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CECÍLIA GALLOTTINI DE MAGALHÃES

Sinalização BMP e modulação da matriz extracelular durante a embriogênese óptica

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CECÍLIA GALLOTTINI DE MAGALHÃES

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RESUMO

Magalhães CG. Sinalização BMP e modulação da matriz extracelular durante a embriogênese óptica [tese]. São Paulo: Universidade de São Paulo, Instituto de Ciências Biomédicas; 2023.

O desenvolvimento da lente é um modelo experimental clássico de morfogênese. A primeira evidência morfogênica da formação da lente é o placóide que surge pelo crescimento apical-basal da ectoderme cefálica em contato com a vesícula óptica. O processo de espessamento converte o epitélioo cuboidal em pseudoestratificado e ocorre apenas na região óptica, enquanto o ectoderma não placoidal circundante permanece cuboidal. A matriz extracelular (MEC) desempenha um papel importante na regulação dos processos morfogenéticos. No desenvolvimento inicial dos olhos, a Fibronectina é essencial para a formação do placóide da lente em camundongos. Nossos resultados anteriores mostram que a MEC entre a vesícula óptica e o placóide da lente evolui durante o espessamento do placóide. O padrão de marcação de Fibronectina e Laminina α1 muda na transição do epitélio cuboidal para o espesso. Antes do espessamento placoidal, a Fibronectina e a Laminina α1 exibem um padrão fibrilar homogêneo em todo o ectoderma cefálico, incluindo a região óptica. Durante o espessamento placoidal, a MEC torna-se difusa apenas na região óptica. Na região extra-placoidal, os componentes da MEC exibem um padrão fibrilar. Hipotetizamos que a MEC evolui junto com as mudanças da forma celular no placóide e sofre rearranjos restritos à região óptica. Como as células e a MEC mantêm uma relação dinâmica de interdependência e modulação, hipotetizamos que a MEC evolui juntamente com as mudanças de formato celular durante a diferenciação do placóide da lente e sofre rearranjos restritos à região óptica. Assim, nosso primeiro objetivo foi investigar se a evolução da MEC óptica depende da sinalização de BMP. A sinalização de BMP é necessária para a formação do placóide. Sem sinalização BMP na região óptica, a formação do placóide não ocorre e o desenvolvimento óptico é interrompido. Inibimos separadamente a sinalização BMP no ectoderma placodal e na vesícula óptica por meio da superexpressão de uma forma dominante negativa do receptor de BMP. Nossos resultados mostraram que a formação do placóide da lente e a evolução da MEC dependem da sinalização BMP no ectoderma placoidal, mas não na vesícula óptica. Nossos resultados mostraram que a formação do placóide do cristalino e a evolução da MEC dependem da sinalização de BMP no ectoderma placoidal, mas não da sinalização de BMP na vesícula óptica. Nosso segundo objetivo foi investigar quais fatores da MEC poderiam estar envolvidos à remodelação da MEC óptica. Para isso, analisamos genes associados à MEC expressos especificamente pelo placóide da lente e a investigamos a atividade proteolítica no tecido óptico. Esses dois processos envolvem importantes mecanismos de remodelação da MEC: deposição diferencial da MEC e a sua degradação. Para isso, realizamos análises *in silico*

de dados publicados de scRNAseq e Microarray. Nós identificamos diversos genes da MEC transcritos por células do placóide da lente, mas não por células da vesícula óptica. Este resultado sugere que o placóide é responsável pela regulação da MEC óptica. Em seguida, examinamos a atividade da metaloproteinases (MMPs) por meio de zimografia in situ e analisamos a expressão de Timp2, um inibidor de MMP. O ensaio de zimografia in situ sugeriu que a atividade de MMPs é inibida na região óptica durante o espessamento do placóide da lente, no memso estágio em que ocorre um aumento na expressão de Timp2 especificamente nas células do placóide da lente. Além disso, observamos a presença significativa de Adamts18 (uma desintegrina e metaloproteinase com motivos de trombospondina) especificamente na porção basal do placóide durante seu espessamento. Esses resultados sugerem que a atividade de protease é modulada de maneira específica na região do placóide da lente durante sua formação. Além disso, a inibição da sinalização BMP manteve a atividade da protease e diminuiu a expressão de Timp2 na região óptica. Esses resultados mostram que a expressão de Timp2 e a inibição das proteases dependem da sinalização BMP e da diferenciação do placóide da lente. Com isso, propomos que o placóide da lente desempenha um papel ativo na remodelação da matriz extracelular óptica.

Palavras-chave: Desenvolvimento embrionário. Placóide da lente. Matriz exrtacelular. BMP. Single-cell RNAseq

ABSTRACT

Magalhães CG. BMP signaling and modulation of the extracellular matrix during optical embryogenesis [thesis]. São Paulo: Universidade de São Paulo, Instituto de Ciências Biomédicas; 2023.

Lens development is a classic morphogenesis experimental model. The first morphogenic evidence of the lens formation is the placode. It appears through apical-basal growth of the single cuboidal epithelial layer (ectoderm) when it contacts the underlying optic vesicle. The thickening process converts the epithelium from cuboidal to pseudostratified and it happens only in the optic region, while the surrounding non-placodal ectoderm remains cuboidal. Extracellular matrix (ECM) plays a major role in regulating morphogenetic processes. In eye development, Fibronectin is essential for lens placode formation in mice. Our previous results show that the ECM between the optic vesicle and lens placode evolves during placode formation. Fibronectin and Lamininα1 labeling pattern changed in the transition from cuboidal to thick epithelium. Prior to placodal thickening, Fibronectin and Lamininα1 display a homogeneous fibrillar pattern throughout the cephalic ectoderm and optic region. During placodal thickening, ECM becomes diffuse only in the placodal region. In the extra-placodal region, ECM components display a fibrillar pattern. Since cells and ECM have a dynamic relationship of interdependence and modulation, we hypothesized that more components of the ECM evolve together with the cell shape changes during lens placode differentiation and undergo rearrangements restricted to the optic region. Thus, our first aim was to investigate if the optic ECM evolution depends on BMP signaling. BMP signaling is another necessary factor for lens formation. Without BMP signaling in the optic region, placode formation does not occur and eye development is interrupted. We inhibited BMP signaling separately in the placode ectoderm and in the optic vesicle through the overexpression of a dominant negative form of BMP receptor. Our results showed that lens placode formation and ECM evolution depends on BMP signaling in the placodal ectoderm but not on BMP signaling in the optic vesicle. Together, our results suggest that evolution of optic ECM architecture and composition depends on the lens placode ectodermal cells. Our second goal was to investigate which ECM factors could be involved in optic ECM remodelling. For this, we analyzed ECM associated genes specifically expressed by the lens placode and protease activity in the optic tissue. These two process drive important mechanisms of ECM remodelling: differential ECM deposition and ECM degradation. Thus, we performed *in silico* analyses of publicly available scRNAseq and microarray data. We identified several ECM genes transcribed by lens placodal cells but not by optic vesicle cells. This result suggests that the placode is modulates the optic ECM actively. Next, we examined extracellular protease activity through in situ zymography and analyzed the expression of Timp2, a metalloprotease (MMP) inhibitor. The in situ zymography assay

suggested that protease activity is downregulated in the optic region during lens placode thickening, concurrently with Timp2 expression increase specifically in the lens placode. In addition, we observed a significant presence of Adamts18 (a desintegrin and metalloproteinase with thrombospondin motifs) specifically in the basal portion of the placode during its thickening. These results suggest that protease activity is specifically modulated in the lens placode region during its formation. Furthermore, inhibition of BMP signaling maintained protease activity in the optic ECM and decreased Timp2 expression decreased in the optic region. These results suggest that Timp2 expression and protease inhibition depend on BMP signaling and lens placode differentiation. With that, we propose that the lens placode plays an active role in remodeling the optic ECM.

Key words: Embryo development. Lens placode. Extracellular matrix. BMP. Single-cell RNAseq.

LIST OF ACRONYMS

PCA: Principal Component Analysis

tSNE: t-distributed stochastic neighbor embedding

UMAP: Uniform Manifold Approximation and Projection

LIST OF FIGURES

Figure 1 - Simplified schematics of an adult human eye……..24

Figure 2 - Early eye development phases in chick embryo…….25

Figure 3 - Actin labeling in histological section and 3D reconstruction in the early stages of eye development……..26

Figure 4 - BMP and FGF regulate Pax6 expression at the lens placode region…….29

Figure 5 - BMP expression during early phases of eye development in chick and mouse embryos……31

Figure 6 - Noggin overexpression suppresses lens placode thickening……..32

Figure 7 - The ECM evolves during lens placode formation……...37

Figure 8 - Noggin overexpression inhibits optic ECM evolution……....39

Figure 9 - Analysis of BMP signalling through BRE-driven expression……...58

Figure 10 - BMP signaling is active in the lens placode between phases 0 and 2a……....60

Figure 11 - BMP signaling is active in the optic vesicle between phases 0 and 2b……61

Figure 12 - Type 1 BMP receptor in the pre-placodal ectoderm is required for the formation of the lens placode……..64

Figure 13 - Overexpression of tBMPr together with BRE show a delay in the BMP signalling inhibition……...66

Figure 14 - tBMPr in pre-placodal cells inhibits ECM evolution…….68

Figure 15 - The expression of tBMPr in the optic vesicle did not affect ECM evolution…….69

Figure 16 - Lamininα1 labeling has a degraded aspect in placodal region in mouse…….71

Figure 17 - Lamininα1 labeling is more intense in the optic vesicle basal domain than lens placode during invagination in mouse……...72

Figure 18 - Workflow of the scRNAseq data analysis……74

Figure 19 - Identification of optic tissue cells in clusters 10 and 21 among Pax6-positive cells……..78

Figure 20 - Identification of cluster 5 as containing lens placode cells……82

Figure 21 - Lens placode cluster shows specific expression of several ECM genes……..84

Figure 22 - In silico analysis of differentially expressed genes in Pax6lens-/- mice from publicly available Microarray data……86

Figure 23 - Identification of ectodermal cells in clusters of the eye region dissected from mouse embryo at stage E9.5……90

Figure 24 – Identification of lens placode cells and non-placodal cephalic ectoderm cells from Krt8-positive cells……..91

Figure 25 - Tenascin and Nidogen1 are present in the ECM between lens placode at phase 0 in mouse embryos……93

Figure 26 - Tenascin and Nidogen1 are expressed in the chick embryonic lens at later stages……94

Figure 27 - Tenascin is present in the ECM between lens placode and optic vesicle at phase 0 and 2b……96

Figure 28 - Nidogen and Lamininα1 are present in the ECM between lens placode at phase 0 and 2b……96

Figure 29 - Timp2 is expressed in the lens placode at phase 2a……..98

Figure 30 – Detection of gelatinase activity with in situ zymography……100

Figure 31 – Gelatinase activity decreases during lens placode formation in the optic region……..101

Figure 32 - Timp2 expression in lens placode depends on BMP signalling…..102

Figure 33 - Inhibition of BMP signalling maintains protease activity in the optic ECM…….103

Figure 34 - Conserved domains close to Timp2 gene show several transcription factors binding sites for lens-associated transcription factors…….105

Figure 35 - Adamts18 is expressed in the lens placode and changes its subcellular location between phases 0 and 2a……..107

Figure 36 - Adamts18 and Pax6 are expressed in the lens placode from phase 0 to phase 2b……..110

