ANALYSIS OF THE ROLE OF BMP SIGNALING IN EARLY EYE DEVELOPMENT

by

VIJAY KUMAR KALASKAR

(Under the Direction of JAMES D. LAUDERDALE)

ABSTRACT

Bone morphogenetic proteins (BMP) and their receptors are expressed in ocular tissue during the stages of early eye specification and development, and are required for normal eye development. Recent screening of individuals with ocular malformations revealed mutations in the gene locus of one of the BMP members, BMP4. Compared to other BMP members, BMP4 expression is more predominant in ocular tissues during development. Understanding the role of BMP4 has been difficult as $BMP4^{-/-}$ homozygous mouse embryos die at mid-gestation, precluding the study of BMP4 in early eye development. However, co-culture studies revealed that BMP4 signaling in the optic vesicle was essential for its lens inducing activity. Although BMP4 is expressed at later stages, its role in mammalian eye development following lens induction is not clear.

My dissertation research was directed towards elucidating the role of BMP signaling in mammalian eye development. To circumvent the problems associated with embryonic lethality, I developed a mouse embryo culture system capable of supporting eye development in a manner consistent with that observed in utero. I manipulated BMP signaling utilizing both gain and loss of function approaches through ectopic expression of BMP4 and Noggin proteins, respectively. I showed that BMP signaling plays several separable roles in eye development including

specification, patterning and development. In cultured mouse embryos, ectopic BMP4 prevented development of pigmentation in retinal pigmented epithelium (RPE) through down-regulation of genes involved in RPE specification and development such as *Mitf*, *Otx2* and *Pax6*. In neural retina (NR), ectopic BMP4 altered expression of genes involved in dorsal-ventral patterning, such as *Tbx2*, *Tbx5*, *Msx1* and *Vax2*, and down-regulated the expression of genes involved in NR specification and development, including *Vsx2*, *Pax6*, *Sox2*, *Rx* and *Lhx2*. In contrast, loss of BMP signaling resulted in upregulation of genes such as *Mitf* and *Pax6* and changed the expression domains of genes involved in dorsal-ventral patterning. Further analysis revealed that BMP signaling mediated these effects on NR and RPE by modulating the members of WNT and SHH signaling pathways. My results suggest a predominant role for BMP signaling in regulating the mechanisms important in NR and RPE specification and development.

INDEX WORDS: Mouse embryo, Rolling bottle culture, Serum-free medium, Bone Morphogenetic Protein 4 (BMP4), Retinal Pigmented Epithelium (RPE), Neural Retina (NR), Eye Development, Specification, Pigmentation, Signaling.

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VIJAY KUMAR KALASKAR

B.V.Sc. & A.H., Sri Venkateshwara Veterinary University, India, 2002

B.Sc., Osmania University, India, 1998

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VIJAY KUMAR KALASKAR

Major Professor:	James D. Lauderdale
Committee:	Gaylen L. Edwards Scott T. Dougan Douglas B. Menke

Electronic Version Approved:

Maureen Grasso
Dean of the Graduate School
The University of Georgia
May 2014

DEDICATION

This dissertation is dedicated to my brother and my parents. Their constant encouragement and support made me realize my dream of becoming a scientist. Every time I speak with my brother his words sound like an inspiration to focus on my research. I try to follow him to be true to myself and to others in whatever I do. I remember my parents in every step of my life and their advice and support have always helped me overcome the difficult times in my path of life and research.

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Chapter 1

General Introduction

Overview of Early Eye Development in Vertebrates

The vertebrate eye is a complex organ system derived from neural and non-neural ectodermal tissues. During gastrulation, the embryonic ectoderm differentiates into neural plate and non-neural ectodermal tissues with an intermediate zone, which contribute to formation of neural crest and the pre-placodal region (Chow and Lang, 2001; Graw, 2010; Saint-Jeannet and Moody, 2014). The neural plate forms into neural tube, which in the head region organizes into brain and continues posteriorly as spinal cord. In late gastrulation, a highly coordinated interaction between different signaling mechanisms induces the expression of a set of transcription factors that establish a single eye field in the anterior forebrain region; the eye field later splits into two separate fields after the formation of the midline.

The first morphological evidence of eye structure appears during neurulation at embryonic day 8.5 days post coitus (E8.5dpc; 11 – 13 somite stage (ss)), when the lateral walls of the diencephalon bulge out to form the optic pits. Concurrent with these events, the intermediate zone that borders the neural plate differentiates into pre-placodal and neural crest regions. The pre-placodal region, upon interaction with underlying anterior neural structures, becomes specified into different placodes (Saint-Jeannet and Moody, 2014). By E9.5 in mouse development, the optic pits further evaginate to form optic vesicles, which interact with the non-neural pre-placodal region to induce the formation of lens placode. In parallel, reciprocal interaction from the presumptive lens placode induces the formation of neural retina in the distal

part of the optic vesicle while signaling interactions from surface ectoderm and periocular mesenchyme induces the formation of retinal pigmented epithelium (RPE) in the proximal part of the optic vesicle. The lens placode begins as an ectodermal thickening which under the influence of signaling interactions from the optic vesicle starts to invaginate to form the lens vesicle. The optic vesicle also invaginates to form the optic cup. The distal optic cup eventually differentiates into neural retina (NR), and the proximal optic cup differentiates into RPE. The lens vesicle separates from the surface ectoderm by E10.5 dpc in mouse embryos and shows an anterior layer of proliferating lens epithelial cells and a posterior layer of lens fiber cells. The surface ectoderm overlying the lens transforms into multilayered cornea with the neural crest and mesoderm derived migrating mesenchymal cells underneath contributing to the formation of corneal stroma and other structures during later stages of eye development.

Signaling Mechanisms and Genes involved in Vertebrate Eye Specification and Development

Eye field specification in vertebrates is preceded by neural induction followed by anterior neural plate specification. Several lines of evidence indicate that the embryonic epiblast starts as a pre-neural tissue as revealed by the expression of neural markers such as *Sox2* and *Otx2* in the inner cell mass / epiblast (Avilion et al., 2003; Levine and Brivanlou, 2007). During early gastrulation, non-neural tissues such as mesoderm, endoderm and ectoderm are induced by BMP, WNT and Nodal signals arising from the proximal extra-embryonic and epiblast tissues (Robb and Tam, 2004). Once these tissues are formed, neural plate is established by signals arising from the gastrula organizer center, anterior visceral endoderm and the node regions. These tissues secrete inhibitory factors such as cerberus, noggin, chordin, follistatin, DKK1 and

lefty to antagonize the action of BMP, Nodal and WNT signaling required for anterior neural tissue induction (Bachiller et al., 2000; Kimura et al., 2000; Kinder et al., 2001; Levine and Brivanlou, 2007; Stern, 2005; Yang and Klingensmith, 2006). In addition FGF signaling was shown to act as a maintenance factor for neural tissue and as well inhibit BMP signaling through MAPK mediated phosphorylation of *Smad1* (Kuroda et al., 2005; Pera et al., 2003).

Continued repression of BMP signaling was suggested to be required during later stages of neurulation to maintain neural identity and anterior neural tissue development (Hartley et al., 2001). Studies in *Xenopus* and zebrafish indicate a role for *Six3* in anterior neural plate specification and maintenance through regulation of cell proliferation and continued suppression of BMP and WNT signaling (Gestri et al., 2005; Liu et al., 2010). While suppression of BMP signaling was required to maintain neural identity, restriction of caudalizing WNT signals was shown to be essential to maintain anterior neural identity. This is accomplished by the secretion of WNT antagonists by the anterior neuroectoderm and underlying mesendodermal tissues and a graded activity of WNT's and WNT antagonists establishes the anterior-posterior polarity and developmental fates in the neural plate (Wilson and Houart, 2004). Absence of WNT or low levels of WNT activity was shown to promote development of prospective forebrain, telencephalon and eye fields in the anterior neural plate whereas increased WNT levels were shown to promote mid brain and diencephalic fates (Braun et al., 2003; Lagutin et al., 2003; Nordstrom et al., 2002).

Eye field specification in the anterior neural plate marked by the expression of *Pax6*, *Six3*, *Otx2* and *Sox2* begins with the expression of *Rx* transcription factor. Studies involving ectopic expression of BMP antagonists, noggin and chordin in *Xenopus* animal caps revealed upregulation of transcription factors such as *Pax6*, *Six3*, *Rx1*, *Lhx2*, *Six6* and *tll* which are

required for eye field specification. This indicates that genes initially expressed in the anterior neural plate could induce the expression of eye field specific genes. However ectopic *Otx2* alone could not upregulate the expression of these transcription factors (Zuber et al., 2003). Later studies in *Xenopus* showed that Otx2 and Sox2 proteins interact each other and bind to a conserved noncoding sequence (CNS1) region ~2kb upstream of the *Rx* gene to directly regulate its expression (Danno et al., 2008). The eye field marked by the expression of *Rx* shows repression of *Otx2* and studies with ectopic expression of *Rx* revealed downregulation of *Otx2* in *Xenopus* animal caps indicating that *Otx2* may not have a role in early eye specification (Andreazzoli et al., 1999). At later stages *Otx2* is expressed in the optic vesicle and eventually gets restricted to RPE regulating its development (Bovolenta et al., 1997; Simeone et al., 1993). *Rx* expression initially seen throughout the eye field and optic vesicle and later gets restricted to the neural retina. $Rx^{-/-}$ mouse embryos are anophthalmic and lack optic vesicle with absence of *Pax6, Six3* and *Otx2* expression while misexpression of *Rx* expanded neural retinal region (Mathers et al., 1997; Zhang et al., 2000).

The single eye field thus formed is split with the introduction of the ventral midline under the influence of SHH signaling. *Six3* was shown to regulate the expression of SHH in the rostral diencephalon ventral midline and loss of *Six3* or SHH resulted in holoprosencephaly with cyclopia (Geng et al., 2008). Studies in *Xenopus* and other vertebrates indicate that genes important in eye field specification exhibit overlapping expression and yet their regulation is not totally interdependent. It is shown that initiation of *Pax6, Six3* and *Otx2* occurs in absence of *Rx* and that of *Rx, Six3* and *Otx2* occurs in absence of *Pax6* (Oliver et al., 1995; Stoykova et al., 1996; Zhang et al., 2000). Though these genes are expressed at early stages, loss of function studies indicate the requirement of *Rx, Six3* and *Otx2* in the initial stages of eye field

specification while the loss of *Pax6*, *Lhx2* and *Six6* affects the eye development at later stages after optic vesicle formation (Chow and Lang, 2001; Graw, 2010; Porter et al., 1997). In *Lhx2*$^{-/-}$ and *Pax6*$^{-/-}$ mouse embryos, the optic vesicle is formed but the formation of optic cup and lens placode is not observed resulting in the arrest of further development of the eye (Grindley et al., 1995; Hogan et al., 1986; Porter et al., 1997).

While the eye field is specified and patterned in the anterior neural plate, the pre-placodal region is specified into different placodes along the anterior-posterior axis under the influence of different signaling factors such as BMP, FGF, WNT and Retinoic acid (Brugmann et al., 2004; Park and Saint-Jeannet, 2008; Saint-Jeannet and Moody, 2014). Anterior placodes such as adenohypophyseal, olfactory and lens are induced at high levels of FGF and low levels of BMP and WNT signaling, while posterior placodes such as trigeminal, epibrachial and otic are induced at low BMP and high levels of FGF and WNT signaling (Brugmann et al., 2004; Ladher et al., 2010; Park and Saint-Jeannet, 2008; Saint-Jeannet and Moody, 2014). The induction of lens placode in the anterior pre-placodal region occurs when the evaginating optic vesicle interacts with the presumptive lens ectoderm. Gain and loss of function studies indicate that *Pax6* expression in the presumptive lens ectoderm is necessary for lens induction (Altmann et al., 1997; Ashery-Padan et al., 2000; Chow et al., 1999; Fujiwara et al., 1994). Transcription factors such as *Six3* and *Pax6* downstream genes such as *Mab21l1*, *FoxE3*, *Prox1* and *Sox2* were all shown to affect lens development to different extents (Lang, 2004). Apart from these genes, BMP and FGF signaling were shown to affect the expression of genes involved in lens induction as well as its later development (Furuta and Hogan, 1998; Garcia et al., 2011; Rajagopal et al., 2009; Wawersik et al., 1999). BMP4$^{-/-}$ mouse embryos do not show lens induction and explant culture experiments indicated BMP4 signaling along with other factors to

be essential for the optic vesicle to manifest its lens inducing activity. In BMP4$^{-/-}$ embryos *Sox2* expression was absent in the surface ectoderm while Pax6 expression was not affected (Furuta and Hogan, 1998). In BMP7$^{-/-}$ embryos *Pax6* expression is affected in the presumptive lens placode and a range of effects on lens formation observed with severe cases showing no lens induction (Wawersik et al., 1999). Studies involving conditional deletion of BMP Type1 receptors, *Bmpr1a*, *Acvr1* and *Smads* -1, -5 and -4 revealed that lens formation, placodal cell proliferation, expression of *FoxE3* and *αA-crystallins* were regulated by BMP signaling in a smad-independent manner while expression of *Sox2* in the lens placode was regulated by canonical smad pathway (Rajagopal et al., 2009). Similar studies with conditional knockout of FGF receptors implicated FGF signaling in lens cell survival but not in initial lens induction (Garcia et al., 2011).

While the interaction of optic vesicle with the preplacodal region induces lens placode formation, a reciprocal signaling mechanism operates from the presumptive lens placode to specify the optic vesicle into neural retina and RPE domains. The optic vesicle in mouse embryos at this stage expresses a set of early transcription factors required for specification and later development of NR and RPE. These early genes include, *Rx*, *Pax6*, *Lhx2*, *Six3*, *Six6*, *Sox2*, *Mitf*, *Otx2* and *Otx1* (Fuhrmann, 2010). Once the optic vesicle gets specified into neural retina and RPE layers, these transcription factors get segregated and restricted either to neural retina or RPE. The transcription factors *Rx*, *Pax6*, *Lhx2*, *Six3*, *Sox2* and *Six6* play an important role in later development of NR and are retained in the neural retinal layer while the genes such as *Mitf*, *Otx2*, *Pax6* and *Otx1* gets restricted to RPE layer and are important for RPE development (Bovolenta et al., 1997; Fuhrmann, 2010; Martinez-Morales et al., 2001; Ohuchi et al., 1999; Teraoka et al., 2009). The signaling trigger for NR induction in optic vesicle was shown to

come from the surface ectoderm in the form of FGF's (Hyer et al., 1998; Pittack et al., 1997). FGF signaling induces *Vsx2* expression in the presumptive neural retina and differentiates the distal region from the proximal RPE layer. When the surface ectoderm was removed at this time in mouse embryos, the distal optic vesicle that usually forms neural retina gets converted into RPE like structure with expression of RPE markers (Nguyen and Arnheiter, 2000). Apart from FGF's, retinoic acid (RA) signaling from temporal mesenchyme was shown to be required for both lens placode and optic vesicle invagination. In mouse *Raldh2*$^{-/-}$ embryos that lack RA synthesis in optic vesicle the invagination does not occur and no optic cup is formed (Cvekl and Wang, 2009; Mic et al., 2004).

The newly specified neural retina expresses *Vsx2* (*Chx10*) as a specific marker and retains expression of early genes such as *Rx, Six3, Six6, Pax6, Lhx2* and *Sox2* which also play an important role in later stages of retinal development. *Vsx2* determines neural retinal identity and *Vsx2* null mutant retina shows decreased proliferation of retinal progenitor cells and absence of bipolar cells while other cell types form normally in the retina (Burmeister et al., 1996). *Rx* is expressed throughout the neural retina in embryonic stages and until postnatal stage 6.5 where it gets restricted to photoreceptor cells (Chow and Lang, 2001; Mathers et al., 1997). Gain and loss of function studies implicate *Rx* in initial specification and later maintenance of retinal progenitor cells in a proliferative and multipotent state throughout development and as well as in retinal regeneration in *Xenopus* (Andreazzoli et al., 1999; Andreazzoli et al., 2003; Casarosa et al., 2003; Martinez-De Luna et al., 2011; Mathers et al., 1997). *Six3* is another early gene that shows expression at later stages in retina development. *Six3* at later stages was shown to be involved in proximal-distal patterning of retina and its overexpression resulted in ectopic retinal primordial in *Medaka* and *Xenopus* with ectopic expression of *Pax6*, *Rx* and endogenous

Six3 (Carl et al., 2002; Loosli et al., 1999). Another related gene, *Six6* (*Optx2*) is expressed in the developing retina and its misexpression induced *Vsx2* expression in the RPE and expanded retinal territory indicating a role in proliferation of retinoblasts (Bernier et al., 2000; Zuber et al., 1999). *Sox2*, a neural progenitor gene is also expressed in early stages of eye field specification and as well in the developing retina. Conditional deletion and studies involving hypomorphic allelic expression of *Sox2* reveal that *Sox2* determines the competence of retinal progenitor cells to proliferate and terminally differentiate by modulating Notch signaling (Taranova et al., 2006). *Pax6* is important in eye development and regulates many other genes involved in retina, lens, cornea and RPE development in the eye. *Pax6* levels are critical for eye development as loss of function of *Pax6* arrested eye development at optic vesicle stage and overexpression disrupted eye development (Grindley et al., 1995; Kim and Lauderdale, 2008; Schedl et al., 1996). Misexpression of *Pax6* was shown to induce ectopic eye formation in drosophila imaginal discs, mouse, squid, ascidian and *Xenopus* with ectopic upregulation of genes such as *Otx2, Rx, Six3* and endogenous *Pax6* (Chow et al., 1999; Chow and Lang, 2001; Glardon et al., 1997; Halder et al., 1995; Tomarev et al., 1997). Retina specific loss of *Pax6* resulted in loss of all retinal cell types except the non-glycinergic amacrine cells in the neural retina (Marquardt et al., 2001). Apart from the transcription factors, signaling mechanisms involving members of BMP, SHH, WNT and Notch pathways regulate retinal patterning and development. BMP4 localized dorsally regulates expression of dorsal retinal genes such as *Msx1* and T-box genes like *Tbx5, Tbx3* and *Tbx2* while SHH localized ventrally regulates expression of ventral retinal and optic stalk genes like *Vax1, Vax2, Pax2* and as well restricts the expression domains of dorsal genes and *Pax6* (Behesti et al., 2006; Kobayashi et al., 2010; Zhao

et al., 2010). Taken together BMP and SHH signaling pattern the neural retina along the dorsal-ventral axis by regulating the expression domains of different genes.

Concurrent with the establishment of neural retina domain in the distal optic vesicle, the expression of *Mitf, Otx2* and *Otx1* genes get restricted to the presumptive RPE layer in the proximal part of the optic vesicle. The RPE specification and development was shown to be regulated by the signals arising from surface ectoderm and the periocular mesenchyme. Studies in chickens report that WNT, BMP and TGFβ related activin signals arising either from surface ectoderm or mesenchyme are involved in RPE induction and development (Fuhrmann et al., 2000; Muller et al., 2007; Steinfeld et al., 2013). Optic vesicle explant cultures in the absence of these tissues resulted in lack of RPE specification in the optic vesicles (Fuhrmann et al., 2000; Kagiyama et al., 2005). Ectopic implantation of beads treated with either BMP's or Activins was shown to induce the formation of RPE tissue in the proximal as well as distal optic vesicle as revealed by the expression of *Mitf* and other RPE specific markers and down-regulation of NR specific genes (Fuhrmann et al., 2000; Muller et al., 2007).

Transcription factor *Mitf* has been shown to play a central role in RPE specification and development. *Mitf* regulates the expression of key melanogenic genes in the RPE such as tyrosinase, its related genes tyrosinase related proteins-1 (TRP-1) and TRP-2 and *MMP115* (Bharti et al., 2008; Martinez-Morales et al., 2004; Muller et al., 2007). *Mitf* was shown to exist in multiple isoforms and among them *Mitf-H* and *Mitf-D* were indicated to be more important than other isoforms in the RPE (Bharti et al., 2008). Studies indicate that apart from *Mitf*, transcription factors such as *Otx2*, *Pax6* and *Pax2* and factors related to WNT signaling are important in RPE development and pigmentation (Baumer et al., 2003; Bharti et al., 2012; Bharti et al., 2008; Bharti et al., 2006; Bumsted and Barnstable, 2000; Fujimura et al., 2009;

Martinez-Morales et al., 2004; Martinez-Morales et al., 2001; Westenskow et al., 2009). *Mitf* expression was shown to be affected in *Pax2*$^{-/-}$;*Pax6*$^{-/+}$, *Otx1*$^{-/-}$;*Otx2*$^{-/+}$ mice and that *Pax2* and *Pax6* regulate *Mitf* expression by binding to promoter regions on *Mitf* gene (Baumer et al., 2003; Martinez-Morales et al., 2001). *Pax6* in RPE was shown to act as anti-retinogenic in conjunction with *Mitf*/*Tfec* transcription factors and was shown to interfere with RPE transdifferentiation into retina in *Mitf* hypomorphic and null mutant mouse embryos (Bharti et al., 2012). *Otx2* was shown to interact with *Mitf* in a regulatory loop and as well bind and transactivate pigmentation genes such as Tyrosinase, TRP-1 and TRP-2 (Martinez-Morales et al., 2003; Takeda et al., 2003). Chromatin immunoprecipitation and luciferase assays reveal that β-Catenin binds near to and activates potential *TCF/LEF* sites in the *Mitf* and *Otx2* enhancers to regulate the RPE development (Fujimura et al., 2009; Westenskow et al., 2009). Thus a regulatory loop of signaling factors including members of BMP, WNT, FGF and SHH pathways tightly regulate the expression patterns of different transcription factors within distinct domains of the optic vesicle to enable proper specification and development of NR and RPE during early stages of embryonic eye development.

Culture Systems to Study Mammalian Early Eye Development

Vertebrate eye development involves a complex series of inductive interactions right from the time the eye field is specified in the anterior neural plate. Organogenesis of eye and other organ systems in mammals occurs during embryonic development and unlike avians and most other species these stages are inaccessible due to the *in utero* development of embryos. Most of the earliest studies on vertebrate eye development were performed in chicks as the developmental stages are accessible in the egg. The development in egg enabled easy

manipulation of the embryonic tissues and as well permitted development under natural conditions. The chick eyes were also cultured in media containing plasma clot and embryo extract with certain degree of success (Dorris, 1938; Strangeways, 1926; Strangeways and Fell, 1926). These culture methods were applied to mammalian eye tissues with initial attention to support the survival and maintenance of the mammalian tissues in culture (Lucas and Trowell, 1958; Tansley, 1933). Later successful culture of intact and trypsinized optic rudiments was performed utilizing different media conditions (Muthukkaruppan, 1965a). However, the introduction of whole embryo culture systems enabled the study of mammalian eye development in the context of whole embryo.

The first successful attempt to culture post-implantation whole embryos from mouse and rats came from studies performed by New and Stein in 1964. They cultured the early post-implanted mouse and rat embryos on plasma clots supplemented with medium containing rat plasma and embryo or uterine extract of rat or chick. They showed different survival rates depending on the initial developmental stage and time period of culture (New and Stein, 1964). These experiments revealed that static culture systems were not supportive of embryonic growth and resulted in embryonic death over short periods of culture. Later New and his colleagues developed several methods using rolling bottles, tubes or circulating serum culture systems to support mammalian embryonic development. These culture systems showed promising results when media supplemented with heat inactivated serum was used (New, 1978; New et al., 1976a; New et al., 1976b; New et al., 1973). Following these advancements in mammalian in vitro culture systems, the eye development was studied in a variety of perspectives advancing our understanding of the mammalian eye development.

