Control of early development of the lens and the retina

Tanushree Pandit

Umeå Center for Molecular Medicine
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The greatest progressive minds of embryology have not looked for hypotheses; they have looked at embryos.
Jane Oppenheimer (1955)

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Abstract

The nervous system is composed of two separate compartments, the central and the peripheral nervous system. The peripheral nervous system (PNS) composed mainly of sensory organs transmits sensory information to the central nervous system (CNS) comprising the brain and the spinal cord. The CNS then processes this information and modifies the behavior of the organism appropriately. To understand the functioning of these systems one has to understand how the different cell types belonging to these systems are generated during the course of embryonic development. Using the chick eye with the lens, which arises from the region that gives rise to components of PNS, and the retina, belonging to the CNS, as an embryonic model tissue the following questions were addressed: how do the BMP and the FGF signalling pathways regulate developmental processes within the lens and retina? When do retinal cells get specified and how do the lens and the retina interact with each other during early development? These questions were addressed by using a combination of *in vitro* and *in vivo* assays in chick embryos.

We show in chick that prospective lens cells are committed to a lens identity, concomitant with the up-regulation of the lens specific marker, L-Maf. Before the onset of L-maf, or in the absence of ongoing BMP activity, lens cells switch to an olfactory fate. However, after cells have up-regulated L-Maf, they are no longer dependent upon BMP signalling for the next step of lens primary fiber differentiation, which is characterized by the onset of δ-crystallin.

We provide evidence that the FGF signalling pathway is critical for regulating proliferation within the developing lens, while FGF and BMP signals cooperate with each other to regulate cell cycle exit. In addition we have characterized the expression of *Equarin* restricted to the prospective differentiating population within the lens, and we show that this gene is subject to regulation by both FGF and BMP signalling. In the absence of FGF and BMP signals, *Equarin* expression is down-regulated similar to down-regulation of the cell cycle exit marker p27kip1. Over activation of BMP, but not FGF signals is sufficient to up-regulate *Equarin* expression within the lens.

Concerning retinal cells, we provide evidence that retinal cells are specified at stage 13. Prior to stage 13, retinal cells are initially specified as telencephalic cells. Our results indicate that prospective retinal cells require

either BMP signals from the lens tissue, to maintain a retinal identity and to promote further development of retinal cells.

Abbreviations

BMP	Bone Morphogenetic Protein
BrdU	Bromo-deoxy-uridine
CNS	Central nervous system
FGF	Fibroblast growth factor
GFP	Green Fluorescent Protein
HH	Hedgehog
L	Lens
PNS	Peripheral nervous system
R	Retina
RA	Retinoic acid
Smad	Small mothers against dpp
TGF-β	Transforming growth factor- beta

Papers in this thesis

This thesis consists of following papers and a manuscript

1) **Pandit, T.,** V. K. Jidigam and L. Gunhaga (2011). "BMP-induced L-Maf regulates subsequent BMP-independent differentiation of primary lens fibre cells." Dev Dyn **240**(8): 1917-1928.

2) Jarrin, M., **T. Pandit** and L. Gunhaga (2012). "A balance of FGF and BMP signals regulates cell cycle exit and Equarin expression in lens cells." Mol Biol Cell **23**(16): 3266-3274.

3) **Pandit, T**., C. Patthey and L. Gunhaga. "Specification of neural retina character by lens derived BMP signalling". *Manuscript*

INTRODUCTION

One of the most interesting aspects of multi cellular organisms, including human beings, is the vast array of diverse cell types that are correctly ordered into different tissues and organs that function simultaneously, like a machine. As an example, one of the sensory organs, the eye transmits visual information to the brain which then in turn, modulates our behavior enabling us to respond appropriately to the visual stimuli we receive. It is difficult to comprehend that the process of building a complex multi cellular organism begins with a single cell fertilized zygote. For this to be achieved, the zygote goes through a transition period known as an embryo. Embryonic development is a dynamic process whereby cells receive instructions to differentiate into specific cell lineages that ultimately are organized into complex tissues and organs of the adult organism. Developmental biology deals with questions like a) when and how cells receive instructions to become part of a specific structure b) how do tissues and organs undergo morphogenesis c) how do these tissues and organs achieve the right size and shape.

If one wants to understand how mature organs perform their functions, it is essential to understand the initial steps that lead to formation of these organs during the course of embryonic development. This knowledge can also be used to devise better strategies to cure developmental disorders. An interesting application of developmental biology is in the field of stem cell biology. Stem cell based therapies are being looked at as attractive tools to cure diseases. Since the field of developmental biology deals with instructions that cells receive to acquire a specific cell lineage, this knowledge can be applied to direct stem cells towards specific cell lineages which can then be used to replace missing tissues in the body.

Phases of embryonic development

Within a growing embryo, labeled groups of cells can be followed over a period of time to monitor which adult structure they become a part of - this is defined as the cell fate. The subsequent process by which a cell receives instructions to form a specific cell lineage can be broken down into following steps:

a) Specification: This is the step whereby cells receive instructions to become restricted to a specific cell lineage (such as the lens of the eye). To know whether a cell has been specified to achieve its particular fate, one can dissect a small piece of tissue based on fate maps and culture it

in vitro for a specific period of time. If the cells achieve their fate, they are specified. However, if specified cells are exposed to ectopic signals, activators or inhibitors, their identity can most often be altered.

b) Determination/commitment: When the fate of the cell can no longer be altered even after exposing it to ectopic signals, these cells are committed. Hence cell commitment is an irreversible step during development.

c) Differentiation: Differentiation is most often coupled to exit of the cell cycle, and is the process whereby cells acquire molecular and biochemical characteristics of a mature cell. For instance, lens cells start elongating and accumulating crystalline proteins that give the lens its transparency. Once cells have finished their terminal steps of differentiation they usually cannot differentiate into another cell type, although exceptions do occur in biology.

Signalling pathways and acquisition of cellular identities

The set of molecular instructions that a cell requires to achieve specific identities are given by a small group of signalling pathways. Some of the major signalling pathways mediating fate decisions in embryonic development are comprised of the Fibroblast growth factor (FGF), transforming growth factor–β superfamily (TGF-β) including Bone Morphogenetic Proteins (BMP), Hedgehog (HH), Retinoic acid (RA) and Notch-delta pathways etc. These signals can act in a paracrine, autocrine or juxtacrine manner. An obvious question that comes to mind is, how can such a small group of signalling pathways, instruct such a diverse range of cellular fates? To answer this question, one has to look into the mechanisms by which signalling pathways mediate their functions. Signalling molecules can form concentration gradients, also called morphogen gradients, over a particular range of distance. Depending upon the particular concentration of the ligand the cells are exposed to cells acquire a specific identity (Ashe and Briscoe, 2006). In addition to morphogen gradients, different cellular identities can also be induced by the same signalling pathway depending upon the time of exposure to the signalling molecule (Sjodal et al., 2007). Signalling pathways can also interact with each other in a cooperative or antagonistic manner to induce different cellular identities (Boswell et al., 2008; Maier et al., 2010; Ohyama et al., 2008). Furthermore, the sequential order in which cells are exposed to the signalling molecules also affects cellular identity (Gunhaga et al., 2003; Patthey et al., 2009). During development these signalling pathways activate or repress various genetic programs depending on time point and context, thereby giving cells different instructions at different time points. It is to be noted that the mechanisms

discussed above are not mutually exclusive and a combination of these eventually leads to acquisition of different cellular identities.