Figure 37 - Conserved domains close to Adamts18 gene show several transcription factors binding site for lens-associate transcription factors……111

Figure 38 - In the lens placode, activated IntegrinB1 concentrates in the apical domain of the cells……..113

Figure 39 - There is no Integrinα9 in the lens placode in mouse embryo at stage E11.0 (phase 2b)……...114

Figure 40 - Diagram summarizing the results obtained in this study……..127

SUMMARY

ABSTRACT..... 13

LIST OF ACRONYMS..... 15

LIST OF FIGURES 16

1 INTRODUCTION 23

1.1 EARLY EYE DEVELOPMENT IN VERTEBRATES..... 23

1.2 MORPHOLOGICAL EVENTS OF EARLY EYE DEVELOPMENT……... 24

1.3 MOLECULAR EVENTS OF EARLY EYE DEVELOPMENT..... 28

1.4 INTERACTION BETWEEN OPTIC VESICLE AND LENS PLACODE..... 33

1.5 EXTRACELLULAR MATRIX ROLE IN DEVELOPMENT..... 34

1.6 EXTRACELLULAR MATRIX DURING VERTEBRATE EYE DEVELOPMENT……..... 36

1.7 EXTRACELLULAR MATRIX REMODELING 39

1.7.1 ECM protein deposition 40

1.7.2 ECM degradation..... 41

1.7.3 Force-mediated ECM modification..... 42

1.8 HYPOTHESIS 44

1.9 OBJECTIVES..... 44

2 MATERIAL AND METHODS 45

2.1 OBTAINING AND INCUBATING EMBRYONATED EGGS..... 45

2.2 OBTAINING MOUSE EMBRYOS..... 45

2.3 ELECTROPORATION IN OVO 45

2.4 IN SITU WHOLE-EMBRYO HYBRIDIZATION 46

2.4.1 Cloning Timp2 sequence 47

2.5 IMMUNOFLUORESCENCE OF WHOLE-MOUNT CHICK AND MOUSE EMBRYOS..... 47

2.6 EMBRYOS CRYOSECTION 48

2.7 In situ zymography using DQ-gelatin..... 48

2.8 MOUNTING CHICK AND MOUSE EMBRYOS FOR IMAGING …….. 49

2.9 IMAGING OF WHOLE-MOUNT EMBRYOS AND IMMUNOFLUORESCENCE OF CRYOSECTION 50

2.10 IMAGE PROCESSING..... 51

2.11 ANALYSIS OF SINGLE-CELL TRANSCRIPTOME DATA OF MOUSE EMBRYOS AT STAGE E9.5 AND 10.5 FROM THE MOUSE ORGANOGENESIS CELL ATLAS..... 51

2.11.1 Downloading data 51

2.11.2 Data analysis 52

2.12 ANALYSING MICROARRAY DATA OF WILDTYPE AND PAX6LENS-/- MOUSE EMBRYOS AT STAGE E9.5..... 54

2.13 ANALYSIS OF SINGLE-CELL TRANSCRIPTOME DATA OF DISSECTED EYE FROM MOUSE EMBRYOS AT STAGE E9.5..... 55

2.13.1 Downloading data 55

2.13.2 Data analysis 56

3 RESULTS 57

3.1 SPATIO-TEMPORAL ANALYSIS OF BMP SIGNALING ACTIVATION 57

3.2 BMP SIGNALING INHIBITION..... 62

3.2.1 Tissue-specific BMP signaling inhibition in neural and non neural ectoderm during lens placode development..... 62

3.2.2 Delay between expression of tBMPr and inhibition of BMP signaling...... 65

3.2.3 ECM modulation upon tissue-specific BMP signaling inactivation... 66

3.3 IN SILICO INVESTIGATION OF LENS PLACODE OVEREXPRESSED GENES WITH MOUSE SINGLE-CELL RNASEQ DATA FROM CAO ET AL., 2019 70
3.3.1 Evolution of optical ECM in mouse embryos 70
3.3.2 Obtaining the scRNAseq data 72
3.3.3 Data quality and filtering 75
3.3.4 Normalizing expression values and clustering the cells 75
3.3.5 Analyzing the clusters and finding eye cells cluster 77
3.3.6 Analyzing eye cells clusters 80
3.3.7 Differently expressed extracellular matrix genes in the lens placode 83
3.4 In silico investigation of Pax6-regulation of ECM during lens placode development with mouse Microarray data from Huang et al., 2011 84
3.5 IN SILICO INVESTIGATION OF LENS PLACODE OVEREXPRESSED GENES WITH MOUSE SINGLE-CELL RNASEQ DATA FROM YAMADA ET AL., 2021 87
3.5.1 Obtaining and processing the data 88
3**.5.2 Analysis and classification of the clusters** 89
3.5.3 Analysis of ectodermal subclusters: lens placode and surface ectoderm clusters 89
3.6 EXPRESSION OF NID1 AND TNC IN THE OPTIC REGION 92
3.7 INVESTIGATION OF PROTEASE ACTIVITY AND ITS INHIBITION IN THE OPTICAL REGION 97
3.7.1 Timp2 expression in the optical region during lens placode development 97
3.7.2 Metalloprotease activity analysis in the optical region 99

3.7.3 Timp2 expression and metalloprotease activity after BMP signaling inhibition..... 102
3.7.4 In silico investigation of lens placode transcription factor binding sites around Timp2 gene..... 104
3.7.5 Adamts18 expression during lens placode development……..... 105
3.7.6 Comparing Pax6 and Adamts18 expression 108
3.7.7 In silico investigation of Pax6 and Pax6-downstream transcription factor binding sites in Adamts18 gene 111
3.8 ANALYSIS OF INTEGRINB1 ACTIVITY IN MOUSE LENS PLACODE…. 112
4 DISCUSSION 115
4.1 BMP SIGNALING IS NECESSARY FOR LENS PLACODE FORMATION AND OPTIC ECM MODULATION..... 115
4.2 OPTIC ECM EVOLUTION DEPENDS ON BMP SIGNALING IN THE LENS PLACODE 116
4.3 OPTIC ECM CHANGES DEPEND ON THE LENS PLACODE DEVELOPMENT..... 118
4.4 REGULATION OF ECM DEGRADATION DEPENDS ON LENS PLACODE FORMATION 118
4.5 MMPS ACTIVITY DECREASES WHILE TIMP2 EXPRESSION INCREASES DURING LENS PLACODE THICKENING 122
4.6 REGULATION OF TIMP2 EXPRESSION 123
4.7 SUBCELLULAR LOCALIZATION OF ACTIVE INTEGRINB1 CHANGES DURING LENS PLACODE EARLY FORMATION 124
5 CONCLUSIONS 127
5.1 FUTURE DIRECTIONS..... 129

1 INTRODUCTION

1.1 EARLY EYE DEVELOPMENT IN VERTEBRATES

Morphogenetic processes are responsible for organs and tissues shapes and position. During embryonic development, morphogenetic movements highly occur repeatedly in different events of organogenesis and are driven by conserved cellular mechanisms. For example, invagination consists in coordinated deformation of a epithelial sheet. It occurs and has been studied in several process (ETTENSOHN, 1985). It is involved in neural tube formation of vertebrates, in sea urchins and Drosophila gastrulation, cranial placodes development and trachea formation (ETTENSOHN, 1985; SCHOENWOLF, 1988; KIMBERLY; HARDIN, 1998; JIDIGAM; GUNHAGA, 2013; JIDIGAM et al., 2015; DAVIDSON et al., 2018).

Eye development is a classical study model for tissues morphogenesis. The shape and anatomy of the eye is crucial for its function. For light rays to transverse properly through the distal optical tissues to reach the retina, all the eye structures must be perfectly aligned and shaped. Optic shape is formed through a series of complex tissue changes. The molecular factors and morphogenesis processes involved in early eye development are highly conserved among vertebrates.

These same morphogenetic events are also observed during formation of other embryonic tissues that are less accessible to experimental procedures and observation. Thus, the eye has the advantage of being an external tissue, easily accessible and visible. For this reason, it is a popular classical model to investigate tissue-shape changes and induction events during embryogenesis.

There are three main tissues that compose the adult eye: retina, cornea, and lens (Fig. 1). These three tissues are derived from two primary embryonic tissues: the cephalic ectoderm forms the non-neural portion of the eye (cornea and lens) and the optic vesicle forms the neural portion (retina). We will now briefly describe the morphogenesis of these elements during early eye development in chick embryo. For this, we will use the stages of chick development defined by Viktor Hamburger and Howard Hamilton in 1992 and, therefore, the stages are given the letters "HH" (HAMBURGUER, VIKTOR.

HAMILTON, 1992). Specifically, we will describe events that happen during neurulation (between stages HH8 and HH9) and move on to placode formation at stage HH14.

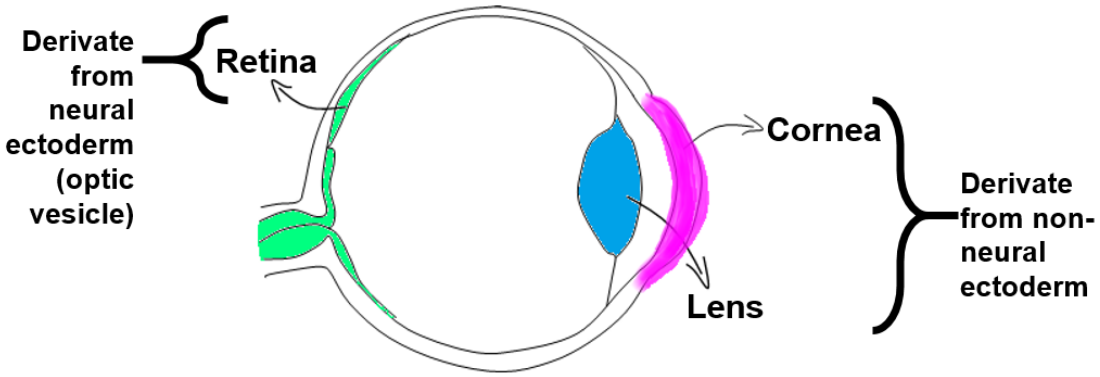


Figure 1. Simplified schematics of an adult human eye. Three main cellular tissues are in the light path of the adult vertebrate eye: the cornea (pink), the most distal transparent and convex layer that covers its outer surface; the lens (blue), also a transparent tissue that is avascular, non- innervated and biconvex; and the retina (green), the third and most proximal structure that is the neural sensitive component of the eye. The cornea and lens derive from the non-neural ectoderm and the retina derive from the optic vesicle, a neural ectodermal tissue. The cornea allows the light to enter in the eye chamber and is the first refractive element that light encounters. It is composed by a simple layer of epithelium cells, stroma and an innermost endothelium layer (LWIGALE, 2015). The lens refracts the light rays and directs them onto the retina. The retina is a cup -shaped tissue composed by the neural retina. It contains the photoreceptors (rods and cones), who transduce the luminous stimulus into neural signals that are further processed by additional neural layers of the retina (CHOLKAR et al., 2013).

