Review

The lens: a classical model of embryonic induction providing new insights into cell determination in early development

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The lens was the first tissue in which the concept of embryonic induction was demonstrated. For many years lens induction was thought to occur at the time the optic vesicle and lens placode came in contact. Since then, studies have revealed that lens placodal progenitor cells are specified already at gastrula stages, much earlier than previously believed, and independent of optic vesicle interactions. In this review, I will focus on how individual signalling molecules, in particular BMP, FGF, Wnt and Shh, regulate the initial specification of lens placodal cells and the progressive development of lens cells. I will discuss recent work that has shed light on the combination of signalling molecules and the molecular interactions that affect lens specification and proper lens formation. I will also discuss proposed tissue interactions important for lens development. A greater knowledge of the molecular interactions during lens induction is likely to have practical benefits in understanding the causes and consequences of lens diseases. Moreover, knowledge regarding lens induction is providing fundamental important insights into inductive processes in development in general.

Keywords: lens; induction; BMP; FGF; Shh; Wnt

1. INTRODUCTION

One of the fundamental goals in developmental biology is to understand the molecular mechanisms that regulate the induction and patterning of different tissues and organs. In the beginning of the twentieth century Hans Spemann introduced the concept of inductive interactions by studying lens development [1], in which induction is a process by which one group of cells or tissue regulates the development of another group of cells or tissue. Since then several studies have used the lens as a developmental model system to better understand the role of specific signalling molecules, the interplay of different signals and tissue interactions in regulating lens induction and patterning events. The acquired knowledge is not only important for understanding normal lens development, but also key to defining general mechanisms in cell specification, as well as to better understanding of lens function and lens diseases.

The lens is a component of the peripheral nervous system, which arises from the neural plate border. Lens development is morphologically first visualized by the thickening of the ectoderm into the lens placode, in the vicinity of the prospective optic vesicle (figure 1*a*,*b*) [3]. In higher vertebrates, including chick, mouse and humans, the lens placode invaginates

and forms the lens pit (figure 1*c*). Subsequently, the lens pit deepens and the connection with the overlying surface ectoderm, the prospective cornea, is abolished resulting in the formation of the lens vesicle (figure 1*d*). In lower vertebrates, such as fish and frog, lens formation proceeds via delamination of the lens placodal cells [4,5]. After formation of the lens vesicle, cells at the centre of the posterior side of the lens will elongate and differentiate into primary lens fibre cells, whereas cells of the lens epithelium at the anterior side of the lens retain their ability to proliferate and will generate fibre cells throughout life (figure 1*e*) [3].

Induction of a tissue or organ seldom occurs in one signalling step, but rather as a result of multi-step processes. Subsequently, it can be difficult to point out a single developmental stage for the initiation of induction of a tissue or organ, including lens induction. Thus, it is often more informative to describe 'specification' of cell fates, which denotes the step whereby cells have received a signal that will instruct the cells to become a specific cell type unless exposed to signals that divert them to alternative fates. In this review, I will highlight known signalling events that control the initial specification of lens placodal cells and progressive development of lens cells between late blastula to lens vesicle stages, and also discuss proposed tissue interactions important for lens development during these stages. I will not address the role of various transcription factors in early lens specification, since this topic has been reviewed in detail elsewhere [6–9].
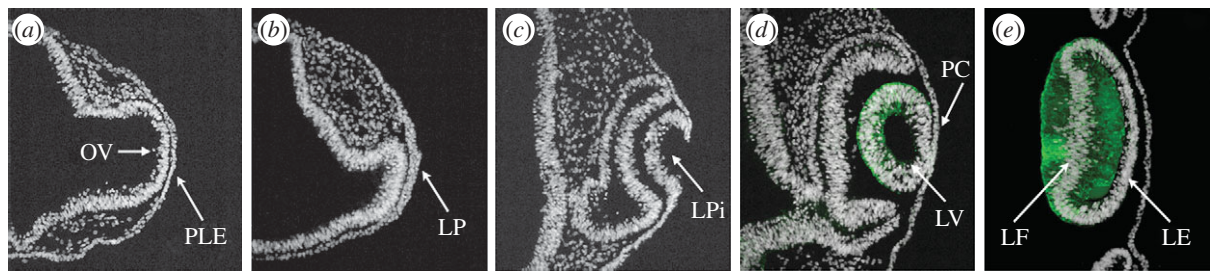
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Figure 1. Morphological changes during early lens development. Immunohistochemistry performed on sections of developing chick lens. (*a*) The prospective lens ectoderm (PLE) lies close to the optic vesicle (OV), and (*b*) will subsequently thicken and form the lens placode (LP). (*c*) Next the lens placode invaginates, leading to the formation of the lens pit (LPi). (*d*) The lens pit deepens and the connection with the overlying surface ectoderm, the prospective cornea (PC), is lost, shaping the lens vesicle (LV). (*e*) Lens cells in the posterior part of the lens will elongate and differentiate into primary lens fibre cells (LF), whereas cells at the anterior side consist of lens epithelial cells (LE). (*d,e*) Differentiated primary lens fibre cells upregulate crystallin proteins, here detected by δ-crystallin [2] shown in green. Nuclei in white are detected with DAPI staining.