Composition of the nervous system

The vertebrate nervous system which is subdivided into the central and peripheral nervous systems (CNS and PNS) is derived from the embryonic ectoderm. The ectoderm is subdivided into non-neural ectoderm and the neural ectoderm which gives rise to the CNS. In addition, the non-neural ectoderm is subdivided into the epidermis, giving rise to the skin, and the border region which gives rise to the PNS. The border region develops between the prospective neural and epidermal domains (figure 1). The PNS is composed of placodes and neural crest that, among other cell types and structures, form the sensory organs and the cranial nerves. Specific sensory cells in the PNS sense external stimuli and send the information to the CNS, which then modulate the behaviour of the organism appropriately. To better understand the connectivity between the CNS and PNS, one must define how each individual component of these two systems develops in relation to each other.

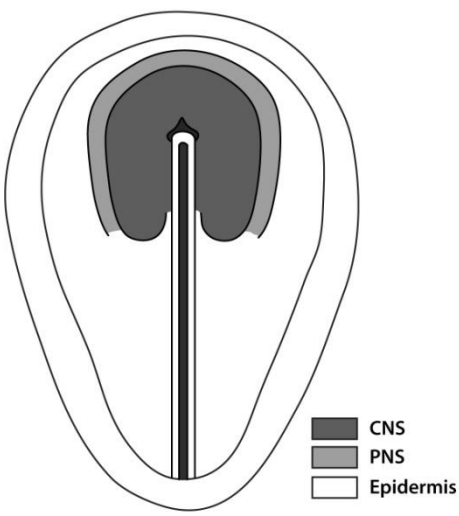

Figure 1: Schematic diagram of an embryo of a stage 4, late gastrula in chick embryonic development. The dark grey region in the medial part of the embryo gives rise to the central nervous system (CNS) and the white region gives rise to epidermal ectoderm. Between these two, the light grey region gives rise to the neural plate border domain from which the peripheral nervous system (PNS) arises.

The central nervous system

The formation of the CNS begins with the process of neural induction. This is the step at which a group of cells within an embryo is instructed to acquire neural identity. This process is initiated at the blastula stage in chick embryos (Wilson et al., 2001). Several lines of evidence show that interplay

between Wnt, Fgf and Bmp signalling pathways is involved in this process (Streit et al., 2000; Wilson et al., 2001). In the medial part of the chick blastula embryo, which gives rise to neural ectoderm, FGF signalling is involved in repression of genes encoding BMP ligands which enables cells to acquire a neural identity (Wilson et al., 2001). In the lateral epiblast region, which gives rise to non-neural ectoderm, Wnt signalling is active which represses FGF signalling relieving BMP repression thereby inducing an epidermal fate(Wilson et al., 2001).

Following neural induction, cells of the neural plate are subdivided into distinct identities with respect to their position along the antero-posterior axis, such as forebrain, midbrain, hindbrain and spinal cord. The identities of these cells belonging to different classes of the CNS are defined by distinct expression profiles of transcription factors. Initially, cells within the neural ectoderm are of an anterior forebrain character (Stern, 2001). Cells in the posterior part of the neural plate are then re-specified as cells of posterior neural character (midbrain, hindbrain and spinal cord) (Nieuwkoop and Nigtevecht,1954), which takes place at the late gastrula stage in chick embryos (Muhr et al., 1999; Nordstrom et al., 2002). The progressive posteriorization of the neural plate is mediated by a combined action of Wnt, FGF and retinoic acid activity (Kiecker and Niehrs, 2001; Muhr et al., 1999; Nordstrom et al., 2002; Storey et al., 1998). After the broad division of the neural plate, each compartment is refined into smaller regions.

The forebrain gets further subdivided into telencephalon, hypothalamus, diencephalon and the retina (Wilson and Houart, 2004). How segregation of the forebrain into these distinct territories is achieved is not well established. Studies conducted in zebrafish suggest that prospective telencephalic and eye field regions are exposed to low levels of Wnt activity, and that ectopic activation of Wnt signals in this region leads to the expansion of the diencephalon (Heisenberg et al. (2001). In mouse mutants lacking the transcription factor Six3, an anterior expansion of Wnt ligands is observed, indicating that Six3 is involved in repressing Wnt activity within the anterior forebrain region (Lagutin et al., 2003). Moreover, in the Six3 mutants, the anterior forebrain, including the telencephalon and the eye field, is severely truncated, and there is an expansion of posterior diencephalic markers. Thus, it is possible that the same signalling pathways used during the initial processes of antero-posterior patterning of the neural tube are reemployed to distinguish between different forebrain fates.

Nevertheless, a detailed understanding of how acquisition of different cellular identities within the forebrain is achieved is lacking. A key to understanding the differential specification of these identities would be to

look into the expression patterns, timing and the combinatorial actions of various signalling pathways during early forebrain development.

The peripheral nervous system

The neural plate border

The peripheral nervous system is composed of sensory organs and glia that transmit information to processing centers within the CNS as well as cells of the autonomic and enteric nervous systems. During early embryonic development, the PNS arises from a region located between the prospective neural ectoderm and the prospective epidermal ectoderm. This region is referred to as the neural plate border region or simply, the border. The border region gives rise to cells of placodal and neural crest character. In vertebrates, the most common placodes are; adenohypophysis, olfactory, lens, epibranchial, otic and trigeminal. Placodes are transient thickenings of ectoderm in the head region that form the sensory organs and parts of the cranial nerves. With the exception of the lens, all other placodes arising from the border region give rise to neuronal populations. The neural crest cells are a migrating population of cells, which occupy various positions in the body and give rise to distinct neurons, glia and other non neuronal cell types such as melanocytes, bone and cartilage.

At anterior levels, the border region gives rise to adenohypophysis, olfactory and lens placodal cells. At more posterior levels, neural crest cells are generated and the epibranchial, otic and trigeminal placodal progenitors are located between the neural crest cells and epidermal ectoderm (Baker and Bronner-Fraser, 2001; Patthey et al., 2009). At late blastula stages, border cells along the entire antero-posterior axis of the border region are specified as neural crest cells (Carmona-Fontaine et al., 2007; Patthey et al., 2009). This specification event involves a sequential exposure to Wnt activity followed by BMP signals (Nguyen et al., 1998; Patthey et al., 2009)

According to fate maps (Bhattacharyya et al., 2004) , prospective lens and olfactory cells are intermingled in the anterior border region at gastrula stages. Consistently cells of anterior border character, comprising cells of olfactory and lens identity, are specified at gastrula stages (Sjodal et al., 2007) (Figure 2). In order for olfactory and lens placodal cells to be specified, anterior placodal progenitors require BMP signals and in the absence of BMP signals, prospective lens and olfactory cells switch to a neural fate (Sjodal et al., 2007). In addition, anterior placodal progenitors must be prevented from being further exposed to Wnt activity, since ectopic exposure of Wnt activity induces neural crest character in prospective

anterior border cells (Dutta and Dawid, 2010; Litsiou et al., 2005; Patthey et al., 2009). The differential specification of olfactory and lens placodal cells is mediated by different durations of exposure to BMP signals (Sjodal et al., 2007). Consistently, by the neural fold stage, when prospective olfactory and lens cells are spatially segregated, prolonged exposure to BMP signalling to olfactory cells results in a switch to lens identity, whereas a shorter exposure to BMP activity in lens cells results in a switch to an olfactory identity (Sjodal et al., 2007). Thus, at neural fold stage, the generation of olfactory placodal cells is independent of BMP signals. However, at what stage prospective lens cells become independent of BMP activity was not investigated in this study.

