also undefined.

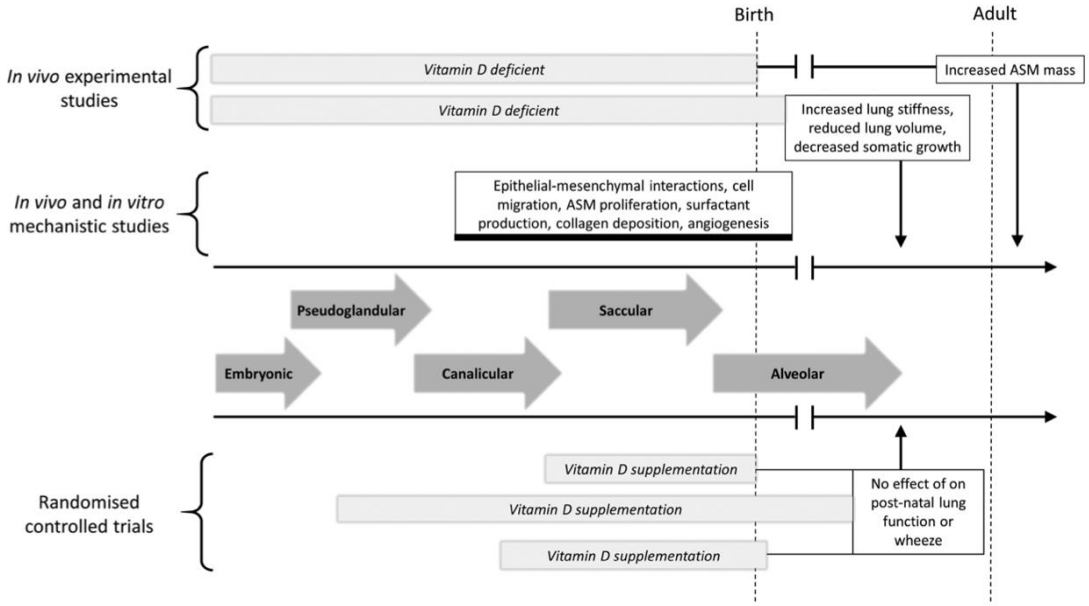


Figure 7 – Summary of the relationship between Vitamin D and lung development. *In vitro* studies have shown that Vitamin D deficiency (VDD) impairs lung growth and results in reduced lung volume. Both *in vitro* and *in vivo* models have shown that these observations are related to the impact of Vitamin D in epithelial-mesenchymal interactions, cell proliferation and differentiation, collagen synthesis and surfactant production. However, supplementation cohort trials are not conclusive on the effects on post-natal outcomes. Retrieved from [11].

CHAPTER II

RATIONALE AND AIMS

II. Rationale and aims

Vitamin D is a key regulator of calcium and phosphate homeostasis in the body; however, it has been shown to have other biological functions beyond its canonical role in bone mineralization. Vitamin D signaling regulates cellular events such as proliferation, differentiation, and apoptosis in nearly every tissue and cell. Regarding the developing lung, it has been demonstrated that Vitamin D is crucial for lung maturation processes, but little is known about its role in early branching stages.

In this sense, this project aims to investigate the role of Vitamin D during early lung development using the chicken embryo as a model and bring new insights into the regulatory roles of vitamin D in embryo development.

To achieve this goal, this project aims to fulfill the following specific objectives:

I: Characterize the expression pattern of the vitamin D processing machinery, in the early stages of chick lung development, by in situ hybridization.

II: Assess the impact of Vitamin D supplementation on lung growth using an in vitro lung explant system.

III: Determine the impact of Vitamin D on cellular responses such as proliferation.

CHAPTER III

MATERIALS AND METHODS

III. MATERIALS AND METHODS

III.1. Ethical Statement

The experiments presented in this work were performed at early stages of the chicken embryo (*Gallus gallus*) in accordance with the Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes. The Portuguese Directive 113/2013 of 7 August 2013 does not hold any constraint to the use of non-mammalian embryos.

III.2. Embryo and Tissue Collection

Fertilized chicken eggs were obtained from a local commercial source and subsequently incubated in a 38 °C incubator, with 49% humidity, for 4.5-5.5 days. After the incubation period, the embryo was accessed by opening a small window in the eggshell. Embryonic chick lungs were carefully dissected in a Petri dish with PBS 1x, under the stereomicroscope (Olympus SZX16, Japan), and staged as b1, b2, or b3, according to the number of secondary buds that emerge from the main bronchus: 1, 2 or 3 respectively. Whole embryos were also collected and staged according to Hamburger and Hamilton developmental table (HH24-26) [16]. Dissected tissues were then processed for whole mount *in situ* hybridization or lung explant culture. For whole mount *in situ* hybridization, lungs and embryos were fixed at 4 °C overnight in PBS 1x containing 4% formaldehyde, 2 mM EGTA, pH 7.5. Afterwards, embryos and lungs were rinsed with PBT, PBT/methanol (50/50), and then with methanol 100% (twice). The tissues were subsequently stored at -20 °C in 100% methanol. For lung explant culture, lungs were transferred into a 24-well plate filled with PBS 1x, using a sterile transfer pipette, and immediately used for culture (see section III.4).

III.3. *In situ* Hybridization

In situ hybridization (ISH) is a semi-quantitative technique that uncovers messenger RNA (mRNA) spatial distribution and expression levels in different tissue compartments using a labeled antisense RNA probe. Briefly, the probe hybridizes with the corresponding mRNA in the tissue producing a stable hybrid. This RNA-RNA hybrid is then detected by an antibody that recognizes

the specific labeling, followed by an enzymatic reaction that produces a chromogenic precipitate, thus revealing the expression sites.

III.3.1. Probe Preparation

III.3.1.1. RNA extraction and cDNA synthesis

cDNA was produced from total RNA obtained from HH24 embryos. Total RNA was extracted using the TripleXtractor directRNA Kit (GRISP, Portugal) according to the manufacturer's protocol. Total RNA quantification was performed using Nanodrop Spectrophotometer (NanoDrop ND-1000, USA). Afterwards, 1 µg of purified RNA was treated with DNase I (Thermo Fisher Scientific, USA) to eliminate genomic DNA in the sample, following the manufacturer's instructions. After incubating the samples for 30 minutes, at 37 °C, the reaction was placed on ice and stopped with 1 µL EDTA; lastly, the enzyme was heat-inactivated for 10 minutes at 65 °C. Subsequently, 1 µg of Dnase-treated RNA was reverse transcribed to cDNA using Xpert cDNA Synthesis Kit (GRISP) and following oligo(dT) recommended protocol. cDNA quantification was determined by spectrophotometry (Nanodrop). To assess cDNA's integrity, the housekeeping gene *gapdh* was amplified by conventional PCR using Supreme NZYTaq II 2x Green Master Mix (NzyTech, Portugal) and specific *gapdh* primers (**Table 1**). Amplification was confirmed by agarose gel electrophoresis.

III.3.1.2. Primer Design

Specific primers were obtained to amplify part of the coding region of *vdr, cyp24a, rxr-alpha, rxr-gamma,* and *pdia3*, which were already annotated in the chicken genome. The primers were designed in-house using Primer-BLAST designing tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi) or obtained from the literature (**Table 1**).

Table 1 - PCR primer sequences used for conventional amplification of key Vitamin D signaling members, *pdia3*, *cyp24a1*, *vdr*, *rxr-α*, *rxr-γ* and *gapdh*.

Gene	Orientation	Accession Number	Sequence (5'-3')	Product size (bp)	Annealing Temperature (°C)	References
PDIA3	Forward Reverse	NM_204110	GGCGCAACCGAGTTATGATG GGCGGCAGGAAATTAAACAGA	841	58,3	*
CYP24A1	Forward Reverse	NM_204979	GAGTTGAAACGACGGCCAAC TCCCCTTCTGTTAAGGATGTGA	1086	59,5	*
VDR	Forward Reverse	NM_205098	AACGATGTCTTTGGCTCCGAT AACAATGTACAGCCTCCTCCC	848	61,2	*
RXR-α	Forward Reverse	XM_003642291	CGCCGCTCTCCACCG CATCCCCATGGCAAGACACT	659	59,3	[139]
RXR-γ	Forward Reverse	NM_205294	CGCAGTGAGAACGAAGCTGA CGCAGGAGGAGTTTGGCA	611	59,3	*
GAPDH	Forward Reverse	NM_204305.2	ATATGACAAGTCCCTGAAAATTGTCAG GCATCAAAGGTGGAGGAATGG	500	51,1	*

**, designed in-house*

III.3.1.3. Cloning

cDNA fragments of the desired gene transcript were amplified by conventional PCR, and the size was confirmed by agarose gel electrophoresis. The fragments with the expected size were cloned in the pCR®II-TOPO TA vector containing T7 and SP6 promoters (Thermo Scientific, USA), according to the manufacturer's instructions. After performing the TOPO cloning reaction, the vector constructs were transformed into *Escherichia coli* competent cells, following the manufacturer's protocol (Nzytech). 50-150 µL from each transformation were plated on selective LB agar plates supplemented with 100 µg/mL ampicillin, 0.1 mM IPTG (NZYTech), and 20 µg/mL X-gal (NZYTech) and incubated overnight at 37 °C.

White positive clones were then selected and inoculated in 800 µL of sterile LB media supplemented with 100 µg/mL ampicillin at 37 °C, 200 rpm overnight. Afterwards, bacteria were pelleted, and plasmid DNA was isolated using an Alkaline Lysis protocol, as described by [140]. For positive clone selection, DNA was used as a template for conventional PCR using Supreme NZYTaq II 2x Green Master Mix and M13 universal primers, and fragment size was verified by agarose gel electrophoresis. Once the positive clones were identified, each single bacterial colony was once inoculated in 6 mL of sterile LB media containing 100 µg/mL ampicillin at 37 °C, 200 rpm overnight. Plasmid DNA was isolated using the GeneJET Plasmid Miniprep Kit (Thermo Scientific) and stored at -20 °C until further use. DNA concentration was determined using the

Nanodrop. Next, plasmid DNA was used as a template for conventional PCR using Supreme NZYTaq II 2x Green Master Mix and M13 universal primers, and the presence of the insert was verified by agarose gel electrophoresis. Finally, plasmid DNA was sent for sequencing (GATC Sequencing Service, Germany) to confirm the sequence and orientation of the insert. Determining the orientation of the fragment is crucial to select the RNA polymerase required for *in vitro* transcription to synthesize the antisense probe.