1.2 MORPHOLOGICAL EVENTS OF EARLY EYE DEVELOPMENT

The first morphogenetic event of eye development is the formation of the optic vesicle. At stage HH9 (corresponding to ~30h of incubation) of chick embryo development, a specific portion of the diencephalon extends outwards bilaterally and forms the early optic vesicle. After complete evagination of the optic vesicle, this neural tissue approaches the superficial epithelia at stage HH11 (~43h incubation; Fig. 2). For clarity, we will classify the early stages of eye formation based on changes in lens placode histology (MAGALHÃES; DE OLIVEIRA-MELO; IRENE YAN, 2021). At stage HH11, the region of the cephalic ectoderm in contact with the optic vesicle is defined as pre-placodal ectoderm. We will call this phase of lens development as phase 0 (Fig. 2). At phase 0, the pre-placodal ectoderm is composed by cuboidal cells, where the actin filaments are homogeneously distributed the cytoplasm (Fig. 3A, E, I and L).

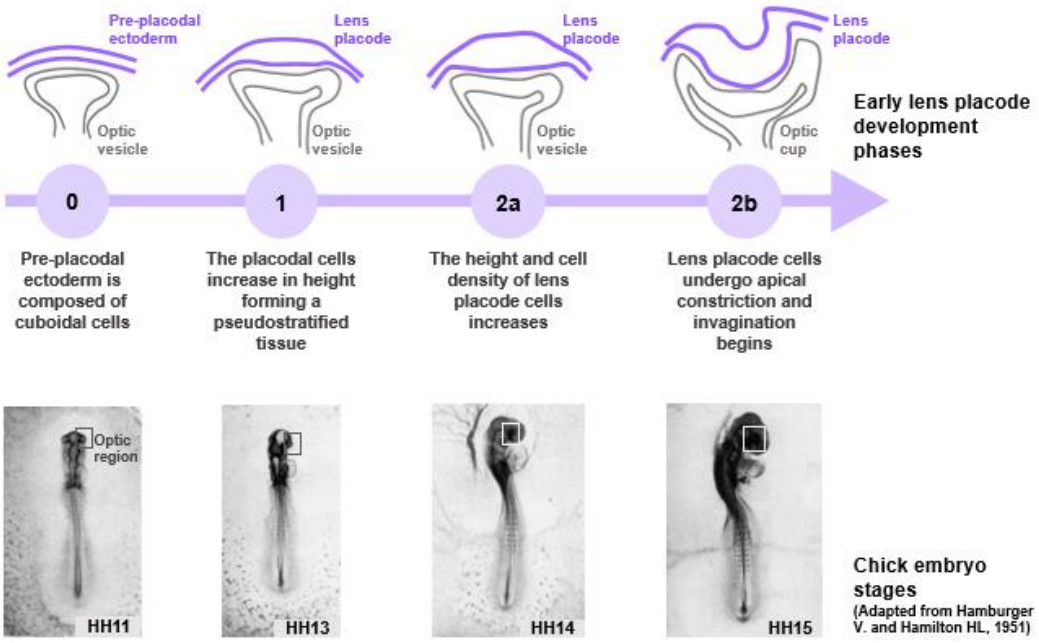
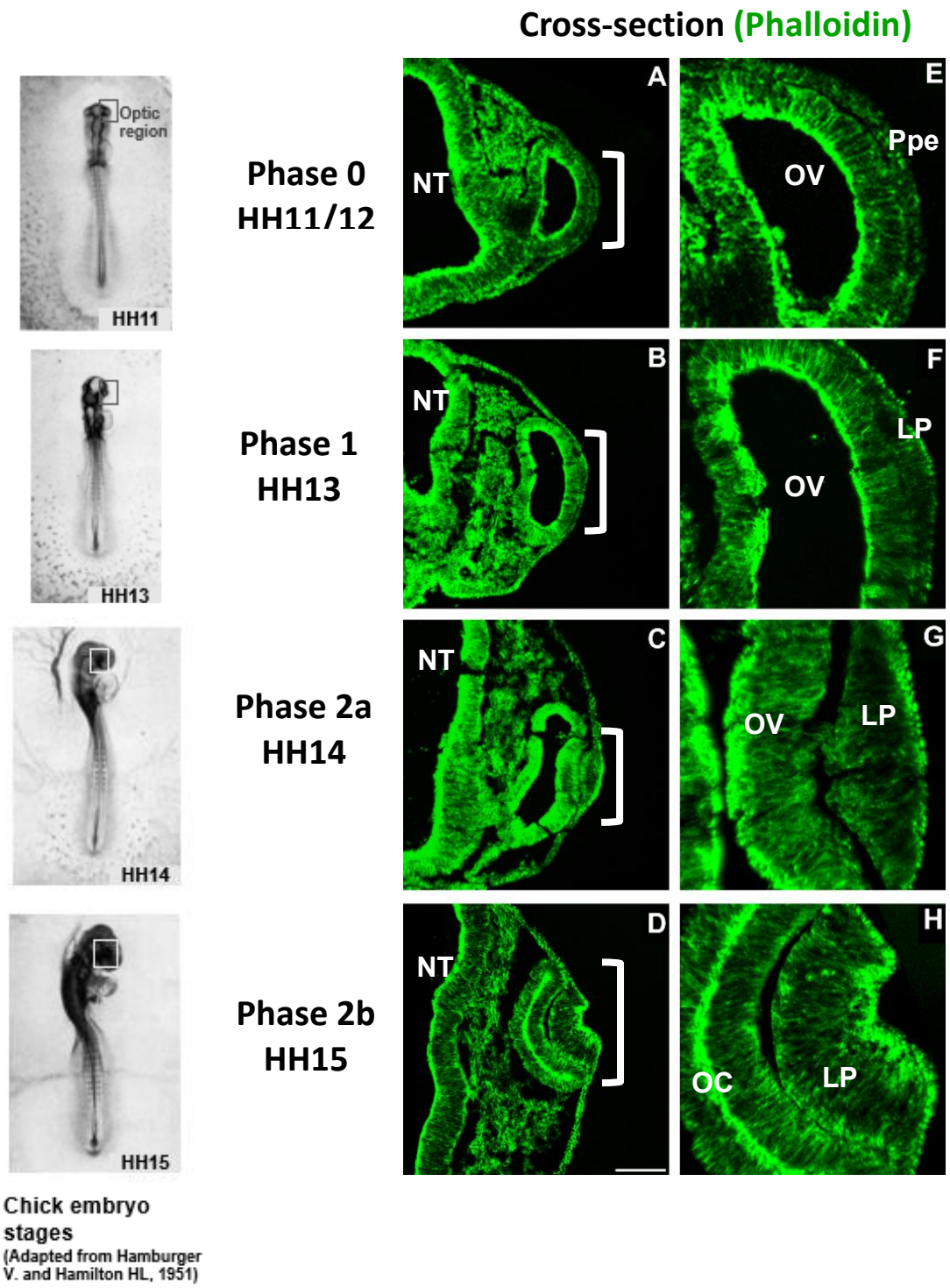


Figure 2. Early eye development phases in chick embryo. The morphogenetic events involved in lens placode formation and invagination are represented here in 4 steps. At the top of the image, there are simplified representations of the optic vesicle and lens placode shape at each phase. At the bottom of the image, there are pictures of the chicken embryo at stages corresponding to the drawings (HAMBURGER and HAMILTON, 1951). (Phase 0) Pre-placodal ectoderm is composed of cuboidal cells. The optic vesicle is evaginated and interacts with the surface ectoderm. (Phase 1) Early thickening of lens placode. The placodal cells increase in height forming a pseudostratified tissue. (Phase 2a) Late thickening of lens placode. The height and cell density of lens placode increases. (Phase 2b) Lens placode begins to invaginate. Placodal height does not increase and the apical constriction begins, forming the early lens pit.

The second crucial morphogenetic event for eye development is the formation of the placode. The pre-placodal ectoderm undergoes an apical-basis axis elongation between stages HH13 and 14 (Fig. 2, phase 1 and 2a). This thickening defines the lens placode morphologically and is divided by two phases, phase 1 and 2a (Fig. 2). At phase 1, the lens placode in converted from a simple cuboidal epithelium into a pseudostratified epithelium that reaches a thickness of about 20 µm in chick embryos (Fig. 2 and Fig. 3B and F) (SCHOOK, 1980; MAGALHÃES; DE OLIVEIRA-MELO; IRENE YAN, 2021). In contrast, the non-placodal ectoderm remains at the height of 7 µm (SCHOOOK, 1980; reviewed in MAGALHÃES, OLIVEIRA-MELLO, YAN, 2021). At phase 2a, cell height and density increase further, and the lens placode reaches 36 µm (Fig. 2 and Fig. 3C and G) (SCHOOOK, 1980; reviewed in MAGALHÃES, OLIVEIRA-MELLO, YAN, 2021). This transition from a cuboidal monolayer tissue into a thickened pseudostratified placode is characterized by extensive cytoskeletal reorganization (Fig. 3 C and J). Electron microscopy images demonstrate a

rearrangement of the cytoskeleton during lens placode thickening where the microtubules become organized parallel to the apico-basal axis of the cells, following cell elongation (reviewed in MAGALHÃES, OLIVEIRA-MELLO, YAN, 2021).