In most studies, mammalian embryo culture or ocular tissue explant cultures were used as a method to study a specific aspect of development which was otherwise not possible to study with currently available in vivo approaches and in many other cases these culture methods were used to supplement genetic studies (Furuta and Hogan, 1998). Some of the advantages of using culture methods to study eye development include the following, (1) Eye tissue in culture is accessible for manipulation with external factors like signaling proteins and their antagonists to determine their role in development. Culture approach was used to determine the role of BMP4 signaling in dorsal-ventral patterning of retina and to show that it is essential for optic vesicle to manifest its lens inducing activity (Behesti et al., 2006; Furuta and Hogan, 1998). (2) It is easy to supplement the culture with different proteins to study their individual or combined effect on eye development in a short span of time. The role of BMP's in initiation of lens fiber differentiation and the combined effect of DKK1 and FGF signaling in RPE pigmentation was quickly determined utilizing a culture system (Belecky-Adams et al., 2002; Bharti et al., 2012). (3) The tissue development in culture can be monitored, altered or terminated at the required specific time point and (4) the requirement of a specific tissue for development of whole organ or other tissues or its role in tissue-tissue interactions can be easily determined by different methods in culture. For example, surgical removal of different tissue parts in the eye region or within optic vesicle helped to determine the dynamics of optic vesicle patterning and revealed that the anterior-ventral optic vesicle region was involved in retinal regeneration in chickens (Hirashima et al., 2008; Kagiyama et al., 2005). Thus the culture systems have the potential to understand, (1) the tissue inter-dependence in the ocular tissue during embryonic stages for mutual development (Hyer et al., 1998), (2) the role of secreted signaling molecules in eye development

(Wawersik et al., 1999) and (3) the effect of signaling molecules on expression pattern of other genes in the eye tissue during development (Behesti et al., 2006).

All through these years, the ocular development has been studied utilizing ocular tissue explants or optic rudiments which encompass the entire optic primordium and also in whole embryo culture systems. Both of these culture approaches reveal certain advantages and disadvantages when considered to study complex organ systems such as the eye. Ocular explants or optic rudiments are mainly limited by the availability of tissue. The stages of ocular organogenesis are critically dependent on inductive tissue interactions and cellular migration. The tissues in explants or optic rudiments are not totally intact as in whole embryos and the inductive interactions although occur for certain limited time period may not be as proportional to those that occur in intact eyes in whole embryos. Added to this, cellular migration as required for corneal, iris and ciliary body development in the eye may not be possible due to the absence of enough mesenchymal cells. Balancing these conditions by using explants encompassing optic rudiments with more surrounding tissue may show deleterious effect on tissue survival as these explants lack intact blood circulation. On the other side, explant tissue cultures are well suited to study differentiated tissues such as mature retina, lens or cornea, and mostly used to quickly determine the role of certain proteins or factors at specific time points during development (Bharti et al., 2012; Furuta and Hogan, 1998). The limitations encountered in explant culture systems can be overcome by using the whole embryo culture systems. Whole embryos provide the tissue integrity and support appropriate for the tissue interactions that are crucial for timely occurrence of signaling mechanisms essential for different cellular processes during ocular organogenesis. However, currently utilized embryo culture systems are mainly dependent on serum for proper growth and maintenance of the embryos in the culture (Behesti et al., 2006;

Gray and Ross, 2011; Kitchin and Ebron, 1984; Miura and Mishina, 2003; Osumi and Inoue, 2001; Sadler, 1979; Takahashi et al., 2008; Tam, 1998; Yokoo et al., 2005).

Serum has been utilized as one of the major components ranging from 10% to 100% of the culture media (Cuthbertson and Beck, 1990; Kitchin and Ebron, 1984; New, 1967; Sadler, 1979; Yokoo et al., 2005). However, the composition of serum is not well defined and can differ from animal to animal and each time the serum is collected. While laboratory preparation of serum is time consuming and involves stringent procedures, serum procured commercially exhibits considerable variability among different lots and raises experimental costs. Added to these, the serum may contain unknown factors such as growth factors, hormones, or other proteins, which can potentially affect the outcome of certain experiments, especially those that involve the study of signaling molecules important in tissue interactions. Studies have shown that addition of serum to culture can potentially alter the intracellular levels of certain signaling molecules such as cyclic adenosine monophosphate (cAMP) and proteins involved in mitogenic signaling and phosphoinositide 3 (PI 3)-kinase signaling pathways (Chotani et al., 2005; Chung et al., 1999; Sasaoka et al., 1994).

These studies necessitate the use of a defined culture system to study the signaling interactions important in organogenesis. A serum-free culture system provides the advantages of antigen free environment, abstinence from biologically active enzymes, proteins or other factors that can alter the cellular processes and enables consistency among experiments. Recent study by Kalaskar and Lauderdale provided these defined culture conditions appropriate to support the mammalian embryonic development comparable to that observed in utero (Kalaskar and Lauderdale, 2014). This study utilized a serum-free culture medium prepared from commercially available stem cell media supplements to culture mid-gestation stage whole embryos in an

atmosphere of 95% O_2 / 5% CO_2 in a rolling bottle culture apparatus at 37 ^oC (Gordon et al., 2014; Moore-Scott et al., 2003). They showed that mouse embryos cultured for 16 to 40 hrs under these defined conditions exhibited progression in morphological development of overall embryonic body and different structures such as the heart, limbs, brain and eyes indicating appropriate levels of cellular proliferation, migration, differentiation and tissue interactions. Molecular analysis of the embryonic development in the culture for eye tissue revealed the ocular development to be consistent with that observed in ocular tissue in embryos developing in utero (*Kalaskar and Lauderdale, in preparation*).

BMP Signaling in Early Eye Development

Bone Morphogenetic Proteins belonging to TGF-β superfamily have been implicated at multiple levels during early stages of ocular specification and development. During early specification events, studies show that the presumptive neural ectoderm and later the anterior neural plate both are under continuous influence of BMP signaling from the surrounding ectodermal and mesendodermal tissues (Levine and Brivanlou, 2007). Members of BMP signaling were shown to mainly inhibit neural tissues and support development of non-neural epithelium and mesendodermal tissues (Kuroda et al., 2005; Levine and Brivanlou, 2007; Liem et al., 2000; McMahon et al., 1998; Pera et al., 2003). This is accomplished by the ubiquitous expression of BMP receptors such as *BMPR1A* (*ALK 3*) throughout the neural and non-neural tissues (Dewulf et al., 1995; Mishina et al., 1995). The inhibition of BMP signaling in these tissues was shown to be essential for initial neural induction and later for maintaining the anterior neural plate identity (Hartley et al., 2001). During neural induction BMP signaling was shown to be inhibited by antagonists such as noggin, chordin and follistatin from the organizer

and also by FGF signaling through MAPK mediated inhibition of *Smad1* (Kuroda et al., 2005; Pera et al., 2003). Later in the anterior neural plate continued suppression of BMP signaling was shown to be mediated by BMP antagonists along with anterior neural genes such as *Six3* (Gestri et al., 2005; Hartley et al., 2001). After neural tube closure, BMP signaling was shown to be involved in patterning of the dorsal-ventral axis in the brain and spinal cord, establishing differentiation of neural crest cells and regulating gene expression, cellular proliferation and apoptosis during morphogenesis of forebrain structures including the eye region (Furuta et al., 1997; Golden et al., 1999; LaBonne and Bronner-Fraser, 1998; Liem et al., 2000; Marchant et al., 1998; McMahon et al., 1998; Selleck et al., 1998).

The eye field specification in the anterior neural plate requires expression of specific transcription factors such as *Rx*, *Pax6* and *Six3* and misexpression studies in chickens with BMP4 revealed down-regulation of these eye field transcription factors affecting the development of optic primordia (Teraoka et al., 2009; Zuber et al., 2003). When optic vesicle begins to form from the evaginating forebrain, BMP's were not detected in the eye region but later BMP4 and BMP7 expression was detected in distal optic vesicle, surface ectoderm and surrounding periocular mesenchyme (Dudley and Robertson, 1997; Furuta and Hogan, 1998; Wawersik et al., 1999). BMP receptor, *Alk3* (*BMPR1A*) was shown to be ubiquitously expressed from early stages in the eye region until E10.5-11.5 dpc while *Alk6* (*BMPR1B*) was shown to be expressed from 16 ss in a part of future retina, optic stalk and in mesenchyme region (Furuta and Hogan, 1998). Conditional knockout of these receptors in neural retina revealed functional redundancy of these receptors and indicated a role for BMP signaling in dorsal-ventral patterning of neural retina (Murali et al., 2005). Similar conditional knockout of *Alk3* and *Acvr1* (*Alk2*) and downstream *Smad* genes in the lens placode indicated BMP signaling to be involved in placodal

cell survival and proliferation and also in regulation of *Sox2* and *αA-crystallin* gene expression (Rajagopal et al., 2009). Studies in BMP4$^{-/-}$ mouse embryos showed that BMP4 signaling was essential for optic vesicle to manifest its lens inducing activity (Furuta and Hogan, 1998). After specification of neural retina and RPE from the optic vesicle, BMP4 expression gets restricted to the dorsal neural retina and BMP7 to the presumptive RPE with both expressed in the surrounding mesenchyme (Dudley and Robertson, 1997; Furuta and Hogan, 1998). Though the role of BMP signaling in early stages of neural and eye field specification are well characterized, its role in early stages of eye development after specification of neural retina and RPE from optic vesicle is not clear.

Studies in chickens show that members of TGF-β signaling, BMP's and Activins expressed in the periocular mesenchyme and / or surface ectoderm are essential for RPE development. Optic vesicle explant cultures in the absence of these tissues resulted in lack of RPE specification in the optic vesicles (Fuhrmann et al., 2000; Kagiyama et al., 2005). Ectopic implantation of beads treated with either BMP's or Activins was shown to induce the formation of RPE tissue in the proximal as well as distal optic vesicle as revealed by the expression of *Mitf* and other RPE specific markers and down-regulation of NR specific genes (Fuhrmann et al., 2000; Muller et al., 2007). Though the studies in chickens indicate the role of BMP signaling in inducing *Mitf* expression and thereby in RPE development, it is not clear whether similar mechanisms exists in the mammalian RPE development.

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Chapter 2

Mouse Embryonic Development in a Serum-Free Whole Embryo Culture System[1]

[1] Kalaskar V. K., Lauderdale J. D., J. Vis. Exp. (85), e50803.
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Short abstract

Serum utilized in embryo cultures contains unknown components that can affect the outcome of experiments especially in studies involving signaling interactions. Here we utilized a serum-free oxygenated culture system and show that mid-gestation mouse embryos cultured for 16 to 40 hrs exhibit morphological development comparable to embryos developing *in utero*.

Long abstract

Mid-gestation stage mouse embryos were cultured utilizing a serum-free culture medium prepared from commercially available stem cell media supplements in an oxygenated rolling bottle culture system. Mouse embryos at E10.5 were carefully isolated from the uterus with intact yolk sac and in a process involving precise surgical maneuver the embryos were gently exteriorized from the yolk sac while maintaining the vascular continuity of the embryo with the yolk sac. Compared to embryos prepared with intact yolk sac or with the yolk sac removed, these embryos exhibited superior survival rate and developmental progression when cultured under similar conditions. We show that these mouse embryos, when cultured in a defined medium in an atmosphere of 95% O_2 / 5% CO_2 in a rolling bottle culture apparatus at 37 ^oC for 16 to 40 hrs, exhibit morphological growth and development comparable to the embryos developing *in utero*. We believe this method will be useful for investigators needing to utilize whole embryo culture to study signaling interactions important in embryonic organogenesis.

Introduction

In vitro culture methods utilizing whole embryos are well suited to study signaling mechanisms involved in embryonic organogenesis that are otherwise difficult to access *in utero.*

Whole embryos provide the tissue integrity and support appropriate for the tissue interactions that are crucial for timely occurrence of signaling mechanisms essential for different cellular processes during organogenesis. While whole embryo cultures provide a platform for a plethora of applications such as transplantation studies, genetic and tissue manipulations, bead implantation studies, toxicological studies, *etc*., currently utilized embryo culture systems are mainly dependent on serum for proper growth and maintenance of the embryos in the culture (Behesti et al., 2006; Gray and Ross, 2011; Kitchin and Ebron, 1984; Miura and Mishina, 2003; Osumi and Inoue, 2001; Sadler, 1979; Takahashi et al., 2008; Tam, 1998; Yokoo et al., 2005).

Serum has been utilized as one of the major components ranging from 10% to 100% of the culture media (Cuthbertson and Beck, 1990; Kitchin and Ebron, 1984; New, 1967; Sadler, 1979; Yokoo et al., 2005). However, the composition of serum is not well defined and can differ from animal to animal and each time the serum is collected. While laboratory preparation of serum is time consuming and involves stringent procedures, serum procured commercially exhibits considerable variability among different lots and raises experimental costs. Added to these, the serum may contain unknown factors such as growth factors, hormones, or other proteins, which can potentially affect the outcome of certain experiments, especially those that involve the study of signaling molecules important in tissue interactions. Studies have shown that addition of serum to culture can potentially alter the intracellular levels of certain signaling molecules such as cyclic adenosine monophosphate (cAMP) and proteins involved in mitogenic signaling and phosphoinositide 3 (PI 3)-kinase signaling pathways (Chotani et al., 2005; Chung et al., 1999; Sasaoka et al., 1994). Contrary to these, a serum-free culture system provides the advantages of antigen free environment, abstinence from biologically active enzymes that can alter the cellular processes and enables consistency among experiments.

In the present study, we utilized a serum-free culture medium prepared from commercially available stem cell media supplements to culture mid-gestation stage whole embryos in an atmosphere of 95% O_2 / 5% CO_2 in a rolling bottle culture apparatus at 37 ^oC (Gordon et al., 2014; Moore-Scott et al., 2003). Mouse embryos cultured for 16 to 40 hrs under these defined conditions exhibited progression in morphological development of overall embryonic body and different structures such as the heart, limbs, brain and eyes indicating appropriate levels of cellular proliferation, migration, differentiation and tissue interactions. Molecular analysis of the embryonic development in the culture for one of the complex organ systems such as the eye revealed the ocular development to be consistent with that observed in ocular tissue in embryos developing *in utero* (*Kalaskar and Lauderdale, in preparation*). Thus we show that mouse embryos cultured at mid-gestation stage, exhibit progressive growth and morphological development comparable to that observed in embryos developing *in utero*.

Protocol

Mouse embryo culture

All experimental procedures were conducted in strict accordance with National Institutes of Health guidelines following protocol # A2010 07-119, which was reviewed and approved by the University of Georgia Institutional Animal Care and Use Committee, which maintains continued regulatory oversight.

1) Preparation of culture media

1.1) Prepare culture medium using commercially available stem cell media and supplements in the following amounts: KnockOut DMEM, KnockOut Serum Replacement (KSR) (10%), N-2 Supplement (1x), Albumin, from Bovine Serum (2%), Penicillin (50 IU/ml), Streptomycin (50

µg/ml) and Amphotericin-B (1.25 µg/ml). The culture medium prepared is similar to that previously described (Moore-Scott et al., 2003) with the following changes: addition of anti-mycotics and using twice the concentration of antibiotics (For details see: Table 2.2, Table of specific reagents and equipment).

Note: Antibiotic concentration as previously used (Moore-Scott, *et al*., 2003) was sufficient for embryo cultures carried up to 24 hrs time period. However, when culture was continued beyond 24 hrs, we experienced contamination of the culture and this was successfully controlled by doubling the antibiotic concentration.

1.2) Store the media components at 4 ^{o}C or at -20 ^{o}C as per manufacturer recommendations. KSR and N-2 Supplement should be stored in aliquots at -20 ^{o}C to avoid repeated freezing and thawing. KnockOut DMEM once opened should be utilized within 30 days as per manufacturer's recommendation. Prior to use, warm the components in a water bath to 37 ^{o}C.

1.3) Prepare the media under sterile conditions. Disinfect the surface of all the equipment and bottles containing the media components with 70% alcohol spray before placing them in the culture hood.

1.4) The media components can be mixed either in a sterile beaker or directly in the filter system (Corning). First add the albumin powder to the KnockOut DMEM. Mix thoroughly by gently shaking until the albumin completely dissolves. Then add the N-2 Supplement, KSR and the antibiotics and mix thoroughly using a pipetter. Then filter sterilize the media using a 0.22 µm pore size filter with vacuum suction. Dispense the media into 50 ml conical tubes and store at 4 ^{o}C until used. The media once prepared should be used within 4 to 5 days for culture.

2) Preparation of culture equipment

2.1) Sterilize the glass culture bottles and rubber corks. Wrap the culture bottles in an aluminum foil and place the rubber bottle corks in a water beaker and sterilize by autoclaving.

2.2) Sterilize the culture chamber (Precision Incubator Unit) with 70% alcohol spray and warm to 37 ^{o}C before starting the culture. The culture chamber is equipped with a rolling disc at the center which holds the culture bottles. Gas flow into the chamber is regulated by a pressure gauge and the flow rate can be adjusted by observing the outflow of air bubbles through water in a glass tube at the end. This rolling bottle culture apparatus is a modified version of the original apparatus introduced by New and Cockroft (New and Cockroft, 1979).

2.3) Connect a gas cylinder containing a gas mixture of 95% O_2 / 5% CO_2 to the culture chamber and adjust the gas flow to "one air bubble per second" which gives a flow rate of 50 cc/min and ensures adequate oxygen supply to the culture embryos.

3) Mouse embryo collection and preparation for culture

3.1) To obtain wild type mouse embryos, we utilized mice of C57BL/6J (Jackson Labs) and CD-1 (Charles River Laboratories) genetic background strains.

3.2) Check the female mice for vaginal plugs every day morning. The noon on the day of finding the vaginal plug should be considered as E0.5 dpc (days post coitus).

3.3) Collect mouse embryos at E10.5 dpc by euthanizing the pregnant mice following standard protocols. Mice were euthanized using a CO_2 inhalation device as specified by the American Veterinary Medical Association (AVMA) Guidelines for the Euthanasia of Animals (2013). To insure death, cervical dislocation was performed after exposure to CO_2.

3.4) Spray 70% ethanol on the ventral abdominal surface to avoid sticking of abdominal hair to the instruments. Then open the abdominal cavity ventrally using a sharp-blunt operating scissors

(Roboz, RS-6812) and a pair of 4-3/4" micro dissecting forceps (Roboz, RS-5237) to locate the uterus. Using a 4" micro dissecting forceps (Roboz, RS-5211) lift the entire uterus and separate it from the body by cutting with a light operating scissors (Roboz, RS-6702) at the uterine body and at the tips of the uterine horns.

3.5) Quickly rinse the entire uterus in 1x PBS warmed to 37 ^{o}C to remove any blood sticking to the uterus and immediately place in DMEM warmed to 37 ^{o}C in a petri dish. Sterilize the instruments by 70% ethanol spray before further use.

Note: Preheating the solutions including the PBS, DMEM and the culture medium and maintaining them at 37 ^{o}C during the entire procedure is recommended.

3.6) Under a dissecting microscope, the uterus should be segmentally dissected with a light operating scissors. This results in small openings on either side of the segmented uterus through which a pair of modified (blunted ends) micro dissecting tweezers (Roboz, RS-5060 / RS-5063) can be gently inserted to widen the opening. This permits the exposure of the placental decidua, which can then be removed by gently tearing with a pair of micro dissecting tweezers to expose the parietal yolk sac (PYS) with the Reichert's membrane. Using one edge of the tweezers gently pierce the Reichert's membrane along with the PYS and separate from the underlying visceral yolk sac (VYS) layer to expose the embryos with intact VYS. The ectoplacental cone and the trophoectoderm derivatives can be removed either with the placental decidua or with the Reichert's membrane. Care must be taken to avoid rupture of the VYS as the embryos pop out immediately.

3.7) The embryos with intact VYS should then be transferred to a petri dish containing the culture medium warmed to 37 ^{o}C using a plastic transfer pipette.

Note: Transfer to culture medium immediately after separation from uterus helps for better development of the embryo in culture.

3.8) A small opening should be made in the yolk sac with a pair of micro dissecting tweezers avoiding major blood vessels. A sharp pair of tweezers can be used to make the opening by gently piercing in an area adjacent to the head region. Alternatively two pairs of blunt tweezers can be used to hold the yolk sac and gently tear to make a small opening. Then gently widen the opening by expanding with the tweezers to a size just enough to fit the embryonic head. The amniotic membrane which is closely wrapping the embryo should be gently held away from the body and torn using a pair of tweezers. The embryonic head and later the whole embryo should be gently exteriorized from the yolk sac while maintaining the integrity of the embryonic vasculature with that of the yolk sac (Moore-Scott et al., 2003).

Note: Any damage to the yolk sac vasculature can potentially affect the development of the embryo in culture. Such embryos with damaged vasculature should not be used for culture and can be used for staging the embryos which can later be discarded.

3.9) **Embryonic staging criteria:** Examine the embryos under the dissecting microscope and group by morphological criteria including body and head size, limb and eye morphology and stage by counting the number of somites (s) for at least two embryos from each group.

Note: Usually embryos obtained from the same litter show differences in development *in utero* and differ in body size, morphological features and number of somites. However, we found that embryos grouped by our morphological criteria usually exhibited similar somite number (±1). For this reason, it is not required to count somites for each embryo as this would delay the time for starting the culture.

Note: Do not use the embryos that were used to count the somites for culture. Count the somites after starting the culture for other embryos in order to avoid the time delay in starting the culture. The embryos that were delayed to culture after separation from the uterus usually exhibit poor development.

3.10) Transfer the embryos immediately to a petri dish with fresh culture medium warmed to 37 ^{o}C and take it to the culture hood where the embryos should again be transferred to a petri dish with sterile culture media before putting them into the culture bottles with medium to start the culture.

Note: The multiple media transfers for the embryos before culture helps in preventing contamination as the different steps of embryo collection and dissection were performed under a dissecting microscope which was not installed in the culture hood.

Note: When the litter size is large (>12 embryos), process half the embryos and start the culture or put them in a dish with culture medium and place it in a CO_2 incubator at 37 ^{o}C (CO_2 Water Jacketed Incubator). When embryos were left outside for longer periods (>35 - 40 min) we observed the heartbeat to slow down and this will affect the later development in the culture.

4) Culturing mouse embryos

4.1) Open the autoclaved culture bottles in the culture hood and properly label them. Transfer 3 ml of culture medium warmed to 37 ^{o}C to each of the bottles and then the mouse embryos should be transferred gently into the culture bottles using sterile plastic transfer pipettes. The culture bottles should then be capped with the rubber bottle corks which help to hold the culture bottles to the rolling disc in the culture apparatus.

Note: The culture bottles with the media can be prepared and placed on the rolling disc of the culture apparatus before euthanizing the mice. This shortens the time for transfer of embryos into the culture bottles.

Note: Mouse embryos can be cultured individually or as co-cultures with two or three embryos in the same culture bottle.

4.2) The culture bottles should be aseptically carried to the culture apparatus at 37 ^{o}C and tightly hook them to the holes on the rolling disc with the rubber corks. Turn on the rolling disc to rotate at a constant speed of 35 rotations per minute (rpm), which enables the free floatation of the embryos in the media and also helps in free gas exchange by the embryonic tissue. The gas from the cylinder connected to the culture chamber flows through the rolling disc and the rubber corks into the culture bottles.

4.3) Culture the embryos for 16 to 40 hrs and regularly check for the gas outflow and culture chamber temperature.

Note: Mouse embryo manipulation in culture: Let the embryos get adapted to the culture conditions for at least 30 min. Then embryos that perform poorly and showing feeble heartbeat can be discarded and the remaining embryos can be utilized for further manipulation studies. Embryo manipulations such as electroporation (Ref 5, 9, 16), micro-injections (Ref 18), bead implantation (Ref 16), drug treatment and other procedures can be performed at this time. We successfully performed implantation of affi-gel agarose beads treated with certain signaling molecules to study their role in early eye development. The embryos after manipulations can be returned back in a fresh medium and continued in culture.

4.4) Replace the culture media totally with fresh media at specific intervals after 9 to 10 hrs and 18 to 19 hrs of culture when the culture was continued for 40 hrs.

Note: Culture media replacement may not be required if the culture is stopped by 16 - 18 hrs.

4.5) **Measures of success in culture:** Embryonic survival in culture should be determined by visible heartbeat and blood circulation in the body while embryonic growth in culture should be assessed by increase in body size, somite number and morphological development of head, limbs, heart and eyes.