III.3.1.4. *In vitro* Transcription

Antisense Digoxigenin (DIG)-labeled RNA probes were obtained by an *in vitro* transcription reaction using DIG RNA Labeling Mix (Roche Applied Sciences, Germany). Probes were synthesized in the presence of uridine-5′-triphosphate nucleotides conjugated to DIG, which are efficiently incorporated by RNA polymerases. During the ISH procedure, DIG is recognized by a specific anti-DIG antibody coupled to an enzyme that catalyzes a colorimetric reaction, generating a visible signal that uncovers mRNA spatial distribution.

100 µL of PCR-amplified fragments of interest were electrophoresed on a 1.2% agarose gel. Using a sterile razor blade, the desired DNA fragment was sliced and excised from the gel. The GRS PCR & Gel Band Purification Kit (GRISP) was then used to obtain the DNA fragments from agarose gel, according to the manufacture's protocol. Purified DNA fragments were then quantified by spectrophotometry and subsequently visualized by gel electrophoresis for quality control. The samples were stored at -20 °C until used for probe synthesis.

In vitro transcription reaction was carried out by adding the equivalent volume of 750 µg of template DNA recovered from agarose gel slices to 4 µL of DTT 100mM, 3.5 µL of 10x transcription buffer (or 7 µL of 5x buffer; Table 2), 2 µL Dig RNA labeling mix 10x, 2 µL RNA polymerase (SP6 or T7; Table 2) and 2 µL RNAse inhibitor to a total volume of 35 µL and incubated at 37 °C, for 3 hours. The reaction was then stopped, placing the tubes on ice. 2 µL of DNAse I and 1 µL of RNAse inhibitor were added and incubated again for 30 minutes, at 37 °C. Then, 200 µL of TE, 20 µL of LiCl 4 M, and 600 µL of ice-cold ethanol 100% were added. It was allowed to precipitate at -80 °C for 45 minutes. Afterwards, it was centrifuged at 13000 rpm, for 30 minutes, at 4 °C. 1 mL of ice-cold ethanol 70% was added to the pellet and centrifuged again

at 13000 rpm, for 15 minutes, at 4 °C. The pellet was resuspended with 50 µL of 10 mM EDTA to prevent polymerase activity. Probes were immediately used or stored at -20 °C.

Table 2 - RNA polymerases, supplier and transcription buffers used for *in vitro* transcription, for RNA probe synthesis.

Probe	RNA polymerase	Transcription Buffer	Supplier
PDIA3	SP6	10x	Roche
CYP24A1	SP6	10x	Roche
VDR	T7	5x	Promega
RXR-α	SP6	10x	Roche
RXR-γ	SP6	10x	Roche

III.3.2. Whole mount *in situ* hybridization

The embryos and lungs, stored in methanol at -20 °C, were rehydrated by washing in a graded series of PBT/MeOH (25%, 50%, and 75%) and then twice in PBT (n≥9 per gene/stage). Tissues were permeabilized with proteinase K (pK) solution (PBT with 0.05% proteinase K) (Roche): HH26 embryos for treated for 40 minutes and the lungs for 2 minutes. Afterwards, tissues were repeatedly rinsed in PBT to stop the pK reaction and placed in a post-fixing solution (PBT with 0.4% glutaraldehyde, 10% formaldehyde) for 20 minutes at room temperature. Embryos and lungs were, once again, rinsed in PBT solution and incubated with PBT with hybridization solution (50:50) (50% formamide, 1% EDTA 0.5 M pH 9.8, 6.5% SSC, 0.2% heparin, 0.25% t-RNA, 0.5% CHAPS, 0.2% Tween 20). Afterwards, tissues were rinsed with fresh hybridization solution and incubated at 70°C for 2 hours. Concomitantly, specific probe solutions (5 µL of each antisense RNA probe per mL of hybridization mixture) were placed at 70 °C to eliminate RNA secondary structures. The hybridization solution was promptly replaced with the prewarmed probe solution and incubated at 70 °C, overnight, to enable efficient penetration of the probe. On the following day, embryos and lungs were washed with prewarmed hybridization solution and incubated at 70 °C for 30 minutes, to be then rinsed in hybridization solution with MABT (50:50) (5.8% $C_4H_4O_4$, 4.4% NaCl, 7% NaOH,

1% Tween 20, pH 7.5) and incubated for 10 minutes at 70 °C. Subsequently, tissues were abundantly washed with only MABT.

Next, tissues were pre-blocked with MABT with 20% blocking reagent (Roche) and with MABT with 20% blocking reagent plus 20% goat serum (Invitrogen) for 3 hours each, at room temperature. This step ensures that all the nonspecific binding sites are blocked, thus minimizing background staining. Finally, embryos and lungs were incubated in 1:2000 anti-digoxigenin antibody (Roche) diluted in MABT with 20% blocking reagent, 20% goat serum solution, with gentle shaking, overnight at room temperature. Afterwards, embryos and lungs were repeatedly rinsed in MABT solution at room temperature and with gentle shaking overnight. On the last day, embryos and lungs were washed twice in an alkaline phosphatase buffer, NTMT solution (0.1 M NaCl, 0.1 M Tris–HCl, 50 mM $MgCl_2$, 1% Tween 20) for 15 minutes at room temperature and then incubated with a developing solution (NTMT containing NBT and BCIP) (Roche), acting as a chromogenic substrate for alkaline phosphatase, protected from the light at 37 °C. Periodically, the reaction was monitored, and when a strong signal was detected, the reaction was stopped by washing several times with PBT.

Each set of lungs and probes was processed simultaneously and developed for the same amount of time. Hybridized lungs were photographed in PBT, with an Olympus U-LH100HG camera coupled to Olympus SZX16 stereomicroscope and then stored in PBT/azide at 4 °C. For each probe, hybridization specificity was confirmed by assessing the probe's expression pattern in HH26 embryos and determining whether it matched the patterns predicted from previous studies of the corresponding mRNA in *Gallus gallus* embryos.

III.3.3. Cross section

Hybridized chicken lungs were dehydrated through a graded ethanol series (70%, 96%, and 100%), embedded in 2-hydroxyethyl methacrylate (Heraeus Kulzer, Germany), and processed for sectioning at 25 µm using a rotary microtome (Leica RM 2155, Germany). Afterwards, slide lung sections were photographed with an Olympus DP70 camera coupled to an Olympus BX61 microscope.

III.4. Lung Explant Culture

In vitro lung explant culture allows the evaluation of the interactions between epithelial and mesenchymal compartments that ultimately control growth, differentiation, and patterning through

specific signaling pathways. This technique, in which lung structure is preserved in an air-liquid interface for up to 2 days, allows the manipulation of a chemically defined culture medium by adding compounds of interest. Briefly, after dissection in PBS 1x, stage b2 lungs were transferred to nucleopore polycarbonate membranes with 8 µm pore size (Whatman, USA) and incubated in a 24-well culture plate. The membranes were presoaked in 400 µL of Medium 199 (5.5 mM glucose; Sigma, USA) 1 h prior to lung placement. Floating explants were cultured in a 5% CO_2 incubator at 37 °C for 48 hours in 200 µL of Medium 199 supplemented with 5% (V/V) heat-inactivated fetal calf serum (Invitrogen), 10% (V/V) chick serum (Invitrogen, USA), 1% (V/V) penicillin 5000 IU/mL plus streptomycin 5000 IU/mL (Invitrogen), 1% (V/V) L-glutamine (Invitrogen) and 0.25 mg/ mL of ascorbic acid (Sigma). Lungs were randomly assigned one of four experimental groups (n ≥ 15 per condition): the culture medium was supplemented with increasing doses of the active biological form, $1,25(OH)_2D$ (679101, Calbiochem), selected according to the literature (50, 150 and 300 nM), or 0.1% (1 µL/mL) DMSO as a control since this solvent was used to resuspend $1,25(OH)_2D$. The medium was replaced by fresh supplemented medium after 24 hours.

After 48 hours of culture, lung explants were rinsed in PBS1x and processed for *in situ* hybridization, as previously described. Additionally, a different set of explants were collected and processed for EdU proliferation assay (n=5/group).

III.4.1. Morphometric analysis

Branching morphogenesis was monitored by photographing the explants at Day 0 (0h), Day 1 (24h), and Day 2 (48h), as previously described [12]. Afterwards, lung explants were analyzed morphometrically. Lung branching was determined by counting the total number of peripheral airway buds present at D0 and D2 of culture. The results are expressed as D2/D0 ratio, which is then used as a measure of lung branching. As for morphometric analysis, this was achieved by outlining the internal and the outer perimeter of the lung at D0 and D2, using the imaging analysis software AxionVision Rel. 4.3 (Carl Zeiss GmbH, Germany). Results are expressed as D2/D0 ratio and disclose the impact on lung growth. Both results of branching and morphometric analysis were expressed as D2/D0. All quantitative morphometric data are presented as mean ± SD.

III.5. Proliferation assay and confocal microscopy

To assess the proliferation status during branching morphogenesis, a DNA-synthesis-based cell proliferation assay was performed using the Click-iT™ Plus EdU Cell Proliferation Kit for Imaging (Invitrogen). EdU (5-ethynyl-2´-deoxyuridine) is a nucleoside analog of thymidine that is incorporated into newly synthesized DNA and fluorescently labeled with a bright dye. Briefly, after 48h of culture, half of the explant's media was replaced by fresh supplemented media containing EdU at a final concentration of 150 μM. Tissues were incubated with EdU in a 5% CO_2 incubator at 37 °C for 90 min. After incubation, explants were collected and fixed in a PBS solution containing 3.7% formaldehyde for 15 minutes at room temperature. Tissues were then washed twice with 3% Bovine serum albumin (BSA) in PBS 1x and subsequently permeabilized with 0.5% Triton® X-100 in PBS for 90 min at room temperature. Tissues were washed twice again with 3% BSA in PBS 1x and processed for Click-iT Plus EdU reaction, according to the manufacturer's instructions. Detection of the incorporated EdU was performed using Alexa Fluor 488. Additionally, nuclei were counterstained with Hoechst 33342. For that, tissues were washed in PBS 1x and then incubated with PBS 1x containing Hoechst® 33342 (1:2000) for 90 min at room temperature, protected from the light. Images were obtained using an Olympus LPS Confocal FV3000 microscope (Olympus).