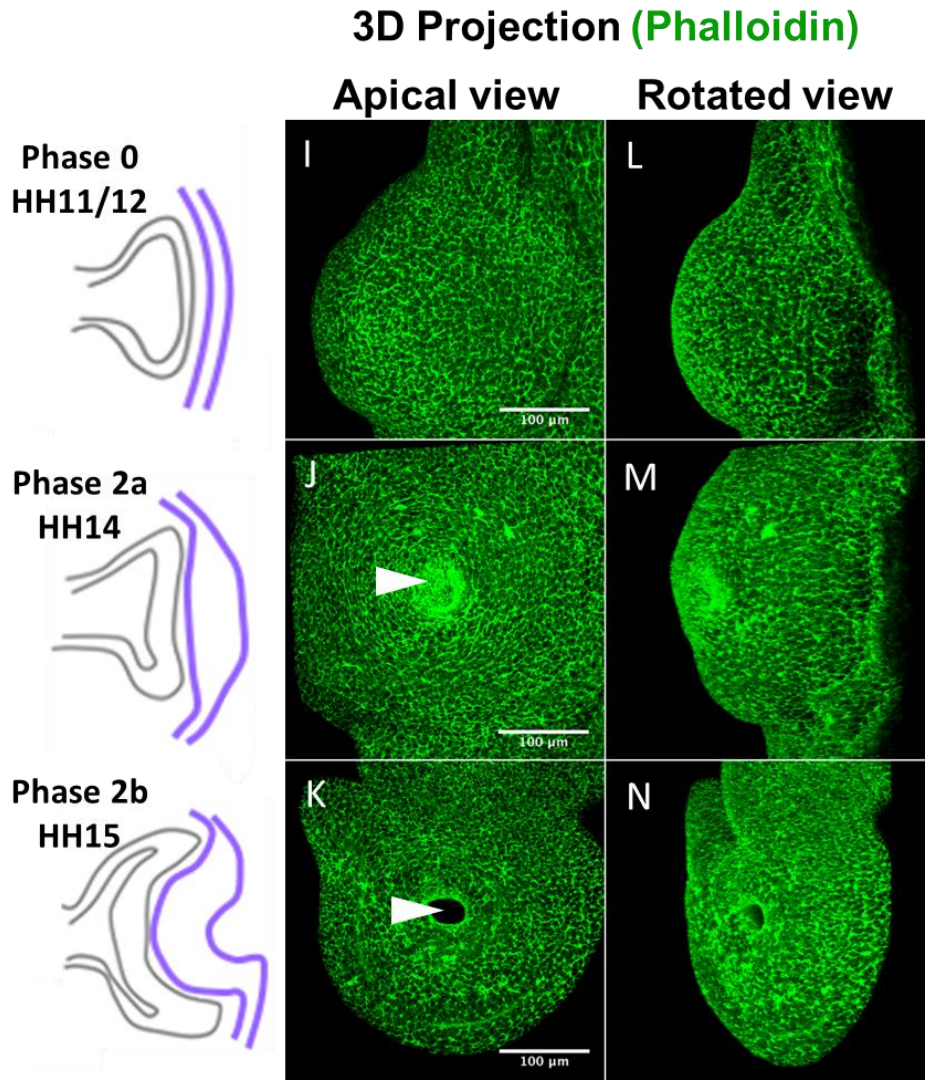


Figure 3. Actin labeling in histological section and 3D reconstruction in the early stages of eye development. (A-H) Cross section of the chick embryo at the eye region in different stages (HH11-HH15) and stained for actin with phalloidin (green), the white bracket indicates the optical region. (A-D) Lower magnification of the right side of the head with the neural tube on the left and the optic vesicle, ectoderm and lens placode on the right side. (E-H) Higher magnification of eye from the same section. (A and E) Stage HH11/12: the pre-placodal cells (cephalic ectoderm) adjacent to the optic vesicle are cuboidal and actin labeling is diffuse. (B and F) Stage HH13: thickening of placodal cells begins and the transition from pre-placode to placode starts to become visible. Also, actin labeling in the placodal cells is apical. (C and G) Stage HH14: the placode is now morphologically defined and the cells have grown in the apical-basal axis. Actin remains apical. (D and H) Stage HH15 lens placode undergoes apical constriction and starts to invaginate while the optic vesicle bends inwards following its movement. Scale bar in D: 100 µm. NT: neural tube; OV: optic vesicle; OC: optic cup; Ppe: pre placode ectoderm; LP: lens placode. (I-L) 3D projection of images obtained in confocal microscopy of chick embryo stained with phalloidin in different eye development stages. The optical slices were taken apically and the 3D projection was turned. (I and L) Stage HH11, the placode is not formed and the surface has a dome shape. (J and M) Stage between HH14 and HH15 where placode is formed and the surface starts to deform in the centre of the placode where the lens pit appears (with arrowhead). (K and N)The lens placode invaginates and the lens pit deepens (white arrow points to the invagination centre). Images were taken on confocal microscope. Scale bar (bottom right corner of images I-K): 100 µm. **Data published in the Master's dissertation: Magalhães, 2019**

After cell height increase (phase 2b), actomyosin filaments accumulate in the apical domain (Fig. 3C and G) (BORGES et al., 2011). Together with accumulation of actin in the apical domain, Rho GTPses also are concentrated in the apical surface and their activity is necessary for the accumulation of myosin that activates apical constriction (Fig. 2 phase 2b, Fig. 3 D, H, J and M) (BORGES et al., 2011; PLAGEMAN et al., 2011). Apical constriction triggers the invagination of the lens placode at stage HH15 (Fig. 2 and Fig. 3D, H, J-N). Consistent with the requirement for an activation step, the accumulation of actin in the apical domain is not sufficient for the onset of apical constriction and the ensuing placode invagination (JIDIGAM et al., 2015; MELO; MORAES BORGES; YAN, 2017). Inhibition of actin-myosin contraction prevents apical constriction and interferes with epithelial invagination (BORGES et al., 2011).

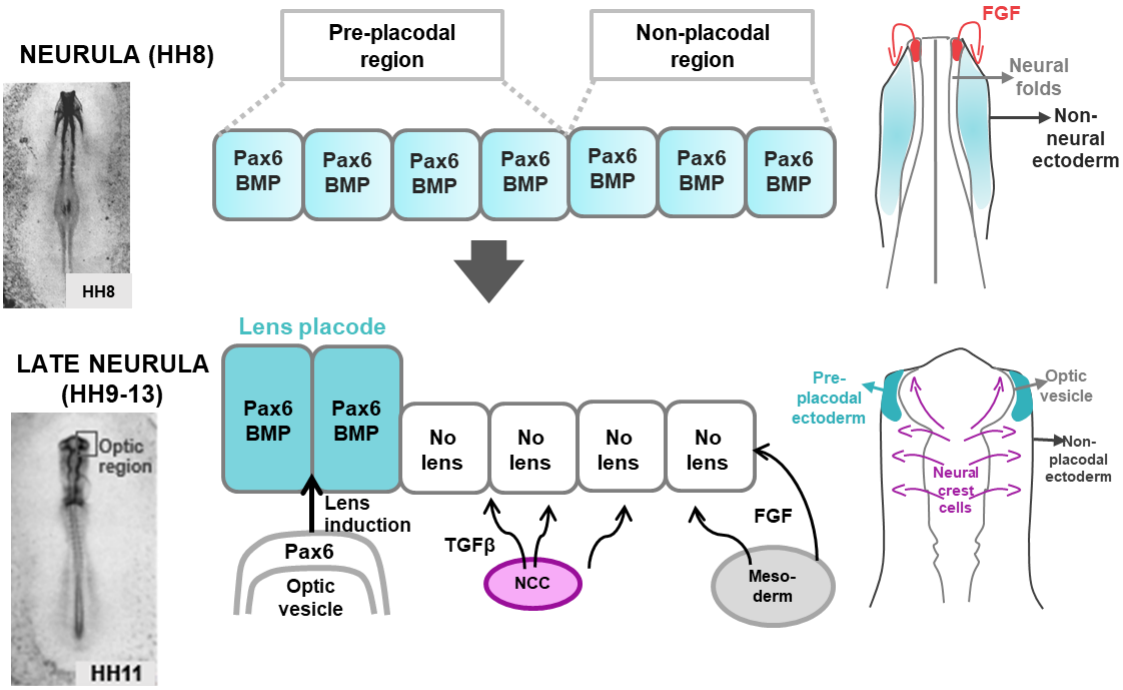
1.3 MOLECULAR EVENTS OF EARLY EYE DEVELOPMENT

The lens placode field is defined molecularly in the ectoderm through a series of molecular signaling events. First, the presumptive lens placode region is defined before the contact between the optic vesicle and the pre-placodal ectoderm (phase 0).

In chick embryos (stages HH8-9), the pre-placodal region is restricted to a specific portion of the cephalic ectoderm. Although it is morphologically like the surrounding non-placodal ectoderm, the pre-placodal region can be identified molecularly by the expression field of Pax6 after HH9. Pax6 is a transcription factor important for eye and neural development. Pax6 expression is sufficient and necessary for lens placode development. Ectopic expression of Pax6 induces ectopic lens in amphibians (ALTMANN et al., 1997), and lack of Pax6 expression in the pre-placodal ectoderm at phase 0 inhibits lens development (ASHERY-PADAN et al., 2000; PLAGEMAN et al., 2010; ANTOSOVA et al., 2016a).

Before optic vesicle formation, at stage HH8, Pax6 is already expressed in the pre-placodal region (Fig. 4) (BAILEY et al., 2006). But Pax6 is also expressed at the anterior portion of neural folds and the non-placodal ectoderm (LLERAS-FORERO et al., 2013; MAGALHÃES; DE OLIVEIRA-MELO; IRENE YAN, 2021). After optic vesical evagination, at stage HH9, the expression of Pax6 becomes

restricted to the optic vesicle and future lens placode (Fig. 4) (ASHERY-PADAN et al., 2000; PLAGEMAN et al., 2010; ANTOSOVA et al., 2016a; MAGALHÃES; DE OLIVEIRA-MELO; IRENE YAN, 2021).



Adapted from Magalhães et al., 2021 and Streit et al., 2008

Figure 4. BMP and FGF regulate Pax6 expression at the lens placode region. At neurula stages, the pre-placodal region (PPR) is defined at the surface ectoderm. The anterior portion of non-neural ectoderm express Pax6 and BMP. Later, the balance between FGF (red) and BMP signal defines the specific placodes fields in the PPR. The maintenance of FGF inhibits lens placode fate, while BMP signal maintains lens placode fate. At late neurula stages, the optic vesicle contacts the overlying PPR and reinforces lens formation. Thus, lens fate is suppressed everywhere except at ectoderm overlying the optic vesicle (blue). FGF is secreted by the anterior neural ridge at the anterior portion of the PPR and the head mesoderm (gray circle), thus restricting the lens placode fate by repressing lens characteristics. At late neurula stages, BMP and Pax6 is also required for definition of lens placode fate. The optic vesicle approaches the pre-placodal ectoderm, and induces lens placode-specific gene expression. After the approximation of optic vesicle to the ectoderm, the restriction of lens character continues with the migration of neural crest cells (NCC, purple circle and purple arrows). Neural crest cells secrete TGFb, which inhibit Pax6 activity in the ectoderme around the optic region.

The restriction of Pax6 expression domain after HH8 depends on the levels of bone morphogenic protein (BMP) and fibroblast growth factor proteins (FGF) (Fig. 4), which are crucial for the pre-placodal region specification (STREIT, 2008; MAGALHÃES; DE OLIVEIRA-MELO; IRENE YAN, 2021). BMP is a ligand from the TGF-β family and FGF is a large family of polypeptide growth factors. Both are important morphogens involved in various developmental processes. At

neurula stages, HH8 and 9, intermediate levels of BMP signaling are required to maintain Pax6 expression in the optic region (LITSIOU; HANSON; STREIT, 2005; HINTZE et al., 2017). In contrast, FGF plays an important role in delimiting the lens placode region by repressing lens fate in the surrounding ectoderm (Fig. 4) (BAILEY et al., 2006; SJÖDAL; EDLUND; GUNHAGA, 2007).

After the optic vesicle approaches the overlying epithelia (HH10), the restriction of lens character continues with the migration of neural crest cells (Fig. 4) (BAILEY et al., 2006; STREIT, 2008). Neural crest cells derive from the neural plate and migrate after neural tube invagination. They restrict development of lens fate outside pre-placodal region, and their absence results in ectopic lens formation (LITSIOU; HANSON; STREIT, 2005; BAILEY et al., 2006; GROCOTT et al., 2011). With this, at phase 0, Pax6 expression is restricted to the optic region and is only expressed at the lens pre-placodal ectoderm and optic vesicle (Fig. 4). This pattern remains constant during the next phases of optic development (phase 1 to 2b) (HUANG et al., 2011; ANTOSOVA et al., 2016a).

BMP signals are also crucial for lens formation from phase 0 to phase 2b (FURUTA; HOGAN, 1998; SJÖDAL; EDLUND; GUNHAGA, 2007; PANDIT; JIDIGAM; GUNHAGA, 2011; HUANG et al., 2015a; JIDIGAM et al., 2015). At phase 0 in chick embryos, BMP4 is expressed only in the pre-placodal ectoderm, but not in the optic vesicle (Fig. 5) (TROUSSE; ESTEVE; BOVOLENTA, 2001; MÜLLER; ROHRER; VOGEL-HÖPKER, 2007; PANDIT; JIDIGAM; GUNHAGA, 2011). Despite the absence of BMP4 expression labeling in the optic vesicle in phase 0, both pre-placodal ectoderm and optic vesicle harbour phosphorylated Smad1/5/8- positive cells (GUNHAGA, 2011; PANDIT; JIDIGAM; GUNHAGA, 2011; JIDIGAM et al., 2015). Contrarily, in mouse embryos, BMP4 is expressed in both tissues (Fig. 5) (BEHESTI; HOLT; SOWDEN, 2006; HUANG et al., 2011, 2015a).