4.6) *In utero* developed embryos at E11.0 dpc (~40 – 41 s) and E12.0 dpc (~49 – 50 s) should be used as controls for comparing embryos cultured for 16 to 18 hrs and 38 to 40 hrs respectively.

4.7) Embryonic development at different time points during culture and for *in utero* stages can be captured under dissecting microscope. Spot imaging software was utilized to capture the images and later processed using image processing software.

Representative Results

Development of mouse embryos *ex utero* depends on multiple factors starting from the time the uterus is isolated from the body to the time the embryos are cultured. As depicted in Figure 2.1, the procedure involves a series of steps including, separation of the gravid uterus from the body (Figure 2.1A), isolation of the embryos with intact yolk sac (Figure 2.1B), exteriorization of the embryos from the yolk sac (Figure 2.1C) and culturing the embryos in a serum-free media in an atmosphere of 95% O_2 / 5% CO_2 in a rolling bottle culture apparatus at 37 ^oC. Embryos exhibited 100% survival when transferred into culture media immediately after separation from the uterus. Under these culture conditions, mouse embryos when cultured for 16 – 40 hrs exhibited growth and morphological development comparable to that observed in equivalent stage embryos developing *in utero* (Figure 2.2).

Embryos at E10.5 (~34 - 35 s) (Figure 2.2A) when cultured for 16 to 40 hrs survived and advanced in development well beyond the stage of 35 s. The embryos after 16 - 18 hrs in culture (Figure 2.2D, 2.3B; Table 2.1) added about 5 – 6 s exhibiting a growth rate of one somite for every two and a half hours in culture and were morphologically comparable to E11.0 (~40 - 41 s) embryos developed *in utero* (Figure 2.2B, 2.3A). Though the development was delayed by about an hour, these embryos in culture exhibited an overall increase in body size and a proportionate head with distinct brain vesicles. The embryos also showed developing limb buds, formation of a well chambered heart and appearance of pigmentation in the retinal pigmented epithelium. However, this type of development was observed in about 58% of the embryos cultured while 28% of the embryos exhibited an intermediate development (Figure 2.3C). These later embryos showed a smaller body size though they had a similar somite count compared to the *in utero* developed embryos. They had a relatively smaller head, although the brain vesicles were demarcated. The heart chambers were not distinct and the limb buds in some embryos exhibited dark areas at the extremities. At the other end of the spectrum, about 14% of the embryos exhibited poor development in the culture (Figure 2.3D). These embryos had shorter body and head sizes and showed dark areas in some parts of the body indicating degenerative changes. Though the heart was beating it was ill developed and lacked differentiation into chambers in some embryos while in others the limbs appeared dark and retarded in growth.

Embryos continued in culture for 38 - 40 hrs (Figure 2.2E; Table 2.1) showed about 49 - 50 s and were comparable to E12.0 embryos (Figure 2.2C) developed *in utero*. Although delayed by 2 - 3 hrs, these embryos in culture exhibited proportionate increase in body size and showed appropriate organogenesis and tissue differentiation as observed in the development of the pigmentation in the retinal pigmented epithelium and formation of a well demarcated snout in the

head region. Though some parts at the extremities like the limb buds and the tail appeared to be under-developed, the embryos appeared to be developing comparable to the *in utero* embryos. However, this kind of development was observed in only 30 – 40% of the embryos cultured (Table 2.1) while the rest of them exhibited a range of developmental progression with areas of retarded growth or under-development in some parts of the body or the whole embryo.

Apart from the above differences in development, we observed a significant difference in development when the embryos were co-cultured in the same culture bottle. Compared to the other embryo (Figure 2.4A, A') in the co-culture, the well-developed embryo (Figure 2.4B, B') exhibited better growth in all aspects of the embryonic development. Major differences were observed in the overall body size and head size and sometimes in the heart and limb development. While the well-developed embryo from the co-culture was morphologically comparable to the embryos developing *in utero*, the other embryo in the co-culture has always appeared to be less developed compared to the well-developed embryo. This kind of difference in development was observed in 45% of the co-cultures (N=27), while the rest of the co-cultures (N=33) showed almost similar development in both the embryos in the co-culture.

Discussion

Mid-gestation stage mouse embryos were cultured in a serum-free culture media in an atmosphere of 95% O_2 / 5% CO_2 in a rolling bottle culture apparatus at 37 ^{o}C. Embryo development *ex utero* was critically dependent on multiple factors at each step during the procedure from the time the uterus is isolated from the euthanized mice to the completion of the culture (Figure 2.1). The most important factor that influenced the development was the time taken to start the culture. The other critical points during the procedure which require most care

include the steps such as the separation of embryos with intact yolk sac from the uterus and the exteriorization of the embryos from the yolk sac. As the rodents have discoid placenta, segmental dissection of the uterus did not damage the blood supply to the embryo even if the placental decidua is slightly damaged at the corners. In fact this left a small opening at either end through which a pair of blunt forceps can be inserted to gently tear open the uterus and the placental decidua enabling easy isolation of the embryos with intact yolk sac. However, the next step of exteriorization of the embryos from the yolk sac is critical in that any damage to the major blood vessels in the yolk sac would potentially effect the development of the embryo in the culture. Proper embryonic development was supported when embryos were exteriorized from the yolk sac maintaining the intactness of the vasculature in the yolk sac. Previous studies have indicated the opening of the yolk sac when mouse embryos beyond embryonic stage E10 were cultured (Behesti et al., 2006; Cockroft, 1973; Moore-Scott et al., 2003; Zeeb et al., 2012). Accordingly, we observed degenerative changes and the embryos could not survive the culture period when E10.5 embryos were cultured with intact yolk sac. We assume the exteriorization of the embryos would facilitate effective nutrient absorption through the tissues while maintaining the continuity in vasculature with the yolk sac. These embryos in culture exhibited effective heart beat and blood circulation in the embryonic body parts compared to the embryos cultured without the yolk sac.

Maintaining the embryos in the culture medium warmed to 37 ^{o}C right from the time they are isolated from the uterus to the time of culture would immediately provide the best suitable environment to sustain their ability for later development in the culture. This also prevents contamination due to presence of antibiotics in the medium and avoids incorporation of even minute quantities of PBS / DMEM into the culture. The defined medium we have utilized

yielded superior embryonic development compared to other established defined media previously described in Wawersik *et al*., 1999 (Thut et al., 2001; Wawersik et al., 1999) (data not shown). The difference in embryonic development could mainly be due to the unique components in our defined medium such as, KnockOut Serum Replacement (KSR) and N-2 Supplement. While N-2 Supplement was shown to support the growth and expression of post-mitotic neurons, KSR was shown to be a direct replacement for fetal bovine serum (Cheng et al., 2004; Greenfield et al., 2002). KSR was used in cultures to support the growth of undifferentiated pluripotent stem cells as well as human and mouse embryonic stem cells which were shown to be stable and germline competent (Cheng et al., 2004; Kim et al., 2009; Totonchi et al., 2010). Apart from the culture medium, the oxygen concentration in the culture bottle plays a critical role in embryonic development. Embryos could not survive the conditions of 5% CO_2 in a conventional incubator. Though we have not tested other oxygen concentrations and different flow rates as previously suggested (Ref 5, 9), the use of 95% O_2 / 5% CO_2 at constant flow rate has sustained the survivability of the embryos in the culture. While rotation of culture bottles is necessary, embryos when cultured under the same conditions but with no rotation of the culture bottles resulted in non-survival of the embryos with no visible heartbeat. We assume that constant rotation (35 rpm) in the rolling bottle culture apparatus enabled free suspension of the embryos in the medium and as well enhanced the absorption of nutrients and oxygen through the embryonic tissues. The growth of these embryos in the culture in terms of overall body and head size, morphology, formation of a well-chambered heart, brain vesicles and ocular development was comparable to that of embryos developing *in utero*. In our hands, we were able to replicate the embryonic development that was observed by *Moore-Scott et al., 2003*.

The embryos in the culture exhibited a range of growth and morphological development depending on multiple factors (Figure 2.3; Table 2.1). Under the defined culture conditions we used, embryos cultured for 16 – 18 hrs showed comparable development to that observed in embryos developing *in utero* in about 58% of embryos, while 28% showed intermediate and 14% showed a poor development. Extension of culture to 38 - 40 hrs further decreased the ability to show comparable development to only 30 – 40% of the total embryos cultured. Apart from the inherent factors that potentially influence the embryonic development *ex utero*, many external factors that can be managed to certain extent may play critical role when the embryos are cultured for prolonged length of time. One such potential factor that can be controlled is the time taken from the isolation of embryos from the uterus to the start of the culture. It can be interpreted that the first isolated embryos are exposed to less oxygen in the culture dish compared to the embryos isolated at the end as all the embryos go into the culture bottle and receive 95% O_2 / 5% CO_2 at the same time. However the oxygen available in the blood that is left in the placenta after segmental dissection may not support for extended period of time and for this reason most of the culture equipment and apparatus should be set up ready to be used before euthanizing the mouse. We usually get the embryos into the culture in 25 – 30 min from the time the mouse was euthanized. This short time period to start the culture would not be possible if embryos were to be processed one at a time as suggested in Zeeb, M. *et al*., 2012 (Ref 18). When embryos are processed individually, grouping of embryos is not possible and each embryo should be counted for somite number. This procedure would take at least 5 - 6 min for each embryo to isolate, exteriorize from yolk sac, count somites and transfer to the culture bottle. For a litter size of 10 - 12 embryos, which we usually get from our CD-1 mice, the entire process would take 60 - 72 min for the last few embryos to get into the culture. This is pretty long period

and we observed the heart beat to slow down and almost stop when embryos were exteriorized and left in PBS and delayed to culture. One other potential factor could be the handling / mishandling of the embryos during the procedure. During handling, you may induce an accidental damage to some part of the body or blood vessels in the embryo or yolk sac which is not easily detected. This can affect the development of that part of the body or sometimes the whole embryo as it was observed that even a well-developed embryo sometimes shows a kind of under-development at the extremities like the limb buds and the tail indicating insufficient blood supply. It is better to discard such embryos that appear to be compromised or damaged during the procedure. The other problem usually encountered during the procedure was, some of the embryos with intact yolk sac prop out abruptly from decidua upon segmental dissection of the uterus. This sometimes results in more blood loss at the separation point from the placenta although the vasculature with the yolk sac remains intact. This blood loss can affect the later development of the embryo due to less amount of blood retained in the embryonic body during separation.

Apart from the above factors certain conditions like, the stage of embryo at the time of starting the culture influenced the development in the culture. We successfully cultured mid-gestation mouse embryos starting with stages that ranged between 28 - 37 s for 16 – 40 hrs and observed some differences in development. Embryos at 34 – 36 s stage exhibited better development in the culture compared to the embryos at 30 – 33 s stage indicating an increase in adaptability to culture conditions in embryos cultured at later stages. The other surprising fact that was observed during culture was the development of embryos when two or more embryos were co-cultured (Figure 2.4). Co-culture resulted in significant improvement in development in one of the embryos compared to the other embryo in 45% of the co-cultures. Though the

embryos from the same uterus look similar morphologically and in somite count, inherently no two embryos can be at the same stage of development and this could be one of the reasons for developmental difference in one of the embryos compared to the other in the co-culture system.

Thus we show that mid-gestation stage mouse embryos cultured *ex utero* in a serum-free medium in an atmosphere of 95% O_2 / 5% CO_2 in a rolling bottle culture apparatus at 37 ^oC exhibit growth and morphological development comparable to that observed in embryos developing *in utero*. We believe this method of mouse embryo culture system will be useful for laboratories needing to utilize whole embryos to study signaling interactions important in early embryonic organogenesis.

Acknowledgements

We would like to thank Dr. Julie Gordon and Dr. Nancy Manley for their helpful advice with the culture technique. This work has been supported by the Children's Glaucoma Foundation and Sharon-Stewart Aniridia Research Trust.

Disclosures

We do not have any competing financial interests.

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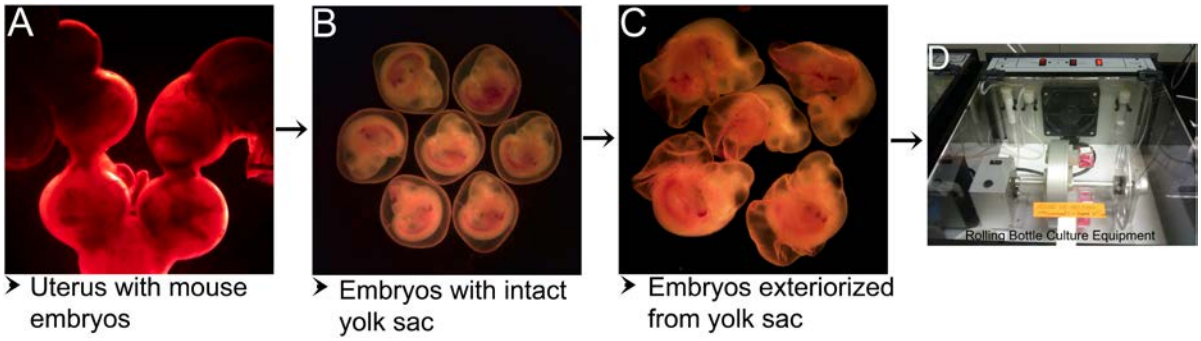
Figure 2.1: Steps in mouse embryo culture protocol. (A) Gravid uterus with embryos isolated from the mother mouse. (B) Embryos with intact yolk sac separated from decidua after segmental dissection of uterus. (C) Embryos exteriorized from the yolk sac. (D) Rolling bottle culture apparatus at 37 ^{o}C and supplied with 95% O_2 / 5% CO_2 for culturing mouse embryos.



➤ Uterus with mouse embryos

➤ Embryos with intact yolk sac

➤ Embryos exteriorized from yolk sac

Figure 2.2: Embryos in culture replicate *in utero* development. *In utero* development at E10.5 (A), E11.0 (B) and E12.0 (C). Development in culture after 18 hrs (D) and 40 hrs (E). Scale bar in (E) applies to all panels.

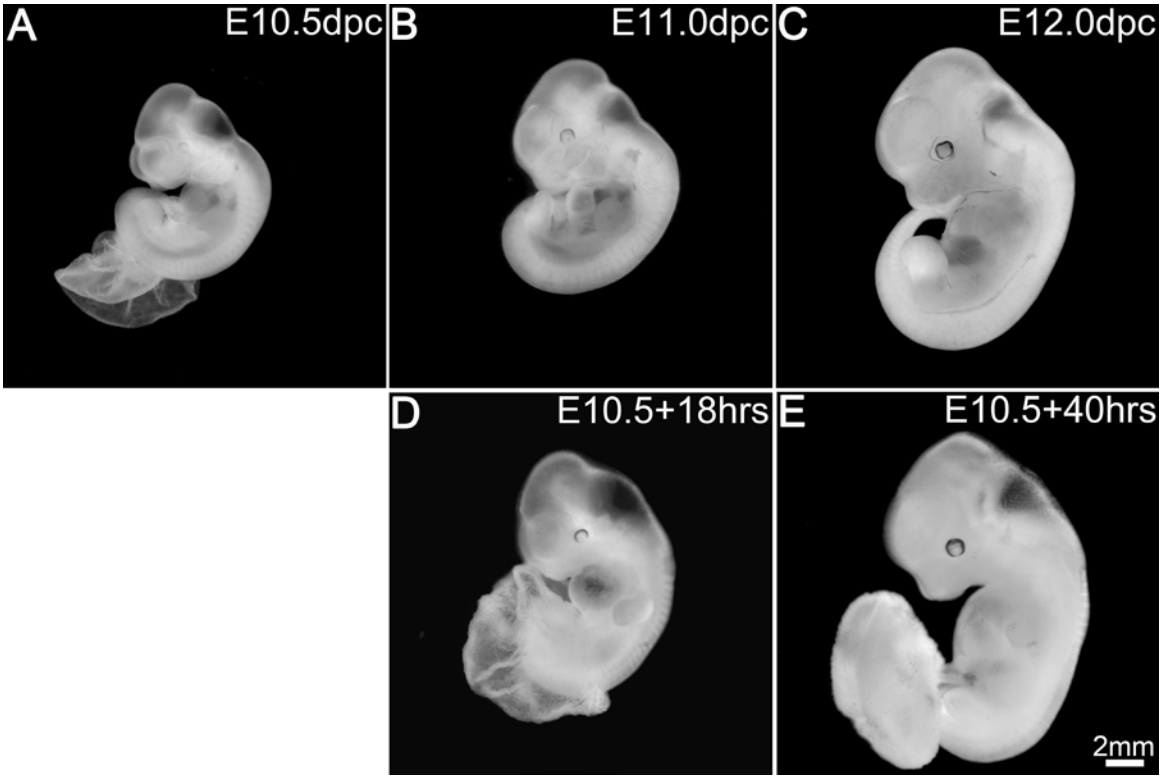

A
E10.5dpc
B
E11.0dpc
C
E12.0dpc
D
E10.5+18hrs
E
E10.5+40hrs
2mm

Figure 2.3: Embryonic development in culture. Embryo developed *in utero* at E11.0 dpc (A). Development in culture (B, C, D). (B) Representation of good embryonic development in culture. About 58% of total embryos cultured exhibit comparable development to *in utero* developed embryos. (C) Representation of intermediate development in culture. About 28% of embryos cultured show intermediate development. (D) Representation of poor embryonic development in culture. About 14% embryos show poor development in culture. Scale bar in (D) applies to all panels.

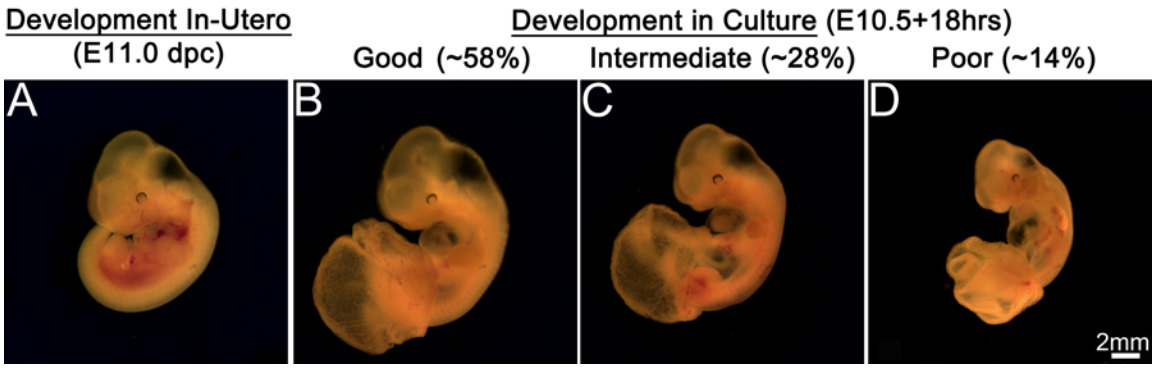

Development In-Utero
(E11.0 dpc)
Development in Culture (E10.5+18hrs)
Good (~58%)
Intermediate (~28%)
Poor (~14%)
A
B
C
D
2mm

Figure 2.4: Developmental differences in mouse embryo co-culture system. Embryos at the start of co-culture (A, B). Embryonic development after 16 hrs of co-culture (A', B'). One of the embryos in the co-culture (A') appears under-sized compared to the other embryo (B') in the co-culture. Scale bar in (B') applies to all panels.

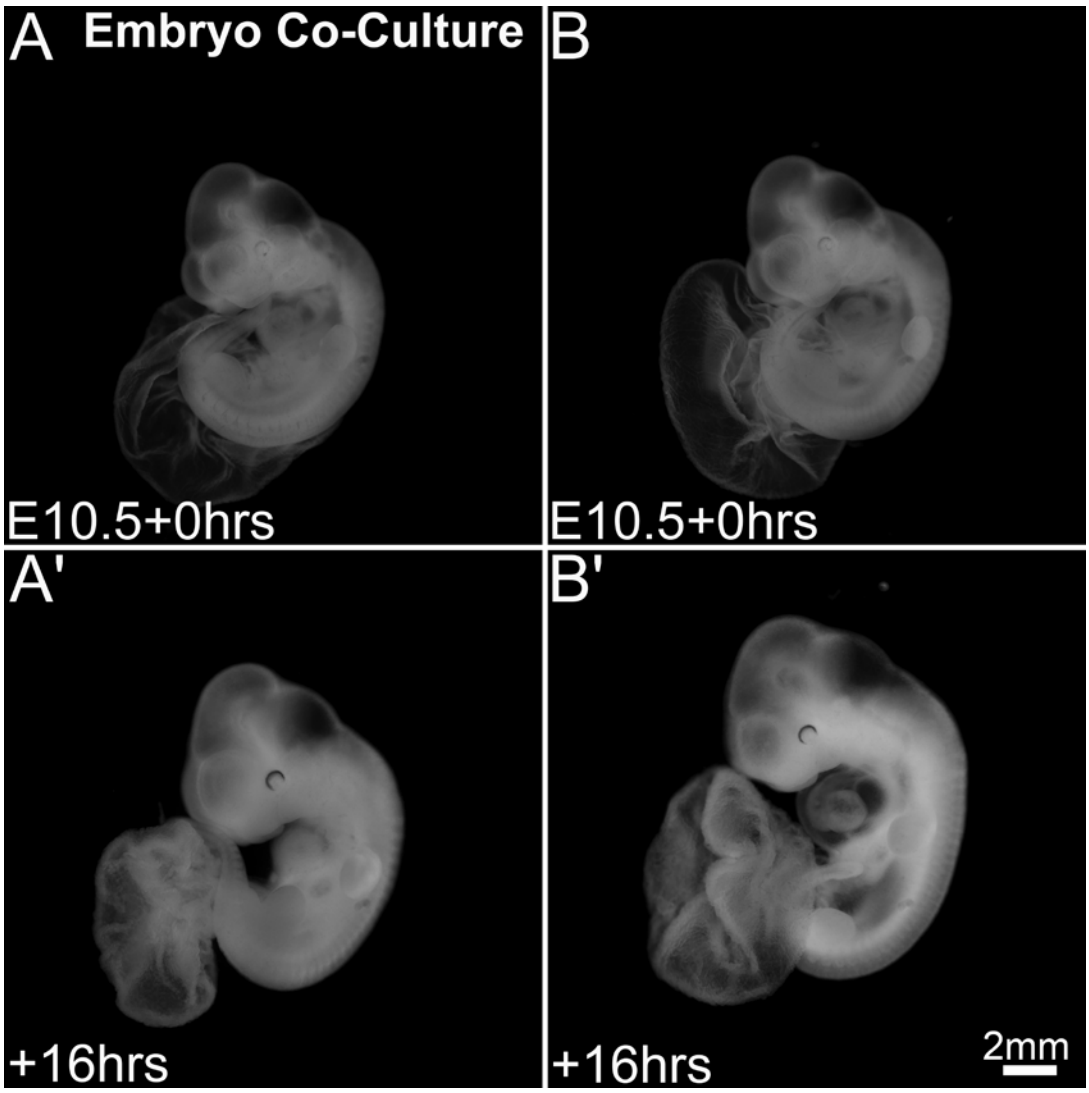

A Embryo Co-Culture
B
E10.5+0hrs
E10.5+0hrs
A'
B'
+16hrs
+16hrs
2mm

Table 2.1: Development of embryos in mouse embryo culture system. Embryos cultured for either 16 – 18 hrs or 38 – 40 hrs. Of the total 112 embryos cultured for 16 – 18 hrs, 65 (58%) embryos exhibited comparable development to embryos developing *in utero*, 31 (28%) showed intermediate and 16 (14%) showed poor development. Of the total 12 embryos cultured for 39 – 40 hrs, only 4 (~35%) embryos showed good development while the remaining showed poor development.