III.5.1. Proliferation status analysis

Confocal stacks analysis and measurements were performed with ImageJ image analysis software (NIH, Bethesda, MD, USA). Briefly, proliferation levels were determined by quantifying EdU intensity by manual thresholding the corresponding images to include the adequate EdU signal. To ensure biological representativity, five images were analyzed per sample (25 images in total per supplementation group). Threshold adjustment settings were replicated in every image within the same sample. % Area fraction was quantified using ImageJ software tools. Total lung area was determined by defining the outer perimeter of the lung for each image. All quantitative proliferation status data were presented as fold variation and as mean ± SD.

III.6. Statistical analysis

Statistical analysis was performed using GraphPad Prism 6 (GraphPad Software, USA). Normality of distribution was assessed using the Kolmogorov–Smirnov test. One-Way ANOVA was

performed and followed by Fisher's Least Significant Difference (LSD) post hoc test for multiple comparisons. All experimental data are presented as mean ± standard deviation (SD) with a statistically significant level considered of 5% ($p < 0.05$).

CHAPTER IV

RESULTS

IV. RESULTS

IV.1. Expression pattern of key signaling members of Vitamin D processing machinery during chick embryonic lung development

To determine the mRNA spatial distribution of key Vitamin D signaling pathway members, whole mount *in situ* hybridization was performed in b1, b2, and b3 stage embryonic chick lungs. *rxr-α*, *rxr-γ*, *vdr*, *cyp24a1* and *pdia3* expression patterns were characterized. When possible, representative examples of hybridized lungs from the three stages studied were selected and processed for sectioning.

rxr-alpha is mainly expressed in the most proximal region of the respiratory tract, specifically in the mesenchyme of the tracheal region (**Figure 8b**, dark arrowhead); it is also diffusely expressed in the mesenchyme surrounding the secondary buds. On the other hand, it is absent from the most distal mesenchyme (**Figure 8c**, dagger) and epithelium but mildly expressed in the epithelium of secondary bronchi (**Figure 8c**, asterisk). This expression pattern is constant for all the three stages studied; nonetheless, b2 stage lungs seem to present higher *rxr-α* expression levels, namely in the tracheal region. Histological sectioning of hybridized lungs showed that *rxr-α* is expressed in the mesenchyme and the epithelial compartment (**Figure 8d**, asterisk). This gene is expressed in the limbs of the HH26 embryo (**Figure 15**, Appendix I).

rxr-gamma is weakly expressed in the three stages studied. It is possible to observe some expression in the secondary bronchi (**Figure 8g**, asterisk) and also in the epithelium of the main bronchus except its distal tip (**Figure 8g**, black arrow). Also, the mesenchymal compartment exhibits a diffuse expression in the ventral region. Whole embryos displayed a clear expression in the somites as expected (**Figure 16**, Appendix I) thus validating the results obtained for the lung. This expression pattern is maintained throughout all studied stages, although b3 stage lungs seem to present higher expression.

vdr is faintly expressed in pulmonary epithelium throughout all studied stages. Its presence was detected after a long developing process. It is worth mentioning that this gene is weakly expressed in the whole embryo (**Figure 17**, Appendix I).

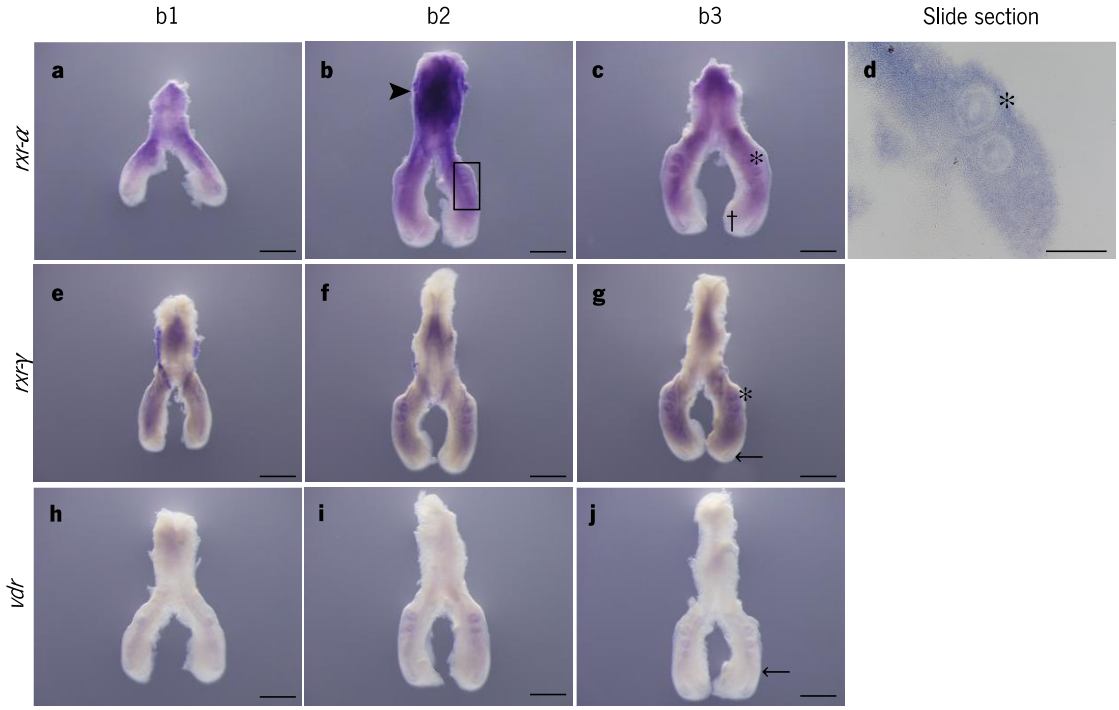


Figure 8 – *rxr-α*, *rxr-γ* and *vdr* expression pattern at early stages of chick lung development. Representative examples of *in situ hybridization* of **a-d** *rxr-α*, **e-g** *rxr-γ* and **h-j** *vdr* of stage b1, b2, and b3 lungs; n ≥ 12 per stage. Scale bar: whole mount, 500 µm; slide section, 100 µm. Black rectangle in image **b** represents the region shown in the corresponding cross section. *Dark arrowhead* trachea region. *Dagger* distal mesenchyme. *Asterisk* secondary bronchi. *Black arrow* main bronchus epithelium.

cyp24a1 expression is faint and limited to the chick pulmonary epithelium; eventually, it is more evident in b3 stage lungs, but still, lungs display low expression levels. (**Figure 9c**). Its presence was detected after a long developing process. Embryos display a very distinct expression pattern in the limbs, hence validating the results obtained for the lung (**Figure 18**, Appendix I).

pdia3 is present throughout the pulmonary epithelium (**Figure 9d**, black arrow) and in the dorsal mesenchyme (**Figure 9e**, dashed arrow), surrounding the secondary bronchi (**Figure 9e**, asterisk). This spatial mRNA distribution was maintained in all three stages studied. Histological sections confirmed the epithelial expression (**Figure 9g**, asterisk) and revealed a distinct mesothelial expression (**Figure 9g**, section sign).

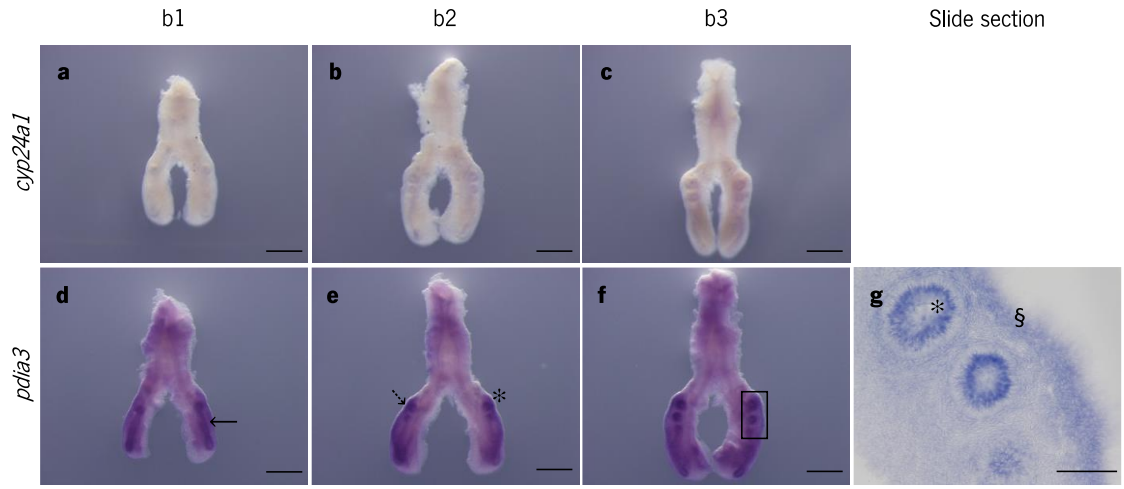


Figure 9 – *cyp24a1* and *pdia3* expression pattern at early stages of chick lung development. Representative examples of *in situ* hybridization of **a-c** *cyp24a1* and **d-g** *pdia3* of stage b1, b2, and b3 lungs; n ≥ 12 per stage. Scale bar: whole mount, 500 µm; slide section, 100 µm. Black rectangle in image **f** represents the region shown in the corresponding cross section. *Asterisk secondary bronchi. Black arrow main bronchus epithelium. Dashed arrow dorsal mesenchyme. Section sign mesothelium.*

IV.2. Impact of *in vitro* Vitamin D supplementation on lung branching and growth

To study the potential role of Vitamin D in the embryonic chick lung, b2 lung explants were cultured, *in vitro* for 48 h, and supplemented with three different doses of calcitriol, the active biological form of Vitamin D, selected according to the literature: 50, 150, and 300 nM $1,25(OH)_2D$. A set of b2 lungs supplemented with DMSO served as the control group. The representative examples of lung explants at 0 h (Day0) and 48 h (Day2) are represented in figure 3.