At placodal phases 1 and 2a, BMP4 is expressed in both tissues in mouse and chick embryos (Fig. 5). However, in chick, BMP4 expression is detected only in the dorsal domain of optic vesicle (TROUSSE; ESTEVE; BOVOLENTA, 2001; MÜLLER; ROHRER; VOGEL-HÖPKER, 2007; PANDIT; JIDIGAM; GUNHAGA, 2011). The presence of phosphorylated Smad1/5/8 remains in both tissues during phases 2a and 2b (PANDIT; JIDIGAM; GUNHAGA, 2011). This data

suggests that the canonical BMP pathway is active in the entire optic region even during early placodal stages.

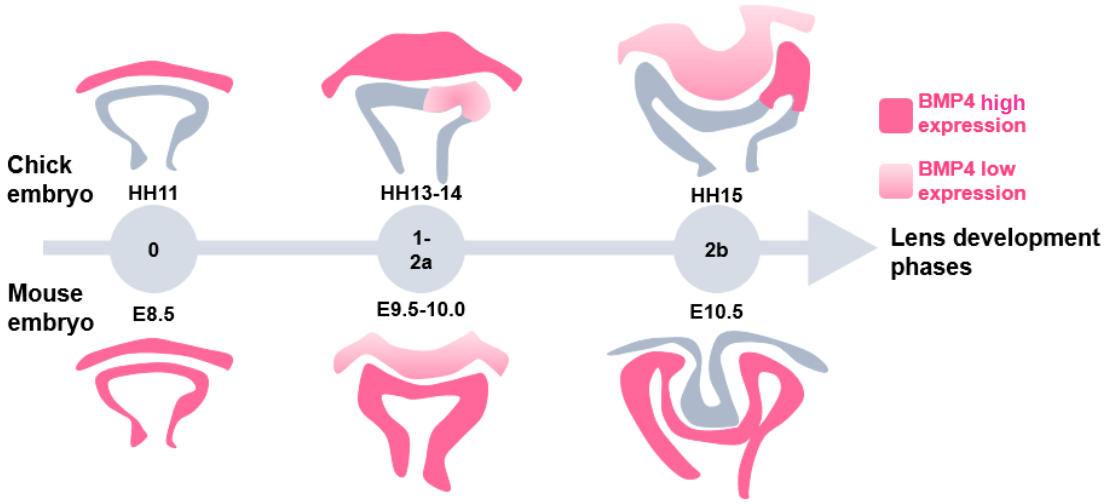


Figure 5. BMP expression during early phases of eye development in chick and mouse embryos. Simplified schematics showing BMP4 expression pattern (pink) in chicken embryo (top row) and mouse embryo (bottom row) at different stages of lens placode development. At phase 0 in chick embryos, BMP4 is expressed only in the pre-placodal ectoderm. In mouse embryos, BMP4 is expressed in both tissues. At placodal phases 1 and 2a, BMP4 is expressed in both tissues in mouse embryos and chick embryos. In chick, it is detected only in the dorsal domain of optic vesicle. In mouse embryos BMP4 expression decreases in the placode at phase 2a and is absent at phase 2b. In chick embryos, BMP4 expression remains at both tissues at phase 2b. In the lens placode, BMP4 expression continues throughout the tissue and, in the optic vesicle, the expression is concentrated in the dorsal domain of the optic cup.

Our previous results show that lack of BMP signaling in the optic region at phase 0, inhibits lens placode thickening (Fig. 6). We inhibited BMP by Noggin overexpression, a secreted protein that interacts with extracellular BMP, preventing its binding to the BMP receptor (WALSH et al., 2010). After Noggin overexpression at the pre-placodal ectoderm, the placode failed to thicken and eye development was arrested at phase 0 (Fig. 6). This confirms previous studies have also demonstrated that BMP signaling is required for lens placode thickening. In mice with lens-specific knockout of BMP4, pre-placodal ectoderm does not undergo cell height increase (FURUTA and HOGAN, 1998). In addition, when BMP receptors are conditionally deleted in the pre-placodal ectoderm of mouse embryos, lens placode fail to undergo morphogenesis (RAJAGOPAL et al., 2009). In summary, inhibition of BMP in either chick or mouse embryos, arrests lens development in the pre-placodal stage.

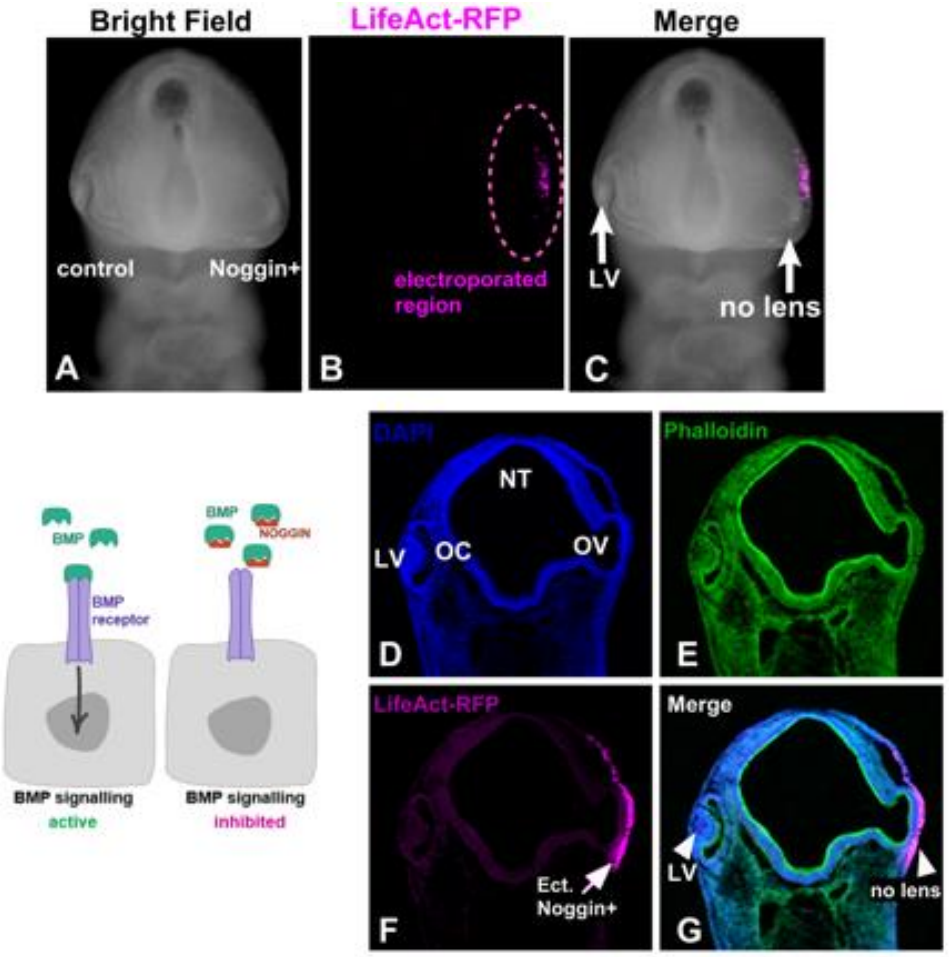


Figure 6. Noggin overexpression suppresses lens placode thickening. (A-C) Frontal view of HH15 stage chicken embryo electroporated with Noggin in the right eye. (A) Bright field image showing the morphological difference between control eye (left) and Noggin+ eye (right). (B) Fluorescence channel showing Noggin+ cells detected by the presence of the LifeAct-RFP electroporated along with Noggin (see Methods). (C) In the control eye, the lens vesicle (LV) is formed (phase 2b) and in the Noggin+ eye, the eye is arrested at phase 0). in the lower left corner of the image, there is a schematic showing active BMP signalling and inactivated BMP signalling by the presence of Noggin in the extracellular space. (D-G) Coronal cross section of chick embryo head at stage HH16 electroporated with Noggin in the right eye. (D) Nucleus stained with DAPI. On the left side the lens vesicle in formed and in the right side the optic vesicle (OV) is present but there is no lens placode formation. NT: neural tube; OC: optic cup. (E) Actin stained with phalloidin. (F) Noggin+ cells in magenta showing the eletroctroporation of the pre-placodal ectoderm in the optic region (Ect. Noggin+). (G) Merge of all channels showing the absence of the lens placode in the Noggin+ ectoderm.
Data published in the Master's dissertation: Magalhães, 2019

The canonical BMP pathway acts through phosphorylation of Smads1/5/8, which interact with Smad4 and are translocated to the nucleus (WANG et al., 2014). Despite of the presence of phosphorylated Smads in both lens placode and optic vesicle, they do not seem to be necessary for placode formation. Knockout mice for Smad1, 5 and 4 show no change in lens placode thickening or invagination (HUANG et al., 2011; JIDIGAM et al., 2015; RAJAGOPAL et al.,

2009). This suggests that BMP signaling acts through a non-canonical pathway during eye development.

BMP signaling and Pax6 act independently through parallel pathways in lens placode thickening. The knockout of BMP or its receptors in mice do not inhibit Pax6 expression (FURUTA; HOGAN, 1998; WAWERSIK; EVOLA; WHITMAN, 2005; RAJAGOPAL et al., 2009; HUANG et al., 2015a). However, lens development in these situations does not progress to phase 1 (FURUTA; HOGAN, 1998; WAWERSIK; EVOLA; WHITMAN, 2005; RAJAGOPAL et al., 2009; HUANG et al., 2015a).

1.4 INTERACTION BETWEEN OPTIC VESICLE AND LENS PLACODE

Following optic vesicle evagination, a region of contact with the overlying epithelia is established where the lens placode will appear. The extracellular space between these two tissues is filled with extracellular matrix. Histological assays show accumulation of glycoproteins and dense fibrous matrix (HENDRIX; ZWAAN, 1975; HILFER; RANDOLPH, 1993). Immunoassays for Fibronectin show intense labeling between the lens placode and optic vesicle at phase 2a (HILFER and RANDOLPH, 1993). At phase 2b, the intensity decreases (HILFER and RANDOLPH, 1993). Collagen IV is also present; however, at phase 2b, it becomes asymmetrically distributed. It is more intense near the lens placode basal membrane than near the optic vesicle side of the ECM (HILFER and RANDOLPH, 1993).

Pre-placode ectoderm is necessary for initial optic vesicle morphogenesis, but not at later stages. Between phase 0 and phase 1, the optic vesicle depends on lens placodal development to form the optic cup (HYER et al., 2003; OLTEAN et al., 2016). When pre-placodal ectoderm is removed at this stage, the optic vesicle does not evolve into the optic cup (HYER et al., 2003). When the ectoderm is removed after lens placode thickening, the optic cup develops normally in the chick embryo (HYER et al., 2003) removal of phase 2a lens placode did not affect optic vesicle morphogenesis and it maintained a concave shape (OLTEAN et al., 2016). Thus, the ectoderm is not important for the maintenance of optic vesicle development. Importantly, the ECM is essential for

the maintenance optic cup formation at this stage (OLTEAN et al., 2016). Placode removal at phase 2b followed by collagenase treatment interrupted optic vesicle morphogenesis and reversed concavity of the nascent optic cup (OLTEAN et al., 2016).