2. LENS CELLS ARE INITIALLY SPECIFIED AT GASTRULA STAGES

Specification can be defined under experimental settings (explants assays), such that specified cells will maintain their fate if removed from the embryo and cultured *in vitro* in the absence of exogenous factors. After specification to a particular cell fate, cells most often have the ability to respond to external signals to acquire another cell identity, before cell fate commitment. Thus, it is important that these experiments are conducted in serum-free conditions in the absence of surrounding tissues to avoid uncontrolled addition of signalling molecules or other components that may affect cell fate.

Early in development, neural plate border cells develop at the junction between the neural and epidermal ectoderm and give rise to placodal and neural crest cells [10–13]. Besides the lens placode, the hypophyseal and olfactory placodes are generated in the rostral part of the neural plate border, whereas the otic, trigeminal and epibranchial placodes, as well as neural crest cells, are formed at a more caudal position of the neural plate border region [14,15]. The well-known rotation transplantation experiment performed by Jacobson in amphibians, in which the neural plate border region was rotated along its rostrocaudal axis at neural plate and late neurula stages, analysed the competence and commitment of the olfactory, lens and otic placodes [16]. The main finding in this paper is that at the neural plate stage, prospective placodal cells are competent to acquire an olfactory, lens and otic placode in response to specific external signals, whereas at the late neurula stage, the different placodal cells appear to be committed to their respective fate. In agreement, transplantation studies in *Xenopus* also suggest that lens cells are committed to a lens fate at the end of neurulation [17]. Moreover, another study in *Xenopus* embryos suggests that at gastrula stages the head ectoderm has a 'lens-forming bias' [18], however, in this study 'lens bias' is defined as the ability of the head ectoderm to respond to external signals from the optic vesicle to acquire lens identity, indicating that these studies actually address competence similar to Jacobson's study. Neither of

these studies, however, address when lens cells initially are specified.

A recent study using explant assays in chick has provided evidence that the specification of neural plate border cells is initiated at the late blastula stage [12]. However, at this stage independent of rostrocaudal position, prospective neural plate border cells are specified as neural crest cells, but no placodal cells are detected [12], indicating that at the late blastula stage lens cells are not yet specified. Shortly thereafter, at the late gastrula stage, rostral neural plate border explants cultured *in vitro* generate cells of lens character, providing evidence that the initial specification of lens cells occurs at the late gastrula stage [19]. In contrast, in *Xenopus* embryos, lens cells are suggested to be specified as the neural tube closes [20], which can be due to differences in experimental settings or a species-specific difference. Using markers of both differentiated lens and olfactory epithelial cells in specification maps, the study of Sjödal *et al*. has shown that late gastrula stage and head fold stage rostral neural plate border explants generate both lens and olfactory placodal cells in a non-overlapping manner (figure 2*a*) [19]. This is consistent with fate maps of gastrula stage chick and zebrafish embryos showing that prospective lens and olfactory placodal cells are intermingled in a domain of the rostral neural plate border [10,11], whereas cells in the caudal neural plate border region are fated to give rise to neural crest and caudal placodal cells [13]. At the late gastrula stage to head fold stages caudal neural plate border explants give rise to neural crest, but no placodal cells ([21,22]; M. Sjödal, C. Patthey, L. Gunhaga 2007, unpublished results), indicating that caudal placodal cells are specified at later stages, and strengthen the argument that lens progenitors are situated in the rostral neural plate border. In contrast to these findings, a study of Bailey *et al*. [23], using explant assays of head fold stage chick embryos, has suggested that lens specification is the 'default' state of all sensory placodes and neural crest cells. However, in this study prospective neural plate border explants from different rostrocaudal regions were analysed by using only lens, but no olfactory placodal or neural
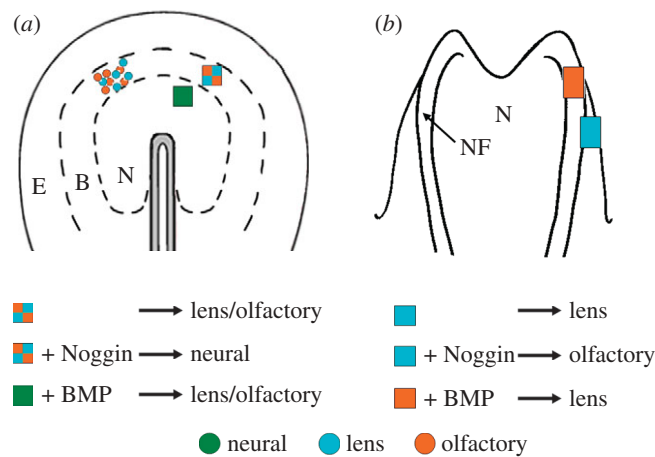
Figure 2. Sustained BMP activity is critical for lens development. (*a*) Specification maps of gastrula stage chick embryos; lens (blue circles) and olfactory (orange circles) placodal progenitors are situated in the rostral neural plate border. (*a,b*) Blue/orange, green, orange and blue boxes indicate regions of explants. (*a*) Gastrula stage lens/olfactory explants (blue/orange box) generate cells of lens and olfactory character when cultured alone, and cells of neural character if cultured together with Noggin. Gastrula stage forebrain explants (green box) generate cells of lens and olfactory character when cultured together with BMP4. (*b*) Neural fold stage lens explants (blue box) generate cells of lens character when cultured alone, and cells of olfactory character if cultured together with Noggin. Neural fold stage olfactory explants (orange box) generate cells of lens character when cultured together with BMP4. Modified from [19]. E, epidermal; B, neural plate border; N, neural plate; NF, neural fold. See text for more details.
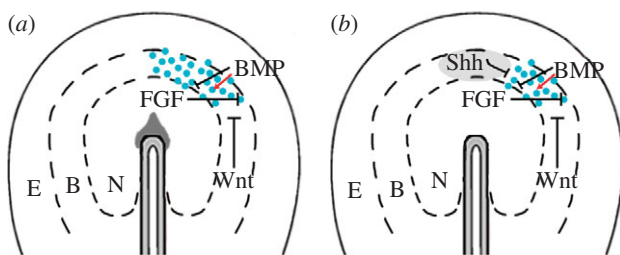