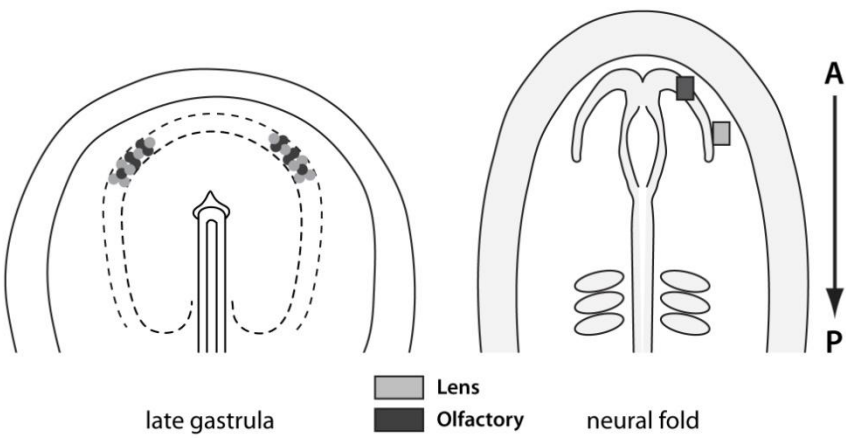

Figure 2: Differential specification of olfactory and lens placodes. Schematic diagram of the embryo on the left shows the prospective lens and olfactory placodes intermingled in the anterior border region at gastrula stages. This is the stage at which prospective lens and olfactory cells get specified. Lens cells are shown in light grey and olfactory cells are in dark grey. On the right, is a schematic diagram of stage 8 or the neural fold stage chick embryo. At this stage, prospective olfactory cells and prospective lens cells spatially segregate. At this stage, olfactory cells are independent of BMP signalling while lens cells are still dependent upon BMP signals.

The vertebrate eye

Following neural tube closure, prospective lens cells comes into close contact with the optic vesicle, an outgrowth of anterior forebrain region (figure 3) (Gunhaga, 2011). At this time, prospective lens cells start thickening to form a placode. During placode formation, cells undergo a change in shape, resulting in cell elongation and apical constriction (Plageman et al., 2011). Then the thickened lens placode and optic vesicle invaginate simultaneously. This is followed by formation of a spherical structure called the lens vesicle. In the anterior part of the lens vesicle, lens epithelial cells retain their

proliferative capacity. In the posterior part of the lens vesicle, cells leave the cell cycle, start elongating to form the first differentiating population within the lens called the primary fiber cells (Gunhaga, 2011). Molecularly, the generation of lens fiber cells is characterized by up-regulation of various crystallin proteins (Piatigorsky, 1989). At later stages and continuously throughout life, cells from the peripheral lens epithelium migrate to the equatorial region of the lens, exit the cell cycle and differentiate into secondary lens fibers.

Several studies have shown the importance of FGF and BMP signals in regulating cellular proliferation and cell cycle exit within the lens (Adler and Belecky-Adams, 2002; Le and Musil, 2001; McAvoy and Chamberlain, 1989). These studies have mainly demonstrated the importance of these signalling pathways during the secondary fiber differentiation process. Cells in the equatorial region of the lens are exposed to FGF and BMP signals and induced to form secondary fiber cells (Boswell et al., 2008). The molecular mechanisms regulating the transition of proliferating lens cells to differentiated primary lens fiber cells are, however, not well characterized.

Simultaneous with the invagination of the lens placode, the optic vesicle also undergoes morphological changes to form a bilayered optic cup. The inner layer of the optic cup gives rise to the neural retina and the outer layer of the optic cup gives rise to the non-neural retina or the retinal pigment epithelium (Fuhrmann, 2010). The optic cup is connected to the forebrain by means of an optic stalk. The optic cup will undergo neurogenesis to give rise to six different types of neurons and glia. The molecular mechanisms regulating the initial specification of retinal cells, and when this occurs during embryonic development have not been determined.

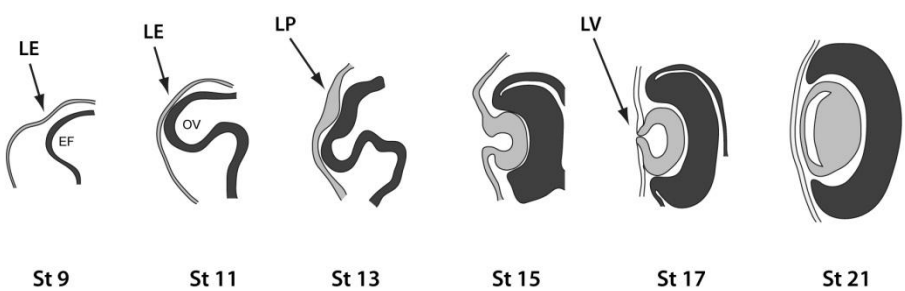

Figure 3: Morphological changes in the prospective lens and retina during chick embryonic development. Around neural tube closure stages, stage 9 the prospective retinal cells referred to as the eye field at this stage begin to evaginate. Following neural tube closure, at stage 11,

prospective lens ectoderm (LE) in light grey, comes into contact with evaginating eye field cells referred to as the optic vesicle at this stage shown in dark grey. After contact of the two tissues, the lens ectoderm thickens to form the lens placode (LP) around stage 13. After thickening, the lens placode and the optic vesicle invaginate simultaneously around stage 15. By stage 17, a spherical structure called the lens vesicle (LV) is formed. The optic vesicle is transformed into an optic cup. Lens cells in the posterior part, close to the optic cup start elongating to form the primary fiber cells. At stage 21, elongated primary fiber cells can be detected in the posterior compartment while a thin layer of proliferative cells called the lens epithelium can be seen in the anterior compartment. The ectoderm above the lens breaks away to form the cornea.Figure modified fom (Lovicu and McAvoy, 2005)

The eye as a model to study tissue- tissue interactions

The lens and the retina of the embryonic eye have served as a classical model to study the process of induction. In classical amphibian experiments performed by Spemann whereby the optic cup was surgically removed, lens tissue failed to form (Spemann, 1901). This led to a model of lens induction in which the optic vesicle was the primary inducer of lens. However, this model was not found to be consistent within the different amphibian embryos tested (Okada, 2004). In addition, studies conducted in chick embryos have shown that prospective lens cells are specified at gastrula stages (Sjodal et al., 2007), prior to contact with the optic vesicle. To this date, the issue of how prospective lens and retinal tissues interact with each other remains controversial.

According to fate maps in chick, around neural fold stages cells fated to give rise to the retina are located roughly in the medial part of the anterior neural plate (Sanchez-Arrones et al., 2009).At this stage, the retina is referred to as the eye field. At this stage, prospective lens and retinal cells are not in close proximity to one another. Simultaneous with the neural tube closure, the prospective lens ectoderm comes in contact with the eye field that evaginates to form the optic vesicle. Apart from the retina, other structures of the forebrain that arise from the neural plate are the telencephalon, hypothalamus and the diencephalon (Wilson and Houart, 2004) (Figure 4). One of the earliest markers to be expressed within the eye field is the transcription factor called Rx/Rax. In mice, inactivation of this transcription factor within the anterior neural plate leads to complete absence of eyes as a result of failure to form optic vesicles. Moreover, lens also fails to form in these mutants (Swindell et al., 2008). Apart from Rx, several other transcription factors have been shown to play important roles at different stages of retinal development. These set of transcription factors are expressed in an overlapping pattern within the developing eye field (Grindley et al., 1995; Lagutin et al., 2003; Swindell et al., 2008; Zuber et al., 2003). In addition, inactivation of a transcription factor, Vsx2 known to be expressed specifically within the neural domain of the developing retina,

results in reduced proliferation of neural progenitors within the optic vesicle (Burmeister et al., 1996). However, the mechanisms by which these transcription factors are regulated during early stages of retinal development remain to be characterized.