Together these data suggest that the relationship between the optic vesicle and the lens placode, as well as the regulation of the extracellular environment between these two tissues, is crucial for early eye development.

1.5 EXTRACELLULAR MATRIX ROLE IN DEVELOPMENT

The extracellular matrix (ECM) is one of the major drivers of morphogenesis. The ECM plays an important role in tissue shape and cell differentiation. The composition and physical characteristics of the ECM can influence cell differentiation, behavior and polarity. Differences in the ECM architecture, density and porosity regulate tissue shape and organization (KLEINMAN; PHILP; HOFFMAN, 2003; ROZARIO; DESIMONE, 2010; MOUW et al., 2015). Likewise, the cells in the overlying tissues are constantly changing and can affect ECM composition through secretion of extracellular molecules, remodeling the ECM and its physical characteristics. Thus, cells regulate the ECM and vice-versa, in an interrelated dynamic relationship.

Here, we will focus our analysis to two major ECM components, Fibronectin and Laminin. The presence of Fibronectin and Laminin has already been described in several embryonic tissues during their formation. These glycoproteins are one of the main components of the ECM and have been extensively studied. Fibronectin is a large glycoprotein with binding motifs recognized by cell surface receptors, Collagen, Proteoglycans and other Fibronectin molecules (SCHWARZBAUER; DESIMONE, 2011; MOUW; OU; WEAVER, 2014). It plays an important role in cell adhesion, migration, and differentiation (SCHWARZBAUER; DESIMONE, 2011). It can regulate cellular cytoskeleton organization through interactions with cell receptors and transmits mechanical signals from the ECM to the cell and vice-versa (MAO; SCHWARZBAUER, 2005). Thus, Fibronectin acts as an extracellular mechanoregulator of cell behavior. Consistent with the relevance of mechanoregulation in morphogenesis, Fibronectin plays important role in

embryonic development. For instance, during heart development, pre-cardiac cells migrate along gradients of Fibronectin, which act as a guide for specific migration (LINASK; LASH, 1988). Also, during neural crest cell migration, Fibronectin stablishes migration routes.

Another important component of the ECM is Laminin. Laminin is the main component of basement membrane, and it is a heterodimer composed of three long polypeptide chains. In early embryos, basement membrane support the entire epithelium in the organism and has a critical role in body architecture (ALBERTS et al., 2008). Disruption of Laminin interactions with other ECM components disturbs the basement membrane and affects early morphogenesis processes (KADOYA et al., 1997). For instance, mouse embryos with knockout for Laminin Ƴ1 chain fail to develop and die after stage E5.5 (SMYTH et al., 1999). Laminin, similar to Fibronectin, interacts with Integrin and Collagen.

Mechanoregulation is important for embryonic morphogenesis. Modulation of the extracellular environment triggers cell and tissue changes. Thus, the ECM actively controls cell differentiation and rearrangements during morphogenesis. An example of this is the branching morphogenesis events that occur in the formation of glands and lungs (ROZARIO; DESIMONE, 2010). Briefly, in branching morphogenesis, the epithelia grows linearly when bud flanks extend and radially when bud tip branch. After branching, tissue growth converts a bud tip into a bud branch. The equilibrium between branching and growth of buds requires a specific control of the physical properties of the ECM. A thick ECM is formed around the bud flanks, whereas a thinner ECM is formed at the end bud tips (reviewed in ROZARIO and DESIMONE, 2010). In other words, the ECM changes in specific domains, allowing the formation of branches. These differences in ECM composition are transferred to the cells through ECM receptors, such as Integrins. The differentiated expression of Integrins regulates branching in this scenario by maintaining pro-migratory signals in growing buds and anti-migratory and anti-proliferative signals in flanks and ducts (reviewed in ROZARIO and DESIMONE, 2010). This study shows the importance of ECM dynamics during development.

1.6 EXTRACELLULAR MATRIX DURING VERTEBRATE EYE DEVELOPMENT

The presence of Fibronectin, Laminin and Collagen IV throughout late eye development is described in detail (reviewed in KWAN, 2014). However, little is known about the ECM during early eye morphogenesis. PAS-staining (Periodic acid-Schiff staining detects polysaccharides such as glycogen, and mucosubstances such as glycoproteins) of the ECM. The staining between the placodal ectoderm and the optic vesicle in chick embryo becomes more intense during placode formation (HENDRIX and ZWAAN, 1975). Immunostaining with Laminin and Fibronectin antibodies followed the presence of these proteins in cross sections during chick lens development. At phase 1, both Laminin and Fibronectin are labelled between the optic vesicle and pre-placode ectoderm (HILFER and RANDOLPH, 1993). At phase 2b, Fibronectin staining is punctate between the optic cup and lens placode (HILFER and RANDOLPH, 1993). Also, after lens placode invagination, both Laminin and Fibronectin staining are less intense between lens vesicle and optic cup when compared to phase 0 (HILFER and RANDOLPH, 1993).

One of our long-term goals is to understand if ECM changes during early lens development. Our previous data followed the dynamics of Lamininα1 and Fibronectin expression pattern at different stages of the lens placode formation (Fig. 7) (MAGALHÃES, 2019. At phase 0, Fibronectin and Lamininα1 have a fibrillar pattern in the ECM between the optic vesicle and the pre-placodal ectoderm (Fig. 7A-F). At phase 1 and 2a, both proteins show a diffuse and punctate pattern between the thick placode and the optic vesicle (Fig. 7G-P). At this stage, the fibrillar pattern is restricted to non-placodal regions, corresponding to cells that do not undergo thickening (Fig. 7G-P). This organization is maintained during placode invagination (Fig. 7Q-V). The ECM evolution is BMP dependent. After Noggin overexpression, lens placode formation is inhibited and Fibronectin and Lamininα1 show an intense and fibrillar pattern, similar to the non-placodal regions (Fig. 8).

In addition, mice with lens-speficic Fibronectin ($Fn^{lens-/-}$) knockout fail to develop lens placodes (HUANG et al., 2011). In these mutant mice, the lack of Fibronectin interrupted placodal growth. Placode thickening only occurred until phase 1, and did not evolve to phase 2a (HUANG et al., 2011). The pre-placodal ectoderm remained cuboidal but, interestingly, there was actin accumulation in the apical membrane (HUANG et al., 2011). This suggests that while Fibronectin in the basement membrane is necessary for the increase in cell height it may not be required for apical actin accumulation.

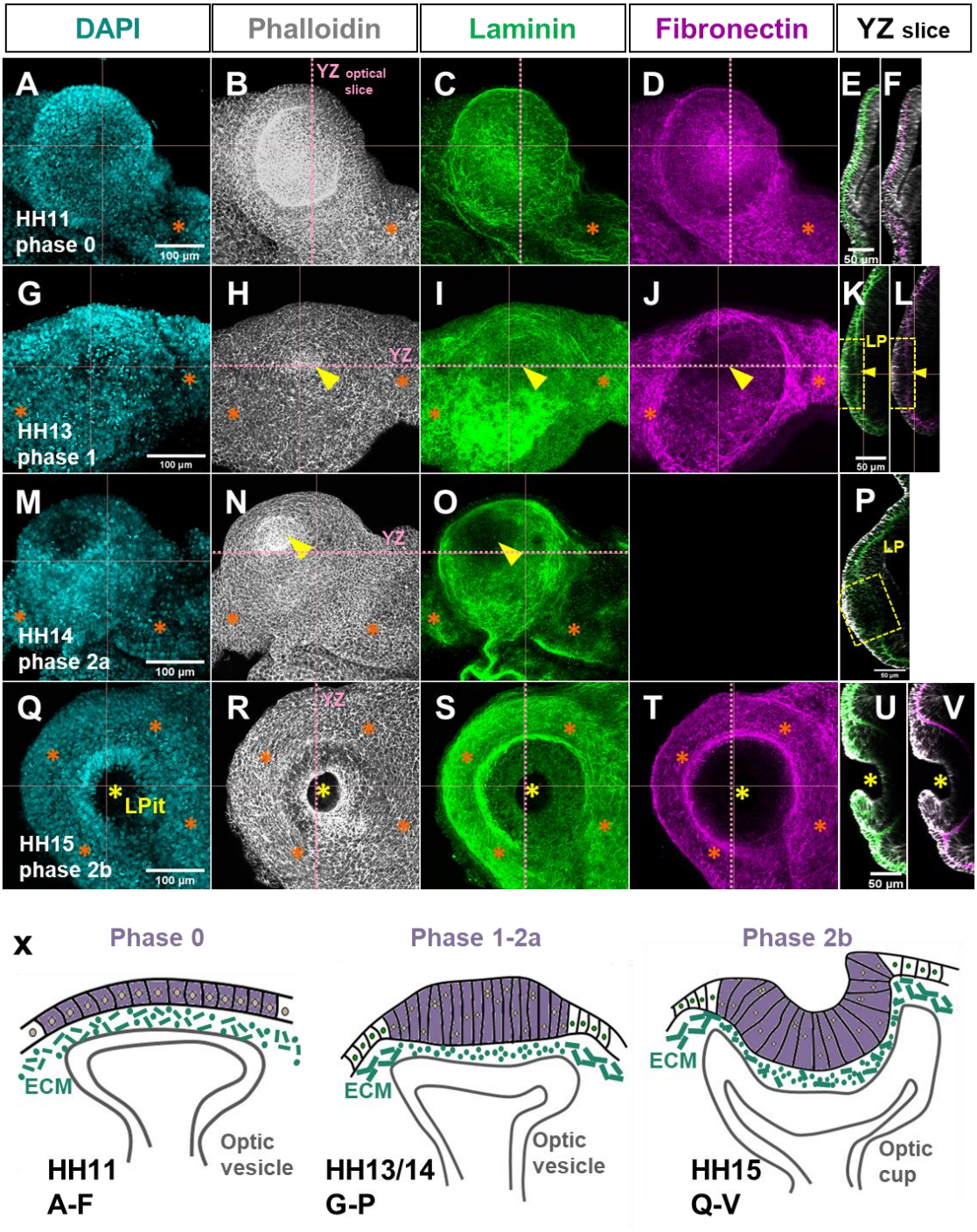


Figure 7. The ECM evolves during lens placode formation. (A-V) 3D reconstructed images taken by confocal microscopy of the apical surface of the eye of HH11 to HH15 stage chicken embryos. The dotted pink line shows the level of the YZ orthogonal slice in the two last columns. (A, G, M and Q) Nucleus stained with DAPI (cyan). (B, H, N and R) Actin staining with phalloidin (grey column). (C, I, O and S) Immunostaining for Lamininα1 (green). (D, J and T) Immunostaining for Fibronectin (magenta). (E, F, K, L, P, U and V) Orthogonal slice from YZ axis showing Fibronectin (magenta) or Lamininα1 (green) co-labelled with Phalloidin (grey) staining. (A-F) At phase 0, the ECM between the pre placode ectoderm and the optic vesicle displays a fibrillar immunostaining pattern for both Fibronectin and Lamininα1 in the optic region and also in the surrounding non-placodal ectoderm (orange asterisk). (E and F) The YZ optical slice shows an intense labeling of Fibronectin and Lamininα1 between the optic vesicle (right side tissue) and the pre-placodal ectoderm (left side tissue). (G-L) At stage HH13, (phase 1) the lens placode starts to thicken and actin accumulates in the apical surface (yellow arrowhead). The immunostaining pattern of Fibronectin and Lamininα1 under the centre of the placode (identified by apical accumulation of actin) becomes more diffuse and punctate. The ECM of regions outside of the optic vesicle present a fibrillar organization (orange asterisk). (K and L) The YZ optical slice shows a weak staining for both proteins between the lens placode (LP) and the optic vesicle (yellow dotted line region). In contrast, the staining is stronger outside the lens placode region. (M-P) At HH14 stage, (phase 2a) the placode is formed and the difference of Lamininα1 immunostaining pattern is more evident. There is a diffuse and punctate Lamininα1 immunostaining pattern at the ECM immediately overlying the optic vesicle and under the region of the placode with apical accumulation of actin (yellow arrowhead). The periplacodal regions (orange asterisk) present a fibrillar organization. (P) The YZ optical slice shows a strong Lamininα1 staining outside the lens placode region and a weak staining between the thick lens placode and the optic vesicle (yellow dotted line). (Q-V) At stage HH15 (phase 2b), during lens placode invagination, the diffuse and punctate pattern of Fibronectin in the placodal region is maintained around the lens pit (yellow asterisk, LPit). The ECM in the periplacodal region has a fibrillar and intense Fibronectin and Lamininα1 pattern (orange asterisk). (U and V) The YZ optical slice shows a weak staining of both proteins between the lens placode and the optic cup. Fibronectin and Lamininα1 staining is stronger in ECM underlying the base of the cuboidal periplacodal epithelium. (X) Scheme of the ECM evolution during lens placode development.