Figure 3. Model of the initial specification of lens cells. Proposed signalling events at gastrula to head fold stages in the rostral neural plate border during the initial specification of lens cells. (*a*) From gastrula stages, FGF activity represses the generation of epidermal cells. BMP signals inhibit neural formation, and in the context of FGF activity induce lens/olfactory placodal progenitor cells. Wnt activity restricts caudal expansion. (*b*) From head fold stages, Shh emanating from the underlying mesoderm (grey domain) restricts rostral expansion of lens formation. (*a,b*) The broken lines indicate that there is not a strict boundary, but actually an overlap between prospective neural, rostral placodal and epidermal cells [10,13]. E, epidermal; B, neural plate border; N, neural plate; blue circles, prospective lens placodal cells. See text for more details.

crest markers, and although cells of non-lens fate were detected they were not further characterized [23].

3. DIRECTED OR RANDOM MIGRATION

Since prospective lens and olfactory placodal cells are intermingled at gastrula stages [10], it still remains unclear whether the differential specification of lens and olfactory cells occurs in the rostral neural plate border or at their final spatial destinations. Fate and specification maps have shown that at neural fold stages, olfactory and lens placodal progenitor cells are spatially separated (figure 2*b*) [10,19,24]. This

process strongly resembles the development of the eye and the odour-detecting antenna in *Drosophila*, where the visual and olfactory cells arise from a common imaginal disc, and at later stages separate and acquire their distinct identities [25]. In *Drosophila*, it has been suggested that the transcription factors Eyeless (Ey) (Pax6 homologue) and Distalless (Dll) (Dlx homologue), in which Ey negatively regulates Dll [26], play a role in the differential specification of eye and antennal cells, respectively [25]. In chick, Pax6 and Dlx5 remain co-expressed until the lens and olfactory placodes are morphologically visualized, and the differential expression of Pax6 in the lens and Dlx5 in the olfactory placode occurs only at later stages [10], indicating that these molecular cues cannot be involved in restricted cell migration. However, lens cells forced to maintain Dlx5 expression in chick [10] and *Pax6*−/− cells in mouse chimeras [27] are expelled from the developing lens, implicating these transcription factors in regulation of cell sorting during lens development.

It is possible that cells in the rostral neural plate border are specified as rostral placodal progenitor cells, and retain the capacity to adopt either lens or olfactory placodal fate until exposed to additional molecular signals from the surrounding environment. At neural fold stages, Pax6, Dlx5, *Six1* and *Sox2*, are expressed in both lens and olfactory placodal precursor cells [10,19,28]. In addition, at this stage no molecular marker has been shown to distinguish the morphologically separated lens and olfactory placodal precursors, supporting the idea that rostral placodal precursors may initially comprise a common fate. Nevertheless, at the neural fold stage, presumptive lens placodal cells cultured *in vitro* acquire lens but not olfactory placodal character [19,23], providing evidence that at this stage lens placodal precursors have been exposed to signals that direct them towards a lens fate. Thus, the question of whether the differential

specification of lens and olfactory placodal cells occurs in the rostral neural plate border followed by a cell restricted migration, or at their final spatial destinations remains to be determined.

4. TISSUE INTERACTIONS

Without doubt the development of mature lens cells from presumptive lens ectodermal cells requires tissue interactions. When and how specific tissues might regulate lens development has also been discussed elsewhere [29–31]. Spemann's classical studies in 1901 suggested that the optic vesicle, which will give rise to the retina, is required for the development of the lens [1]. This statement was later challenged by findings that a lens or lens-like structures can form even in the absence of retinal tissue [32,33]. These first studies concerning lens induction were based exclusively on morphology. Since then, accumulating results using cytological criteria and molecular markers indicate that structures resembling a lens develop independent of interactions with the neural retina [19,23,34]. However, at later developmental stages the optic vesicle appears to play an important role for further maturation of the lens [35–38].