IV.2.1. Validation by *in situ* hybridization for *cyp24a1*

To validate the *ex vivo* lung explant system and confirm that the observed morphological alterations were due to Vitamin D signaling pathway activation, b2 treated explants were probed with *cyp24a1*, the regulator of calcitriol intracellular levels and direct readout of the pathway. As expected, Vitamin D supplementation leads to an increase in *cyp24a1* expression, independently of the dose, compared to the DMSO-treated explants (**Figure 10**). In DMSO-treated explants, *cyp24a1* is faintly present and displays an expression pattern in accordance with the previous results (**Figure 8**). On the other hand, after 48 h of supplementation, *cyp24a1* becomes expressed

in the mesothelium, the outermost region of treated explants. These results imply that vitamin D supplementation was effective and accounts for the observed morphological phenotype.

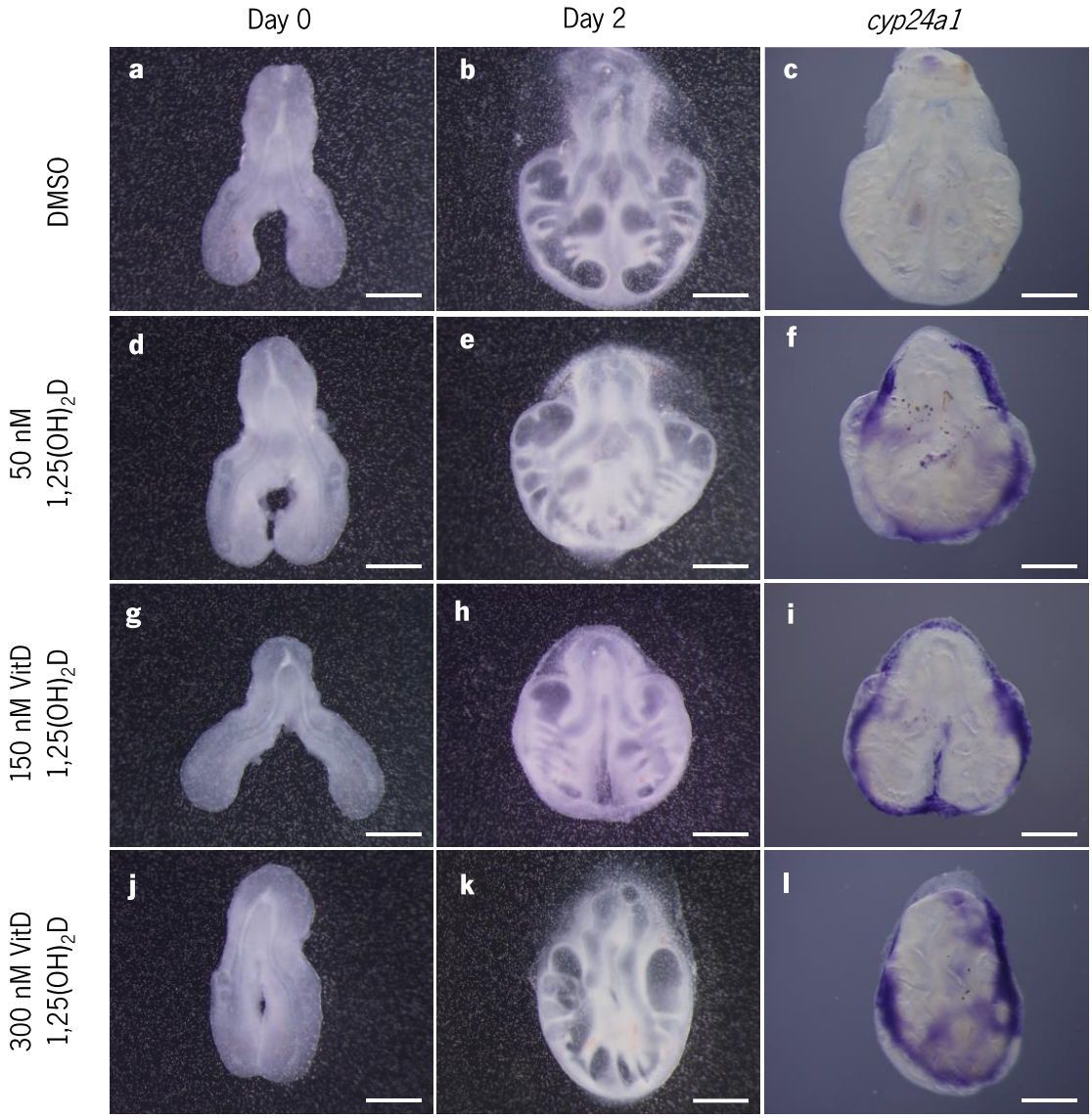


Figure 10 – *In vitro* Vitamin D supplementation of chick lung explants and *cyp24a1* expression analysis. a, **d**, **g**, **j** Representative examples of stage b2 lung explant culture at D0 (0 h) and **b**, **e**, **h**, **k** D2 (48 h), **a**, **b** treated with DMSO; **d**, **e** 50 nM $1,25(OH)_2D$; **g**, **h** 150 nM 1,25(OH)2D and **j**, **k** 300 nM $1,25(OH)_2D$. **c**, **f**, **i**, **l** Corresponding *in situ* hybridization for *cyp24a1* (n ≥ 4). Scale bar 500 µm.

IV.2.2. Morphometric and branching analysis

To evaluate the impact of Vitamin D supplementation on lung growth, lungs were morphometrically analyzed. Morphometric analysis was performed by determining the total and epithelial lung explant area/perimeter and assessing the number of peripheral airway buds formed after 48h in culture. The results are expressed D2/D0 ratio and summarized in figures 11 and 12. Vitamin D treatment induced a statistically significant reduction in the total area compared to DMSO-treated explants, pointing to a reduction in total lung size (**Figure 11a**); in these conditions, no significant differences were found between groups treated with 50, 150, and 300 nM of $1,25(OH)_2D$. Total perimeter results (**Figure 11b**) revealed that 50 nM and 300 nM dose treatment induced a significant reduction in mesenchymal perimeter ($p<0.05$) when compared to the control group. No significant differences were found within the calcitriol treatment groups.

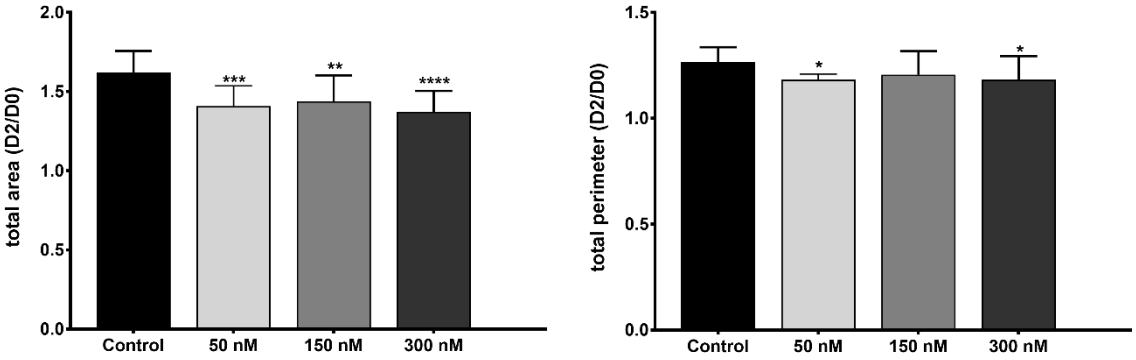


Figure 11 – Morphometric analysis of stage b2 lung explants treated with DMSO or 50, 150, and 300 nM of $1,25(OH)_2D$. DMSO and Vitamin D-treated lung explants were evaluated at D0 and D2 for **a** Total area and **b** Total perimeter. Results are expressed as D2/D0 ratio ($n \geq 14$/condition). Data is represented as mean ± SD. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$.

The significant reduction in total lung area was accompanied by a decrease in the number of secondary buds in $1,25(OH)_2D$-treated lungs compared with control DMSO-treated explants (**Figure 12a**). Branching analysis revealed that increasing doses of $1,25(OH)_2D$ induced a statistically significant reduction ($p<0.0001$) in the number of secondary airway buds formed when compared to DMSO-treated explants (**Figure 12a**). On the other hand, there is no statistically significant difference between the three tested doses (**Figure 12a**). In addition, the D2/D0 ratio of the epithelial perimeter and area were determined (**Figures 12b and 12c**). Vitamin D treatment induced a statistically significant reduction in the epithelial perimeter compared to

DMSO-treated explants (**Figure 12b**). No statistically significant differences were observed within treatment doses. Considering the epithelial area, only the higher dose of supplementation, 300 nM, induced a statistically significant decrease ($p<0.01$) when compared to both the DMSO treatment group and calcitriol 50 and 150 nM supplementation group (**Figure 12c**).