Table 2.1

Mouse Embryo Culture Experiments

Total embryos cultured for 16 - 18 hours	Development after Culturing for 16 -18 hours			Total embryos cultured for 38 - 40 hours	Development after Culturing for 38 - 40 hours	
	Good	Intermediate	Poor		Good	Poor
112	65	31	16	12	4	8

Table 2.2			
Table of Specific Reagents and Equipment			
Name of the Reagent / Equipment	**Company**	**Catalog Number**	**Comments**
KnockOut DMEM	Invitrogen	10829-018	
KnockOut Serum Replacement	Invitrogen	10828-028	
N-2 Supplement	Invitrogen	17502-048	Stock: 100x
Albumin, from Bovine Serum	Sigma	A9418-50G	Stock: 100%
Antibiotic - Antimycotic Solution	Cellgro	30-004-Cl	Stock: Penicillin (10000 IU/ml); Streptomycin (10000 µg/ml; Amphotericin (250µg/ml)
DMEM	Cellgro	15-013-CV	
Precision Incubator Unit	B.T.C. Engineering Milton Cambridge England	Id.No. 840-374	
Glass Bottles for Rotating Unit	B.T.C. Engineering		
Silicone Rubber Cork	B.T.C. Engineering		
95% O_2/5% CO_2 Cylinder	AirGas Inc.		
Stemi SV11 Apo Dissecting Microscope	Zeiss		
Stemi SV6 Microscope	Zeiss		
CO2 Water Jacketed Incubator	Forma Scientific	Model: 3110	
Culture Hood	Nuaire Biological Safety Cabinets Class II TypeA2	Model: Nu-425-600	
Water Bath	Fisher Scientific	IsoTemp205	
Weigh Balance	Mettler Toledo	AG285	
Centrifuge Tube – 50ml	Corning	430291	
Light Operating Scissors	Roboz	RS-6702	

Operating Sharp-Blunt Scissors	Roboz	RS-6812	
Micro Dissecting Forceps – 4”	Roboz	RS-5211	
Micro Dissecting Forceps - Hudson (cWALD) – 4-3/4”	Roboz	RS-5237	
Micro Dissecting Tweezers (5/45)	Roboz	RS-5005	Modified - Sharp ends were made blunt
Micro Dissecting Tweezers (5)	Roboz	RS-5060	Modified - Sharp ends were made blunt
Micro Dissecting Tweezers (55)	Roboz	RS-5063	Modified - Sharp ends were made blunt
Instrument Tray	Roboz	RT-1401S	
Instrument Tray Lid	Roboz	RT-1401L	
Petri Dish-100mm	Fisher Scientific	087571Z	
Petri Dish-60mm	Fisher Scientific	0875713A	
Petri Dish-35mm	Fisher Scientific	0875711YZ	
Filter System (0.22um Cellulose Acetate)-150ml	Corning	431153	
Filter System (0.22um Cellulose Acetate)-250ml	Corning	430756	
Filter System (0.22um Cellulose Acetate)-500ml	Corning	430758	
Pipet-aid Pipetter	Drummond Scientific Co.	D57849	
Serological Pipette-10ml	VWR	89130-898	
Disposable Serological Pipette-25ml	Corning	4251	
Transfer Pipette - 7.7ml	Thermo Scientific	202-20S	

Chapter 3

Evaluation of Embryonic Mouse Eye Development in Culture[1]

[1] Kalaskar V. K., Lauderdale J. D. To be submitted to *Molecular Vision*.

Abstract

Purpose: To evaluate embryonic mouse eye development under defined culture conditions and utilize this system to identify signaling interactions that are important in early eye development.

Methods: Mouse embryonic tissue explants and whole embryos at mid-gestation stage were cultured in a standardized serum-free culture medium comprised of commercially available stem cell media supplements in an oxygenated rolling culture system. Ocular development was assessed using morphological and gene expression criteria, including *Pax6*, *Sox2*, *Prox1*, *αA-crystallins* and *FoxC1*.

Results: Mouse embryos at mid-gestation stage when cultured for 16 to 40 hours in a serum-free oxygenated culture system, exhibited morphological and molecular correlates of early eye development consistent with those observed in utero.

Conclusions: In vitro culture methods utilizing whole embryos are well suitable to study the tissue interactions involved in early eye development that are otherwise difficult to access in utero.

Introduction

Tissue interactions are critical for organogenesis of complex organs such as eye and often involve multiple signaling mechanisms. In vivo studies to elucidate these interactions using genetic or pharmacological approaches are difficult to design and are time consuming (Salaun, 1982). To overcome these limitations, in vitro methods have been extensively designed and utilized as an alternative approach to study the different aspects of early eye development (Armstrong and Elias, 1968a; Armstrong and Elias, 1968b; Coulombre, 1965; Faber et al., 2001;

Furuta and Hogan, 1998; Osakada et al., 2009; Wawersik et al., 1999). One of the most commonly used in vitro methods involved the use of embryonic ocular tissue explant cultures. These explant cultures have been used to understand the tissue inter-dependence and the importance of inductive interactions in early eye development (Furuta and Hogan, 1998; Hyer et al., 1998; Muthukkaruppan, 1965b; Nieuwkoop, 1963). For example, ocular explant cultures revealed that BMP4 signaling was required for optic vesicle to manifest its lens-inducing activity (Furuta and Hogan, 1998) and that TGFβ signaling from extraocular mesenchyme was shown to be required for retinal pigmented epithelium development (Fuhrmann et al., 2000). Similarly, optic rudiment cultures, which include the entire optic primordium separated from the periocular region, were employed to study the role of secreted signaling molecules in eye development (Nishiguchi et al., 1998; Thut et al., 2001).

Although cultures employing explants or optic rudiments have been utilized to elucidate certain signaling mechanisms, they are not effective in supporting certain processes like cellular migration, tissue interactions and are not well suited for studies requiring the eye development in the context of the whole embryo. These limitations can be overcome by culturing whole embryos which provide tissue integrity and support appropriate tissue interactions during development. Currently utilized whole embryo cultures for studying ocular development are mainly dependent on serum for maintenance and survival of the embryos in the culture (Behesti et al., 2006). Serum has been shown to be a source of certain signaling molecules and may contain unknown factors which can potentially affect the outcome of experiments especially those that involve signaling mechanisms (Chotani et al., 2005; Chung et al., 1999; Sasaoka et al., 1994). These studies emphasize the need for a serum-free whole embryo culture system to study the embryonic eye development.

In the present study, we have first set a criterion in order to determine the appropriate development of ocular tissue in culture. The criteria included, (1) morphological development comparable to in utero growth with appropriate cellular orientation and proper migration, (2) expression of tissue specific markers consistent with early eye development, (3) cellular proliferation and differentiation comparable to in utero conditions and (4) no significant increase in cellular apoptosis. To achieve these criteria for ocular tissue development in culture, we initially tested the already established culture conditions employing optic rudiments, embryonic heads and compared to the development in whole embryo culture. We utilized a serum-free culture medium prepared from commercially available stem cell media supplements to culture the embryonic explants and whole embryos in an atmosphere of 95% O_2 / 5% CO_2 in a rolling bottle culture apparatus at 37 ^oC (Kalaskar and Lauderdale, 2014; Moore-Scott et al., 2003). We show that mouse embryos cultured at mid-gestation stage, exhibit morphological development and tissue specific gene expression maintaining appropriate levels of cellular proliferation, migration and differentiation in the ocular tissue consistent with that observed in utero.

Methods

Mouse embryonic explant and whole embryo culture

Mice were maintained and handled following standard protocols in accordance with institutional policies. Mouse embryos at E10.5 dpc (days post coitus) were obtained by breeding mice of C57BL/6J (Taconic) and CD-1 (Charles River Laboratories) genetic background strains considering the noon on the day of finding the vaginal plug as 0.5 dpc. Mouse embryo culture method was performed following our protocol in Journal of Visualized Experiments and as previously described (Kalaskar and Lauderdale, 2014; Moore-Scott et al., 2003). Briefly, the

procedure involves segmental dissection of the uterus followed by the removal of the decidua to isolate the embryos with intact yolk sac. A small opening was made in the yolk sac and the amnion to exteriorize the embryos while maintaining the integrity of the major blood vessels (Moore-Scott et al., 2003). The embryos were immediately transferred to a defined culture medium prepared as previously described (Moore-Scott et al., 2003) with changes made including, addition of anti-mycotics and using twice the concentration of antibiotics. The embryos were cultured in 3ml medium in a rolling bottle apparatus (Moore-Scott et al., 2003) at 37 ^{o}C and 95% O_2 / 5% CO_2 for 16 to 40 hours and the media was changed after 9 and 18 hours of culture. In utero developed embryos at E11.0 and E12.0 dpc were used as controls for comparing embryos cultured for 16 and 40 hours respectively. Embryonic ocular development at different time points during culture and in utero stages were captured under dissecting microscope using Spot imaging software and processed. Mouse embryonic optic rudiment culture was performed as described previously (Thut et al., 2001; Wawersik et al., 1999). Embryonic heads were collected by dissection from the embryos and cultured as a whole explant or as sagittal sections either complete or partial with attachment at the dorsal or ventral part of the head regions.

Histology

The explants and embryos were fixed in 4% paraformaldehyde for 40 – 60 min at room temperature, washed in 1x PBS and dehydrated in a series of alcohols and xylene. The embryos were then processed for paraffin embedding following standard methods. Sections were cut at 6□m thickness and stained in hematoxylin and eosin for histology.

Immunohistochemistry and Immunofluorescence

Expression of ocular tissue specific markers like Pax6, Sox2, Prox1, αA-crystallins and Foxc1 was detected by immunohistochemistry. Sections were deparaffinized in xylene and dehydrated in a series of alcohols. Antigen retrieval was performed by placing the slides in sodium citrate buffer (pH6) and boiling in a water bath for 30min. Endogenous peroxidase activity was quenched by incubating in 3% hydrogen peroxide. The sections were blocked in 1% BSA / 0.1% Tween20 / 10% serum for 1 hour followed by overnight incubation with primary antibody. The primary antibodies used include, goat anti-αA crystallin (1:200; Santa Cruz sc-22389), rabbit anti-C-terminal Pax6 (1:500; Covance), rabbit anti-Prox1 (1:2000; Chemicon AB5475), goat anti-FoxC1 (1:200; Abcam) and rabbit anti-Sox2 (1:1000; Chemicon, AB5603). After several washes the slides were incubated in biotinylated secondary antibody followed by ABC reagent (Vector Labs) as per manufacturer's protocol. The signal was amplified by incubating with DAB followed by quick dehydration and mounting in cytoseal.

Cellular proliferation and apoptosis were detected by immunofluorescence on cryosections prepared from sucrose infiltrated tissues using primary antibodies, rabbit anti-histone H3 (1:100; Abcam ab-5176), rabbit anti-cleaved PARP (1:100; Cell Signaling Technology #9544), rabbit anti-cleaved Caspase3 (1:200; Cell Signaling Technology) followed by donkey anti-rabbit Alexa 594 secondary antibody (1:1000; Jackson labs) following standard protocols. Proliferating and apoptotic cells were counted in the ocular region in three sections from each eye for four eye tissues obtained from different embryos developed in culture and in utero. Bright field and fluorescent images were captured using AxioVision software and processed.

In Situ Hybridization

Whole mount in situ hybridization was performed on mouse embryos developed in utero and in culture following the protocol as described (Carpenter et al., 1993; Manley and Capecchi, 1995; Wei et al., 2011). DIG labeled sense and antisense RNA probes for *Pax6* were prepared as described (Kim and Lauderdale, 2006). Hybridization with the probes was performed overnight at 65 ^{o}C and posthybridization washes were done at 70 ^{o}C. DIG-labeled probes were detected using Alkaline phosphatase-conjugated anti-digoxigenin Fab fragments at 1:2000 dilution followed by color reaction with NBT/BCIP substrate (Roche) carried out for 4 - 5 hours at room temperature or overnight at 4 ^{o}C. After color development the embryos were postfixed in 4% paraformaldehyde / PBS overnight at 4 ^{o}C. The embryos were imaged under bright field dissecting microscope using AxioVision software and processed.

Results

Optic rudiments do not support proper ocular development in culture

We first tested the ocular growth and development in isolated optic rudiment cultures utilizing established defined conditions as previously described (Thut et al., 2001; Wawersik et al., 1999). Optic rudiments in culture exhibited varied growth and development in the culture depending on the stage of start of the culture (Table 3.1). Optic rudiments when cultured before the stage of lens vesicle separation from the surface ectoderm did not survive in the defined media at 37 ^{o}C and 5% CO_2. When cultured at E10.5 dpc or E11.5 dpc, the optic rudiments survived but showed abnormal morphological features. The lens fiber cells were highly disorganized with no alignment along the anterior-posterior direction and lacked the specific orientation of their nuclei along the equatorial region of the lens after one day in culture (data not

shown). The cornea was observed as a thin epithelial layer with no further development due to the absence of migrating mesenchymal cells. However the tissues showed expression of differentiation markers such as, αA-crystallins and β-crystallins in the lens fiber cells and Pax6 in the lens epithelium, corneal epithelium and retina (data not shown). Adding serum to the above media at 5% or 10% did not improve the ocular development in culture.

Oxygen plays a vital role in tissue survival in culture

In order to improve the corneal and lens development, we tested the embryonic head cultures using whole head or head sagittal sections either complete or partial with attachment at the dorsal or ventral part of the head regions (Table 3.2). When the embryonic heads or head sections were cultured at 37 ^{o}C and 5% CO_2 starting at different time points from E8.5 to E11.5, the tissues did not survive and exhibited degenerative changes. However, when the embryonic heads were cultured at 95% O_2 / 5% CO_2 in a rolling bottle culture apparatus at 37 ^{o}C, the tissues survived and showed morphological growth indicating the importance of oxygen for tissue survival in culture. This is consistent with previous studies that indicated the use of higher oxygen concentrations when embryonic stages beyond E10.5 dpc were cultured (Osumi and Inoue, 2001; Takahashi et al., 2008; Zeeb et al., 2012).The ocular development in the embryonic head culture was better than that observed in optic rudiment cultures with improvement in overall size of the eye and lens tissue. However the migration of mesenchymal cells was poor and the lens fiber cell orientation and polarity were disturbed (data not shown).

Ocular tissue in mouse embryo culture exhibits morphological development

Ocular tissue in mouse embryo culture exhibited far better growth and morphological development compared to all the culture conditions we have tested (Figure 3.1, 3.2). Morphological development of the eye in the mouse embryos in culture was visible externally by a gradual increase in pigmentation of the retinal pigmented epithelium (RPE) and increase in size of the ocular tissue (Figure 3.1). At 34 - 35 somite stage, pigmentation in the RPE is initiated in the dorsal region of the eye which then gradually extends to other regions of RPE in later stages of development (Figure 3.1A-C). A comparable increase in pigmentation of RPE was observed after 16 hrs and 40 hrs of culture indicating the differentiation of optic cup into neural retina and RPE (Figure 3.1D-F).

In the embryonic eye, the lens vesicle separates from the surface ectoderm at E10.5 stage (Figure 3.2A) which permits the migration of periocular mesenchyme cells under the presumptive corneal epithelium. The lens then develops an anterior proliferating epithelial layer and a posterior layer of differentiating fiber cells (Figure 3.2B). The lens vesicle is eventually closed by the elongation of the posterior lens fiber cells with their nuclei starting to align along the equatorial region of the lens. At this stage, the optic vesicle differentiates into the optic cup, with an inner multi-layered neural retina and an outer single-layered RPE (Figure 3.2C). The ocular development in culture proceeds similar to that observed in utero. Starting at E10.5, the ocular tissue after 16 hrs in culture (Figure 3.2D) showed a well separated lens vesicle with distinct anterior epithelial and posterior fiber cell layers which was comparable to stage E11.0 embryo developed in utero (Figure 3.2B). Further continuation of culture for 40 hrs (Figure 3.2E) revealed closing of lens vesicle with the elongating lens fiber cells whose nuclei start

towards the equatorial region of the lens. The optic vesicle showed the differentiation into optic cup with formation of inner multi-layered neural retina and outer RPE.

Tissue specific gene expression maintained in the ocular tissue in embryos developed in culture

We evaluated the embryonic development at the level of gene transcription and protein formation by examining the tissue specific genes at early and later stages of ocular development. Expression of pan-ocular genes such as *Pax6* was examined using whole mount in situ hybridization and immunohistochemistry. During embryonic stages E10.5 to E12.5, *Pax6* gene has been shown to be expressed in the developing eye and as well in other brain regions such as cerebral vesicles, diencephalon, hindbrain, nasal pit and also in the spinal cord and the endocrine pancreas (Grindley et al., 1995; Kim and Lauderdale, 2006; Walther and Gruss, 1991). Similar to the expression observed in the E11 embryos developed in utero (Figure 3.3A), *Pax6* transcripts were detected in the brain regions, spinal cord, developing eye and nasal pit in the E10.5 embryos cultured for 18 hrs (Figure 3.3B). In the eye, *Pax6* is necessary for normal development and during embryonic stages E10 to E12, *Pax6* has been shown to be strongly expressed in the inner optic cup, lens and overlying surface ectoderm and at a lower level in the RPE (Ashery-Padan et al., 2000; Walther and Gruss, 1991). The ocular tissue from embryos developed in culture exhibited a similar pattern of expression. The Pax6 protein was detected in the nuclei of cells in the corneal epithelium, lens and the developing optic cup including the neural retina and the RPE (Figure 3.4A, B).

We then evaluated the expression of other early markers during ocular development such as Sox2 and Prox1in these cultured embryos and compared to that observed in utero. Sox2 is an

early marker of ocular tissue and its activation was shown to be essential for lens placode formation (Furuta and Hogan, 1998; Kamachi et al., 1998). Sox2 has been implicated in lens differentiation and has a dose-dependent regulatory role in retinal neural progenitor competence (Kamachi et al., 1995; Taranova et al., 2006). Sox2 protein was shown to be detected in the nuclei of cells of both the optic cup and the lens pit at E10.5 and later observed in the lens fiber cells (Nishiguchi et al., 1998). In the embryos cultured for 18 hours from E10.5, Sox2 expression was observed in the nuclei throughout the lens and the optic cup (Figure 3.4C, D). Knockout studies in mice show that lens fiber cell differentiation and elongation were dependent on Prox1 activity during lens development. Prox1 was shown to be normally expressed in the entire lens at E10.0 and later in the anterior proliferating lens epithelium and posterior lens fiber cells (Wigle et al., 1999). The ocular tissue in the culture exhibited similar Prox1 protein expression in the lens epithelium and fiber cells (Figure 3.4E, F).

We next tested the ocular tissue in culture for its ability to exhibit timely differentiation and support appropriate cellular migration by evaluating the expression of genes such as crystallins and FoxC1. αA-crystallins are structural proteins in the lens required for survival of lens epithelial cells and differentiation of fiber cells (Andley, 2007; Xi et al., 2003). αA-crystallins are not expressed at E9.5 but become visible in the lens pit by E10.5 and its expression is intensified in the lens vesicle at E11.5 (Wolf et al., 2008). Consistent with the expression pattern observed in embryos developing in utero, αA-crystallin protein was detected in the lens vesicle in the E10.5 embryos cultured for 18 hours (Figure 3.4G, H). Neural crest derived periocular mesenchymal cells in the anterior segment and surrounding the optic cup are marked by FoxC1 expression at E11.5 (Gage et al., 2005). E10.5 embryos after 18 hours in culture showed mesenchymal cells in the periocular region around the optic cup. These cells

were observed to be migrating under the corneal epithelium and showed nuclear expression of FoxC1 protein (Figure 3.4I, J).

Apart from the expression of molecular markers, the ocular tissue in culture maintained a comparable level of cellular proliferation as revealed by the Ki-67 and PhosphoHistone-H3 markers (data not shown). When tested for cellular apoptosis using markers like cleaved Caspase3 and cleaved PARP, the ocular region in the embryos after 18 hours of culture showed no significant increase in the cells expressing apoptotic markers (data not shown) indicating the culture conditions to be well suitable for ocular tissue survival and maintenance.

Discussion

This study introduces a serum-free whole embryo culture system that enables the ex utero development of ocular tissue comparable to that observed in embryos developing in utero. We tested the mouse ocular development ex utero in mid-gestation stage embryos under different culture conditions (Table 3.1-3.3). Ocular development ex utero under defined conditions was best observed when embryos were cultured in an atmosphere of 95% O_2 / 5% CO_2 at 37 ^oC in a rolling bottle culture apparatus (Figure 3.1, 3.2). This study reveals the importance of two critical components which are essential for proper development of ocular tissue in the culture. First, the availability of oxygen to all the cells in the embryo, required for cell survival and second, the tissue integrity, essential for proper occurrence of signaling mechanisms important in tissue interactions and organ development. The embryonic explants including the whole heads or head sagittal sections could not survive when cultured in a conventional incubator at 37 ^oC supplied with 5% CO_2. However, when cultured under an atmosphere of 95% O_2 / 5% CO_2 at 37 ^oC in a

rolling bottle culture apparatus, the embryonic explants were able to survive and exhibit developmental progression to certain extent (Table 3.2).

While oxygen plays a vital role for tissue survival in the culture, embryonic intactness is essential for proper development of complex organs like eyes which involve multiple tissue interactions in its formation. Ocular development in optic rudiment culture was very much retarded due to the absence of migrating mesenchymal cells whose signaling interaction has been shown to be important in eye development including the lens and the retinal tissue (Cvekl and Tamm, 2004; Gage and Zacharias, 2009; Ittner et al., 2005). While embryonic head cultures were expected to supplement the source of mesenchymal cells, however, the ocular development in these cultures was not entirely rescued. Though there were some migrating mesenchymal cells under the corneal epithelium, the lens cell orientation was disturbed as they exhibited improper elongation. The overall ocular size in these embryonic head cultures was very small compared to those developing in utero. One of the reasons for under-development could be insufficient oxygen supply to the inner cells in the tissue as there is no intact blood circulation in these explants. These limitations were overcome when whole embryos exteriorized from the yolk sac but maintaining vasculature continuity with the yolk sac were cultured in an atmosphere of 95% O_2 / 5% CO_2 in a rolling bottle culture apparatus (Table 3.3).

The developmental progression and the morphology of the ocular tissue in the mouse embryos in whole embryo culture at different time periods was comparable to the development observed in utero. The critical stages of ocular development including the mesenchymal cell migration under the corneal epithelium, fiber cell orientation in the lens and differentiation of the optic vesicle into optic cup with anterior neural retina and posterior RPE were all timely maintained in the ocular tissues in the mouse embryo culture (Figure 3.2). Molecular analysis of

the ocular tissue for tissue specific gene expression supported the morphological development observed. The tissue specificity and the level of expression of the transcripts and the proteins for different genes was maintained in the cultured embryos comparable to that observed in embryos developing in utero (Figure 3.3, 3.4). For example, the expression of Pax6 was maintained not only in the eye but also in the brain regions, spinal cord and nasal pit in the cultured embryos. The ocular tissue in the cultured embryos also revealed the timely expression of early eye markers such as, Pax6, Sox2, Prox1 and FoxC1 and differentiation markers like αA-crystallins (Figure 3.4). Apart from these, analysis of the cultured embryos showed appropriate levels of cellular proliferation with no increase in apoptotic cell numbers in the ocular tissue although there were some areas in the brain that showed slight increase in number of cells expressing apoptotic markers.

Thus the ocular tissue in mouse embryo culture maintained appropriate levels of cellular proliferation, migration and differentiation indicating the timely occurrence of signaling mechanisms which are crucial in mediating the tissue interactions during development. We show that this mouse embryo culture method provides the right conditions for the developing embryonic ocular tissue which exhibits comparable morphological development and expression of molecular markers consistent with that observed in embryos developing in utero. In our hands, of all the culture methods tested, the mouse embryo culture represents the best possible defined culture system suitable for embryonic ocular development ex utero. We believe this method will be useful for laboratories needing to utilize whole embryo culture to study signaling interactions important in early eye development.

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Figure 3.1: Ocular development in mouse embryo culture replicates in utero development. Ocular development in utero at E10.5 (A), E11.0 (B) and E12.0 (C). Ocular development in culture after 16 hrs (D) and 40 hrs (E). Eye development indicated by pigmentation in the retinal pigmented epithelium. Scale bars in (E) applies to all panels.