Explant assays in chick have provided evidence that gastrula stage prospective lens placodal cells cultured alone, without interactions of the neuroectoderm, epidermal ectoderm or underlying mesoderm, generate cells of lens character [19,22,23], suggesting that the initial specification of lens cells is independent of tissue interactions. In *Xenopus* the situation is somewhat different, since both rostral neural tissue and mesoderm are suggested to be required and/or enhance lens induction [20], once again pointing towards a difference in experimental settings or species-specific difference in lens induction. In chick, it has been shown that in the absence of the underlying mesoderm prospective adenohypophyseal placodal cells generate cells of lens character ([23]; D. Gustavsson and L. Gunhaga 2007, unpublished results), suggesting that the mesoderm underlying adenohypophyseal placodal progenitors restricts lens fate in prospective hypophyseal placodal ectoderm. Consistently, prospective hypophyseal placodal explants cultured in contact with the underlying head mesoderm generate cells of adenohypophyseal character, and under these conditions no lens cells are detected (D. Gustavsson and L. Gunhaga 2007, unpublished results). Taken together, in chick, the initial specification of lens cells does not require interactions with the underlying head mesoderm, but rather the reverse; the most rostromedial part of the head mesoderm appears to restrict specification of lens fate.

The restriction of lens fate in the caudal part of the embryo is regulated by neural crest cells [23]. At neural fold stage, prospective lens ectodermal explants cultured together with prospective, pre-migratory or migratory neural crest cells all fail to generate cells of lens character, analysed by Pax6 and δ-crystallin expression [23]. Moreover, partial ablation of prospective neural crest cells from fore- and midbrain levels at neural fold stage in chick [23] and amphibians [39], resulted in ectopic lens formation in a fraction of the

studied embryos. Why all embryos did not generate ectopic lenses could be due to the fact that cells are exposed to other lens-repressing signals together with lack of lens-promoting signals in the caudal region of the embryo. Taken together, the initial specification of lens cells appears to depend on planar signals within the ectoderm, and one major challenge has been to define when and how different signals regulate this process.

5. BONE MORPHOGENETIC PROTEIN SIGNALS PLAY A KEY ROLE IN THE SPECIFICATION OF LENS PLACODAL CELLS

Bone morphogenetic protein (BMP) signals have been shown to play important roles during lens formation. Already at gastrula stages *Bmp2* and *Bmp4*, as well as the BMP downstream activator, p-Smad-1/5/8, are detected in the rostral neural plate border, where both prospective lens and olfactory placodal cells are positioned [40,41]. Recent results using chick explants and intact embryo assays have provided evidence that at this stage, BMP activity is both required and sufficient to induce lens and olfactory placodal cells, and that sustained exposure of placodal progenitors to BMP signals is required for lens induction [19]. Prospective olfactory/lens placodal explants from gastrula stage chick embryos cultured together with the BMP inhibitor Noggin generate cells of neural character (figure 2a) [19]. Vice versa, prospective forebrain explants cultured in the presence of BMP4 generate cells of lens and olfactory placodal character (figure 2a) [19]. Thus, chick explant assays provide evidence that at gastrula stages, BMP activity inhibits neural fate and regulates the initial specification of lens placodal cells (figure 3a). Consistently, *Bmp4* knockout mouse embryos lack morphological lens placodes, but the expression of *Pax6* and *Six3* is detected in prospective lens ectoderm [42]. These observations indicate that lens placodal progenitor cells are induced in *Bmp4* mutant mice, which might reflect functional redundancy between Bmp members, as *Bmp2* is also expressed in the neural plate border region at gastrula stages [40].

A useful tool to study the roles of signalling molecules in early lens development in mice is the Lens-Cre construct. In this construct the Cre recombinase is driven by a lens-specific enhancer of *Pax6*, expressed in the prospective lens ectoderm and the surface ectoderm near the presumptive lens, generating lens-specific transgenes [43]. By using the Lens-Cre construct to delete either the two type I BMP receptors Alk2/Alk3, or *Smad1/Smad5* or *Smad4*, a recent study has shown that BMP activity regulates lens formation in mice [44], supporting previous findings in chick [19]. This study suggests that lens placodal invagination and upregulation of the lens markers FoxE3 and αA-crystallin is mediated by BMP signalling in a Smad-independent manner, while cell proliferation in the lens is mediated by the Smad pathway [44]. However, in the study of Rajagopal *et al.*, no data for an alternative downstream BMP pathway regulating lens specification was presented, and it is unclear why the significant increase in cell death in *Smad1/Smad5* deficient lens cells does

not prevent or cause disturbed lens formation. Thus, further studies have to be performed to define in detail the downstream pathway(s) of BMP receptor activation that regulate the formation and invagination of the lens placode, and the initial upregulation of lens markers.