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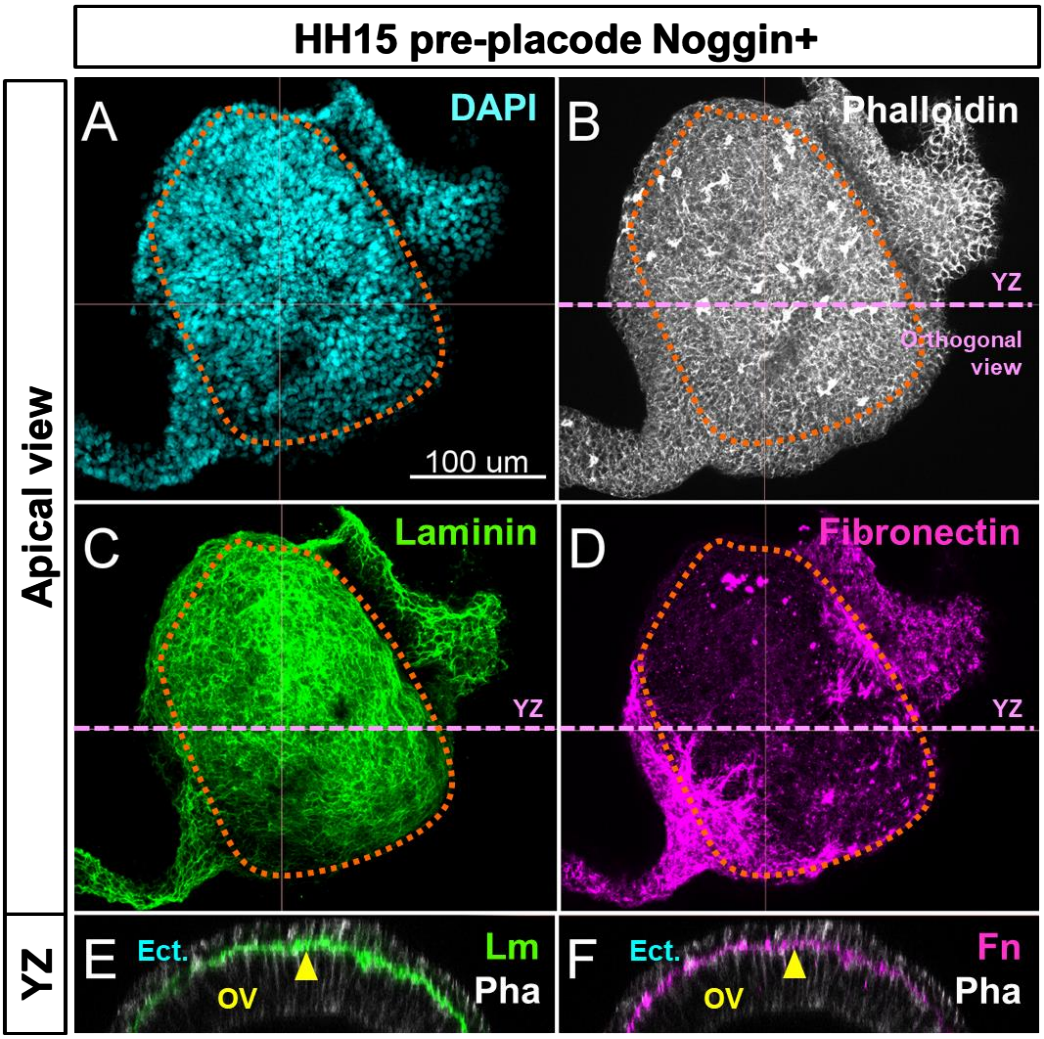

Figure 8. Noggin overexpression inhibits optic ECM evolution. (A-F) 3D reconstructed confocal images of the apical surface of the eye at stage HH15 after Noggin overexpression in the pre-placodal ectoderm. The dotted pink line delimits the YZ orthogonal slice in the bottom row. The orange dotted line delimits the optic region, where the ectoderm (Ect) is in contact with the optic vesicle (OV). (A) Nucleus staining with DAPI (cyan). (B) Actin staining with Phalloidin (grey). (C-D) ECM staining after BMP inhibition. Immunostaining for Lamininα1 and Fibronectin show a fibrillar pattern in the optic region, similar to the fibrillar pattern in the periplacodal region. (E-F) The YZ orthogonal slice shows the inhibition of lens placode formation, where the ectodermal cells remain cuboidal. Phalloidin staining shows a homogenous distribution of actin in the cells. Both Lamininα1 (Lm) and Fibronectin (Fn) are intensely labelled between the Noggin+ ectoderm and the optic vesicle.
Data published in the Master's dissertation: Magalhães, 2019

1.7 EXTRACELLULAR MATRIX REMODELING

ECM is one of the main drivers of morphogenesis. Specifically, in the optic region, the composition of the ECM plays a significant role in its development (HUANG et al., 2011). Our previous results also suggest that the optic ECM is

dynamic and remodels during placode thickening. In this session, we will point the main mechanisms responsible for the ECM remodeling that may be associated to the evolution of optic ECM. The three ECM remodeling mechanisms discussed here are: changes in ECM proteins deposition (which depends on the cell expression and secretion), ECM degradation (which involves proteases), and force-mediated ECM modification (which depends on the cellular-ECM interactions) (LU et al., 2011; BONNANS; CHOU; WERB, 2014; WINKLER et al., 2020).

1.7.1 ECM protein deposition

Each tissue has a specific ECM composition that is crucial for tissue morphology, function and homeostasis. The dysregulated change in ECM composition or architecture is associated with pathological conditions or accelerated disease progression (LU et al., 2011; BONNANS; CHOU; WERB, 2014; WINKLER et al., 2020). For example, in fibrosis and cancer, there is an abnormal ECM deposition and stiffness (WINKLER et al., 2020). This abnormal deposition involves a disturbed balance between ECM synthesis and secretion and changed expression of matrix-remodeling enzymes. In this context, the alteration in ECM deposition is associated with intracellular signaling. In the context of fibrosis, TGFβ activates the transcriptional factors Smad2/3 that induce the expression of ECM-related genes, such as Col1a1, Col3a1 and Timp1, that results in over-accumulation of ECM proteins (VERRECCHIA; CHU; MAUVIEL, 2001).

During development, the unique ECM composition for each tissue and organ is defined (reviewed in BONNANS et al., 2014). Cells are constantly rebuilding the ECM through synthesis and chemical modification. During lung development in mice, branching morphogenesis and alveoli formation depends on elastin production and other specific ECM proteins. The inhibition of elastin expression results in softening of neonatal mouse lung tissue and decreases the expression of important genes for lungs development.

We previously mentioned that the presence of Fibronectin in mouse optical ECM is essential for lens formation and eye development (HUANG et al., 2011). Lack of Fibronectin arrested lens placode development in phase 1 (HUANG et al., 2011). This shows that early eye morphogenesis depends on a specific ECM

composition. Furthermore, $Pax6^{lens-/-}$ mouse embryos do not develop the optic placode and the expression of several ECM genes decreases (HUANG et al., 2011). This data suggests that the composition of optic ECM is regulated by the differentiation of lens placode cells at early eye development.

1.7.2 ECM degradation

Protease degradation is one of the mechanisms involved in ECM remodeling (STERNLICHT et al., 2006; CLAUSE; BARKER, 2013; DIAZ-DE-LA-LOZA et al., 2018). The main enzymes involved in this process are the matrix metalloproteinases (MMPs).

MMPs are a group of proteolytic enzymes that cleave most of ECM components. Metalloproteinase proteolytic activity not only regulate ECM assembly and remodeling, but can also release growth factors retained in the ECM (SIMIAN et al., 2001; reviewed in DRAKE; FRANZ-ODENDAAL, 2018).

Several morphogenic events depend on the action of metalloproteases (MMPs) for modulation of the ECM. In the development of breast cells, for example, branching morphogenesis depends on MMP-mediated degradation of the matrix degradation (SIMIAN et al., 2001). In this context, MMPs control the amount of ECM proteins and, also, regulate morphogenic signals during branching morphogenesis in mouse mammary gland (reviewed in FATA; WERB; BISSELL, 2004).

MMPs are involved in several epithelial morphogenesis process. In Drosophila, ECM degradation is crucial for columnar-to-cuboidal cell shape changes involved in wing and legs elongation (DIAZ-DE-LA-LOZA et al., 2018). The change in cell shape from columnar to cuboidal during facilitate the the accommodation of proliferating cells within the epithelium. In this context, cell height reduction depends on MMP1/2 activity, as ECM degradation allows cells to spread and change their shape (DIAZ-DE-LA-LOZA et al., 2018). The inhibition of ECM degradation maintains the columnar cell shape (DIAZ-DE-LA-LOZA et al., 2018).

Other proteins involved in ECM degradation are the ADAMs (a disintegrin and metalloproteinases) and ADAMTs (ADAMs with a thrombospondin motif). There are several types of ADAMs and ADAMTs described, but not all their

targets have been elucidated. These proteins can cleave transmembrane protein ectodomains that are adjacent to the cell membrane and releases cytokines, adhesion molecules and growth factors (reviewed in KELWICK et al., 2015; MURPHY, 2008). In addition, several proteins in this family cleave gelatin, fibronectin, laminins, collagens, versican and proteoglycans and laminins (reviewed in KELWICK et al., 2015; MURPHY, 2008). In development, several ADAMTS controls the amount of versican in the ECM. Versican provides structural support during dynamic remodeling during morphogenesis. Several types of ADAMTS are associated with the regulation of versican during heart and limb development (reviewed in KELWICK et al., 2015).