The Bone Morphogenetic Protein (BMP) pathway has been implicated in several aspects of eye development. Bmp4 and Bmp7 mutants exhibit severe defects in eye development (Furuta and Hogan, 1998; Wawersik et al., 1999). In the BMP4 mutants, lens induction fails to occur (Furuta and Hogan, 1998) while in the BMP7 mutants, although lens induction is initiated, lens development fails to be maintained due to lack of Pax6 expression (Wawersik et al., 1999). BMP signalling has also been implicated in regulating proliferation and cell death during early stages stages of eye development (Trousse et al., 2001). In addition, in both chick and mouse, BMP activity is known to regulate early dorso-ventral patterning of the optic vesicle ((Kobayashi et al., 2010; Murali et al., 2005). However, the role of BMP signalling in early specification of retinal cells, if any, remains to be determined.

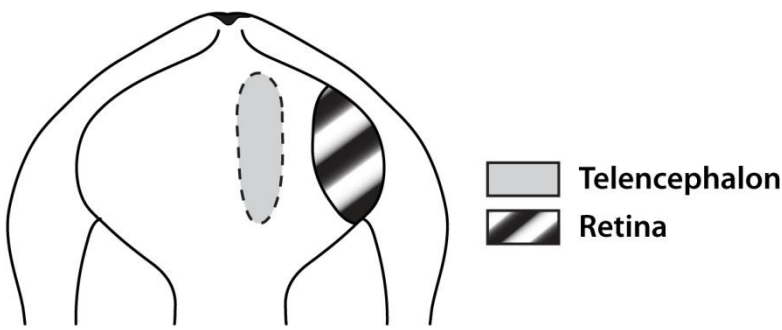

Figure 4: Segregation of anterior forebrain into different structures. Figure shows schematic diagram of a chick embryo around neural tube closure stage /stage 10. For simplicity only telencephalic and retinal precursors have been depicted. The light grey region gives rise to the telencephalon, and the striped region gives rise to the retinal cells during embryonic development.

RESULTS

Paper I: BMP induced L-Maf regulates subsequent BMP independent differentiation of primary lens fiber cells

The expression of components of BMP signalling pathway precedes expression of lens specific markers within the developing eye.

To understand the role of BMP signalling at stages when the prospective lens and optic vesicle come into close contact, we analysed the expression of *Bmp4* and p-Smad 1/5/8 which is an indicator of active BMP signalling. The expression of lens specific markers L-Maf and δ-crystallin was also analyzed at these stages. *Bmp4* and pSmad 1/5/8, but not L-Maf or δ-crystallin are detected within the lens ectoderm at stage 11. By stage 13, in addition to the prospective lens cells, *Bmp4* was also observed in a few cells within the optic vesicle, and p-Smad 1/5/8 was detected more robustly within the prospective lens cells and a few cells were found within the optic vesicle as well. At this stage, when the lens placode starts to thicken, L-Maf positive cells were observed within the thickened lens placode. However, δ-crystallin expression was not detected until stage 17, when a lens vesicle has formed and cells in the posterior part of the lens vesicle begin to elongate. In summary, expression of the members of the BMP pathway precedes the expression of lens specific markers, L-Maf and δ-crystallin.

BMP activity is required for onset of L-Maf expression both in vitro and in vivo

In order to test the requirement of BMP activity for proper lens formation, we used an explant assay of ectodermal cell differentiation where prospective lens cells together with the underlying optic vesicle were dissected out at stage 11. This is the stage before cells start expressing L-Maf or δ-crystallin, and the explants were cultured *in vitro* to a time point equivalent to stage 21 approximately. The lens/retina (LR) explants were cultured either in the presence or absence of Noggin, which is a known inhibitor of BMP signalling (Lamb et al., 1993). The LR explants were then sectioned and processed for immunohistochemistry and expression levels of L-maf and δ-crystallin were analysed. Control LR explants generated L-Maf$^+$ and δ-crystallin$^+$ cells in the lens domain of the explants. However in the presence of Noggin, the

expression of the lens specific markers L-Maf and δ-crystallin were down-regulated. Instead these cells co-expressed Keratin and HuC/D together with Dlx indicative of an olfactory fate.

To test for the requirement of BMP activity within intact chick embryos, we electroporated prospective lens ectoderm at stage 11 with Noggin (Timmer et al., 2002) together with the green fluorescent protein (GFP) construct to monitor the transfection efficiency. These embryos were cultured to approximately stage 21. As controls, embryos were electroporated with only GFP. Embryos electroporated with only GFP displayed a normal morphology of the lens with L-Maf and δ-crystallin expression in the primary lens fibre cells. By contrast, embryos electroporated with Noggin exhibited disrupted lens development. In the more severely affected embryos, lens ectoderm failed to undergo normal morphogenesis, leading to complete lack of any lens structure and in the slightly less severely affected embryos, a small but malformed lens developed. The severity of the lens phenotype was correlated with electroporation efficiency. Lenses that failed to undergo normal morphogenesis also failed to up-regulate L-Maf and δ-crystallin, while the small malformed lenses expressed these markers. In addition to a disrupted morphology of the lens, the optic vesicle also failed to undergo normal morphogenesis to develop into an optic cup.

To understand in more details, why lens morphogenesis was severely disrupted after BMP inhibition, control or Noggin electroporated embryos were cultured to different time points and the levels of proliferation and cell death were assessed by pHistoneH3 and aCaspase3 respectively. In embryos grown to approximately stage 13/14 there were no significant differences in pHistoneH3 levels in Noggin and control electroporated embryos. With increase in culture time, a higher reduction in cellular proliferation was observed in Noggin treated embryos in comparison with controls. At all stages examined, we did not detect any significant changes in the levels of aCaspase3 between Noggin and control electroporated embryos. Thus the disrupted morphology of lens in Noggin treated embryos was in part due to a reduction in cellular proliferation. However, the loss of L-Maf and δ-crystallin expression in Noggin electroporated cells suggests a specific requirement for BMP activity for proper differentiation of lens cells.

Prospective lens fiber cells become independent of BMP signalling after onset of L-Maf

The onset of δ-crystallin expression follows that of L-Maf. The down-regulation of both L-Maf and δ-crystallin observed in stage 11 LR explants cultured with Noggin could be due to a direct requirement of BMP signals for

δ-crystallin expression or an indirect requirement via regulation of L-Maf expression. To distinguish between these two possibilities, stage 13 LR explants were cultured in the presence or absence of Noggin to a time point equivalent to stage 21 in a developing chick embryo. At stage 13, lens ectoderm starts thickening to form a lens placode and cells up-regulate expression of L-Maf but not δ-crystallin. In control explants, L-Maf and δ-crystallin expression was found within the lens domain of the explants. In the presence of Noggin, however, prospective lens cells were committed to a lens fate as assessed by continued expression of L-Maf and δ-crystallin. This suggested that the earliest event of primary fiber differentiation, indicated by up-regulation of δ-crystallin can proceed independently of BMP signalling after L-Maf induction.

BMP activity is sufficient to induce L-Maf expression

At late gastrula stages, BMP4 mRNA is up-regulated in the border region from where lens cells arise (Chapman et al., 2002). To test for the sufficiency of BMP signals to induce L-Maf expression, stage 4/late gastrula neural explants were cultured in the presence or absence of BMP4 and grown to approximately stage 17. Neural explants cultured alone generated L5 and Sox2 positive cells characteristic of a neural fate. By contrast, neural explants cultured together with BMP4 resulted in loss of these markers and up-regulation of L-Maf. Thus, BMP activity is sufficient to induce L-Maf expression.