At the neural fold stages in chick, pSmad-1/5/8 is preferentially detected in the prospective lens ectoderm compared to the prospective olfactory placodal region [19]. At this stage using prospective lens and olfactory placodal explants, cells can switch between lens and olfactory placodal fate in response to changes in BMP activity, providing evidence that at neural fold stages BMP signals promote the generation of lens cells at the expense of olfactory placodal cells (figure 2b) [19]. Furthermore, in intact neural fold stage chick embryos, inhibition of BMP signals in prospective lens cells completely abolishes lens placodal formation and inhibits the onset of *L-Maf* and δ-crystallin expression [19]. Consistently in *Bmp4* mutant embryos, where rostral placodal progenitor cells are generated, although lens placodes fail to develop the olfactory placodes appear normal [42]. At later stages, *Bmp7* is expressed in the lens ectoderm and optic vesicle, while *Bmp4* is expressed in the optic vesicle [45,46]. Since both *Bmp7* − /− and *Bmp4* − /− mice embryos exhibit disturbed lens formation [42,46], it appears that both *Bmp4* and *Bmp7* are required for lens development, and that these Bmp family members cannot subsidize for one another. Collectively, these results indicate that sustained BMP activity regulates the specification and formation of the lens placode. What specific role, if any, BMP signals play in the differentiation of lens fibre cells has, however, not been determined.

6. THE ROLE OF FIBROBLAST GROWTH FACTOR SIGNALLING IN LENS PLACODAL CELL FATE

Many studies have shown that fibroblast growth factor (FGF) signals play an important role in lens development, primarily in secondary lens fibre cell differentiation [6,47,48]. In support of this, several members of the FGF family are expressed in the eye region and all four FGF receptors (FGFR1–4) are expressed in the developing vertebrate lens [48]. Thus, the requirement of FGF signals during lens formation is apparent, but how does FGF activity regulate early lens specification?

A recent study has provided evidence that at the late gastrula stage, when lens placodal cells are initially specified, FGF activity prevents prospective lens/olfactory placodal cells in the rostral neural plate border from acquiring epidermal fate [19]. However, at this stage, FGF8 is not sufficient to induce cells of lens character in either prospective neural or epidermal cells [19]. The above results, taken together with the role BMP signals play at gastrula stages, suggest a possible model of early lens specification (figure 3a), in which FGF and BMP signals act in the neural plate border region in an opposing manner, to restrict neural and epidermal cell fate, respectively. Thus, in the context of FGF signals, which prevent the generation of epidermal cell fate, BMP activity specifies lens/olfactory placodal progenitor cells in the rostral neural plate border (figure 3). In the light of this model previous results can be interpreted in new

ways. Mis-expression of *Fgf8* in the chick head ectoderm at early neural tube stages ectopically induces expression of the early lens marker *L-Maf* [49]. Rather than a direct lens-inducing role of FGF signals, these results indicate that mis-expression of *Fgf8* in the ectoderm inhibits the generation of epidermal cells, thereby enabling BMP signals to ectopically induce cells of lens character. Similar results are observed in *Xenopus* embryos, where placodal *Six1* expression is induced by a combination of FGF8 and low levels of BMP activity, but not by FGF8 alone [50].

Neither at the gastrula stage nor at the neural fold stage do FGF signals contribute to the differential specification of lens and olfactory placodal cells [19,23]. Although Bailey *et al.* proposed that FGF signalling represses lens specification and induces olfactory fate, both their studies and another study reveal that at the neural fold stage, lens progenitor cells do not upregulate olfactory markers when exposed to FGF8 [19,23]. In addition, in the presence of FGF activity, presumptive lens cells still generate *L-Maf* and δ-crystallin positive cells [19]. Moreover, at this stage inhibition of FGF signalling does not induce lens character in prospective olfactory cells [19,23]. Thus, at early stages of development changes in FGF activity are not sufficient to switch between a lens and olfactory placodal fate.

In embryonic day (E) 8.5–E9.5 mouse eye explants, inhibition of FGF signals reduces Pax6 expression in the lens placode and the size of the lens pits formed in culture [51]. Moreover, blocking FGF activity in the presumptive lens by expressing a dominant-negative FgfR1 using the Lens-Cre construct results in reduced Pax6 expression, decrease in placodal thickness and delayed placodal invagination, but nevertheless a lens, although smaller, develops [51]. This phenotype resembles the disrupted lens formation in $Frs2\alpha^{2F/2F}$ mice mutants [52]. $Frs2\alpha^{2F}$ is a docking protein mediating FGF signalling via the ERK pathway, and in $Frs2\alpha^{2F/2F}$ mice mutants Pax6 and Six3 expression are decreased in the presumptive lens ectoderm, the placodal thickness is reduced and approximately 70 per cent of the mutants have smaller lenses [52]. At lens pit stages, both α- and β-crystallins are induced in the lens in Lens-Cre;*FgfR1/2/3*-deficient mouse embryos [53]. Consistently, in chick, the induction of δ-crystallin expression is not directly regulated by FGF signals, whereas the induction of Caprin2 expression and further differentiation of lens fibre cells requires FGF activity [36]. In summary, although FGF activity is required at early stages for preventing lens progenitor cells from acquiring an epidermal fate and for proper lens placodal formation, the initial differentiation of primary lens fibre cells and onset of early lens-specific markers are not dependent on FGF signals.