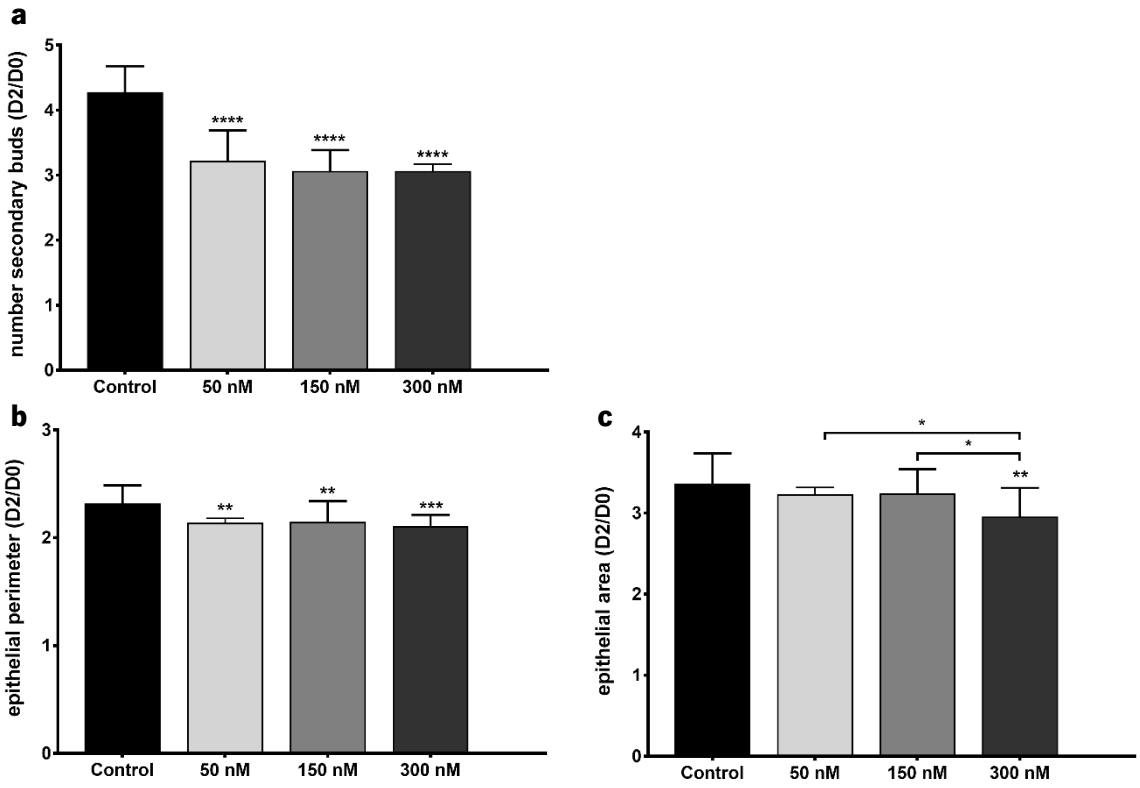


Figure 12 – Branching morphogenesis analysis of stage b2 lung explants treated with DMSO or 50, 150, and 300 nM of 1,25(OH)$_2$D. DMSO and Vitamin D-treated lung explants were evaluated at D0 and D2 for **a** Number of secondary buds; **b** Epithelial perimeter and **c** Epithelial area. Results are expressed as D2/D0 ratio (n ≥ 14/condition). Data are represented as mean ± SD. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001.

IV.3. Effect of Vitamin D supplementation on proliferation status in branching morphogenesis

To evaluate the proliferation status of lung branching morphogenesis, an EdU-based proliferation assay was performed using b2 stage lung explants treated with 50, 150, and 300 nM of 1,25(OH)$_2$D, or DMSO (control group) (**Figure 13**). Proliferation status was determined by assessing EdU incorporation into newly synthesized DNA strands with Alexa Fluor 488 (Green) (**Figure 13b**, **e**, **h**, **k**); nuclei were counterstained with Hoechst 33342 (Red) (**Figure 13a, d**, **g**, **j)**. High proliferation levels were detected in the trachea region (white arrowhead) (**Figure 13b**),

in the secondary bronchi (asterisk) (**Figure 13e**), and the distal tip of the lung (white arrow) (**Figure 13k**). This proliferation pattern was maintained in all conditions.

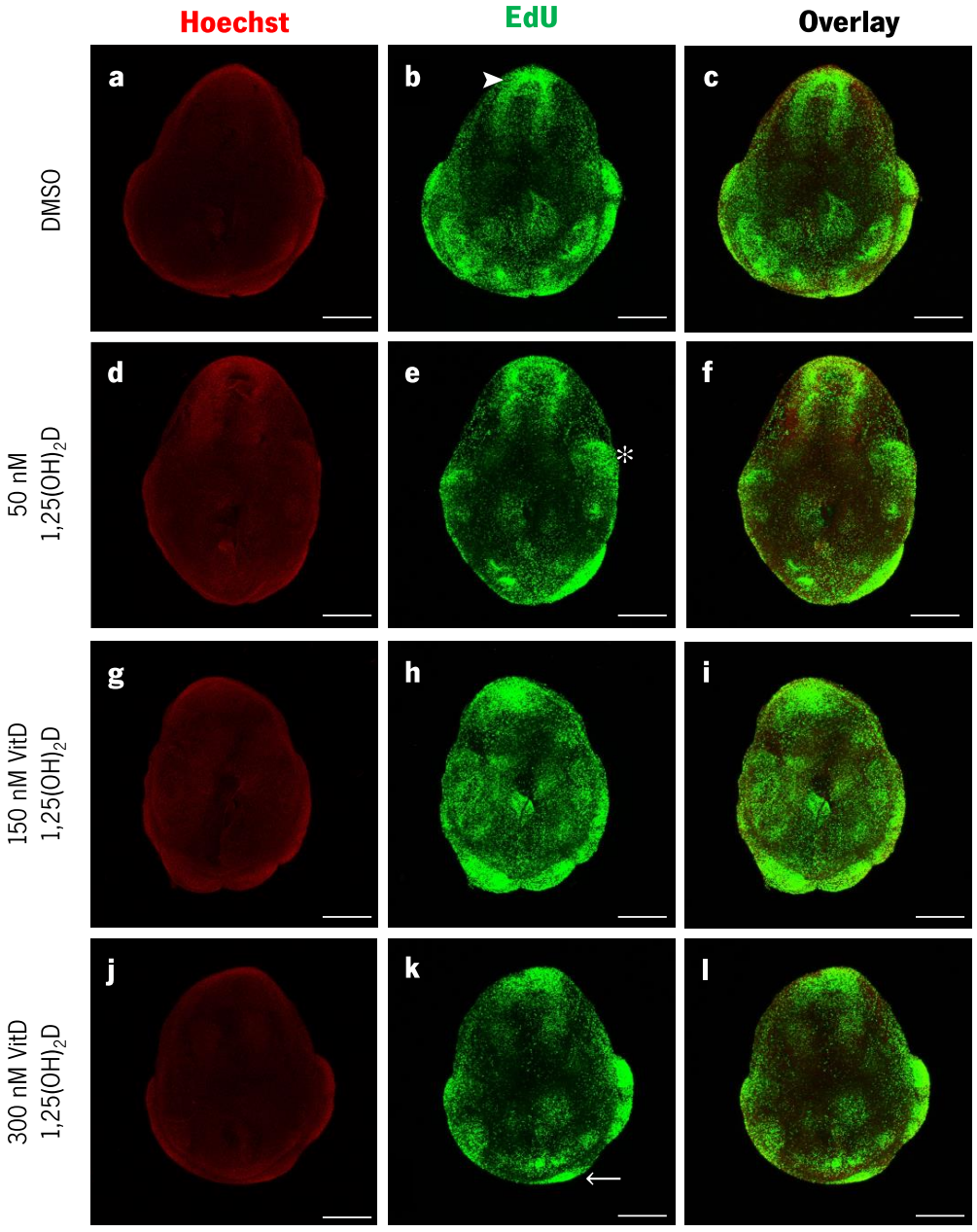


Figure 13 – Proliferation analysis during lung branching morphogenesis. Representative confocal microscopy fluorescence images of b2 lung explants kept in culture for 48 h, treated with DMSO (**a**, **b**, **c**), 50 nM (**d**, **e**, **f**), 150 nM (**g**, **h**, **i**) and 300 nM of calcitriol (**j**, **k**, **l**). **a**, **d**, **g**, **j** Nuclei were counterstained with Hoechst 33342 (Red). **b**, **e**, **h**, **k** Proliferation status was assessed using an EdU-based assay; Detection of EdU incorporation into newly synthesized DNA was performed by using Alexa Fluor 488 (Green). **c**, **f**, **i**, **l** Overlay represents merged images of Hoechst (Red) and EdU (Green). All images are displayed as maximum intensity projection of z-stacks. n ≥ 5 per condition. Scale bar: 500 µm. White arrowhead: tracheal region. Asterisk: secondary bronchi. White arrow: distal tip.

To further evaluate the impact of Vitamin D supplementation on the proliferation status, EdU staining intensity was quantified. For all treatment doses, % area fraction was represented as fold variation, in arbitrary units. Results revealed a significant decrease in proliferation levels, when compared to the control (**Figure 14**). No differences were found within calcitriol supplementation groups.

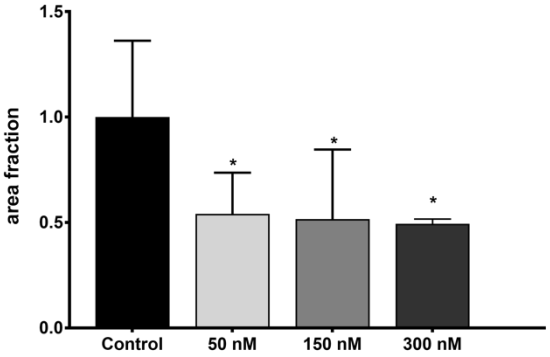


Figure 14 – Quantification of the proliferation levels during lung branching morphogenesis. % Area fraction fold variation from b2 lung explants kept in culture for 48 h and supplemented with DMSO, 50 nM, 150 nM and 300 nM of calcitriol. Proliferation levels were assessed as fractional area of EdU staining in the confocal microscopy fluorescence images. % Area fraction was normalized for number of z-stacks for each lung explant sample. Results are expressed in arbitrary units, as mean ± SD ($n \geq 5$/condition). Significantly different results are indicated as: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$.

CHAPTER V

DISCUSSION

V. DISCUSSION

Lung development is a highly complex process that depends on epithelial-mesenchymal interactions, mediated by a series of conserved signaling cascades culminating with the formation of a fully functional organ capable of effective gas exchange. Several intrinsic and extrinsic factors can influence the normal course of lung development and impact respiratory health and function later in life. During pulmonary organogenesis, branching morphogenesis is an extremely important step since it establishes the prospective airway conducting system [2]. Vitamin D has been implicated in various processes, including proliferation, differentiation, and morphogenesis of multiple organs. Regarding the lung, the active form of Vitamin D is essential in the later stages of development. Nonetheless, little is known about the regulatory role of this vitamin in the early stages of airway development when major morphological events occur. In this sense, this Master thesis aimed to uncover the role of Vitamin D during branching morphogenesis.