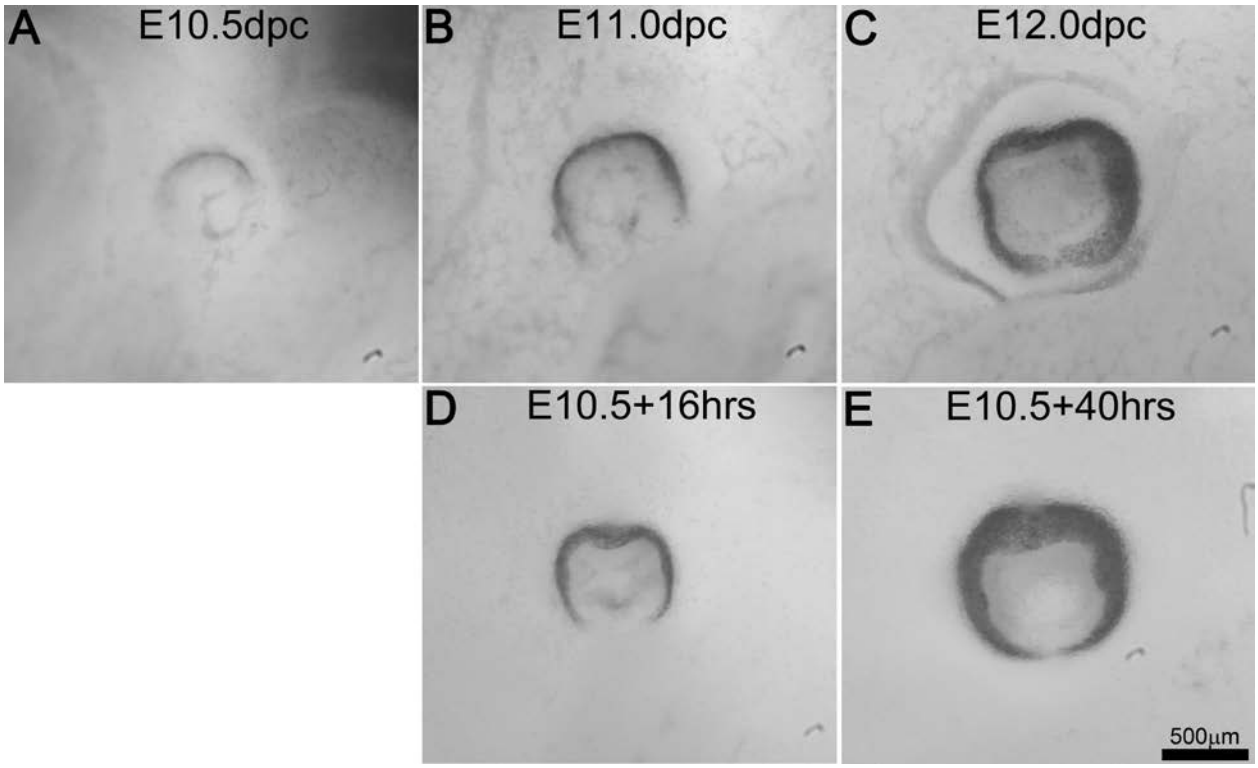
A
E10.5dpc
B
E11.0dpc
C
E12.0dpc
D
E10.5+16hrs
E
E10.5+40hrs
500μm

Figure 3.2: Ocular tissue in culture exhibits morphological development. Eye development in utero at E10.5 (A), E11.0 (B) and E12.0 (C). Eye development in culture after 16 hrs (D) and 40 hrs (E). Oc – Optic Cup, L – Presumptive Lens, C – Presumptive Cornea. Scale bar in (E) applies to all panels.

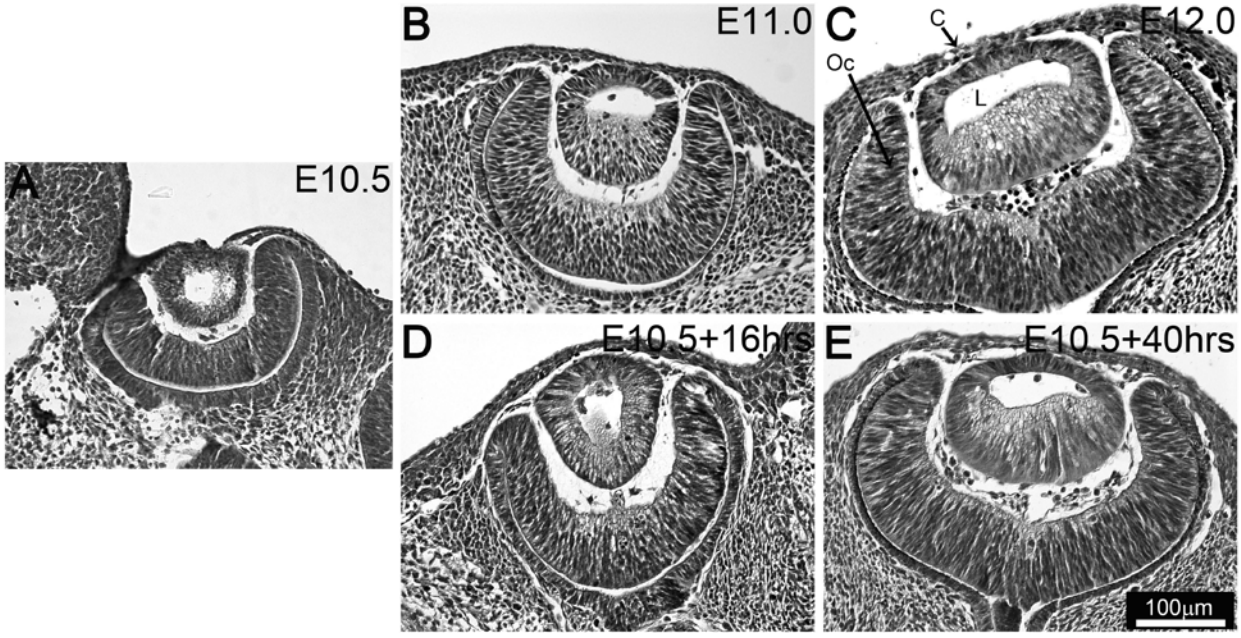
A
E10.5
B
E11.0
C
E12.0
C
Oc
L
D
E10.5+16hrs
E
E10.5+40hrs
100μm

Figure 3.3: Embryos developed in culture exhibit comparable levels of gene transcription. mRNA expression levels for Pax6 gene (A, B) is maintained in the embryonic tissues developed in culture (B) comparable to that observed in utero (A). Pax6 expression observed in brain (Br), hindbrain (HBr), eye (Ey), Nasal pit (Np) and spinal cord (Sc). Arrows indicate mRNA expression. Scale bar in (B) applies to (A).

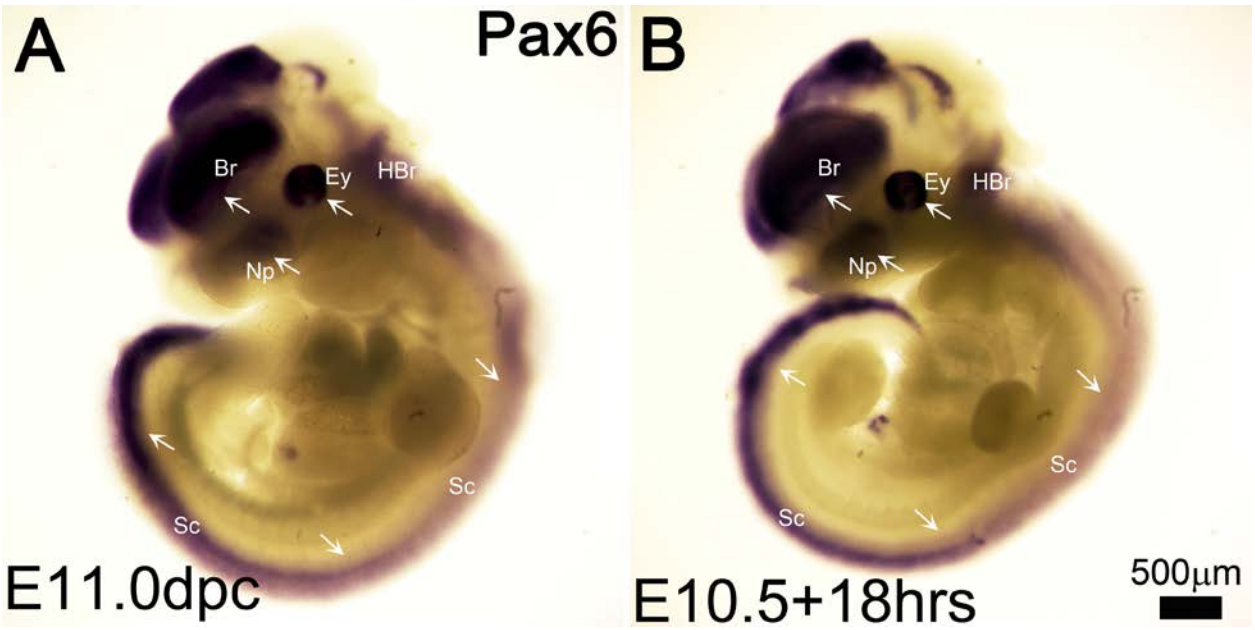
A
Pax6
Br
Ey
HBr
Np
Sc
Sc
E11.0dpc
B
Br
Ey
HBr
Np
Sc
Sc
E10.5+18hrs
500μm

Figure 3.4: Tissue specific gene expression maintained in ocular tissue in embryos developed in culture. Comparable expression pattern observed for tissue specific proteins like Pax6 (A, B), Sox2 (C, D), Prox1 (E, F), αA-crystallins (G, H) and FoxC1 (I, J) in ocular tissues developed in utero (A, C, E, G, I) and in culture (B, D, F, H, J). Oc – Optic Cup, L – Presumptive Lens, C – Presumptive Cornea. Arrow heads indicate cellular antibody staining. Scale bar in (J) applies to all panels.

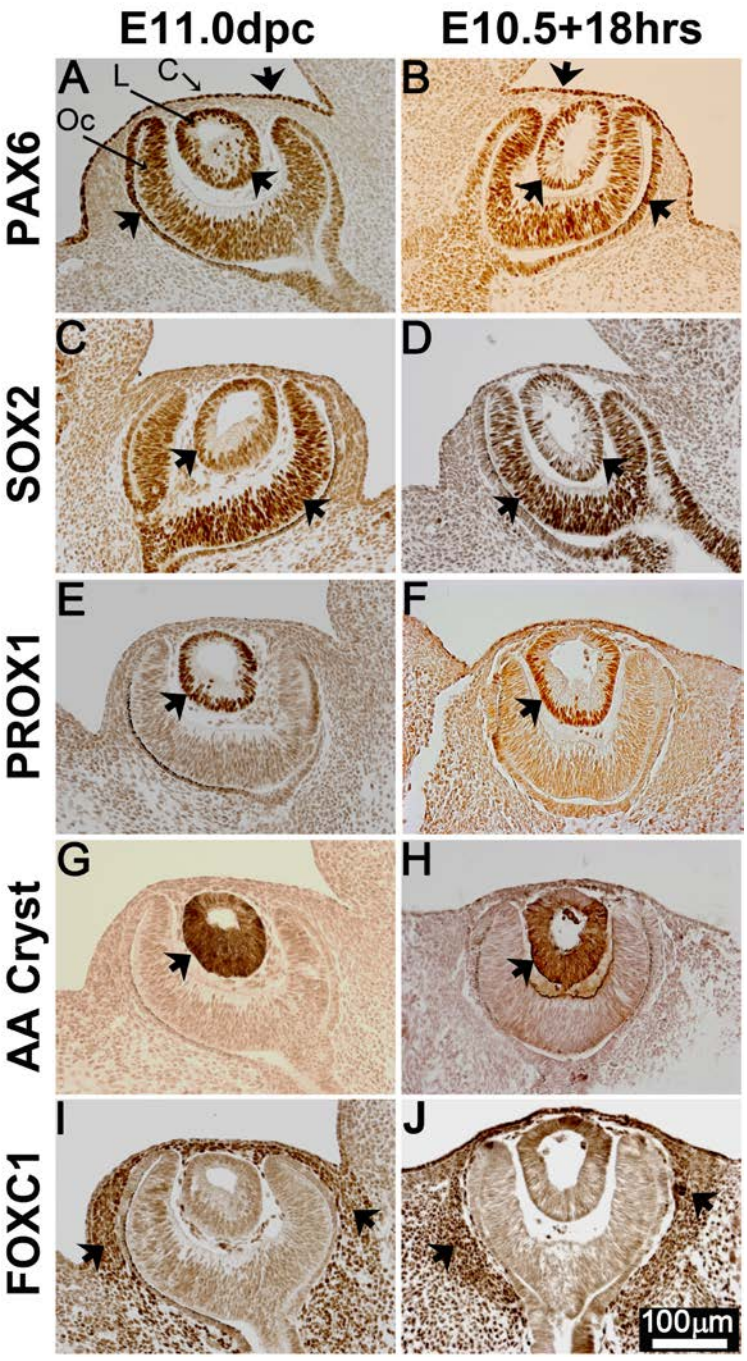
E11.0dpc
E10.5+18hrs
PAX6
SOX2
PROX1
AA Cryst
FOXC1
A
L
C
Oc
B
C
D
E
F
G
H
I
J
100µm

Table 3.1: Tissue integrity required for proper ocular development in culture. Ocular development is incomplete in optic rudiment culture. Optic rudiment culture system do not support corneal development, affects cellular orientation in lens and exhibit retarded growth of the eye tissue.

Table 3.1

Mouse Optic Rudiment Culture			
Type of Tissue	**Stage of Culture**	**Culture Conditions**	**Observation & Interpretation**
Optic Rudiment	E10.5 dpc	Defined media in 5% CO_2 incubator at 37 ^oC	Tissue survived but growth & development was retarded. Optic rudiment showed smaller morphological size. Corneal stromal development was not observed due to lack of migrating mesenchymal cells. Lens cell differentiation was observed but cellular orientation was disturbed.
Optic Rudiment	E11.5 dpc	Defined media in 5% CO_2 incubator at 37 ^oC	Optic rudiment showed better growth and morphological size compared to the optic rudiment cultured at E10.5dpc.
Optic Rudiment	E10.5 dpc	Media with serum in 5% CO_2 incubator at 37 ^oC	Growth and development was similar to the optic rudiments cultured in defined media alone.

Table 3.2: Oxygen plays a vital role in tissue survival in culture. Embryonic whole head tissues cultured in defined media with 95% O_2 / 5% CO_2 at 37 ^oC survived and exhibited developmental progression, while the embryonic whole head tissues cultured in 5% CO_2 incubator at 37 ^oC did not survive and exhibited tissue degeneration.

Table 3.2

Mouse Head Culture			
Type of Tissue	**Stage of Culture**	**Culture Conditions**	**Observation & Interpretation**
Whole head	E10.5 dpc	Defined media with 95% O_2 / 5% CO_2 in rolling bottle incubator at 37 ^oC	Explant tissue survived and ocular region exhibited development and cellular differentiation. Mesenchymal cell migration under corneal epithelium and lens cell orientation were disturbed.
Whole head	E11.5 dpc	Defined media with 95% O_2 / 5% CO_2 in rolling bottle incubator at 37 ^oC	Explant tissue survived and ocular region exhibited development and cellular differentiation. Mesenchymal cell migration under corneal epithelium and lens cell orientation were disturbed. Abnormal elongation of lens fiber cells observed.
Whole head / Head sagittal section / Head sagittal section with ventral or dorsal attachment	E8.5 / 9.5 / 10.5 dpc	Defined media in 5% CO_2 incubator at 37 ^oC	No morphological development of eye observed. Tissue did not survive. Degenerative changes observed.

Table 3.3: Mouse embryo culture system supports proper ocular development. Ocular growth comparable to in utero development was observed in whole embryos grown in oxygenated rolling bottle culture system. Embryos exteriorized from yolk sac with attachment at the umbilical region when cultured in a defined media with 95% O_2 / 5% CO_2 supply at 37 ^oC exhibited better development compared to the embryos without yolksac or with intact yolksac.

Table 3.3

Mouse Embryo Culture			
Type of Tissue	**Stage of Culture**	**Culture Conditions**	**Observation & Interpretation**
Embryo exteriorized with yolk sac attached at umbilical region	E10.5 dpc	Defined media with 95% O_2 / 5% CO_2 in rolling bottle incubator at 37 ^oC	Embryo survived and exhibited growth, morphological development and cellular differentiation. Lens cell orientation and neural crest mesenchymal cell migration to form corneal stroma were observed.
Embryo without yolk sac	E10.5 dpc	Defined media with 95% O_2 / 5% CO_2 in rolling bottle incubator at 37 ^oC	Tissue survived but growth & development was retarded. Smaller morphological size observed.
Embryo with intact yolk sac	E10.5 dpc	Defined media with 95% O_2 / 5% CO_2 in rolling bottle incubator at 37 ^oC	No morphological development of eye observed. Tissue did not survive. Degenerative changes were observed.

Chapter 4

BMP Signaling alters Neural Retina and Retinal Pigmented Epithelium Specification and Development[1]

[1] Kalaskar V. K., Lauderdale J. D. To be submitted to *Development*.

Abstract

Specification and development of neural retina (NR) and retinal pigmented epithelium (RPE) from optic vesicle involves multiple interactions among the members of WNT, BMP, FGF, SHH and other signaling pathways which are well conserved across different vertebrate species. However, significant differences can exist in the mechanisms they operate to regulate the expression of different transcription factors important in tissue specification and development. Using both gain and loss of function approaches, here we show that during early eye development BMP signaling exhibits a significant difference in modulating some of the key genes involved in NR and RPE specification and development in the mammalian system compared to that in avian species. Contrary to the results shown in chickens, we show that ectopic BMP signaling either inhibit or decrease the pigmentation in the mammalian RPE in a stage-dependent manner by down-regulating important genes involved in RPE specification and development such as *Mitf, Otx2* and *Pax6*. In the neural retina, ectopic BMP signaling altered expression of genes involved in dorsal-ventral patterning and down-regulated the expression of *Vsx2*, *Pax6*, *Sox2* and other early genes such as *Rx* and *Lhx2* known to be important in NR specification and development. However, unlike that observed in chicken studies, the NR did not reveal ectopic expression of RPE specific markers indicating no apparent transdifferentiation of the NR tissue. On the other hand, loss of BMP signaling when treated with ectopic Noggin protein resulted in opposite effects with upregulation of genes such as *Mitf* and *Pax6* along with changes in expression domains of genes involved in dorsal-ventral patterning. Further analysis revealed that BMP signaling mediated these effects on NR and RPE by modulating the members of WNT and SHH signaling pathways. Our results suggest a predominant role for BMP signaling in regulating the genes important in NR and RPE specification and development and indicate the

possibility for existence of significant differences in the mechanisms regulating organogenesis among vertebrate species.

Introduction

Vertebrate eye is a specialized neural structure that develops after a series of specification events involving the formation of neural plate from ectoderm, specification of anterior neural plate and eye field and finally the formation of eye structure (Chow and Lang, 2001; Graw, 2010). Though molecular mechanisms that specify the eye field start long back, the first morphological evidence of eye structure begins with the formation of optic vesicle from the evagination of diencephalon brain region during early neurula stage (Chow and Lang, 2001). The outgrowing optic vesicles interacts with the surface ectoderm and a complex series of inductive interactions specify the optic vesicle into neural retina and retinal pigmented epithelium structures. Multiple signaling mechanisms involving members of WNT, TGF-β, FGF and other signaling pathways along with various cell intrinsic factors have been implicated in regulating these specification processes (Chow and Lang, 2001; Fuhrmann, 2010; Graw, 2010; Zuber et al., 2003). Of these signaling mechanisms, Bone Morphogenetic Proteins belonging to TGF-β superfamily, have been implicated at multiple levels of these specification events. Members of BMP signaling were shown to mainly inhibit neural tissues and support development of non-neural epithelium and mesendodermal tissues (Kuroda et al., 2005; Levine and Brivanlou, 2007; Liem et al., 2000; McMahon et al., 1998; Pera et al., 2003). This is accomplished by the ubiquitous expression of BMP receptors such as BMPR1A (ALK 3) throughout the neural and non-neural tissues (Dewulf et al., 1995; Mishina et al., 1995).

The eye field specification in the anterior neural plate requires expression of specific transcription factors such as *Rx, Pax6* and *Six3* and misexpression studies in chickens with BMP4 revealed down-regulation of these eye field transcription factors affecting the development of optic primordia (Teraoka et al., 2009; Zuber et al., 2003). When optic vesicle begins to form from the evaginating forebrain, BMP's were not detected in the eye region but later BMP4 and BMP7 expression was detected in distal optic vesicle, surface ectoderm and surrounding periocular mesenchyme (Dudley and Robertson, 1997; Furuta and Hogan, 1998; Wawersik et al., 1999). BMP signaling was shown to be essential for optic vesicle to manifest its lens inducing activity and later it was shown to regulate the expression of genes involved in dorsal-ventral patterning of the neural retina (Behesti et al., 2006; Furuta and Hogan, 1998). After specification of neural retina and RPE from the optic vesicle, BMP4 expression gets restricted to the dorsal neural retina and BMP7 to the presumptive RPE with both expressed in the surrounding mesenchyme (Dudley and Robertson, 1997; Furuta and Hogan, 1998). Though the role of BMP signaling in early stages of neural and eye field specification was well characterized, its role in later stages of mammalian eye development after specification of neural retina and RPE from optic vesicle is not clear.

The optic vesicle is initially bipotential and can differentiate into NR and RPE in response to extrinsic signals emanating from the surrounding tissues such as the surface ectoderm and the periocular mesenchyme. The unspecified optic vesicle expresses a set of genes such as *Rx, Pax6, Otx2*, *Otx1, Pax6, Six3, Six6* and *Lhx2* which are later differentially distributed to individual domains upon specification into NR and RPE structures (Fuhrmann, 2010). After differentiation of optic vesicle, the NR retains the expression of *Rx, Pax6, Six3, Six6* and *Lhx2* while the RPE retains the expression for *Otx2, Otx1, Pax6* (Bovolenta et al., 1997; Crossley et

al., 2001; Martinez-Morales et al., 2001; Ohuchi et al., 1999; Teraoka et al., 2009; Zimmer et al., 1991). However considerable difference in the optic vesicle initial expression of *Mitf* gene has been shown to exist in mammals compared to chickens (Muller et al., 2007). In chickens, the expression of *Mitf* is restricted to the distal optic vesicle while in mice it is shown to express all over the optic vesicle (Muller et al., 2007; Nguyen and Arnheiter, 2000). This differential expression of *Mitf* could be one of the reasons to consider the chicken optic vesicle to be already specified even before its contact with the surface ectoderm. Studies revealed that, when the dorsal and ventral halves of chicken optic vesicle were cultured separately they were fated to become RPE and NR structures respectively (Kagiyama et al., 2005). The optic vesicles in mice and chickens also exhibit differences in responding to the presence or absence of extrinsic signals from the surrounding environment. For instance, at the time when optic vesicle differentiates into NR and RPE, the surface ectoderm has been shown to be the source of FGF signaling which induces neural retinal markers in the optic vesicle (Hyer et al., 1998; Pittack et al., 1997). When the surface ectoderm or the source of FGF signaling was removed in the mouse embryos, the distal optic vesicle which is near to the surface ectoderm and which usually forms into NR will now form into RPE-like structure with a single epithelium of cuboidal cells (Nguyen and Arnheiter, 2000). While in chickens, removal of surface ectoderm resulted in the proximal optic vesicle to still retain its multilayered structure typical of NR differentiation with very few NR-like cells intermingled with pigmented cells (Hyer et al., 1998). However, in both chickens and mice, the addition of FGF proteins to these optic vesicles which lack surface ectoderm could rescue proper patterning of the optic vesicle into NR and RPE like structures (Hyer et al., 1998; Nguyen and Arnheiter, 2000). While FGF signals have a role in patterning the NR domain from the optic vesicle, studies in chickens indicate that signals emanating from the

surface ectoderm and periocular mesenchyme are important in RPE development (Fuhrmann et al., 2000; Kagiyama et al., 2005; Martinez-Morales et al., 2004; Muller et al., 2007).