7. CAUDAL RESTRICTION OF LENS CELLS BY WNT ACTIVITY

Though, at gastrula stages, the importance of BMP and FGF signalling in ensuring a correct medial–lateral restriction of lens placodal cell character is clear, other signals provide rostral and caudal suppression

of lens fate. Several studies have provided evidence that Wnt signals play a key role in suppressing lens formation in the caudal part of the neural plate border region. As previously described in this review, at the late gastrula stage cells in the rostral neural plate border are specified as lens/olfactory placodal cells, while cells in the caudal border are specified as neural crest cells [19,21,22]. At this stage, Wnt activity imposes caudal character on neural plate border cells, thereby inhibiting lens and olfactory placodal specification, while promoting the generation of neural crest cells (figure 3a) [22,54]. Moreover, gain-and-loss of Wnt activity studies in chick explant assays have provided evidence that cells can switch between a lens/olfactory placodal and neural crest fate in response to changes in Wnt activity [22]. Prospective neural crest cells cultured in the presence of soluble Frizzled receptor, acting as a Wnt inhibitor, acquire lens and olfactory placodal character. On the other hand, prospective lens/olfactory placodal cells cultured together with Wnt3 conditioned medium acquire neural crest fate [22]. Consistently, *Masterblind* and *headless* zebrafish mutant embryos, which exhibit exaggerated Wnt signalling in the rostral neural plate border, lack or have reduced lens and olfactory placodes, while trigeminal placode and neural crest cells have expanded into the rostral part of the embryo [55–57]. Similarly, in the *Drosophila* eye-antennal imaginal disc, the expression of Ey (the Pax6 homolog) is suppressed by Wingless signals [58].

The findings that Wnt signals inhibit the specification of lens cells indicates that prospective lens cells need to develop in a Wnt-free domain. These results are supported by findings in chick and mouse, showing that the Wnt antagonist, *Secreted frizzled-related protein 2 (Sfrp2)* is expressed in the lens placode of mice and chick [46,59]. Moreover, in *Xenopus*, Dickkopf1 (Dkk1), another Wnt inhibitor, is required for the exclusion of neural crest formation in the rostral neural plate border region [60]. In LacZ reporter mouse lines, which express LacZ in response to activation of the canonical Wnt pathway [61,62] there is no indication of X-gal staining, i.e. Wnt activity, in the lens region at E8.5–E15.5 [63,64]. Consistently, even in the presence of Wnt inhibitors prospective lens cells in chick explants still upregulate the lens markers *L-Maf* and δ-crystallin (Sjödal and Gunhaga 2006, unpublished data). Finally, in mice, individual deletions of two Wnt co-receptors, *Lrp5* and *Lrp6* required for Wnt canonical signalling, do not perturb lens fate determination [64–66]. Although, a double knock-out of *Lrp5* and *Lrp6* would determine whether these co-receptors may act redundantly during early lens specification, it seems unlikely that such double mutants would exhibit a lens phenotype, since the above results clearly provide evidence that Wnt signals are not required for lens fate specification.

Also at lens placodal stages, several functional studies in mice have provided evidence that Wnt activity through the canonical pathway represses lens formation. In mice, gain of β-catenin activity through the Lens-Cre system prevents lens formation, suppresses Pax6 expression and upregulates Tuj1 expression [64,67]. Smith and colleagues argue that

surface ectodermal cells have acquired a neural fate, but since studies performed at earlier stages have shown that prospective lens cells can switch to neural crest fate in response to Wnt activity [22], it is also possible that Tuj1 positive neural crest derived neurons are generated. Analyses of specific neural crest and neuroectoderm markers would reveal this uncertainty. In agreement, loss of β-catenin activity in the presumptive lens and extraocular ectoderm using the Lens-Cre construct does not perturb lens fate decision, but leads to formation of small ectopic lentoids expressing Pax6 and crystallin proteins in the extraocular ectoderm [63,64]. The overall conclusion is that between gastrula stages and lens placodal stages Wnt activity restricts caudal expansion of lens formation (figure 3).

8. WNT INDEPENDENT β-CATENIN SIGNALLING AFFECTS LENS MORPHOGENESIS

Although no activation of the canonical Wnt pathway has been detected in the developing lens [63,64], β-catenin is expressed in the lens placode at E9.5 [64], suggesting that Wnt independent β-catenin activity plays a role in lens formation. β-catenin is known to affect cell adhesion and morphogenesis, and is a central component of the cadherin–catenin adhesion complex, which anchors the adhesion complex to the actin cytoskeleton. Conditional deletion of β-catenin, using the Lens-Cre construct, results in breaks in the continuous curve of the epithelium. These breaks corresponds to disruption of cytoskeletal and/or junctional complexes, detected by F-actin and ZO1 labelling, respectively [63,64]. These results indicate that Wnt independent β-catenin activity is required, not for lens fate decision or initial lens placodal invagination, but for further lens morphogenesis.