Expression pattern of Vitamin D processing machinery in the embryonic chick lung

Vitamin D exerts its biological actions by binding to the nuclear vitamin D receptor (VDR) that, upon ligand interaction, activates transcription of target genes, whose promoters contain a vitamin D response element, by forming a heterodimer with the Retinoid X Receptors (RXR-α, β, and γ).

In the chicken developing lung, *rxr-α* is mainly present in the most proximal region of the respiratory tract, namely in the mesenchyme of the tracheal region (**Figure 8a-d**). Retinoid X receptors are not exclusively involved in the transduction of the Vitamin D signaling but are also involved in other hormonal signaling pathways, particularly retinoic acid. Given the high amino-acid conservation vitamin D receptor and retinoic acid receptors (RARs), RXR can functionally interact with both nuclear receptors [141]. Although $1,25(OH)_2D$ and retinoic acid have distinct physiological and developmental roles, both signal through related nuclear receptors to regulate the transcription of specific genes. Moreover, molecular and genomic evidence point that their cognate receptors recognize similar regions on promotor regions, thus regulating common target genes [141]. Specific deletions/mutations of the RXR (α, β, and γ) isoforms have been described in mice and lead to different physiological outcomes. Deletion and/or mutation of RXR-α lead to the most detrimental effects since homozygous null RXR-α mice die *in utero* [142]. Moreover, RXR-α/RAR-α deficiency caused lung hypoplasia or agenesis in the mouse model, proving the

importance of both these receptors in embryonic lung development [143,144]. In embryonic chick lung, RXR-α and RAR-β expression patterns seem to coincide, particularly in stages b2 and b3, where both mRNAs are detected in the mesenchymal compartment, particularly in the tracheal region, and absent from the distal region of the main bronchi [31]. These findings are consistent with the fact that RA activity seems to be restricted to the most proximal region of the lung, supporting the establishment of the proximal airways, most likely through the RAR-β receptor [145]. Overall, considering the similar expression pattern in the embryonic chick lung, RXR-α might be interacting with RAR-β, and their regulatory role is probably conserved in avian lung organogenesis.

Regarding the gamma isoform, we observed that it is faintly expressed in the lung epithelium (**Figure 8e-g**). As secondary bronchi start to elongate, it is possible to detect RXR-γ in the region where they emerge from the main bronchus. RXR-γ expression pattern had already been described during mouse embryonic development. Similar to what we found, RXR-γ has been reported to be less abundant and more restricted in its distribution, in contrast to the other two isoforms [146,147]. A previous study from Kimura*, et al.* [148] has revealed that the mRNA levels of RXR-γ are detected in human fetal lung proximal (trachea and main bronchus) and distal sites. Similarly, Malpel *et al.* [48] found that on days 11 and 12, the mouse lung epithelium expresses both RXR-α and RXR-γ at proximal and distal sites. RXR-γ presence in the whole embryo (**Figure 16**, Appendix I) prompted us to conclude that the low expression levels in the chick lung are specific and not a consequence of a poor-quality probe.

vdr is expressed throughout the pulmonary epithelium of chick developing lung and presents low endogenous expression levels throughout the three stages of chick lung development (**Figure 8 h-j**). We assessed *vdr* expression pattern in the whole embryo (**Figure 17**, Appendix I) and obtained similar low expression levels. VDR is expressed in the metabolic tissues (including the liver and kidneys) and in almost all cell types, although its expression is low in normal cells. For instance, VDR is expressed in human bronchial epithelial cells. Previously, Nguyen *et al.* demonstrated the presence of VDR receptors in the rat lung during the perinatal period [149]. Similar to our results, both human fetal lungs in organ culture and isolated AT2 cells presented very low levels of the VDR that could be induced in cells and tissues incubated with calcitriol [150]. Nevertheless, it has been shown that VDR knockout mice presented emphysema phenotype, reduced alveologenesis and lung function, and developed a COPD-phenotype, proving the role of VDR in late lung development and the pathogenesis of chronic lung diseases [151].

Despite the very low expression levels, we observed that VDR and RXR-gamma are co-expressed throughout the pulmonary epithelial compartment, suggesting that the gamma isoform might preferentially associate with the nuclear vitamin D receptor to stimulate transcription in the developing chick lung. In fact, the interaction of RXR:VDR heterodimers are RXR isotype-specific and affected by other ligands. Transactivation studies using rat 24-hydroxylase Vitamin D response elements (VDREs) revealed that VDR preferentially associates with RXR-α or RXR-γ to stimulate transcription [152].

Vitamin D levels must be tightly regulated to maintain adequate signaling activity and ensure the appropriate tissue response. The catabolic enzyme CYP24A1 (a member of the cytochrome P450 family) is responsible for this regulatory mechanism. In the chick lung, in the stages studied that resemble the early mouse pseudoglandular stage, *cyp24a1* is faintly expressed in the pulmonary epithelium (**Figure 9a-c**). These results are in accordance with the fact that, in fetal rat lungs, at E15, which corresponds to the transition from the pseudoglandular stage to the canalicular stage, *cyp24a1* demonstrated minimal expression, but by E20 expression levels are significantly increased [153]. Moreover, in the airways of COPD patients, *cyp24a1* is present in all layers of the bronchial epithelium [154]. Once again, despite the low expression levels of *cyp24a1*, it is possible to observe that mRNA of the catabolic enzyme is co-expressed with VDR and RXR-gamma throughout the pulmonary epithelial compartment, suggesting that the Vitamin D signaling machinery is, most likely, acting in this compartment.

Vitamin D must undergo a series of enzymatic processing before it reaches the hormonally active form and reaches the nucleus, where it binds to its cognate nuclear receptor. Due to its lipophilic nature, most of the Vitamin D active metabolite enters the cell by crossing the membrane without the need for a membrane receptor. Nonetheless, $1,25(OH)_2D$ can also exert non-genomic effects by rapid membrane-initiated signaling via binding to a membrane receptor, PDIA3, also known as 1,25D-membrane-associated rapid response steroid binding protein. In the chick lung, *pdia3* is present in the pulmonary epithelium and the dorsal mesenchyme surrounding the secondary bronchi, specifically in the mesothelium (**Figure 9d-f**). So far, this gene has only been reported in the developing chick liver, somites, and limb development [155]. Systemic ablation of PDIA3 is lethal in mice, as these animals fail to progress beyond E15 [156]. High PDIA3 expression levels are detected in the lung during the early stages of embryogenesis in the mouse model, suggesting that impaired development of this tissue may in part be responsible for the embryonic lethality, suggesting a putative role for PDIA3 in lung development.

Vitamin D signaling pathway-related genes are up-regulated primarily in the third trimester, both in rat and mouse models, when the distal airways are formed [157]. Kho and colleagues described that vitamin D pathway genes are transcriptionally active during normal mouse and human lung development, but their expression increases throughout fetal lung development, with peak expression just before birth [131]. Moreover, Mandell and coworkers demonstrated in the rat lung that key vitamin D regulatory enzymes, VDR, CYP24A1, and CYP27B1 1α-OHase (which was not assessed in this study), are strongly expressed in the late fetal lung and undergo striking developmental regulation before birth [153]. These results point to a potential role for endogenous Vitamin D activity in normal development, especially in the later stages of fetal life. In fact, mechanistic studies have uncovered that the developing lung is more sensitive to the effects of Vitamin D during the late canalicular/ early saccular and alveolar stages of development through the regulation of surfactant and collagen synthesis and epithelial-mesenchymal transitions [126].

As Weiss and Litonjua [158] mentioned, evidence on the physiological effects of Vitamin D in the pseudoglandular phase of lung development, when the proximal airways are formed, is more speculative but could be of great relevance, particularly in the context of chronic respiratory diseases. Some Vitamin D pathway genes are up-regulated in the pseudoglandular stage, during human lung development. Other related genes with VDRE are also up-regulated during this gestational period, making a plausible connection to Vitamin D and normal embryonic lung development [157]. These findings are consistent with the ones proposed by Bossé and coworkers, that demonstrated that following stimulation of human bronchial smooth muscle cells with $1,25(OH)_2D$, the most significant network of up-regulated genes included genes involved in morphogenesis, cell growth, and survival [159].

Considering the spatial distribution and expression levels of key modulators of the Vitamin D signaling pathway in the chicken developing lung, one can speculate that the role of Vitamin D pathway might also be detectable only in specific lung micro-environment or stage-related contexts in the avian pulmonary system.

Impact of Vitamin D signaling supplementation in the embryonic chick lung

In vitro lung explant culture is widely used to study organogenesis-related events since it maintains native tissue interactions. Vitamin D supplementation is a straightforward method to uncover its impact on lung morphogenesis and branching. b2 stage chick lung explants were cultured for 48h, and supplemented with increasing doses of calcitriol, the active biological form

of Vitamin D, selected according to the literature. As the maximal concentration of $1,25(OH)_2D$ achievable in lung tissue is unknown, 300 nM was chosen as the highest tested dose, based on several *in vitro* studies of Vitamin D in pulmonary and cancer cell types [122]. Additionally, a set of lung explants were treated with DMSO to serve as the control group. The impact of Vitamin D supplementation on lung growth was disclosed by performing a morphometric and branching analysis. Overall, calcitriol supplementation significantly reduced total lung size, compared to the control group, but with no significant alterations within the three different supplementation doses (**Figure 11**). Moreover, $1,25(OH)_2D$ supplementation significantly reduced chick lung branching for all tested doses compared to the DMSO-treated group. No differences were found between supplementation groups (**Figure 12a**). Furthermore, the reduction in lung branching was accompanied by a significant decrease of the epithelial perimeter in all doses tested; nonetheless, the same trend was not observed in the epithelial area, that remained unaltered, except for the higher treatment dose, that induced a significant decrease in this parameter, compared to control and other treatment groups (**Figure 12c**). This could be explained by the cystic appearance of secondary bronchi (cystic wider shape of the lung buds).