Up-regulation of δ-crystallin expression is independent of BMP signals

At stage 13, after onset of L-Maf expression, inhibition of BMP signalling did not result in down-regulation of δ-crystallin. This suggested that this event in primary fiber differentiation was dependent upon L-Maf and not BMP signals. To test for this, an L-Maf expression construct (Ogino and Yasuda, 1998) was electroporated in the prospective olfactory epithelium at stage 9-11 with or without Noggin. Any BMP signalling in this region would be down-regulated as a result of over expression of Noggin. Under these circumstances, L-Maf construct was still able to induce δ-crystallin expression further strengthening the idea that L-Maf regulates δ-crystallin expression independently of BMP signals. In addition, we observed the same effect when we electroporated L-Maf with or without Noggin into the anterior head ectoderm region. Taken together, these results provide evidence that the up-regulation of δ-crystallin proceeds independently of BMP signals in the presence of L-Maf expression.

Paper II: A balance of BMP and FGF signals regulates cell cycle exit and *Equarin* expression within the lens cells

Equarin expression is located in the p27kip1 positive region of the lens

To analyse processes of proliferation and cell cycle exit within the developing lens, we first analyzed the expression of δ-crystallin, p27kip1 an indicator of cell cycle exit, pHistoneH3 an indicator of proliferative cells and *Equarin*. *Equarin* is also known as Ccdc80, and is a secreted protein (Mu et al., 2003) which is up-regulated in the invaginating lens placode. At stage 18, when the lens vesicle is morphologically observed, *Equarin* expression is localized to cells expressing p27kip1 and δ-crystallin. In other words, *Equarin* is localized to cells undergoing cell cycle exit and early events of primary fiber differentiation. This makes it a valuable marker that can be used to study cell cycle exit in the lens. At later stages, *Equarin* expression was localized to the equatorial region of the lens where cells from the epithelium migrate and differentiate to form secondary fiber cells. *Equarin* expression was not detected in the epithelium compartment of the lens, suggesting that *Equarin* is only expressed in cells instructed to leave the cell cycle.

FGF signals promote both mitosis and cell cycle exit of lens cells

To examine whether BMP and FGF signals regulate cell cycle exit and early differentiation of primary lens fiber cells, an explant assay of cell differentiation was developed in which lens-retina (LR) explants from stage 13 were exposed to different modulators of the BMP and FGF signalling pathways. Stage 13 LR explants exposed to SU5402, which inhibits FGF signalling down-regulated levels of both pHistoneH3 and p27kip1 and *Equarin* expression within the lens domain of these explants. Upon addition of FGF2 or FGF8 in stage 13 LR explants, there was an increase in the levels of both pHistoneH3 and p27kip1. However, this ectopic activation of FGF signals was not sufficient to up-regulate *Equarin* expression in these cells suggesting that other molecular players are involved in regulating *Equarin* levels.

BMP activity promotes cell cycle exit and Equarin expression in lens cells

Next we modulated the activity of BMP in stage 13 LR explants by culturing them alone or in the presence of BMP4 or Noggin. Upon inhibition of BMP activity by addition of Noggin, there was a reduction in the numbers of both

proliferating cells and cells undergoing cell cycle exit as seen by decreased numbers of pHistoneH3 and p27kip1 and *Equarin* expression respectively. By contrast, over activation of the BMP signalling pathway led to similar numbers of pHistoneH3 as cells in control explants, but a significant increase in the numbers of cells undergoing cell cycle exit as seen by increase in p27kip1 and *Equarin* expression. Thus, BMP signals were essential in promoting cell cycle exit and sufficient to up-regulate *Equarin* expression.

BMP activity is critical for cell cycle exit but not mitosis within the lens cells

Inhibition of BMP activity in the LR explants assay resulted in a decrease in numbers of proliferating cells, while exogenous addition of BMP4 to LR explants had no effect on the proliferation rate. We speculated that this seemingly contradictory result could be due to the fact that inhibition of BMP activity might cause down-regulation of FGF signals, resulting in lower proliferation. To test this possibility, we examined the expression of *Fgf8*, normally expressed in developing retinal cells. Under control conditions, as expected *Fgf8* expression was detected in the retinal compartment of the explants. By contrast, in explants cultured with Noggin, *Fgf8* failed to be up-regulated. This suggested that the decrease in proliferation in Noggin treated explants resulted from down-regulation of FGF signalling. Further strengthening this idea was the fact that, explants cultured with Noggin in the presence of FGF2 or FGF8 were able to restore proliferation levels to those comparable to control explants. However, addition of exogenous FGF2 or FGF8 was not sufficient to rescue either cells undergoing cell cycle exit or *Equarin* expression. This suggested that BMP signalling had a more important role to play in regulation of cell cycle exit within the lens rather than regulating levels of mitosis.

FGF and BMP interactions promote cell cycle exit

To analyse if BMP signals were sufficient to rescue cell cycle exit in the absence of FGF signalling, LR explants were cultured with SU5402 and BMP4. In this scenario, the numbers of p27kip1 positive cells were still significantly reduced in comparison to control explants. Upon comparison with explants cultured only in the presence of SU5402, there were slightly more p27kip1 positive cells. In the absence of FGF signals, although BMP was sufficient to rescue *Equarin* expression, its pattern of expression was different from control explants. These results suggested that FGF and BMP signals are both critical for cell cycle exit within the developing lens. In the presence of both BMP and FGF activity, the numbers of cells undergoing cell cycle exit was highly up-regulated as seen by levels of p27kip1 in comparison

with LR explants cultured alone or explants exposed to FGF2, FGF8 or BMP4 alone. The *Equarin* levels were also highly up-regulated in BMP/FGF-treated explants in comparison with control explants or explants treated with FGF2, FGF8 or BMP4 alone. These results suggested that BMP and FGF signals cooperate to regulate cell cycle exit and *Equarin* expression.

Lens cells are halted in the cell cycle in the absence of BMP activity

To analyse in detail how modulation of the BMP and FGF pathways affected the progression of the cell cycle in developing lens cells BrdU labeling experiments were performed. Bromo-deoxy-uridine (BrdU) is taken up by cells that are in the S phase of the cell cycle. This can be used in addition to pHistoneH3 which marks cells in the M phase of proliferation to analyse progress through the cell cycle. In LR explants exposed to Noggin, a high level of BrdU incorporation was observed. These results suggested two possibilities: a) Cells were halted in the cell cycle in the early G2 phase or b) Cells were spending more time in S phase. As expected, treatment of LR explants with BMP4 on the other hand, decreased BrdU incorporation significantly and there was an increase in the numbers of p27kip1 positive cells indicative of higher cell cycle exit within the lens.

FGF signals increase BrdU incorporation in lens cells

Next, BrdU incorporation was analysed in explants in which the FGF activity was modulated. In explants treated with SU5402, both the levels of BrdU and pHistoneH3 positive cells were decreased in comparison with controls. In contrast, explants treated with FGF2 showed an increase in BrdU incorporation and the numbers of pHistoneH3 positive cells. In explants treated with both FGF2 and BMP4 the numbers of BrdU positive cells were higher than in explants treated with BMP4 alone. These results suggested that FGF signals are essential for cellular proliferation and BMP4 and FGF signals cooperate to enable cell cycle exit within the developing lenses.