9. ROSTRAL RESTRICTION OF LENS CELLS BY SONIC HEDGEHOG ACTIVITY

Fate maps in chick and zebrafish at gastrula to neural fold stages have shown that lens precursor cells are located in a more caudal–distal domain compared to adenohypophysis progenitors, which arise from the most rostral–medial region of the neural plate border [10,11,68]. Several studies have provided evidence that Hedgehog (Hh) signals play a major role in the development of the adenohypophysis, and have suggested that Hh activity promotes the generation of adenohypophyseal cells, while inhibiting lens formation [69–71]. In agreement with these results, a recent study has observed that at the head fold stage in chick, *Sonic Hedgehog (Shh)* is expressed in the mesoderm underlying the prospective hypophyseal placode, which expresses *Ptc2*, a receptor for Shh signalling [72]. In contrast, at this stage, the mesoderm underlying the prospective lens placode does not express *Shh*, and prospective lens placodal cells do not express *Ptc2* [72]. Thus, the expression patterns of members of the Shh signalling pathway support the idea that Hh signals suppress lens fate and promote the specification of adenohypophyseal cells.

Convincing studies of different vertebrate mutants with disturbed Hh activity have provided evidence that Hh signals direct the choice between adenohypophyseal
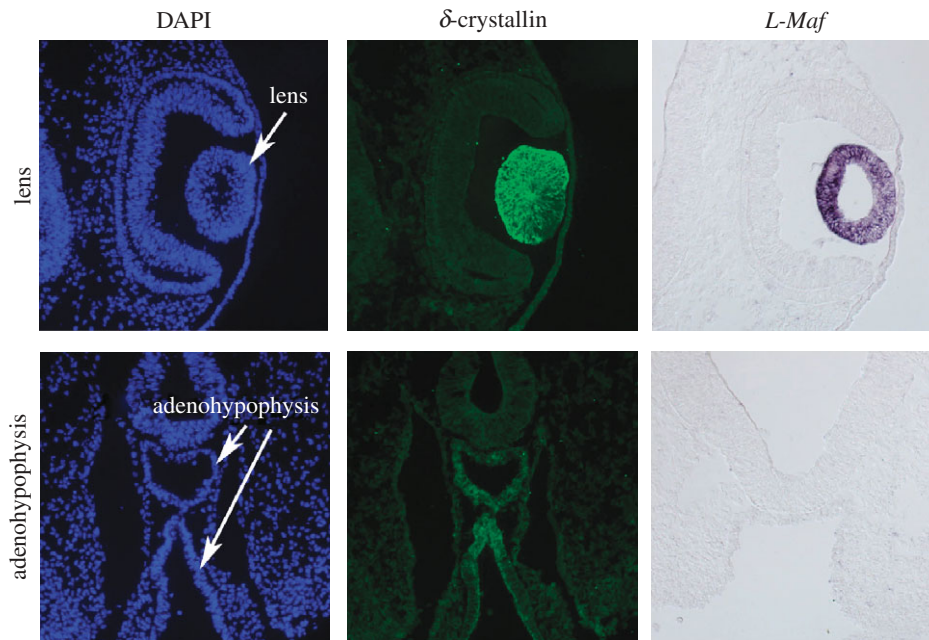
Figure 4. Expression of δ-crystallin and *L-Maf* in lens and adenohypophysis. The expression of δ-crystallin in the lens of a stage 17 chick embryo is strong, while there is barely detectable δ-crystallin expression in the adenohypophysis. *L-Maf* is expressed in the lens but not in the adenohypophysis. The same immunohistochemistry slide using the same magnification and time of exposure was used.

and lens fate. *Talpid³* chicken mutants, which have a defective activation of the Shh pathway, develop ectopic lenses usually located in the midline deriving from or connected to the hypophyseal duct [69,70]. Similarly, several zebrafish mutants with disturbed Hh-signalling have ectopic lenses at the expense of the adenohypophysis [71,73,74]. In these mutants, the head ectoderm that normally forms the hypophyseal placode instead develops into a lens indicated by the repression of adenohypophyseal markers together with upregulation of lens-specific markers [71,73,74]. In addition, transgenic mice that over-express the Shh inhibitor Hip (Huntingtin interacting protein) in the oral ectoderm and in the adenohypophyseal placode only develop a rudimentary Rathke's pouch [75]. In agreement with these studies, in chick explant assays when Shh signals are blocked prospective adenohypophyseal placodal cells generate cells of lens and olfactory epithelial fate (Gustavsson and Gunhaga 2007, unpublished data). Consistently, over-expression of Hh in zebrafish suppresses lens formation [11,76]. In *Xenopus*, *Xhip* (*Xenopus hip1*) is expressed in the prospective lens ectoderm, and loss of *Xhip*, leading to exaggerated Hh activity, results in the suppression of lens placode formation [77]. These observations indicate that the absence or presence of Shh activity in the rostral part of the surface ectoderm mediates a switch between lens and adenohypophyseal placodal cell identity. In summary, at the head fold stage, Shh signals from the underlying mesoderm act on the most rostral neural plate border cells, thereby inhibiting lens specification and inducing adenohypophyseal placodal cell fate. Thus, from head fold stages, Shh activity in the neural plate border region regulates the rostral restriction of lens specification (figure 3b).