The effect of vitamin D supplementation has been reported in other branching organs. For instance, incubation with $1,25(OH)_2D$ inhibits mouse mammary glands branching *in vitro* [160,161]. Moreover, mammary glands from VDR knockout mice exhibit increased branching morphogenesis compared with normal mice [161]. Collectively, these data provide evidence that $1,25(OH)_2D$ and nuclear VDR affect elongation and branching during mammary gland development.

Several studies have demonstrated that $1,25(OH)_2D$ inhibits the transcriptional activity of β-catenin [162,163]. Vitamin D is a multilevel repressor of WNT/β-catenin and several studies have reported different crosstalk mechanisms between these pathways. $1,25(OH)_2D$ antagonizes the Wnt/β-catenin pathway by inducing VDR/β-catenin interaction, thus reducing the amount of β-catenin. Moreover, $1,25(OH)_2D$ induces E-cadherin expression, thus promoting the translocation of β-catenin from the nucleus to the plasma membrane and inducing Dickkopf (DKK)-1 expression, an extracellular inhibitor of WNT signaling, in colon carcinoma cells [162,164].

Furthermore, gene expression pattern analysis from Vitamin D-deficient mice showed up-regulated genes, some of which are involved in embryonic organ development and morphogenesis, including WNT receptor signaling [165]. Additionally, Huang*, et al.* [166] has recently demonstrated that Vitamin D supplementation ameliorated some structural changes of the airway in chronic asthma by down-regulating the activity of the WNT/β-catenin signaling pathway. The WNT/β-

catenin signaling pathway is indispensable during embryonic lung development. Several WNT ligands and β-catenin are expressed at the early stages of chick lung development, and their expression patterns are in agreement with the mammalian counterparts, pointing to a similar function for these ligands in the developing avian lung. As described by Moura *et al.* [18], canonical WNT signaling inhibition in the chick lung induces an impairment of secondary branch formation after 48 h of culture. Moreover, β-catenin knockout studies revealed that mesenchymal and epithelial β-catenin are necessary for branching morphogenesis and lung specification [167]. Inactivation of β-catenin led to trachea and lung agenesis, while conditional deletion of β-catenin in the mesenchymal cells of developing lung resulted in shortened trachea and significantly reduced branching morphogenesis [168]. One can hypothesize that the reduction in lung branching and growth after supplementation with $1,25(OH)_2D$ may be due to the interaction of these two signaling pathways. Antagonization of the WNT pathway by the action of $1,25(OH)_2D$ could explain the reduction in the total number of secondary buds, similar to that observed in [18].

To confirm that the morphological effects were indeed caused by activation of the Vitamin D signaling pathway, chick lung explants were probed for *cyp24a1*, a downstream target of this pathway and the main effector of its catabolic breakdown [169]. This enzyme regulates 25(OH)D and $1,25(OH)_2D$ intracellular levels to prevent detrimental effects and ensure appropriate tissue response. $1,25(OH)_2D$ supplementation led to a clear increase of *cyp24a1* expression level in all supplementation doses (**Figure 10**), confirming the activation of Vitamin D signaling pathway. It was not possible to visually confirm an increase in *cyp24a1* expression level in a dose-dependent manner, as we were expecting. The DMSO-treated explants appear to lack *cyp24a1* expression (**Figure 10c**). This phenomenon may be due to the fact that the developing reaction didn't act long enough for the expression of this gene to be detected, thus excluding the influence of the *in vitro* culture itself or the lack of real expression of *cyp24a1*. In fact, as previously described, the endogenous expression of *cyp24a1*, evaluated by *in situ* hybridization, had already disclosed very low basal levels of *cyp24a1* expression in the early stages of chick lung development. In this sense, if the explants had developed longer, the two expression patterns would have coincided. In contrast, the epithelial *cyp24a1* expression in whole-mount lungs shifted to the outermost region of the lung, the mesothelium, after calcitriol supplementation, in all tested doses (**Figure 10f, i, l**).

During lung development, the mesothelium has a crucial role in regulating lung size and morphogenesis, particularly through the interaction with the submesothelial mesenchyme. A clear example of this interaction is FGF9, which is produced by the mesothelium and signals to the

underlying mesenchyme, where it promotes proliferation in *fgf10*-expressing cells and inhibits airway smooth muscle cell (ASMC) differentiation [170,171]. To the best of our knowledge, to date, there is no description of crosstalk between the Vitamin D signaling pathway and the FGF10/FGF9 pathways. However, the interaction between these pathways in lung mesothelial cells may contribute to the reduction in chick lung branching and growth. The role of Vitamin D in malignant pleural mesothelioma (MPM) has been previously described. A recent study demonstrated that calcitriol reduced cell viability and proliferation in human MPM cells lines [172]. Moreover, in a recent study, which aimed to uncover the role of vitamin D in malignant pleural disease, 25(OH)D levels were found to be higher in pleural fluid than serum. The authors hypothesized that mesothelial and/or immune cells stimulated the sequestration of systemic 25(OH)D into pleura in response to the local insult. The sequestrated 25(OH)D would be the substrate for local production of the biologically active form, $1,25(OH)_2D$. However, there is no evidence in the literature for this "sequestration hypothesis" for the hormonally active form, nor is there a description of a specific relationship for augmented *cyp24a1* expression in mesothelium induced by $1,25(OH)_2D$ supplementation. In this sense, currently, we do not have a plausible explanation for this finding. Nevertheless, the previous experiment confirmed that, in the chick lung, *cyp24a1* is unequivocally a target of the vitamin D signaling pathway since its expression is up-regulated upon $1,25(OH)_2D$ supplementation. Therefore, it is reasonable to assume that the observed effects on lung branching and morphology are a direct result of the activation of Vitamin D signaling pathway.

$1,25(OH)_2D$ has potent effects on gene expression, cells, and biological mechanisms beyond the classical roles of maintaining skeletal integrity. The non-classical effects of calcitriol and its analogs include the regulation of several physiological processes like cell proliferation, differentiation and apoptosis, cell cycle progression, and immune modulation. Several experimental studies have demonstrated that vitamin D has a pro-differentiative and antiproliferative role in numerous normal and pathological cell types [173]. In this sense, we hypothesized that the significant reduction in lung size and branching observed after Vitamin D supplementation could be explained by the antiproliferative effect of vitamin D, particularly during these early stages of development marked by extensive cell proliferation.

To determine proliferation status during branching morphogenesis, an EdU proliferation assay was performed, using b2 staged lung explants, supplemented with 50, 150, and 300 nM of $1,25(OH)_2D$ (or DMSO, for the control group) (**Figure 13**). Results showed that high proliferation levels are detected in the tracheal region (white arrowhead) (**Figure 13b**) and in the distal tips of

the developing chick lung (white arrow) (**Figure 13k**). In addition, high proliferation levels of proliferation were also detected in the secondary bronchi (asterisk) (**Figure 13e**). This proliferation pattern was maintained in all treatment groups and control. These findings are consistent with previously published data that demonstrated that lung active branching sites (secondary bronchi), trachea, and distal lung are associated with high proliferation rates [51]. To further clarify the impact of vitamin D on proliferation levels during branching morphogenesis, a quantitative analysis of cell proliferation levels was performed by measuring the fluorescent labeling of Alexa Fluor and EdU, using an imaging software. The results demonstrated a significant reduction in proliferation levels in all calcitriol-treated explants, regardless of the dose, compared to the control (**Figure 14**). Furthermore, no significant difference was found between the supplementation doses. These results were in agreement with those observed for morphometric parameters, including branching analysis.

The antiproliferative effects and/or pro-differentiative actions of Vitamin D and its analogs are described in a variety of malignant and normal cell types, and in several cancer types, including colorectal [174], breast [175], prostate [176], and melanoma [177]. Although the mechanisms by which calcitriol regulates proliferation and differentiation are not yet completely understood, the pro-differentiative and antiproliferative effects are mostly mediated by the nuclear vitamin D receptor. For instance, studies performed in mammary tumor cell lines derived from VDR-knockout mice revealed a significant reduction of $1,25(OH)_2D$ antiproliferative effect, indicating that VDR mediates this effect [178]. Experimental evidence has uncovered that 1,25-dihydroxyvitamin D causes a G_0/G_1 cell cycle arrest, suggesting that calcitriol can regulate some factors involved in the G_1/S transition [179,180]. The same mechanisms are described in the respiratory system. For instance, in human bronchial airway smooth muscle cells sensitized with asthmatic serum, $1,25(OH)_2D$ had direct antiproliferative activity, and fewer cells proceeded into S (synthesis) phase [181]. In another study, Damera and coworkers have shown that Vitamin D reduced proliferation and growth of airway smooth muscle cells by halting cells in G_0/G_1 phase [182]. Presumably, the same effects are observed at this stage of development after $1,25(OH)_2D$ supplementation. Vitamin D may exert its antiproliferative role in chick lung epithelial and mesenchymal cells, preventing cell cycle progression in some of these cells that, in turn, display a significant reduction in proliferation levels (EdU incorporation); nonetheless, the proliferation patterns are maintained in places already described as high proliferation sites [51].

CHAPTER VI

FINAL REMARKS AND FUTURE PERSPECTIVES

VI. FINAL REMARKS AND FUTURE PERSPECTIVES

It is generally accepted that vitamin D deficiency is a worldwide health problem that affects a wide range of physiological functions. Hence, it is crucial to understand its role. The importance of Vitamin D signaling in mammalian lung development, particularly late development, has been explored. However, the regulatory role of this Vitamin in the early developmental stages is rather unexplored. Moreover, nothing is known about its function in the embryonic chick lung.