Paper III: Specification of neural retina character by lens derived BMP signalling

Characterization of optic vesicle and forebrain markers

In order to analyze when prospective retinal cells acquire retinal identity, various markers expressed in different regions of the forebrain were examined at different embryonic stages. At stage 9, evaginating prospective retinal cells expressed *Rax2* together with prospective hypothalamus. At this stage *FoxG1* expression was confined to prospective telencephalic cells and Nkx2.1 expression was seen within prospective hypothalamic region. By stage 11, *FoxG1* and *Rax2* continued to be expressed in prospective telencephalic and optic vesicles respectively. Nkx2.1 staining was observed within prospective hypothalamic cells at stage 11. By stage 13, when the lens placode thickens and the placode is in contact with the optic vesicle, the neural domain of the optic vesicle expressed *Rax2* and *Vsx2,* and *FoxG1* expression was confined to the peripheral region of the optic vesicle. At stage 13 Nkx2.1 staining was detected within prospective hypothalamic region. By stage 21, *Rax2* and *Vsx2* expression was seen within the neural domain of the developing optic cup. In addition, low levels of *Rax2* expression were also seen within the ventral hypothalamus. In addition, at stage 21, *FoxG1* expression was confined to the developing telencephalon and expression of Nkx2.1 was observed within the ventral telencephalon and ventral hypothalamus. On the basis of these expression patterns, *Rax2* and *Vsx2* were used to identify cells of retinal identity, while *FoxG1* and Nkx2.1 were used to identify cells of telencephalic character.

Prior to stage 13, prospective retinal cells acquire a telencephalic identity in vitro

On the basis of fate maps (Sanchez-Arrones et al., 2009) and morphology, prospective retinal explants from stages 9, 10 and 13 were dissected out and cultured to approximately stage 21. Surprisingly, retinal explants (R) dissected from stages 9 and 10 did not give rise to *Vsx2* and *Rax2* positive cells. Instead, stage 9 and 10 R explants up-regulated *FoxG1* positive cells and a few Nkx2.1 positive cells were also observed. By contrast, stage 13 R explants generated *Rax2* and *Vsx2* positive cells. This suggested that prospective retinal cells are not specified until stage 13 in chick embryo.

Retinal cell identity is dependent upon BMP signals emanating from the lens ectoderm

To identify potential tissues that provide the necessary signal for the proper generation of retinal cells, stage 9/10 prospective retinal explants were cultured together with the lens ectoderm (LR) to approximately stage 21. Under these conditions, δ-crystallin positive lens cells, and *Rax2* and *Vsx2* positive retinal cells were generated in non over-lapping regions within the explants. Consistent with results from our previous publication (Pandit et al., 2011) in the presence of Noggin, the generation of δ-crystallin positive cells was inhibited in LR explants. In addition, the generation of *Rax2* and *Vsx2* positive retinal cells was also inhibited within the prospective retinal domain of the LR explants. Instead prospective retinal cells up-regulated expression of *FoxG1* and Nkx2.1 indicative of telencephalic fate. This indicated that BMP signalling from the developing lens is required for the specification of retinal cells and to prevent these cells from acquiring a telencephalic identity. In agreement, addition of BMP4 to stage 9/10 R explants inhibited the generation of telencephalic cells, maintained *Rax2* expression and up-regulated expression of *Vsx2*. Thus, BMP activity is both required and sufficient to induce retinal cells.

BMP induces Fgf8 expression in prospective retinal cells

Previous studies have shown the importance of FGF signalling in promoting neural retinal character (Hyer et al., 1998; Pittack et al., 1997). Therefore, we analysed if FGF and BMP signals act together to specify and maintain retinal identity. Based on *in vivo* expression analysis, we observed that *Fgf8* expression was induced within the optic vesicle around stages 13-14 and continued to be expressed in the medial part of the optic cup at stage 21. Consistently, in cultured stage 11 LR explants, *Fgf8* expression was detected within the *Vsx2* positive retinal domain. In the presence of Noggin, however, in addition to loss of *Vsx2* expression, *Fgf8* expression was also down-regulated in the prospective retinal domain.

To assess if the inhibition of *Rax2* and *Vsx2* expression within the retinal domain of the explants was due to a secondary effect of loss of FGF activity, stage 11 LR explants were cultured together with Noggin and FGF8. However, FGF8 was not sufficient to rescue *Rax2* or *Vsx2* expression in these Noggin treated explants. In addition, upon treating stage 11 LR explants with SU5402, an inhibitor of FGF signalling, *Rax2* and *Vsx2* expression was still observed within the retinal domain of the LR explants. These results suggested that at early stages FGF signals are not required or sufficient to induce or maintain retinal identity.

DISCUSSION

Temporal requirement for BMP activity within the developing lens

The present study uncovers a temporal requirement for BMP signalling within the developing lens. There is a long time window during which prospective lens cells are dependent upon BMP signals for commitment to a lens fate. It has been shown in chick, that prospective lens cells are specified in response to BMP signals at the late gastrula stage (Sjodal et al., 2007). Our results now extend that knowledge and show that prior to the onset of the lens placodal marker L-Maf, around stage 13, prospective lens cells are still dependent on BMP signals. Absence of BMP signals prior to this stage leads to down-regulation of lens specific markers like L-Maf and δ-crystallin. In addition, cells switch to an olfactory fate characterized by co-expression of Keratin/HuC/D and Dlx. Concomitant with up-regulation of L-Maf within the thickened lens placode, prospective lens cells are committed to a lens fate and become independent of BMP signalling. At these stages in the absence of BMP signals, lens cells continue to express L-Maf and up-regulate δ-crystallin. Our results extend and refine previous observations in which it was found that the differential specification of prospective olfactory and lens cells was mediated by different durations of exposure to BMP activity. A longer duration of exposure to BMP signalling specifies lens cells while a shorter duration of exposure specifies olfactory placodal cells. (Sjodal et al., 2007). The mechanisms by which the duration of exposure to BMP signals regulates these cell fate decisions have not been explored in further detail. It is interesting to speculate how cells respond to different durations of exposure to a particular signal and how this results in the right genes being expressed at the right time. Recent evidence suggests that different durations of exposure to a signalling pathway can result in corresponding levels of intracellular signalling activity, that is, longer durations of exposure to a ligand can generate higher levels of intracellular signalling activity leading to differential gene expression patterns (Tozer et al., 2013). In our study, acquisition of a lens identity is achieved by means of prolonged exposure to BMP signalling until L-Maf is induced. It would be interesting to look into regulation of L-Maf expression which may or may not be transcriptionally regulated by BMP activity. One possibility is that BMP activates a genetic cascade from the late gastrula stages that eventually leads to up-regulation of L-Maf within the thickened lens placode. If BMP activity transcriptionally regulates L-Maf, it is quite possible that a certain threshold of BMP activity is required to fully activate L-Maf expression at lens placode stage. This threshold could be achieved by a longer duration of exposure to BMP activity

within the developing lens. The cellular context within which BMPs act on prospective lens cells also changes during embryonic development. Activation of L-Maf specifically at lens placodal stage could be the result of an epigenetic modification in the regulatory region of L-Maf enabling Smad complex to access their binding sites. In addition, some more as yet unknown factors might also regulate L-Maf expression within the prospective lens.

Up-regulation of δ-crystallin is independent of BMP signals.

Following activation of L-Maf, accumulation of δ-crystallin within the posterior compartment of the lens vesicle is one of the first events to mark primary fiber differentiation. L-Maf, a basic leucine zipper transcription factor is known to regulate expression of δ-crystallin (Ogino and Yasuda, 1998; Reza et al., 2002; Yoshida and Yasuda, 2002). Next, we analyzed if up-regulation of δ-crystallin also is dependent on BMP signals. However, stage 13 lens cells cultured in the absence of BMP signals were able to maintain L-Maf and up-regulate δ-crystallin expression. This is indicative of the fact that the earliest event of primary fiber differentiation is independent of BMP signalling. Consistently, inhibition of BMP signalling in stage 13 lenses led to formation of normal lenses and these cells were able to up regulate δ-crystallin in the same manner as controls. In addition, the initial elongation of primary fiber cells in Noggin electroporated embryos appears to be comparable to those of control embryos.