ECM degradation has an important role in regulating the availability TGFβ family ligands (reviewed in MIGLIORINI et al., 2020). The relationship between BMP signaling and ECM could be a positive feedback loop in that BMP-triggered ECM changes could further enhance BMP signaling in the placode. In other words, the ECM architecture alteration could contribute for towards sustained BMP signaling in the placode. As mentioned previously, lens placode specification in vitro requires long-term exposure to BMP (SJODAL et al., 2007). Indeed, there are examples where ECM modifications can modulate BMP signaling. ECM components can bind to BMP and modulate the steepness of the BMP gradient (reviewed in PLOUHINEC et al., 2011). For instance, Dpp, a BMP2/4 homologue, binds to Collagen IV and this interaction facilitates the Dpp flow necessary for dorsal-ventral patterning in Drosophila (WANG et al. 2008). In this context, changes ECM degradation would increase the availability of BMP in the optic region.

1.7.3 Force-mediated ECM modification

The ECM communicates with cells through membrane receptors, such as Integrins. Integrins are heterodimers composed of two transmembrane glycoprotein subunits (α and β). They play important roles in mechanobiological and morphogenetic processes. On the extracellular domain, those receptors recognize ECM components and, on the intra-cellular domain, they interact with the cytoskeleton and can activate several signaling pathways. Interaction between Integrins and the ECM can regulate the changes in cell shape during

morphogenesis. In summary, Integrins transmit mechanical signals from the ECM to the cell and vice-versa (ALBERTS et al., 2008).

When Integrins transmit the characteristics of the extracellular medium to the cell, the ECM act as a stress sensor (KECHAGIA; IVASKA; ROCA-CUSACHS, 2019). In this context, Integrin activation can be triggered by force application in an outside-in mechanism. Mechanical tension originated from experimental application of forces on cell and tissue activates Integrins and leads to integrin clustering. Integrin clustering activates intracellular pathways, such as FAK, and regulates GTPases activation (KECHAGIA et al., 2019). This process results in a rearrangement of the cytoskeleton and change in cell shape (KECHAGIA et al., 2019). After blocking specific Integrin-binding sites of ECM ligands, the signals induced by shear stress to intracellular pathways are abolished (JALALI, 2001).

Integrin activation can also trigger ECM changes in an inside-out mechanism. In this context, Integrin activity changes the conformation of extracellular matrix components and modulate the physical properties of the extracellular environment. For instance, activation of Integrins induces Fibronectin fibrillogenesis (MAO; SCHWARZBAUER, 2005). The activated integrin binds to Fibronectin in the extracellular environment, changing its conformation and exposing the binding site with other Fibronectins, causing a progressive assembly of large fibrils. In many developmental processes, tissue tension increased by contractility leads to the activation of Integrins and promotes Fibronectin fibril assembly. During Xenopus gastrula, tissue tension promotes Fibronectin fibrillogenesis that is crucial for early morphogenesis process (DZAMBA et al., 2009). In this context, blocking Integrin-binding sites that interact with Fibronectin results in reduction of Fibronectin deposition at the blastocoel roof and abnormal cell organization (MARSDEN; DESIMONE, 2001). Increased cell contractility induces Fibronectin matrix assembly in zebrafish paraxial mesoderm (JÜLICH et al., 2015). Lastly, micro-tissues in 3D cultures that are submitted to high mechanical stress increase extracellular Fibronectin fibril formation and accumulate F-actin intracellularly (LEGANT et al., 2009).

Fibronectin remodeling plays an important role during zebrafish neurulation (ARAYA; CARMONA-FONTAINE; CLARKE, 2016; GUILLON et al.,

2020). The Fibronectin in the ECM between the presomitic mesoderm (presumptive tissue of the somites) and the neural tube show two different conformations: a more diffuse that is classified as ‘small fibrils’ and a fibrillar organization that forms a large assembled network (GUILLON et al., 2020). Fibronectin matrix continually remodels during the neural tube convergence extension and creates a medial-lateral gradient of tension. In the medial portion, closer to the neural tube centre and notochord, there are small fibrils, while in the lateral portion, there are large fibrils and the ECM tension is highest (GUILLON et al., 2020). This remodeling depends on Integrinα5 activation and regulate the shape and extension of the neural tube. Knockout embryos exhibit several neural tube defects (GUILLON et al., 2020).

1.8 HYPOTHESIS

ECM is one of the main drivers of morphogenesis. Our previous data showed that the ECM between the optic vesicle and the lens placode ectoderm evolves during placode formation. ECM is dynamic and its characteristics can both depend on and induce cell differentiation. Thus, we hypothesize that the lens placode cellular changes are followed by modifications in the optic ECM.

I. Changes in optic ECM architecture depends on BMP signaling in the lens placode but not the optic vesicle.
II. The lens placode transcribes ECM modulators prior to and during its invagination.

1.9 OBJECTIVES

1. Investigate optic ECM evolution after interference with BMP signaling in lens and optic vesicle, separately.
2. scRNAseq transcriptome analysis of optic tissues at early stages of development.
3. Investigate modulation of protease activity in the optic tissue during lens placode development.
4. Detect the distribution of Integrinβ1 activity in the different phases of lens placode development.

5 CONCLUSIONS

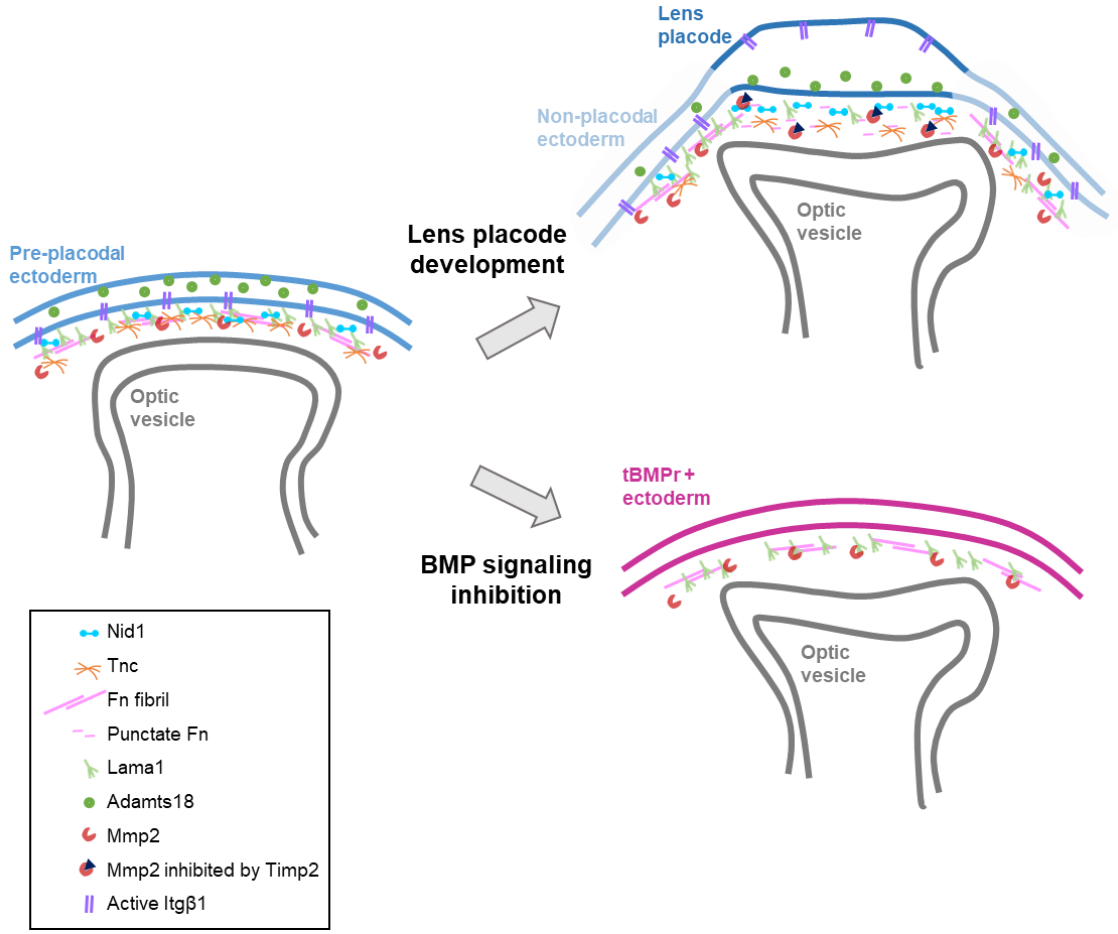

Figure 40. Diagram summarizing the results obtained in this study

In this study, we conclude that the lens placode ectoderm modulates the optical ECM during early eye development. We propose that lens placode differentiation is necessary for regulating the characteristics of the optical ECM and its remodeling. At early lens development, a series of molecular factors and signaling events induce the specification of the lens placode in the ectoderm. Once defined, the placodal cells acquire a unique transcriptomic profile that induces changes in cellular shape and alterations in the ECM. Here, we observed expression of several lens placode-specific ECM genes, such as Timp2, Col13a, Tnc, and Adamts18. Analysis of the TFBS proximal to the Timp2 and Adamts18 genes strengthens our proposal, as they contain binding sites for several transcription factors involved in placode differentiation. Downregulation of Timp2 in the absence of BMP signaling and downregulation of Col13a1 and Tnc in the

absence of Pax6 further support that modulation of the optical ECM depends on placode formation.

The optical ECM factors described here are associated with various mechanisms responsible for ECM remodeling. Production of Tnc, Nid1, Col13a1, P3h2, and other identified genes modify the extracellular matrix composition and alters its physicochemical properties. Thus, the interaction of these proteins with others can result in ECM remodeling.

The expression of Timp2 by the placode occurs concomitantly with inhibition of MMP activity. This result suggests that Timp2 inhibits MMP2 in the optical ECM. Inhibition of MMP2 activity in addition to the presence of Adamts18 in the basal region of the placode may also regulate protease-target protein cleavage, altering ECM properties. For instance, Adamts18 can lead to the release of important cytokines and the inhibition of MMP2 can arrest the breakdown of the matrix.

Finally, we propose here that optic ECM remodeling not only depends on the differentiation of the lens placode, but is also important for its morphogenesis. Further experiments will be necessary to test our proposal in the future.

5.1 FUTURE DIRECTIONS

We observed high expression of Timp2 in the placode cells, and the in situ zymography showed MMP activity inhibition in the optic region during lens placode formation. Therefore, we hypothesize that inhibition of MMPs is necessary for regulating early eye formation. In this case, ectopic inhibition of MMPs over a larger region would form a more extensive or even ectopic lens placode. In contrast, the overexpression of MMP2 would inhibit the correct formation of the lens placode.

Thus, to test whether Timp2 overexpression is sufficient for ectopic placode formation, we cloned the complete Timp2 sequence from the chicken embryo into an expression vector (Appendix D). Also, we are interested in investigating whether MMP2 overexpression inhibits placode formation. Thus, we cloned the complete MMP2 coding sequence from the chicken embryo into an expression vector (Appendix D).

Here we propose as future direction electroporated the chick embryo with the constructed plasmids and evaluate the effects.

In addition, we observed a difference in active-Integrinβ1 distribution in the lens placode compared to non-placodal ectoderm, which might cause the changes in Fibronectin and Lamininα1 pattern. However, we only observed the active-Integrinβ1 distribution in phase 1 in the mouse embryo. Therefore, we propose to analyze active-Integrinβ1 distribution evolves comparing phase 1 with phase 0 and 2a. In addition, we also propose to verify the activity of Integrinβ1 in chicken embryo to understand if this ECM modulation mechanism is conserved.

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