In this study, we characterized, for the first time, the spatial distribution of key signaling members of Vitamin D processing machinery, specifically *rxr-α*, *rxr-γ*, *vdr cyp24a1*, and *pdia3,* throughout b1, b2, and b3 stages of chicken lung development. The similarity between the expression patterns and low expression levels of these genes with the mammalian lung points to a similar role of this hormone in early stages of development and a less relevant role during these stages of development. To further determine Vitamin D function in early organogenesis and morphogenesis of the avian lung, *in vitro* lung explants were supplemented with increasing doses of $1,25(OH)_2D$, and its impact on lung growth and patterning was examined. During this window of exposure, Vitamin D supplementation impaired lung growth and branching. Based on studies performed in other branching organs, we speculate that these effects are probably due to the crosstalk between the Vitamin D signaling and other pathways that control proliferation and differentiation. Given the existing evidence regarding the interplay between Vitamin D and other regulatory lung signaling pathways, it would be important to evaluate possible crosstalk with these signaling pathways. For that, Vitamin D-treated explants could be assessed, by *in situ* hybridization, for components of different signaling pathways entangled in the events underlying early lung morphogenesis: *fgf10*, *fgf9*, *spry2*, and *axin2* that belong to the FGF and WNT signaling pathways, respectively.

One of the limitations of this study was that we did not test lower supplementation doses to determine the minimum dose at which the effects on branching and lung growth are detected. In this sense, future work should include lower $1,25(OH)_2D$ supplementation doses; besides, this could also exclude the possibility that the results reported in this this study were due to toxic supplementation doses. Furthermore, many *in vitro* and *in vivo* and studies have shown that maternal Vitamin D deficiency impacts normal lung development, although less is known about the effect at earlier stages. However, the chicken model does not allow the assessment of the influence of maternal VDD on lung development since the chicken embryo develops independently outside

the mother, in a self-sufficient egg. Nonetheless, it could be interesting to assess the impact of Vitamin D signaling inhibition during branching morphogenesis. Some VDR antagonists have already been described in the literature [183]. Thus, in the same way that the lung explant culture medium supplementation is a straightforward method to uncover the impact on lung morphogenesis and branching, Vitamin D inhibition could also bring new insights into the regulatory role of this vitamin during early lung development.

Furthermore, we explored the hypothesis that the reduction in lung size and branching could be due to the documented antiproliferative activity of Vitamin D. Results showed a clear reduction of proliferation at the sites of active proliferation during branching morphogenesis. Likewise, given the well-documented pro-differentiative role of vitamin D, it would be interesting to evaluate if Vitamin D supplementation promotes differentiation on lung tissue compartments. At this stage of development, cells are still undifferentiated; nevertheless, the spatial distribution and expression levels of differentiation markers such as *sox2* and *sox9*, transcription factors that regulate cell specification and differentiation during pseudoglandular stage, could be assessed. For that, Vitamin D-treated explants would be probed for *sox2* and *sox9* to determine the impact of $1,25(OH)_2D$ supplementation on lung proximal-distal patterning and pulmonary cell fate.

Even though preclinical studies have revealed several mechanistic roles of Vitamin D in lung development, there is still a lot to uncover regarding the effect of Vitamin D on lung microenvironment and resident cell interactions, particularly in the early stages of bronchial airway morphogenesis. It is accepted that pregnancy induces noticeable stress on vitamin D metabolism and that Vitamin D brings health benefits; nonetheless, there is still lacking information concerning the specific dosing and critical timing of vitamin D strategies to prevent lung abnormalities.

Our results highlight the importance of regulating vitamin D levels during normal lung development and reinforce the need to establish appropriate doses and intervention windows to ensure an appropriate physiological response during embryonic development. The relevance of the *in vitro* findings reported in this work needs further validation before translating them to the mammalian model.

In conclusion, this report has brought new insights about the potential role of vitamin D signaling in the early stages of chick pulmonary branching and contributes to a better understanding of this highly complex process.

CHAPTER VII

REFERENCES

VII. REFERENCES

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CHAPTER VIII

APPENDIX I

VIII. APPENDIX I

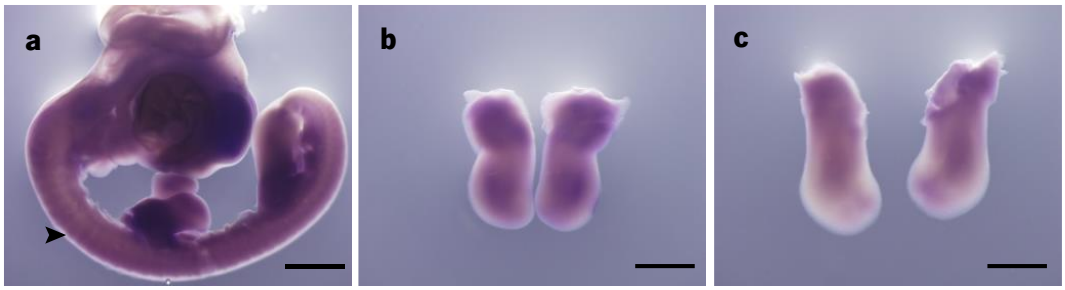

Figure 15 – *rxr-α* expression pattern in the chick embryo. Representative example of whole mount *in situ* hybridization of HH26 embryo. *rxr-alpha* is expressed **a** in the somites, and **b, c** front and hind limbs. Scale bar: 500 µm. *Dark arrowhead* somites.

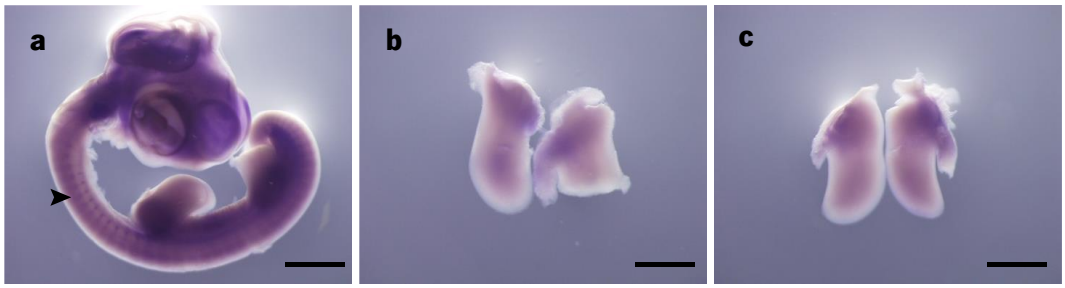

Figure 16 – *rxr-γ* expression pattern in the chick embryo. Representative example of whole mount *in situ* hybridization of HH25 embryo. *rxr-γ* is expressed **a** in the midbrain, forebrain and somites, **b, c** limb buds. Scale bar: 500 µm. *Dark arrowhead* somites.

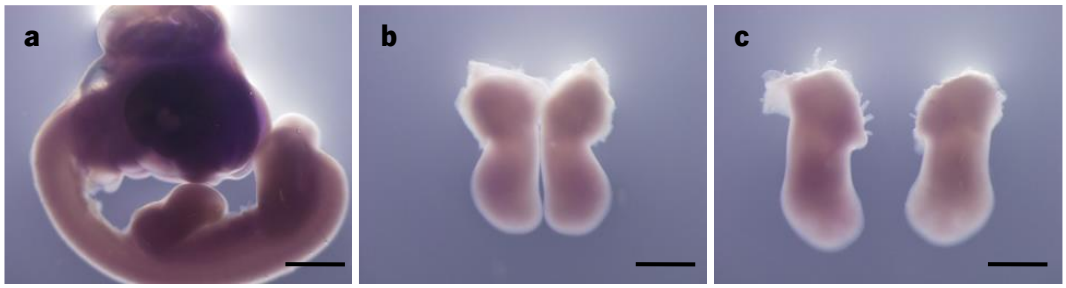

Figure 17 – *vdr* expression pattern in the chick embryo. Representative example of whole mount in situ hybridization of HH26 embryo. vdr is faintly expressed **a** throughout whole embryo body and **b, c** limbs. Scale bar: 500 µm.

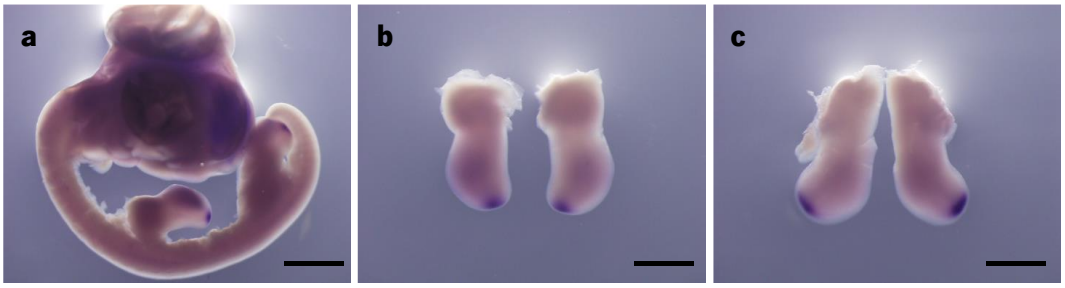

Figure 18 – *cyp24a1* expression pattern in the chick embryo. Representative example of whole mount *in situ* hybridization of HH26 embryo. *Cyp24a1* is expressed in **a** forebrain and **b, c** posterior region of front and hind limbs. Scale bar: 500 µm.

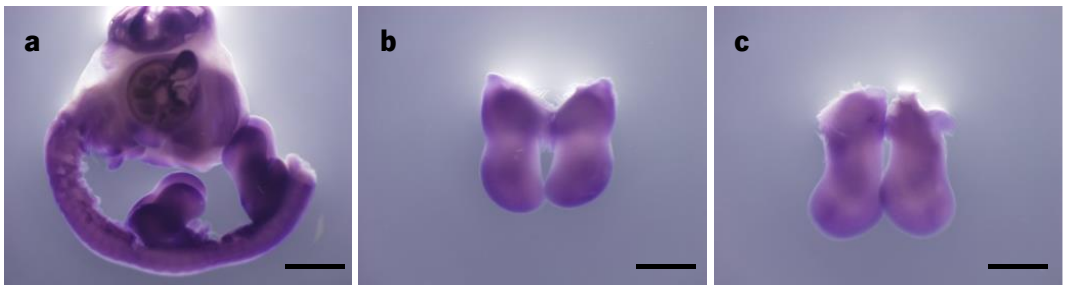

Figure 19 – *pdia3* expression pattern in the chick embryo. Representative example of whole mount *in situ* hybridization of HH25 embryo. *pdia3* is expressed **a** in the somites and **b, c** front and hind limbs. Scale bar: 500 µm.