The specification and development of RPE as well as the expression of genes responsible for pigmentation are sensitive to the expression of specific transcription and signaling factors. Studies indicate that apart from *Mitf*, transcription factors such as *Otx2*, *Pax6* and *Pax2* and factors related to WNT signaling are important in RPE development and pigmentation (Baumer et al., 2003; Bharti et al., 2012; Bharti et al., 2008; Bumsted and Barnstable, 2000; Fujimura et al., 2009; Martinez-Morales et al., 2004; Martinez-Morales et al., 2001; Westenskow et al., 2009). Studies show that RPE development and *Mitf* expression were affected in $Pax2^{-/-}$;$Pax6^{-/+}$, $Otx1^{-/-}$;$Otx2^{-/+}$ mouse embryos and that Pax2 and Pax6 regulate *Mitf* expression by binding to promoter regions on *MITF* gene (Baumer et al., 2003; Martinez-Morales et al., 2001). Otx2 was shown to interact with *Mitf* in a regulatory loop and as well as to bind and transactivate pigmentation genes such as Tyrosinase, TRP-1 and TRP-2 (Martinez-Morales et al., 2003; Takeda et al., 2003). Chromatin immunoprecipitation and luciferase assays revealed that β-Catenin binds near to and activate potential TCF/LEF sites in *Mitf* and *Otx2* enhancers to regulate the RPE development (Fujimura et al., 2009; Westenskow et al., 2009). Apart from cell intrinsic factors, studies in chickens show that members of TGF-β signaling, BMP's and Activins expressed in the periocular mesenchyme and / or surface ectoderm are essential for RPE development. Optic vesicle explant cultures in the absence of these tissues resulted in lack of RPE specification in the optic vesicles (Fuhrmann et al., 2000; Kagiyama et al., 2005). Ectopic implantation of beads treated with either BMP's or Activins was shown to induce the formation of RPE tissue in the proximal as well as distal optic vesicle as revealed by the expression of *Mitf*

and other RPE specific markers and down-regulation of NR specific genes (Fuhrmann et al., 2000; Muller et al., 2007).

Though the studies in chickens indicate the role of BMP signaling in inducing *Mitf* expression and thereby in RPE development, it is not clear whether similar mechanisms exists in the mammalian RPE development. Studies revealed a considerable difference in the expression pattern of *Mitf* gene and the response of optic vesicle to external signaling factors such as FGF's in the chickens compared to mice. Apart from these, though BMP's are expressed in the optic vesicle, their role in NR specification and development in the mammalian embryos is not clear. To address these questions and to elucidate the role of BMP signaling in mammalian NR and RPE development we utilized a mouse embryo culture system and manipulated the BMP signaling with a gain and loss of function approaches. We show that, contrary to the results shown in chickens, ectopic BMP's affect pigmentation in the mammalian RPE in a stage-dependent manner. BMP signaling affected the expression of several genes important in NR and RPE specification and development through modulating the members of WNT and SHH signaling pathways. Our study indicates an important role for BMP signaling in mammalian NR and RPE development and reveals the possibility for significant differences in the mechanisms regulating organogenesis among vertebrate species.

Methods

Mouse embryo culture and Bead Implantation

Mice were maintained and handled following standard protocols in accordance with institutional policies. The following mice strains were used: wild type mice belonging to C57BL/6J (Taconic) and CD-1 (Charles River Laboratories) genetic background, transgenic

mice, Tg293d08#3 or #15 (Kim and Lauderdale, 2006), TCF/LEF-GFP mice (Jackson labs). Mouse embryos at different time points in gestation were obtained by breeding wild type mice and transgenic mice considering the noon on the day of finding the vaginal plug as 0.5 dpc. The mouse embryo culture method was performed following our protocol in Journal of Visualized Experiments and as previously described (Kalaskar and Lauderdale, 2014; Moore-Scott et al., 2003). Embryos were implanted in dorsal eye region with affi-gel blue gel agarose beads treated with proteins such as BMP4 (100 µg/ml), Noggin (1 mg/ml) or DKK1 (Sigma # PHC9214; 400 µg/ml) in left eye and those treated with BSA (1 mg/ml) protein in right eye following protocol previously described (Behesti et al., 2006). Briefly the implantation procedure involved making a small opening in the surface ectoderm by piercing with a micro-tipped glass needle followed by inserting the agarose bead using a pair of tweezers (Roboz # 55; Cat.# RS-5063). The embryos were cultured in 3ml medium in a rolling bottle apparatus (Moore-Scott et al., 2003) at 37 ^{o}C and 95% O_2 / 5% CO_2 for 10 to 18 hours. Embryonic ocular development at different time points during culture was captured under a dissecting microscope using Spot imaging software and later processed with image processing software.

Immunofluorescence

Immunofluorescence was performed on cryosections prepared from sucrose infiltrated tissues using primary antibodies such as, Mouse monoclonal anti-Mitf (1:6000; Abcam, ab12039), Goat anti-Chx10 (Vsx2) (1:300; Santa Cruz, sc21690), rabbit anti-C-terminal Pax6 (1:500; Covance), rabbit anti-Sox2 (1:1000; Chemicon, AB5603), rabbit anti-Phosphorylated Smad 1/5/8 (1:100; Cell Signaling Technology, 9511S), rabbit anti-cleaved PARP (1:100; Cell Signaling Technology, 9544), rabbit anti-cleaved Caspase3 (1:200; Cell Signaling Technology) followed by donkey anti-rabbit Alexa 594 secondary antibody (Jackson labs) following standard

protocols. Fluorescent images were captured using AxioVision software and processed later with image processing software.

Whole mount In situ hybridization

Whole mount in situ hybridization was performed on mouse embryos developed in utero and in culture following the protocol as described (Carpenter et al., 1993; Manley and Capecchi, 1995; Wei et al., 2011). DIG labeled sense and antisense RNA probes were prepared as previously described for *Pax6* (Kim and Lauderdale, 2006), *Mitf* (Open Biosystems, Clone # 40047440), Tyrosinase (*Tyr*) (by PCR primers; Table 4.2), *Otx1* (Kim and Lauderdale, 2008), *Otx2* (Rhinn etal., 1998), *Wnt13* (Kim and Lauderdale, 2008), *Vsx2* (Open Biosystems, Clone # 6492679), *Vax2* (Mui SH, 2002), *Tbx5* (Agulnik etal., 1996; Chapman etal., 1996), *Rx* (Mathers etal., 1997) and *Six3* (G. Oliver, 1995). Hybridization with the probes was performed overnight at 65 ^{o}C and posthybridization washes were done at 70 ^{o}C. DIG-labeled probes were detected using Alkaline phosphatase-conjugated anti-digoxigenin Fab fragments at 1:2000 dilution followed by color reaction with NBT/BCIP substrate (Roche) carried out for 4 - 5 hours at room temperature or overnight at 4 ^{o}C. After color development the embryos were postfixed in 4% paraformaldehyde / PBS overnight at 4 ^{o}C. The embryos were imaged under bright field dissecting microscope using AxioVision software and processed.

Quantitative Real-Time PCR (qPCR)

Mouse embryonic eyes from culture and in utero developed embryos were used to prepare RNA using Trizol reagent following standard protocols. Twenty or more BMP4 or Noggin or BSA treated eyes were pooled together in a single biological sample for RNA extraction. Three such biological replicates were prepared and cDNA was made from 1µg of RNA using Revert Aid first strand cDNA synthesis kit (Thermo Scientific # K1622). Three

technical replicates were performed for each reaction with 10-20 ng cDNA. The gene expression was initially normalized to two or three of the internal genes such as *β-actin, Gapdh* or *α-Tubulin* and later the expression in BMP4 or Noggin treated eyes was shown as relative to the expression observed in control BSA treated eyes. Statistical analysis done using two-tailed student t-test and error bars were calculated using standard error of mean and statistical significance denoted as (***) for P-Value (≤0.001), (**) for P-Value (≤0.01) and (*) for P-Value (≤0.05). For a complete list of primers please see Table 4.2.

Results

BMP4 affects RPE pigmentation in a stage dependent manner

The role of BMP signaling in mammalian RPE development and pigmentation was analyzed by employing both gain and loss of function approaches utilizing a mouse embryo culture system that we established for supporting ex utero embryonic eye development ((Kalaskar and Lauderdale, 2014); *Kalaskar and Lauderdale, in preparation*). Affi-gel blue gel agarose beads treated either with human recombinant BMP4 or Noggin proteins were implanted dorsal to retina in the left eye and those treated with BSA protein were placed in the right eye which served as a control. The embryos were cultured for a time period ranging from 10 – 18 hours to determine the effect of BMP signaling on RPE development and pigmentation (Figure 4.1A).

The development of RPE begins with the differentiation of optic vesicle into neural retina and RPE domains under the influence of signaling interactions from surface ectoderm and surrounding mesenchymal cells (Fuhrmann et al., 2000; Martinez-Morales et al., 2004; Muller et al., 2007). Later the RPE starts to differentiate at E33 – 34 ss with the formation of pigmentation

first visible in the dorsal most region which later extends to other regions of the RPE. We first tested the role of BMP signaling in initial development of RPE by implanting BMP4 treated beads in the dorsal optic vesicle in mouse embryos cultured at E29 – 31 ss before any visible sign of RPE pigmentation is evident (Figure 4.1B, C). Ectopic over-expression of BMP4 at this stage resulted in arrest in development of pigmentation even after 16 hours of embryonic development in the culture (Figure 4.1C). During this time period, the embryos in culture added 5 – 6 somites and exhibited development comparable to embryos at E35 – 37 ss developed in utero. The right eye that was implanted with BSA treated bead showed clear formation of pigmentation in the RPE which was comparable to that observed in embryos developing in utero (Figure 4.1B). The arrest in development of pigmentation indicates three possibilities, first, the development of RPE is halted and the tissue remains in an undifferentiated state; second, the ectopic BMP4 is just inhibiting the mechanisms or genetic pathways involved in RPE differentiation resulting in no pigmentation and the third possibility would be that the RPE cells are converted to other tissue type and in this case most likely to a neural retina like structure. It is evident from similar experiments in chickens and other species that RPE can transdifferentiate into neural retina and vice versa upon exposure to certain signaling proteins (Fuhrmann et al., 2000; Lane and Lister, 2012; Muller et al., 2007). We tested the first two scenarios by ectopic implantation of BMP4 treated beads just after the first appearance of visible pigmentation in the dorsal most region of RPE at E33 - 34 ss (Figure 4.1D, E). In these BMP4 treated eyes, the dorsal patch of RPE cells that already started expression of pigmentation retained their pigment but did not reveal any increase in the intensity of pigmentation in these cells. The adjacent RPE cells that have not started the pigmentation remained unpigmented even after 16 hours of culture (Figure 4.1E). The contralateral eye that had the BSA treated bead implantation showed

increased intensity in pigmentation of the dorsal patch of cells that had already initiated the pigmentation and also revealed the appearance of pigmentation in adjacent RPE cells farther away from the dorsal region (Figure 4.1D). This clearly indicates that differentiation of RPE is inhibited in the undifferentiated cells exposed to BMP4 and in the cells that are already differentiated (pigmented) the mechanisms and genetic pathways involved in generation of RPE pigmentation were arrested from progression. Studies in chickens showed that ectopic BMP4 application resulted in development of RPE in the optic neuroepithelium as revealed by expression of *Mitf*, *Otx2* and other downstream pigmentation genes (Muller et al., 2007). This prompted us to investigate the effect of ectopic BMP4 on the genes involved in regulation of RPE development and pigmentation in the mammalian embryos.

Genes involved in RPE pigmentation affected by BMP signaling

Pigmentation in RPE and other areas on the body such as the skin is mainly regulated by key melanogenic proteins such as tyrosinase (Tyr), a rate limiting enzyme responsible for oxidation of amino acid tyrosine to dopa and dopa quinine, and its related genes such as, tyrosinase related protein-1 (TRP-1) and TRP-2 (Bharti et al., 2008; Park et al., 2009; Slominski et al., 2004). We tested the effect of ectopic BMP4 on the expression of *Tyr* at both the stages before and after the visible sign of pigmentation (Figure 4.2A-D). Similar to the effect on pigmentation, *Tyr* expression was totally lacking in the eyes in which BMP4 treated beads were implanted before the visible sign of pigmentation and partial or decreased expression was observed in the eyes that were implanted after the onset of visible pigmentation (Figure 4.2B, D). The contralateral eyes implanted with BSA treated beads in these embryos showed good expression of *Tyr* in the RPE layer of the eye (Figure 4.2A, C). We confirmed this effect of

BMP4 by quantifying the expression of tyrosinase and one of its related genes, TRP-1 by qPCR. The expression levels for *Tyr* and TRP-1 were significantly decreased in the eyes treated with BMP4 compared to the control eyes treated with BSA protein (Figure 4.2G). This indicates that the genes responsible for pigmentation are affected which could possibly be mediated by effect on other regulatory genes involved in RPE development and pigmentation pathway. *Tyr* is transcriptionally regulated by Mitf, an important gene responsible for RPE specification and development (Bharti et al., 2008; Nakayama et al., 1998; Yasumoto et al., 1994; Yasumoto et al., 1997). In mouse embryonic eyes, *Mitf* is expressed throughout the optic vesicle but RPE is specified only when its expression is later restricted to the proximal domain of the optic vesicle upon its differentiation into NR and RPE (Martinez-Morales et al., 2004). Studies indicate that loss of function mutations in *Mitf* gene resulted in loss of pigmentation, absence of RPE and microphthalmia (Hodgkinson et al., 1993; Nakayama et al., 1998). This indicates the requirement of *Mitf* expression for RPE specification and development and as well as in differentiation by regulating the formation of pigmentation. Exposure to ectopic BMP4 resulted in inhibition of *Mitf* expression in the dorsal region and as well as in lateral regions all along the RPE. However the *Mitf* expression farther away from the region of bead placement still persisted but very much decreased compared to the control (Figure 4.2E, F). A significant reduction in *Mitf* levels was observed upon performing qPCR on cDNA collected from eyes treated with BMP4 compared to the control (Figure 4.2G). The decrease in *Mitf* expression in the RPE in BMP4 treated eyes was also confirmed by immunofluorescence (data not shown). We then tested whether loss of BMP signaling in these eyes by Noggin treatment would result in increased expression of *Mitf* and downstream pigmentation genes like *Tyr*. As expected the *Mitf* and *Tyr* expression levels were upregulated in the noggin treated eyes compared to the control (Figure 4.2H, I and data not

shown). These results clearly indicate that BMP signaling regulates the genes important for RPE development and formation of pigmentation.

BMP signaling regulates early genes involved in RPE specification and development

Studies show that *Mitf* expression in the RPE can be regulated by many transcription factors such as *Otx1*, *Otx2*, *Pax6* and *Pax2* (Baumer et al., 2003; Fujimura et al., 2009; Martinez-Morales et al., 2001). However the role of BMP signaling in modulating the expression of these genes in the mammalian RPE has not been studied. We evaluated the expression of these genes in BMP4 treated eyes and compared to that observed in BSA treated control eyes. Studies show that *Otx1* and *Otx2* genes are initially expressed throughout the optic vesicle and later their expression is restricted to the RPE layer (Bovolenta et al., 1997; Martinez-Morales et al., 2001; Simeone et al., 1993). In $Otx1^{-/-}$ mouse embryos the RPE showed no developmental defects, however in $Otx1^{-/-}$ / $Otx2^{-/+}$ embryos the RPE was almost absent or only a dorsal patch of RPE with *Mitf* expression was observed (Martinez-Morales et al., 2001). This indicates an important role for *Otx2* in RPE development compared to *Otx1* in mouse embryos (Martinez Morales et al 2001). In BMP4 treated eyes, *Otx1* expression in the RPE was not much altered but a slight upregulation was observed in the retinal region compared to the control (Figure 4.3A, B). However this slight increase in *Otx1* expression was not significant as revealed by the qPCR analysis of these ocular tissues (Figure 4.3I). On the other hand, *Otx2* expression in the BMP4 treated eyes was very much down-regulated as revealed by in situ hybridization and was confirmed to be significant by qPCR analysis (Figure 4.3C, D, I). Apart from *Otx* genes, *Pax6* transcription factor which is expressed in RPE and NR has been shown to have binding sites on the *Mitf* gene locus and directly regulate *Mitf* expression (Baumer et al., 2003; Bharti et al.,

2012). However previous studies in chickens with ectopic BMP4 treatment showed differential effects on *Pax6* expression at different stages (Teraoka et al., 2009). While studies in mice revealed that BMP4 and *Pax6* work independently and *Pax6* expression was not altered in BMP4^{tm1} mutant eyes and similarly BMP4 expression was not altered in the eyes of *Pax6*$^{sey\text{-}1Neu}$ mouse embryos (Furuta and Hogan, 1998). We tested this scenario by first utilizing transgenic mouse embryos which carry a *Pax6-GFP* BAC transgenic construct and express *GFP* wherever *Pax6* gene is active in the body (Kim and Lauderdale, 2006). Treatment with ectopic BMP4 in these mouse embryonic eyes revealed a significant reduction in the expression of *Pax6* driven *GFP* in the RPE and retina (Figure 4.3E, F). We confirmed this change in *Pax6* expression in wild type embryos treated with ectopic BMP4 by immunofluorescence and in situ hybridization (Figure 4.3G, H and data not shown). We quantified the *Pax6* expression levels to be significantly decreased in BMP4 treated eyes compared to the control eyes by qPCR analysis (Figure 4.3I). Studies using *Pax2*$^{-/-}$, *Pax6*$^{-/+}$ and *Pax2*$^{-/-}$;*Pax6*$^{-/+}$ double mutant mice showed that *Pax6* and *Pax2* together regulate *Mitf* expression (Baumer et al., 2003). The expression of *Pax2* in BMP4 treated eyes was not significantly altered as revealed by qPCR analysis (data not shown). On the other hand, implantation of noggin treated beads increased the *Pax6* expression levels in the eye compared to the control (Figure 4.3J, K).

Ectopic BMP4 down-regulates *Vsx2* gene expression and alters the expression domains of genes involved in dorsal-ventral patterning of retina.

Pax6 has been shown to be important in retinal development and retinal progenitor cell specification and differentiation (Chow and Lang, 2001; Grindley et al., 1995; Hsieh and Yang, 2009; Zimmer et al., 1991). The evidence that ectopic BMP4 affected the expression of *Pax6* in

NR prompted us to further investigate other key genes involved in NR development and specification at this time point in development. It has been well demonstrated that BMP4 has a role in dorsal-ventral patterning of the retina by altering the expression of genes in dorsal and ventral domains of NR (Behesti et al., 2006; Kim and Lauderdale, 2008; Kobayashi et al., 2010; Zhao et al., 2010). We first investigated some of the BMP4 target genes like *Msx1* and genes in the dorsal domain such as T-box genes like *Tbx5, Tbx2* and in ventral domain such as *Vax2*. Ectopic BMP4 significantly up-regulated the expression of *Msx1*, *Tbx5* and *Tbx2* and down-regulated *Vax2* expression as revealed by qPCR and in situ hybridization (Figure 4.4A-D, K). We cross checked these gene alterations by implanting Noggin treated beads and as expected the expression of *Tbx5* was significantly decreased while that of *Vax2* was up-regulated (Figure 4.4E-H, K). These changes in gene expressions confirms previous studies indicating the role for BMP signaling in regulating dorsal-ventral patterning of the NR (Behesti et al., 2006). We then tested for *Vsx2* (*Chx10*) expression which is the first gene in the distal optic vesicle shown to differentiate the NR domain from the RPE. At the initial stages when NR differentiates from optic vesicle, the *Vsx2* is expressed all over the neural retina (Fuhrmann, 2010; Graw, 2010; Horsford et al., 2005). *Vsx2* expression in BMP4 treated eyes showed significant reduction at both mRNA and protein levels as revealed by the immunofluorescence, in situ hybridization and qPCR (Figure 4.4I-K and data not shown).

Early genes involved in retinal specification and development affected in BMP4 treated eyes

The evidence that the expression of *Vsx2* and *Pax6* genes is altered in the eyes treated with BMP4 indicates the possibility that other early genes involved in NR development might

also be affected in these eyes. Genes expressed in the early eye field such as *Rx, Six3, Six6, Lhx2, Otx2, Sox2* and *Pax6* have been shown to be required for early specification of optic primordium and as well as in later establishment and development of NR in the optic vesicle (Chow and Lang, 2001; Mathers et al., 1997; Porter et al., 1997; Zhang et al., 2000; Zuber et al., 2003). In $Rx^{-/-}$ embryos the eyes do not develop and the expression of *Pax6, Six3* and *Otx2* is not upregulated in the area of the anterior neural plate that would form the primordium of the optic vesicle. While in Small eye embryos that lack functional *Pax6* gene, the expression of *Rx* is not altered in the optic vesicle indicating the dependence of *Pax6* and other genes on functional *Rx* gene in optic vesicle specification (Zhang et al., 2000). In mouse embryos with *Rx*-Cre mediated conditional deletion of *Six3* in the eye region, the NR specification is abrogated and the expression of *Vsx2, Six6, Rx* and *Sox2* was absent and similarly in $Lhx2^{-/-}$ embryos, the eye development is halted at optic vesicle stage (Liu et al., 2010; Porter et al., 1997). Studies in chickens with ectopic BMP4 implantation resulted initially in down-regulation of *Rx* gene only but later both *Rx* and *Pax6* genes were down-regulated (Teraoka et al., 2009). We examined whether the ectopic BMP4 in mouse embryonic eyes had any effect on the expression of some of these genes such as *Rx, Six3, Sox2* and *Lhx2*. In the BMP4 treated eyes the expression of *Rx* in the NR was very much down-regulated when implanted at both early and late stages and a significant reduction was confirmed by qPCR analysis (Figure 4.5A, B, E). Similarly, the expression levels of *Lhx2* and *Sox2* were significantly reduced as revealed by qPCR analysis (Figure 4.5E). However the expression of *Six3* was not altered in these BMP4 treated eyes compared to the control (Figure 4.5C-E).

In addition to the early genes, studies in mice and chickens have established that during specification, FGF signaling from the surface ectoderm induces expression of NR specific

markers (*Vsx2*) in the distal optic vesicle (Hyer et al., 1998; Nguyen and Arnheiter, 2000; Pittack et al., 1997). Concurrent with this event, the expression of BMP4 eventually gets confined to the dorsal region of the optic vesicle (Fuhrmann, 2010; Furuta and Hogan, 1998). This indicates that BMP4 may be antagonistic to FGF signaling but studies in chicks and mice reveal contradictory evidence on their interaction in NR. Studies in chickens have shown that ectopic BMP4 downregulated *FGF8* as well as *Rx* and *Vsx2* expression in NR while that in mice ectopic BMP4 rescued expression of *FGF15* in BMP4$^{-/-}$ optic vesicle explant cultures and analysis of *BMPR1A*$^{-/fx}$;*BMPR1B*$^{-/-}$;Cre double mutant embryos revealed diminished expression of *FGF15* in the NR (Muller et al., 2007; Murali et al., 2005). However when tested for expression of FGF members in our BMP4 treated eyes, we did not find any significant change in the expression of *FGF15* and other members such as *FGFR2*, *Sprouty-1* and *Sprouty-2* as revealed by qPCR analysis (Figure 4.5E, data not shown). These results suggest that the downregulation of *Vsx2* expression may be mediated by BMP4 effect on early markers such as *Rx*, *Pax6*, *Lhx2* and *Sox2* in the NR. These results indicate that BMP signaling regulates the expression of certain early and late genes important in optic vesicle and NR specification and development.