Previous studies have shown that electroporation of L-Maf in the head ectoderm is sufficient to induce δ-crystallin expression (Ogino and Yasuda, 1998; Shimada et al., 2003). Now our results, based on simultaneous electroporation of L-Maf and Noggin, show that the induction of δ-crystallin by L-Maf in the head ectoderm as well as in the prospective olfactory placode, is independent of BMP signalling. These experiments strongly support the idea that BMP signals are critical for the onset of L-Maf expression which can then activate or repress the set of genes that will promote lens identity and one of these genes is the earliest primary fiber differentiation marker δ-crystallin. The role of Maf genes as general regulators of fate determination has been shown in other tissues as well. For instance, in pancreatic tissue, two members of the Maf family, Maf A and C-Maf are shown to be expressed in β- and α- cells, respectively. Moreover, Maf A, and C-Maf differentially regulate levels of insulin and glucagon, respectively, within the different cell types in pancreas ((Artner et al., 2006; Kataoka et al., 2002). Moreover, within the dorsal spinal cord, ectopic activation of BMP signalling results in up-regulation of MafB levels (Chizhikov and Millen, 2004). Hence, in addition to eye development,

members of the Maf family might be general regulators of cellular differentiation during embryonic development and could be regulated by similar mechanisms in different tissues.

Prospective retinal cells initially acquire telencephalic identity in the absence of BMP activity from the lens ectoderm

The mechanisms by which early specification of prospective retinal cells is mediated in embryonic development are not well understood. In the present study we have analysed early specification of prospective retinal cells and found that BMP signalling has an important role to play in this process. The expression patterns of retinal and telencephalic markers were analyzed and expression of *Rax2* and *Vsx2* was used to determine retinal cell identity. The expression of *FoxG1* and Nkx2.1 was used to identify cells of telencephalic identity. *Rax2* is expressed already at stage 9 in prospective retinal cells and *Vsx2* is up-regulated in the optic vesicle around stage 13. Stage 9 and 10 prospective retinal cells were cultured *in vitro* based on fate maps and morphology (Sanchez-Arrones et al., 2009). Surprisingly these cells failed to maintain their retinal identity *in vitro*. In addition, these cells up-regulated expression of telencephalic markers. This indicated that certain signals from surrounding tissues are required for prospective retinal cells to maintain and up-regulate retina specific gene expression. The invagination of the eye field is ongoing at stages 9 and 10 and this causes to the optic vesicle to come in contact with prospective lens ectoderm. Hence, we speculated that the missing signal might be provided by the developing lens. It has been shown in previous studies that members of the BMP pathway like BMP4 and p-Smad, an indicator of active BMP signalling are expressed within the developing eye (Hyer et al., 2003). This suggested to us that BMP signals might be important for proper development of the retina. In agreement with this, addition of BMP4 to prospective retinal cells at stages 9 and 10, inhibited the generation of telencephalic cells and up-regulated the early retinal specific markers *Rax2* and *Vsx2*. Further supporting the notion that BMP signals from lens ectoderm are essential for up-regulation of *Rax2* and *Vsx2* expression, it was observed that culturing prospective lens together with the invaginating optic vesicle led to up-regulation of these markers. In addition, upon BMP inhibition in co-cultures of retina and lens explants, the generation of retinal cells was suppressed and cells of a telencephalic character were induced. In summary, our results suggest that BMP signalling is required for promoting retinal cell fate and suppression of telencephalic identity.

A recent study in zebrafish has implicated BMP signalling in protecting telencephalic cells from acquiring an eye identity through suppression of the eye field transcription factor Rx3 (Bielen and Houart, 2012). BMP signalling was shown to mediate this function around late blastula to early gastrula stages. Although, this seems to contradict with our findings, it can be explained by the fact that, the stages at which the experiments were conducted in the above mentioned study were during the late blastula to early gastrula stages. This is much earlier than the stages at which BMP activity was modulated in our study. In addition, in the previous study, BMP was not required as such for development of the telencephalon, but rather BMP signals were involved in suppressing Rx3 expression within the developing telencephalon. It is very likely, that the function of BMP signalling in mediating these fate decisions changes during the course of embryonic development. Around late blastula to early gastrula stages, BMP signalling is important for protecting telencephalic cells from acquiring a retinal identity while at later stages; BMP signalling promotes retinal identity instead of the telencephalic identity.

Prospective retinal cells become independent of BMP signalling from lens tissues at stage 13

In the present study, we have identified a temporal requirement for BMP signalling in specification and maintenance of retinal identity. Stage 13 prospective retinal cells cultured *in vitro* in the absence of developing lens cells were able to up-regulate expression of *Rax2* and *Vsx2*. The issue of how prospective lens and retina interact with one another has been the subject of intense study for several decades and continues to this day. Several studies have shown the importance of the lens and the ocular mesenchyme in patterning of the optic vesicle (Ashery-Padan et al., 2000; Fuhrmann et al., 2000; Hyer et al., 2003). In Pax6 Le-Cre knockout mice, where Pax6 was specifically inactivated within the lens ectoderm, lens induction was initiated but further lens development was arrested. In these mutants several foldings of the retina were observed, but neural retinal differentiation proceeded normally (Ashery-Padan et al., 2000). In agreement with this, in our explants assay, after stage 13, neural retinal cells can develop independently, even in the absence of the adjacent lens placode. This suggests that normal induction of lens is necessary for proper development of the retina. Consistently, in intact chick embryos, surgical ablation of the lens placode at stage 13 still leads to formation of a normal optic cup (Hyer et al., 2003). However, before this stage, surgically ablating lens tissue led to defective morphogenesis of the optic cup (Hyer et al., 2003). This is in agreement with our finding that the generation of retinal cells in our assays is dependent upon lens ectoderm before stage 13.

Interestingly, a recent study in embryonic stem cells has indicated that a retina can develop *in vitro* even in the absence of the lens or the ocular mesenchyme (Eiraku et al., 2011).This study contradicts with the above mentioned findings where presence of surrounding tissues has been shown to be important for early development and patterning of the optic cup. These contradictory findings can be attributed to a difference in the methods employed to investigate the mechanisms by which retina development is mediated. In summary we show that 9/10 prospective retinal cells are dependent upon BMP signalling from the lens ectoderm, and in the absence of the lens tissue or BMP signalling, prospective retinal cells acquire a telencephalic identity. By stage 13, however, prospective retinal cells do not require the presence of lens tissue to up-regulate the expression of *Rax2* or *Vsx2*, suggesting a time window for the BMP signalling pathway in regulating early retinal development.

FGF signals are not initially involved in specification of prospective retinal cells

In our study, prior to stage 13, absence of BMP signalling led to down-regulation of *Fgf8* expression within the retinal domain of the lens-retina explants. This is in agreement with a study in which retina specific BMP receptor conditional knockout mice also showed down-regulation of *Fgf15* expression (Murali et al., 2005). FGF signalling has been shown to be involved in promoting neural retinal fate (Hyer et al., 1998; Pittack et al., 1997). Hence, it was possible that the loss of *Rax2* and *Vsx2* expression upon inhibition of BMP within the lens-retina explants observed in our assays could be an indirect consequence of loss of FGF activity within the retinal domain of these explants. However, in our study, addition of FGF8 together with inhibition of BMP signals was not sufficient to rescue expression of retinal markers within the lens retina explants. In addition, inhibition of FGF signals within lens retina explants did not result in down-regulation of *Rax2* and *Vsx2* expression. This is indicative of the fact that at least at stage 11 FGF signals are not required or sufficient to induce or maintain retinal character. This finding is seemingly in contrast with the study of Pittack and colleagues in which FGF2 from the surface ectoderm was found to promote neural retinal fates in optic vesicles cultured *in vitro* (Pittack et al., 1997). In this study, it was found that application of FGF2 to optic vesicle cultures containing prospective lens, neural retina and retinal pigment epithelial tissues resulted in up-regulation of Tuj1 staining within the prospective pigment epithelium. Tuj1 is a marker for post-mitotic neurons and are only expressed within neural domain of the retina in intact chick embryos. However, expression of early neural retina markers like *Rax2* and *Vsx2* was not examined in this study. Hence, it is very likely that FGF signals do have

an important role in promoting neural retinal fates, but their roles are more prominent in initiation of retinal neurogenesis. In agreement with this, FGFs have been shown to be involved in initiating retinal neurogenesis in both chick and fish (Martinez-Morales et al., 2005). However our findings indicate that at early stages of retinal development, FGF signalling is not required for inducing or maintaining early neural retina markers like *Rax2* and *Vsx2*.