In zebrafish, the Nodal signalling pathway, belonging to the transforming growth factor (TGF)β-family, has been implicated in directly activating transcription of

the *Shh* gene [78]. Subsequently, in zebrafish embryos with mutations in the *cyclops* gene, encoding for nodal-related protein2, midline expression of *Shh* is lost in the ventral central nervous system [79,80]. Consistently, mutations affecting components of the Nodal signalling pathway result in severe axial defects with two lens placodes in close proximity or a single median eye and lens formed at the expense of the adenohypophysis [79,81,82]. Taken together, as seen in both Hh and Nodal mutants, Hh signals play a key role in generating two separated lens domains via the formation of a medial located hypophyseal placode.

At later stages of development, the characteristic protein of chick lens fibre cells, δ-crystallin, is also transiently expressed in the adenohypophysis, as first demonstrated by Barabanov and Fedtsova [83]. Although the adenohypophysis expresses δ-crystallin, this expression is very weak compared with the δ-crystallin expression in the lens (figure 4), and an estimated concentration of δ-crystallin in the stage 22 chick adenohypophysis is only approximately 1/3000 of the detected amount in the lens [84]. Moreover, lens fibre cells, but not the adenohypophysis, express *L-Maf*, an early lens marker first expressed in the prospective lens ectoderm (figure 4) [85], and the morphological cell elongation characteristic of lens fibre cells is not detected in adenohypophyseal cells [84,86]. These results imply that high levels of δ-crystallin expression in cells of chick is characteristic of lens fibre cells.

10. RETINOIC ACID AND NOTCH ACTIVITY IN EARLY LENS FORMATION

In the last 10 years, several studies concerning how retinoic acid (RA) regulates lens development have been reported, and recently reviewed [87]. RA activity is first

observed in the lens placode around neural fold stage and at later stages in the lens pit and lens vesicle, using *in vivo* reporter systems to visualize RA signalling [88,89]. The invagination of the lens placode appears to depend on RA activity, while inhibition of RA signals using antisense oligonucleotides against cellular retinal-binding protein (CRBP) in early neural fold to early neural tube stages in mouse results in disturbed invagination of the lens placode [90]. A similar phenotype, with failure of lens placodal invagination, is also observed in Pax6[Sey/Sey] mice mutants, which interestingly exhibit reduced RA activity in the lens placode [88]. Since, mice mutants suppressed in BMP or FGF activity, also exhibit reduced or completely blocked Pax6 expression [46,52,91], it is possible that several signalling molecules regulate Pax6 expression, which in turn is critical for proper lens placodal invagination and further lens development.

Notch signalling is known to promote proliferation and inhibit cell differentiation in many embryonic tissues. When the intracellular domain of Notch (Notch[IC]) translocates to the nucleus it acts in a transcriptional complex with the DNA-binding transcription factor Rbpj and Mastermind to promote transcription. Loss of Notch signalling in the lens of mice, by using a Lens-Cre;*Rbpj* construct, results in premature exit from the cell cycle and subsequently accelerated primary lens fibre cell differentiation [92,93]. Consistently, mice with constitutive Notch activity, through Lens-Cre;*Notch1[IC]*, exhibit delayed primary fibre cell differentiation [92]. However, the overall conclusion from studies using the Lens-Cre construct to suppress Notch activity is that Notch signals are not essential for lens formation, but are primarily required during secondary lens fibre cell differentiation [92–95].

To date, studies regarding how Notch signals affect lens development at earlier stages are few. The first direct evidence that Notch activity plays a role in lens induction is that inhibition of the Notch ligand, Delta2, in *Xenopus* results in loss or severe reduction of *FoxE3* expression followed by failure of placodal formation and reduced or absent *γ1-crystallin* expression [96]. Interestingly, the *FoxE3* promoter includes a target sequence for Su(H)-binding motif for Notch signalling and also for Smad1-binding motif for BMP signalling, and both are suggested to be required for proper upregulation of *FoxE3* [96]. This finding beautifully highlights the general knowledge that a combination of signalling factors affects lens specification and proper lens formation.

11. CONCLUDING REMARKS

A model that describes how specific signalling molecules regulate the initial specification of lens cells in vertebrates at gastrula stages has emerged (figure 3). At this stage, planar BMP, FGF and Wnt signal(s) within the ectoderm regulate lens induction. In the context of FGF signals, which repress epidermal ectoderm formation, BMP activity specifies lens/olfactory placodal progenitor cells in the rostral neural plate border region. Wnt activity restricts caudal expansion of lens formation. From head fold stages, Shh activity derived from the underlying mesoderm restricts lens formation in the most rostral part of the neural plate

border. Taken together, around gastrula to head fold stages the activities of BMP, FGF, Shh and Wnt appear to choreograph the patterning of the neural plate border and subsequently restrict the ectoderm fated to become the future lens (figure 3).

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