BMP signaling regulates NR and RPE development by modulating the members of WNT and SHH signaling

Apart from cell intrinsic transcription factors, cell autonomous mechanisms involving members of WNT and SHH signaling pathways were shown to have important role in NR and RPE development (Fuhrmann, 2010). In RPE, members of WNT signaling have been shown to directly regulate the expression of *Otx2* and *Mitf* genes and conditional knockout of WNT/β-Catenin signaling in RPE was shown to affect RPE development and pigmentation (Fujimura et

al., 2009; Westenskow et al., 2009). While in NR, multiple members of WNT family were shown to be expressed and have a role in retinal neurogenesis and regulation of retinal progenitor cell proliferation (Lad et al., 2009; Van Raay et al., 2005). To test whether BMP signaling has any effect on WNT signaling in the eye we utilized a WNT reporter mice wherein GFP is expressed under the control of TCF/LEF genes which are the downstream pathway genes in canonical WNT/β-Catenin signaling. Ectopic BMP4 treatment in these embryos resulted in significant decrease in GFP expression in the RPE and NR cells compared to the control (Figure 4.6A, B). We confirmed this effect on WNT signaling in BMP4 / BSA treated wild type embryos by testing for the expression of *WNT13* (*WNT2b*) which has been shown to be active in the RPE (Fujimura et al., 2009; Zakin et al., 1998). The expression of *WNT13* was significantly reduced in BMP4 treated eyes in wild type embryos compared to the control (Figure 4.6C, D). On the other hand, loss of BMP signaling in noggin treated eyes resulted in significant upregulation of WNT signaling in the NR and RPE compared to the control as revealed by the TCF/LEF-GFP expression (Figure 4.6F, G).

Further analysis of the BMP4 treated eye tissues for other potential genes involved in regulating WNT signaling revealed that *DKK1*, a potent antagonist of WNT/β-catenin signaling, was significantly upregulated (4 fold) while that of another WNT antagonist, *sFRP1* was significantly reduced. In addition there was a significant reduction in the expression of *β-catenin* and *Tcf4* levels indicating a possibility for DKK1 mediated reduction in canonical WNT/β-catenin signaling by the ectopic BMP4 (Figure 4.6E). However when DKK1 treated beads were implanted in the eyes, the expression of TCF/LEF-GFP which represents WNT signaling was reduced but no significant effect on RPE pigmentation was evident (data not shown). This is consistent with previous studies in mice and chickens wherein treatment with DKK1 or DKK3

alone resulted in no effect on RPE expression of *Mitf* but when treated with DKK1 along with FGF2 or with sFRP1 alone, the *Mitf* expression was downregulated (Bharti et al., 2012; Steinfeld et al., 2013). The evidence that DKK1 alone could not downregulate RPE pigmentation indicates that ectopic BMP4 altered some other factor in addition to DKK1 to downregulate the WNT signaling in RPE and NR. These results indicate that BMP signaling regulates the RPE and NR development by affecting the expression of genes involved in WNT signaling.

Apart from WNT signaling, members of sonic hedgehog (SHH) signaling were shown to regulate proliferation and cell cycle exit in retinal cells, development of optic stalk and ventral retina by regulating the expression of genes such as *Pax2*, *Vax1* and *Vax2* and also in maintenance of RPE cell fate in the ventral optic cup (Fuhrmann, 2010; Huh et al., 1999; Lee et al., 2001; Prykhozhij, 2010; Zhang and Yang, 2001; Zhao et al., 2010). We analyzed the effect of BMP signaling on some of the members and targets of SHH signaling in these BMP4 treated eyes (Figure 4.6F). We observed a significant reduction in the expression levels of SHH signaling members such as *Gli2*, Smoothened (*Smo*) and *SHH* and as well as in its target genes such as *Sox8* in the BMP4 treated eyes compared to the control (Figure 4.6F). Thus our results indicate a predominant role for BMP signaling in regulating the genes important in NR and RPE specification and development by tightly modulating the expression levels of members of WNT and SHH signaling.

Discussion

During early stages after specification, NR and RPE are highly sensitive and responsive to extrinsic signals such as FGF's and BMP's as revealed by misexpression studies in chicks and mice (Bharti et al., 2012; Fuhrmann, 2010; Fuhrmann et al., 2000; Muller et al., 2007; Nguyen

and Arnheiter, 2000). In this study we show that these tissues in the mammalian system exhibit a differential response to extrinsic BMP signaling compared to that in avians. Following specification of NR and RPE, members of BMP signaling are expressed in the distal optic vesicle, surface ectoderm and also in periocular mesenchyme along with ubiquitous expression of their receptors *BMPR1A* and *BMPR1B* (Dudley and Robertson, 1997; Furuta and Hogan, 1998; Muller et al., 2007). Though the source of signaling is not clear, studies in chickens have shown that ectopic BMP's support RPE development and when misexpressed can inhibit NR with induction of *Mitf* and other markers of pigmentation (Muller et al., 2007). Contrary to this, here we show that in mice, ectopic BMP signaling interferes with RPE development by downregulating important transcription factors such as *Mitf*, *Otx2*, and other downstream pigmentation genes (Figure 4.1-4.3). In NR, a similar mechanism was observed wherein the ectopic BMP's downregulated *Vsx2*, *Pax6* and other early genes involved in NR specification in the optic vesicle (Figure 4.3-4.5). However, contrary to the results shown in chicks, though most of the tissue specific genes were downregulated, no apparent transdifferentiation of the presumptive NR was evident as none of the RPE specific genes were expressed in the NR domain. The loss of function experiments resulted in upregulation of these key genes and further analysis revealed that the BMP signaling mediated these effects by modulating the members of WNT and SHH signaling (Figure 4.6). Our results in addition to previous reports strongly indicate the existence of considerable differences between avian and mammalian species in the expression pattern of certain genes like Mitf and response mechanisms to external signaling factors in these ocular tissues (Table 1).

In the developing RPE, ectopic BMP signaling has potentially inhibited the process of differentiation as determined by the effect on pigmentation (Figure 4.1). Ectopic BMP4 arrested

the formation of pigmentation in unpigmented and pigmented cells, which was consistent with the effect on expression of key melanogenic proteins such as *Tyr* in the RPE (Figure 4.2). The expression of *Tyr* that was already present at the time of ectopic BMP treatment was not altered but further transcription of the mRNA was arrested. The *Mitf* expression was significantly downregulated in the vicinity of the BMP4 bead although some expression was observed further away in the RPE. Though the differential action of BMP signaling on *Mitf* expression in the RPE in mice compared to chickens is not clear, studies in human melanocytes indicate that BMP4 activates MAPK/ERK which causes a transient increase in *Mitf* expression followed by proteasome-mediated degradation of *Mitf* (Park et al., 2009). The differential response could also be accounted to the dosage of BMP4 used (we used 100 µg/ml per 10 µl of agarose beads compared to 1000 µg/ml per 10 – 15 agarose beads used in chicken studies by Muller et al., 2007), the developmental stage and the length of treatment as it is evident from previous studies that BMP4 specifies different cell fates depending on concentration gradient (Simeoni and Gurdon, 2007; Wilson et al., 1997). We observed similar outcome when treated for a range of time period from 10 to 18 hours. Although some of the BMP4 treated eyes appeared smaller than the control, we observed no apoptosis when stained for cleaved caspase 3 and cleaved PARP in RPE layer except for few cells in the dorsal most region in NR which is consistent with previous reports (data not shown) (Behesti et al., 2006). Apart from *Mitf*, the key upstream genes involved in RPE development and specification such as *Pax6*, *Otx2* and *WNT13* were all downregulated in response to BMP4 treatment. Detailed analysis of the WNT signaling genes revealed four-fold upregulation of *DKK1* with concurrent downregulation of downstream genes such as *β-catenin* and *Tcf4* which strongly indicated the involvement of canonical WNT/β-catenin signaling (Figure 4.6). However, the inability of DKK1 to downregulate WNT signaling as revealed by

TCF/LEF-GFP expression (data not shown) suggests two possibilities, 1) Ectopic BMP4 altered some other factors in addition to DKK1 to downregulate the WNT signaling in RPE and 2) The involvement of a non-canonical WNT signaling as previously indicated (Steinfeld et al., 2013). Despite downregulation of these genes, the RPE did not undergo transdifferentiation as no evidence of expression of NR specific genes was observed.

In the developing NR, downregulation of *Vsx2*, *Pax6*, *Rx*, *Lhx2* and *Sox2* indicates the anti-neural activity of BMP signaling. Apart from these genes, members of FGF signaling have been shown to have a role in induction and subsequent development of NR (Hyer et al., 1998; Nguyen and Arnheiter, 2000; Pittack et al., 1997). However, contrary to results in chicks, our results showed that ectopic BMP4 does not affect the expression of members of FGF signaling in the eye at least at the time point we tested (Muller et al., 2007). Apart from this, we observed significant upregulation of dorsal genes such as *Tbx5*, *Tbx2* and *Msx1* and downregulation ventral genes such as *Vax2* in the NR in BMP4 treated eyes. In some of these eyes the ventral regions in NR showed reduction in size while in others they appeared normal. Studies indicate that BMP4 and SHH induce dorsal and ventral fates respectively and are implicated in regulating the dorsal-ventral patterning of NR and RPE structures (Fuhrmann, 2010; Kobayashi et al., 2010; Sasagawa et al., 2002). The evidence that ectopic BMP signaling affected the expression of some of the members of SHH pathway as analyzed by qPCR strongly support previous reports and indicate BMP signaling in supporting dorsal fates by suppressing SHH mediated ventral fates during dorsal-ventral patterning of the NR and RPE. Taken together our results indicate an important role for BMP signaling not only in dorsal-ventral patterning but also in regulating the expression of early genes involved in NR and RPE specification and development.

From this study we conclude that in the mammalian system, early stages of NR and RPE development are critically dependent on BMP signaling in the optic primordium (Figure 4.7). In the presumptive RPE, BMP signaling regulates the expression of early genes such as *Otx2*, *Pax6* and *Mitf* involved in specification and development. At later stages, BMP signaling regulates the process of RPE differentiation by controlling the expression of genes required for generation of pigmentation. In the presumptive NR, precise levels of BMP signaling are required to establish dorsal-ventral patterning and to maintain the expression of NR specific genes such as *Vsx2*. BMP signaling regulates the expression of early genes such as *Rx*, *Lhx2*, *Pax6* and *Sox2* which are important in NR specification and development. We further show that BMP signaling regulates the development of these tissues by modulating the members of WNT and SHH signaling. Taken together, we propose that, the dorsally localized BMP signaling precisely regulates the expression of early genes important in NR and RPE specification and development by establishing a regulatory signaling network between WNT's in RPE and NR, SHH in ventral NR and optic stalk and FGF's in NR and surface ectoderm. Although most of the regulatory genes are well conserved across different species, our results indicate the possibility for existence of significant differences in the mechanisms regulating organogenesis among vertebrate species.

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Figure 4.1: BMP signaling affects RPE pigmentation in a stage dependent manner. (A) Mouse embryo culture system. Mouse embryos implanted with affi-gel blue gel beads at early (E29-31 ss) and late (E33-34 ss) stages cultured for 10 – 18 hours in a rolling bottle culture apparatus supplied with 95% O_2 / 5% CO_2 at 37^{o}C. (B-E) BMP4 affects pigmentation in a stage dependent manner. (B, C) Bead implantation at early stage. (B) RPE well pigmented in control eye implanted with BSA bead in culture, (C) BMP4 inhibits formation of pigmentation. (D, E) Bead implantation at late stage. Decreased pigmentation in BMP4 treated eye (E) compared to control (D). Arrows indicate the extent of pigmentation. Scale bar in (B) applies to (B-E).

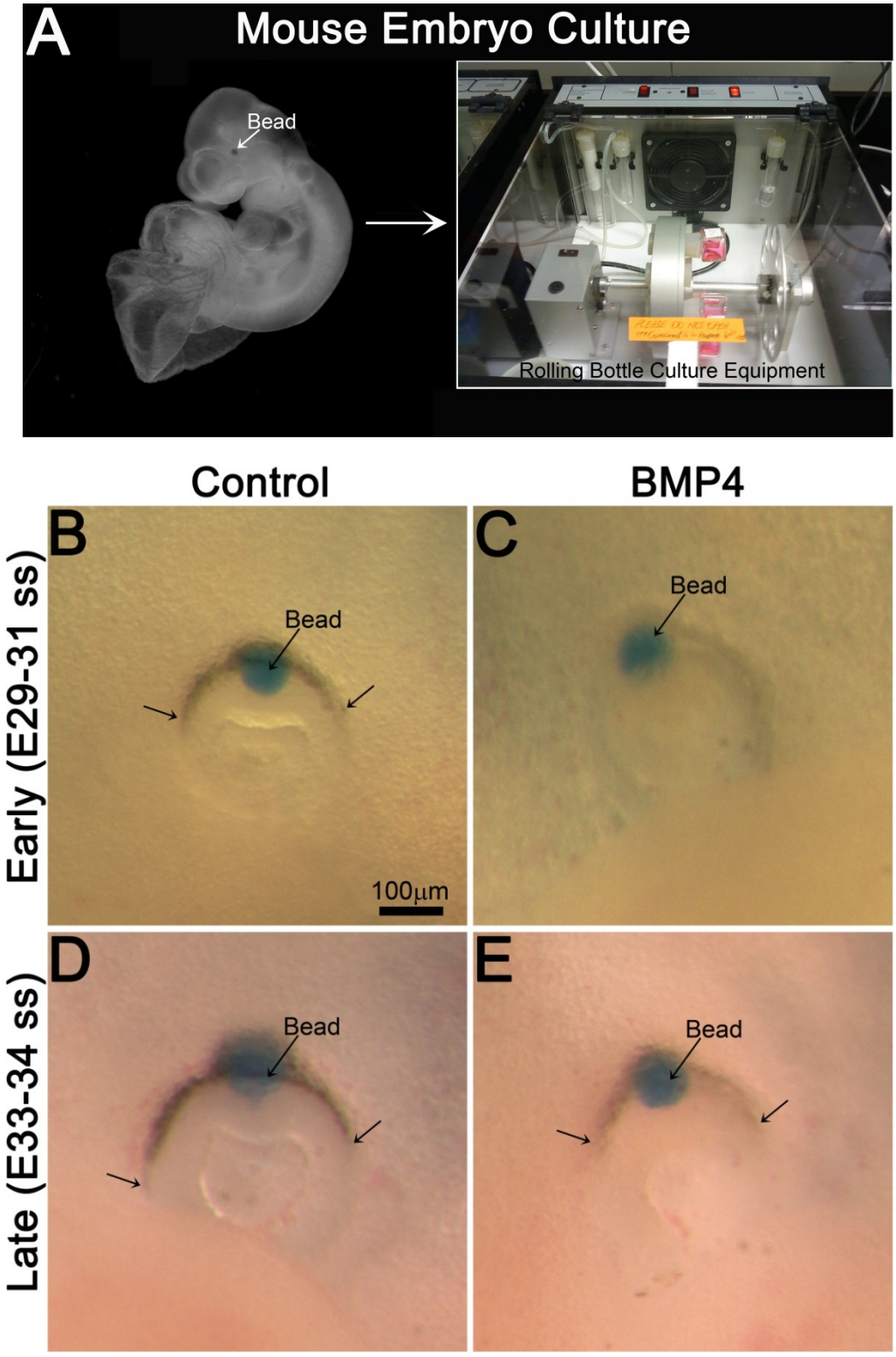

A
Mouse Embryo Culture
Bead
Rolling Bottle Culture Equipment
Control
BMP4
B
C
D
E
Bead
Bead
Bead
Bead
100μm
Early (E29-31 ss)
Late (E33-34 ss)

Figure 4.2: BMP signaling affects genes involved in RPE pigmentation. (A-D) Tyrosinase (*Tyr*) expression at early (A) and late (C) stages in control. Ectopic BMP4 inhibited *Tyr* expression at early stages (B) and decreased at late stages (D) in the RPE (E, F) *Mitf* expression downregulated in BMP4 treated eye (F) compared to control (E). Arrows indicate the extent of mRNA expression. Scale bar in (A) applies to (A-F). (G) qPCR analysis reveals significant reduction in expression levels of *Tyr*, Tyrosinase related protein-1 (TRP1) and *Mitf* in BMP4 treated eyes compared to control. (H-I) *Mitf* expression upregulated in Noggin treated eyes (I) compared to control (H). Scale bar in (H) applies to (H, I).

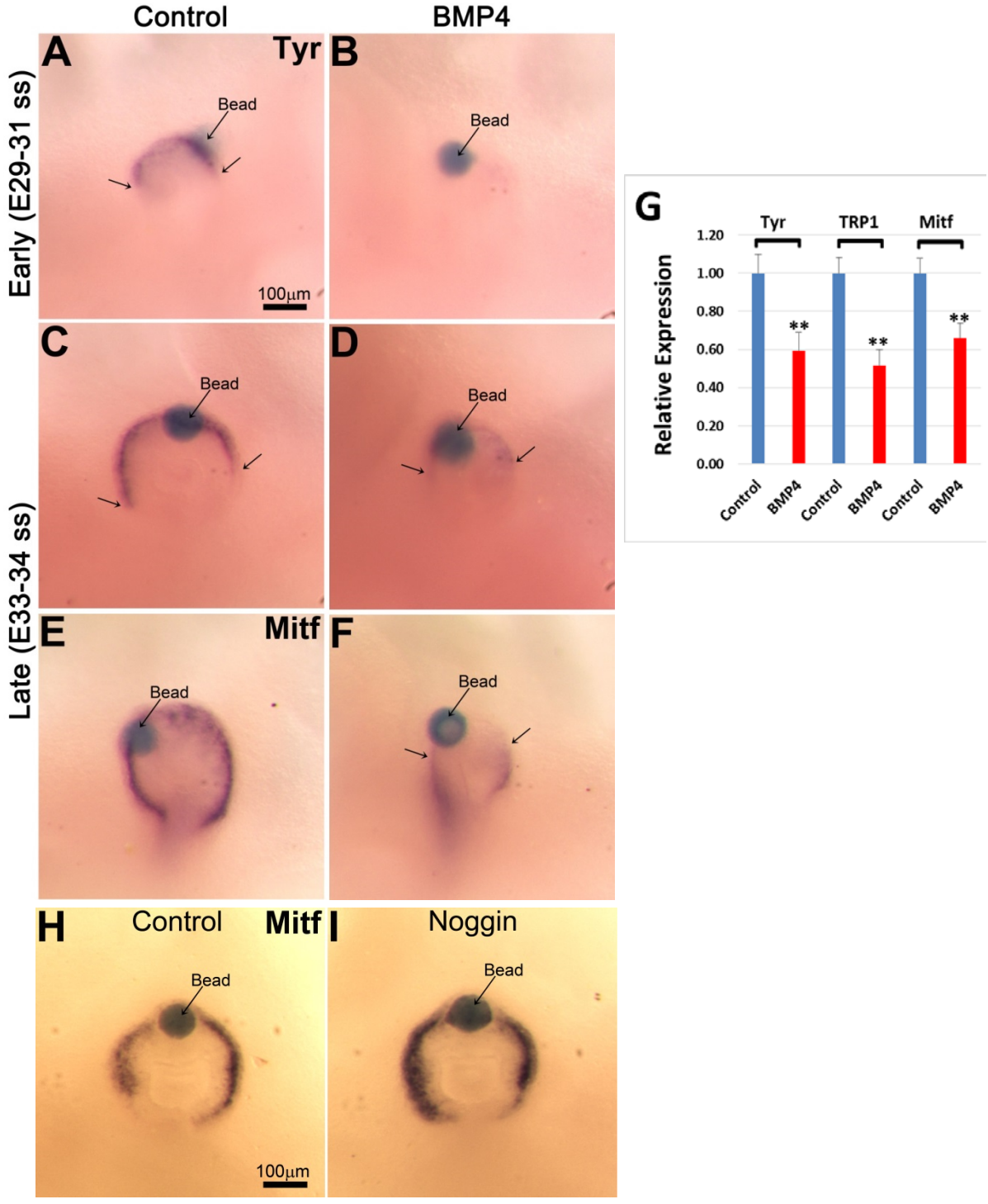

Control
BMP4
A
Tyr
B
Bead
100μm
Early (E29-31 ss)
C
D
Late (E33-34 ss)
E
Mitf
F
H
Control
Mitf
I
Noggin
G
Tyr
TRP1
Mitf
Relative Expression
1.20
1.00
0.80
0.60
0.40
0.20
0.00
**
Control
BMP4

Figure 4.3: BMP signaling regulates genes involved in RPE specification and development. (A, B) *Otx1*. Slight upregulation of *Otx1* in BMP4 treated eye (B) compared to control (A). Arrows indicate expression all over NR and RPE. (C, D) *Otx2*. Significant reduction in *Otx2* in BMP4 treated eye (D) compared to control (C). Arrows indicate extent of expression. (E, F) *Pax6* driven *GFP* expression. Ectopic BMP4 downregulated *Pax6-GFP* expression in RPE and NR and affected ventral retina development (F) compared to the control (E). Arrow heads indicate *Pax6-GFP* expression in RPE. Arrows indicate location of ventral retina. (G, H) *Pax6*. Expression of *Pax6* and development of ventral retina affected in BMP4 treated eye (G) compared to control (H). Arrows in (G) indicate expression all over and in (H) show the extent of expression. (I) qPCR analysis reveals significant reduction in expression levels of *Otx2* and *Pax6* but not of *Otx1* in BMP4 treated eyes compared to control. (J-K) Pax6 expression upregulated in Noggin treated eyes (J) compared to control (K). (Scale bar in (A) applies to (A-D); in (E) applies to (F); in (G) applies to (H); in (J) applies to (J, K).