Regulation of early events in primary fiber differentiation by cooperation between the FGF and BMP signalling pathways

The individual roles of FGF and BMP signalling in early events of primary lens fiber differentiation have been poorly defined. In this study, we have uncovered how FGF and BMP signalling mediate cellular proliferation and cell cycle exit within the developing lenses during early primary fiber differentiation. We find that FGF signals are essential in regulating both cellular proliferation and cell cycle exit as seen by levels of pHistoneH3 and p27kip1 respectively. In the absence of FGF signals, both the levels of proliferation and cell cycle exit are decreased. Consistently, addition of FGF2 or FGF8 leads to an increase in the levels of both proliferation and cell cycle exit, pointing towards an important role for FGF signalling in regulating these processes. Our findings regarding the role of FGF signalling in mediating early events of primary fiber differentiation are in agreement with previous studies regarding the role of FGF signalling in the lens secondary fiber differentiation process (Iyengar et al., 2009; Le and Musil, 2001; McAvoy and Chamberlain, 1989). These studies were mainly conducted using chick and rat lens epithelial explants. Addition of FGFs to chick lens central epithelial explants was shown to stimulate proliferation and to promote expression of fiber differentiation specific markers like δ-crystallin and CP49 (Le and Musil, 2001). Moreover, in a rat lens epithelial cell assay, different concentrations of FGF promoted different responses within the lens epithelial explants. Enhanced proliferation was observed at lower levels of FGF, intermediate levels of FGF promoted migration and higher levels of FGF promoted fiber cell differentiation (McAvoy and Chamberlain, 1989). By contrast in our study, we did not detect differential responses to FGF depending upon different concentrations of FGF. Instead, we found that at a certain threshold of FGF activity the levels of proliferation and cell cycle exit were elevated. This is indicative of certain differences in the mechanisms by which processes of primary fiber differentiation and secondary fiber differentiation are mediated.

We also investigated the role of BMP signalling in regulating cellular proliferation and cell cycle exit during early events of primary fiber differentiation. Absence of BMP signals led to a decrease in the levels of proliferation and cell cycle exit. Although, ectopic BMP activity increased the levels of cell cycle exit in lens cells, the number of proliferative cells remained unchanged or slightly decreased in comparison with control explants. This suggested that in the absence of BMP signalling, prospective lens cells were either halted in the cell cycle or they were spending prolonged time in the cell cycle. Addition of BMP4 led to a decrease in BrdU incorporation within the developing lenses accompanied by higher levels of p27kip1.

The lower levels of proliferation observed within lens cells upon inhibition of BMP signals was found to be a consequence of loss of FGF activity within the lens-retina explants. We observed loss of *Fgf8* expression within the retinal domain of lens-retina explants exposed to Noggin. This is consistent with findings in mice, in which retina specific BMP receptor conditional knockout mice showed a reduction in *Fgf15* expression (Murali et al., 2005). Consistent with the role of FGFs in promoting proliferation, over activation of FGF signals together with BMP inhibition within lens retina explants was sufficient to restore the proliferation levels comparable to those of controls. This indicated that in the presence of FGF signals, BMP signals were dispensable for cellular proliferation. However, over activation of FGF signals simultaneous with BMP inhibition within lens retina explants was not sufficient to restore the levels of cell cycle exit. This suggested that BMP signals were essential in regulating cell cycle exit levels during early events of primary fiber differentiation. In agreement with this, previous findings in chick showed that inhibition of BMP activity in chick epithelial explants resulted in defective lens fiber cell differentiation assessed by lack of fiber cell elongation (Belecky-Adams et al., 2002).

In our study, we also show that FGF and BMP signals cooperate to regulate cell cycle exit during early events of primary fiber differentiation. Absence of either of these signals leads to lower levels of cell cycle exit. Moreover, over activation of FGF signals together with BMP inhibition within lens retina explants is not sufficient to restore levels of cell cycle exit comparable to controls. Similarly, although over activation of BMP signals together with FGF inhibition within lens retina explants increases the number of p27kip1 positive cells, it is not sufficient to restore levels of cell cycle exit in comparison with controls. In agreement with these findings, a previous study in chick using a lens epithelial cell assay has shown that the vitreous body that surrounds the posterior compartment of the lens contains FGF and BMPs which can induce secondary fiber differentiation. Moreover, BMP

activity was shown to be critical in enabling FGFs to induce secondary fiber differentiation, since BMP inhibition reduced the ability of FGFs or vitreous body to induce secondary fiber differentiation process (Boswell et al., 2008). Overall, our results suggest that while FGF signalling is essential in promoting cellular proliferation, BMP and FGF signals cooperate to regulate cell cycle exit during early events of primary fiber differentiation.

Equarin as a novel marker for cell cycle exit within developing lenses

The expression of the secreted protein and novel lens marker *Equarin,* also known as Ccdc80, (Mu et al., 2003) was also examined in our study. *Equarin* expression is up-regulated at stage 18 within the posterior compartment of the lens vesicle that gives rise to the lens primary fiber cells. We found that *Equarin* was localized to the same cells that expressed p27kip1 and δ-crystallin, that is, the early primary fiber differentiating population and was excluded from pHistoneH3 positive proliferative cells. Hence, *Equarin* positive cells define the population of cells undergoing cell cycle exit. Consistently, we found that the up -and down -regulation of *Equarin* corresponded to high and low levels of p27kip1, respectively. In agreement with this, a recent study in chicks has also identified Equarin as a novel molecule involved in lens fiber cell differentiation (Song et al., 2012). In this study,, over expression of Equarin led to up-regulation of fiber differentiation specific markers both *in vivo* and *in vitro*. In addition, inhibition of Equarin resulted in a modest decrease of fiber differentiation markers suggesting that other signalling pathways are important for mediating fiber differentiation (Song et al., 2012). This is in agreement with our finding that both BMP and FGF signals are important for primary fiber differentiation. Our results provide evidence that absence of either BMP or FGF signals results in down-regulation of *Equarin* expression in the lens. In the study of Song and colleagues, Equarin was shown to modulate the activity of FGF by binding to FGF ligands and heparan sulfate proteoglycans that mediate FGF ligand and receptor binding, resulting in up-regulation of phospho-Erk levels within the lens (Song et al., 2012). Thus, it is possible that FGF and Equarin are involved in a positive feedback loop, whereby FGF and BMP signals together induce *Equarin* expression and Equarin in turn promotes FGF activity within the lens. Taken together, our findings provide evidence that BMP and FGF signalling regulate *Equarin* expression, coupled to cell cycle exit and fiber differentiation, however BMP and FGF signals might also regulate cell cycle exit and fiber differentiation via gene targets other than Equarin.

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