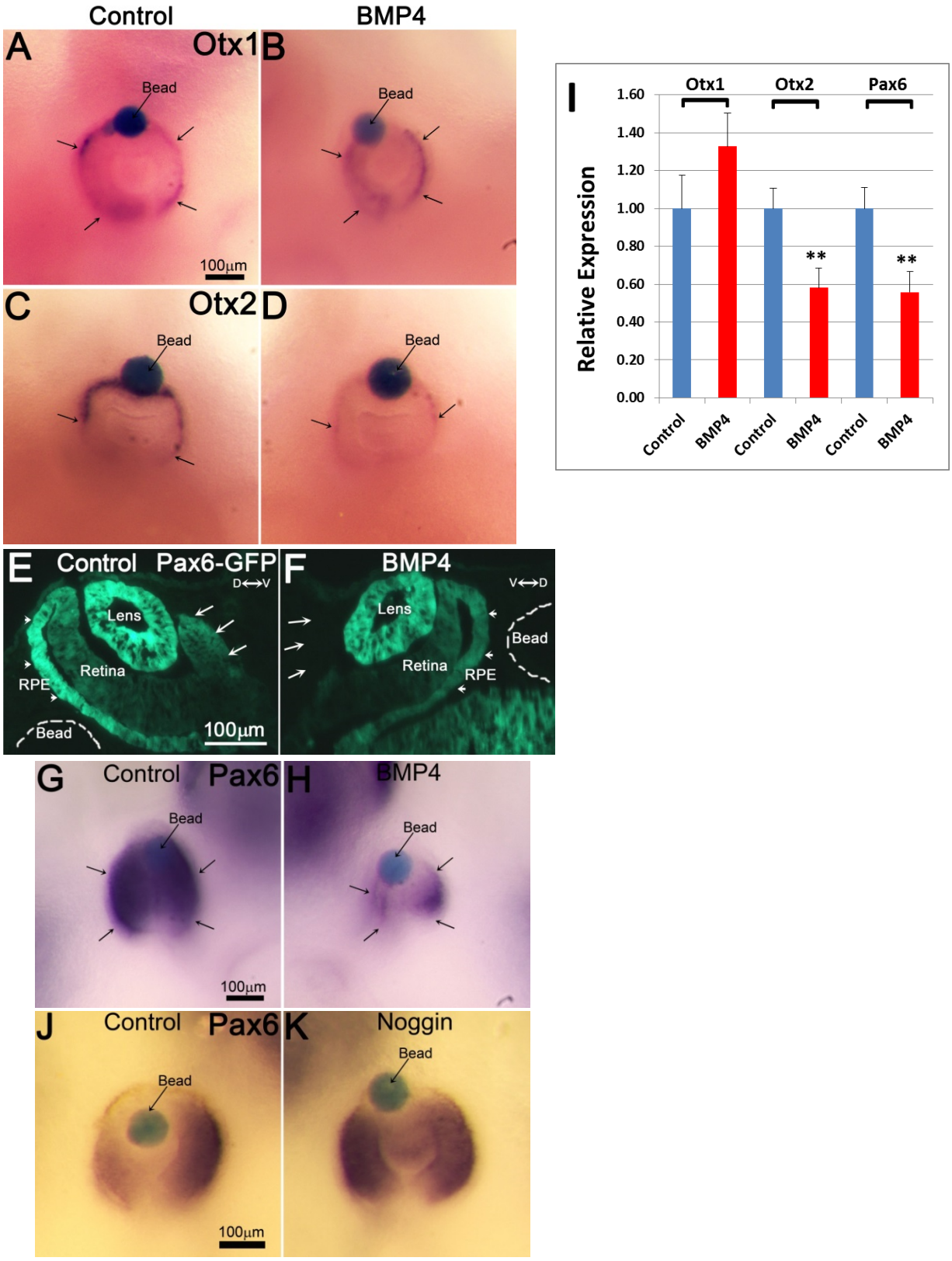
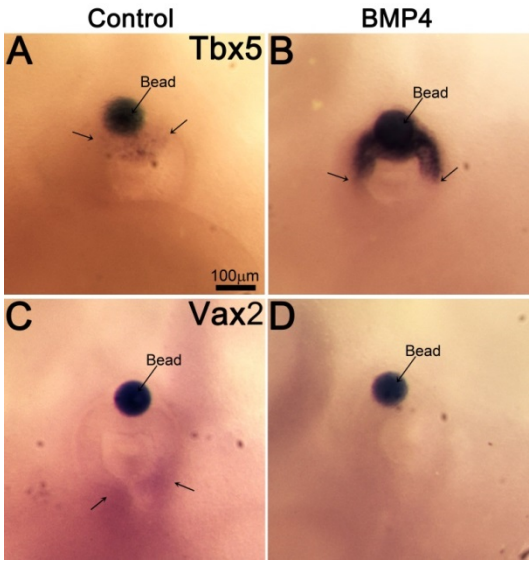
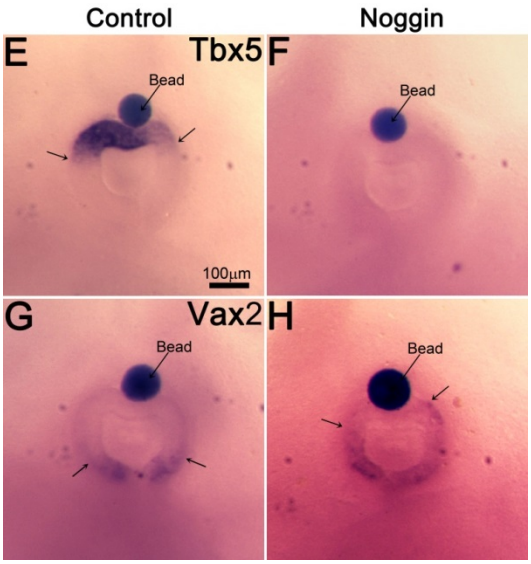
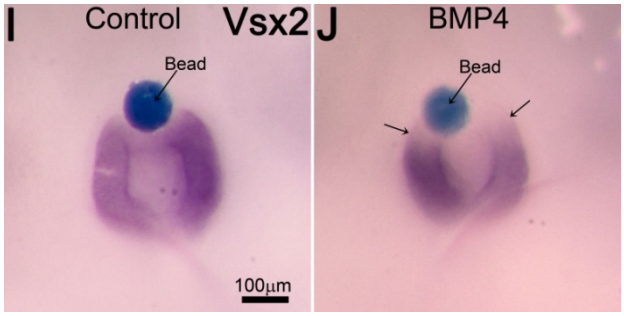

Control
BMP4
A
Otx1
B
Bead
100µm
C
Otx2
D
I
Otx1
Otx2
Pax6
Relative Expression
1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
**
**
Control
BMP4
Control
BMP4
Control
BMP4
E
Control
Pax6-GFP
D↔V
Lens
Retina
RPE
Bead
100µm
F
BMP4
V↔D
Lens
Bead
Retina
RPE
G
Control
Pax6
H
BMP4
J
Control
Pax6
K
Noggin

Figure 4.4: BMP signaling affects NR specific genes and alters dorsal-ventral patterning. Expression of *Tbx5* (A, B, E, F), *Vax2* (C, D, G, H) and *Vsx2* (I, J). *Tbx5* (B) expanded, *Vax2* (D) and *Vsx2* (J) downregulated in BMP4 treated eyes compared to control eyes (A, C, I). Noggin treatment inhibited *Tbx5* (F) and expanded *Vax2* (H) compared to control eyes (E, G). (K) qPCR analysis reveals upregulation of *Tbx2*, *Tbx5* and *Msx1* and significant reduction in *Vax2* and *Vsx2* in BMP4 treated eyes compared to control. Noggin treatment reduced *Tbx5* and increased *Vax2* expression levels compared to control. Arrows indicate extent of mRNA expression. Scale bar in (A) applies to (A-D); in (E) applies to (E-H); in (I) applies to (I, J).


Control
BMP4
A
Tbx5
B
Bead
Bead
100μm
C
Vax2
D
Bead
Bead


Control
Noggin
E
Tbx5
F
Bead
Bead
100μm
G
Vax2
H
Bead
Bead


I
Control
Vsx2
J
BMP4
Bead
Bead
100μm

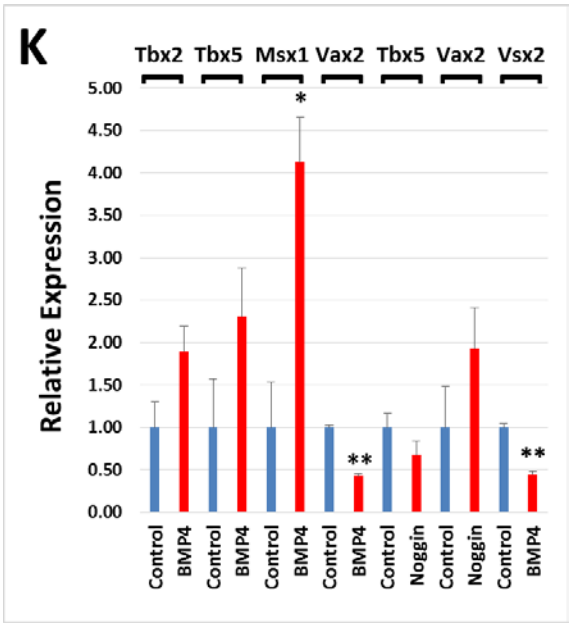
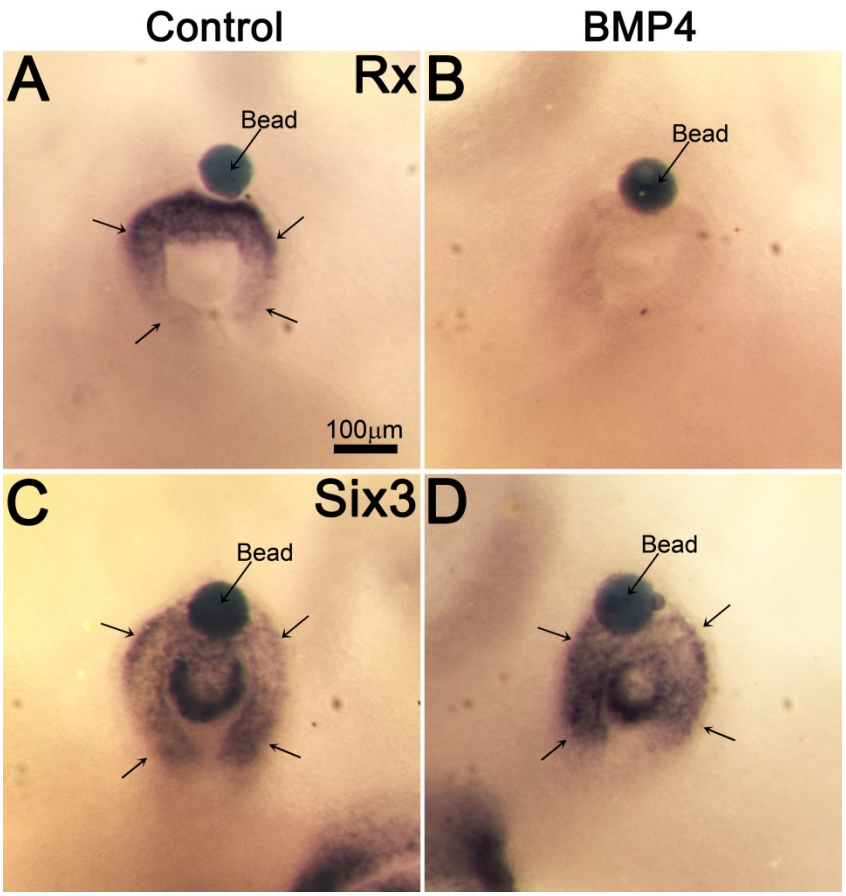

K
Tbx2
Tbx5
Msx1
Vax2
Tbx5
Vax2
Vsx2
Relative Expression
5.00
4.50
4.00
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
*
**
**
Control
BMP4
Control
BMP4
Control
BMP4
Control
BMP4
Control
Noggin
Control
Noggin
Control
BMP4

Figure 4.5: BMP signaling affects early genes involved in NR specification and development. (A, B) *Rx*. Ectopic BMP4 (B) downregulated *Rx* expression compared to the control (A). (C, D) *Six3*. Expression of *Six3* not altered in BMP4 treated eyes (D) compared to control (C). (E) qPCR analysis reveals significant downregulation in expression levels of *Rx*, *Lhx2* and *Sox2* but not of *Six3*, *FGF15* and *FGFR2* in BMP4 treated eyes compared to control. Arrows in indicate expression all over NR. Scale bar in (A) applies to (A-D).


Control
BMP4
A
Rx
Bead
B
Bead
100μm
C
Six3
Bead
D
Bead

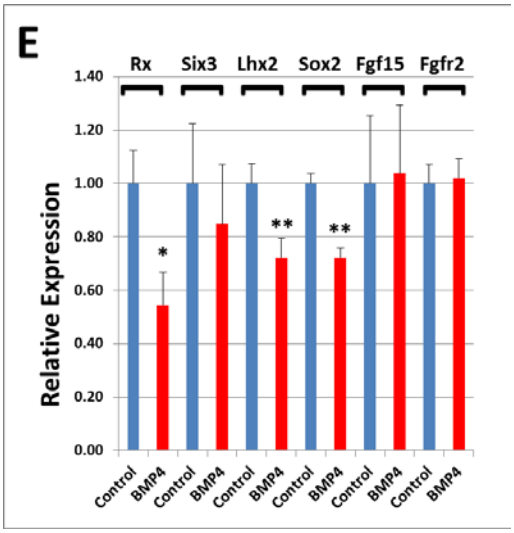
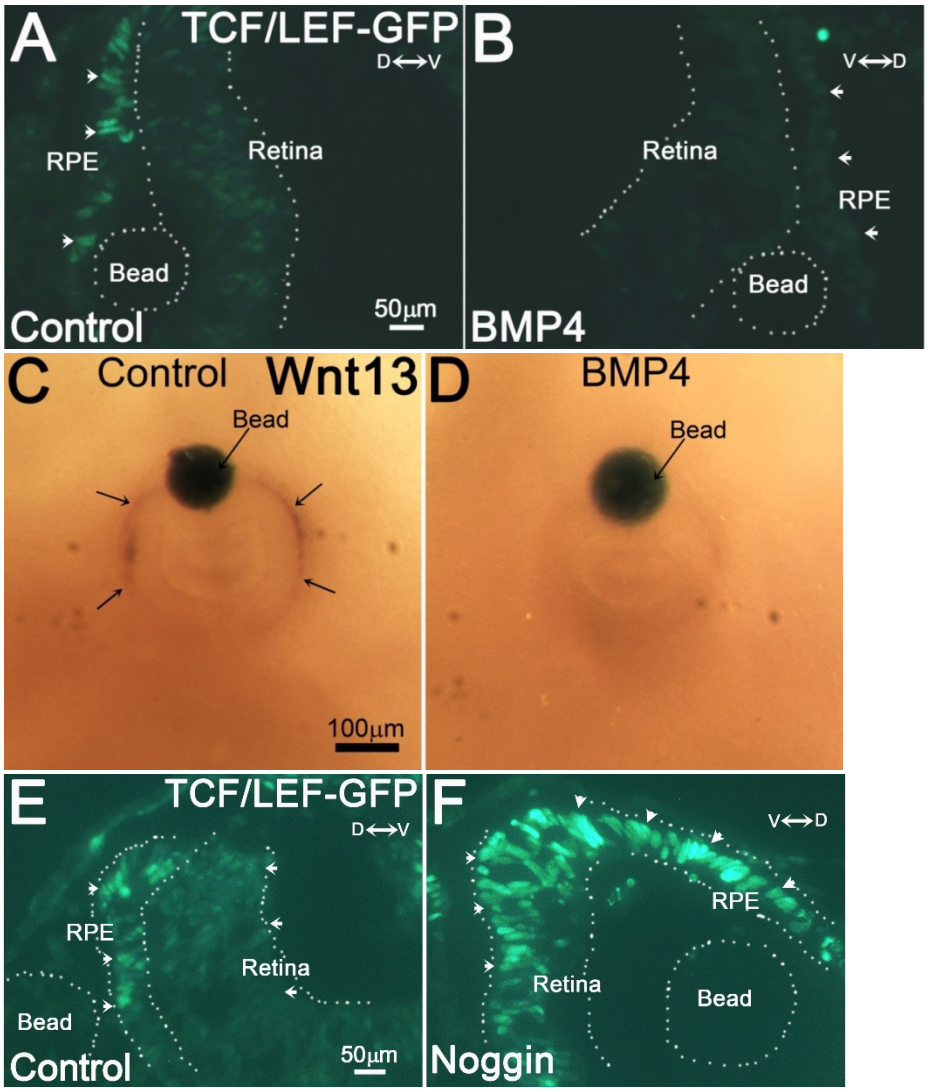
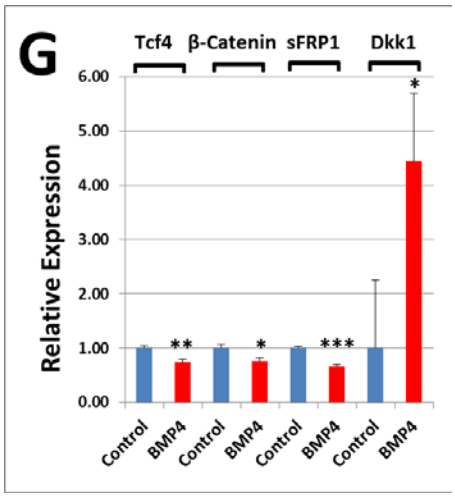

E
Rx
Six3
Lhx2
Sox2
Fgf15
Fgfr2
Relative Expression
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
*
**
**
Control
BMP4

Figure 4.6: Ectopic BMP signaling affects members of WNT and SHH signaling pathways. (A, B) *TCF/LEF-GFP* expression. WNT signaling prominent in RPE and NR in the control eye (A). Ectopic BMP4 significantly downregulated WNT signaling in RPE and NR (B). Arrow heads indicate WNT signaling as represented by *TCF/LEF-GFP* expression in RPE. (C, D) *WNT13* expression. BMP4 significantly downregulated *WNT13* expression in RPE (D) compared to control (C). Arrows in (C) indicate expression all over RPE. (E-F) WNT signaling as represented by *TCF/LEF-GFP* expression. WNT signaling significantly upregulated in RPE and NR in the Noggin treated eye (E) compared to the control (F). (G-H) qPCR analysis. (G) Members of WNT signaling such as *Tcf4* and *β-Catenin* and WNT antagonist, *sFRP1* significantly downregulated while that of *DKK1*, a potent WNT/β-Catenin antagonist upregulated by more than four fold in BMP4 treated eyes compared to control. (H) Members of Sonic hedgehog signaling such as *Gli2*, Smoothened (*Smo*) and *Shh*, and its target gene *Sox8* were all significantly downregulated compared to control. Scale bar in (A) applies to (A, B); in (C) applies to (C, D); in (E) applies to (E, F).


A
TCF/LEF-GFP
D↔V
RPE
Retina
Bead
Control
50μm
B
V↔D
Retina
RPE
Bead
BMP4
C
Control
Wnt13
Bead
100μm
D
BMP4
Bead
E
TCF/LEF-GFP
D↔V
RPE
Retina
Bead
Control
50μm
F
V↔D
RPE
Retina
Bead
Noggin


G
Tcf4
β-Catenin
sFRP1
Dkk1
Relative Expression
6.00
5.00
4.00
3.00
2.00
1.00
0.00
**
*
***
*
Control
BMP4

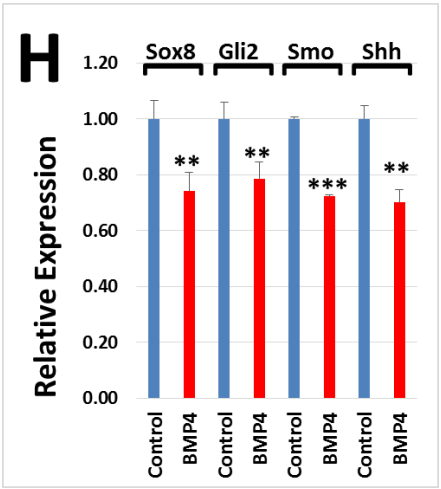

H
Sox8
Gli2
Smo
Shh
Relative Expression
1.20
1.00
0.80
0.60
0.40
0.20
0.00
**
**
***
**
Control
BMP4

Figure 4.7: BMP signaling regulates NR and RPE specification, patterning and development by modulating WNT and SHH signaling. BMP signaling modulates WNT signaling through altering the levels of WNT antagonist, *DKK1* in addition to other unknown factors. In RPE, BMP signaling regulates expression of *Pax6*, *Otx2*, *Mitf* and other downstream pigmentation genes through WNT and SHH signaling. In NR, BMP signaling regulates expression of early genes involved in NR specification and development such as *Rx*, *Lhx2*, *Sox2 Pax6* and *Vsx2*, and alters expression domains of genes involved in dorsal-ventral patterning such as *Tbx2*, *Tbx5*, *Msx1* and *Vax2*.

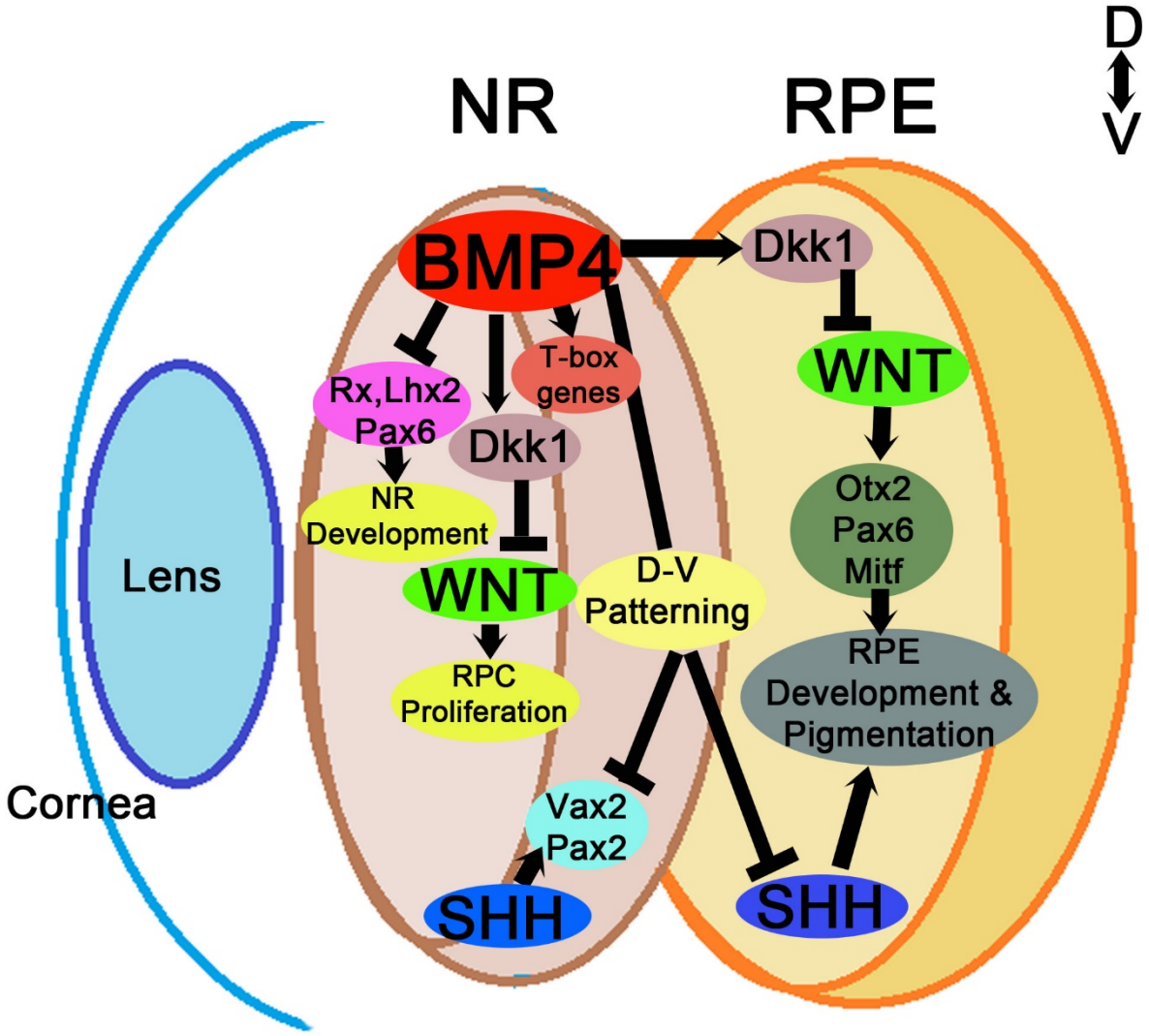

NR
RPE
D
V
BMP4
Dkk1
T-box genes
Rx,Lhx2 Pax6
Dkk1
WNT
Otx2 Pax6 Mitf
NR Development
Lens
WNT
D-V Patterning
RPC Proliferation
RPE Development & Pigmentation
Cornea
Vax2 Pax2
SHH
SHH

Table 4.1: Developmental differences between avian and mammalian species in the ocular tissue. Species specific differences exists between avian and mammalian species in expression pattern of certain genes and response to external signaling factors during early stages of NR and RPE development.

Developmental Differences between Avian and Mammalian Species in the Ocular Tissue

Description	Chickens	Mouse	Reference
Mitf Expression	Restricted mostly to proximal part of the optic vesicle.	Expressed by most cells in the proximal as well as distal parts of the optic vesicle.	Muller et al., 2007; Nguyen and Arnheiter, 2000
Removal of Surface ectoderm or source of FGF signaling	Distal part of optic vesicle (neural retina) retains its multilayered structure with appearance of more pigmented cells and few Vsx2 positive cells.	Distal part of optic vesicle (neural retina) converts to a single layered RPE-like structure with pigmented cells. The proximal part of optic vesicle (RPE) converts into multilayered retina like structure.	Hyer et al., 1998; Nguyen and Arnheiter, 2000
Ectopic BMP4 treatment	Down-regulates FGF8 expression in neural retina.	Up-regulates FGF15 expression in neural retina.	Muller et al., 2007; Murali et al., 2005
Ectopic BMP4 treatment	Supports RPE development and upregulates expression of RPE specific genes. Apparent transdifferentiation of neural retina into RPE like structure with ectopic expression of RPE specific genes.	Do not support both RPE and NR development. Down-regulates expression of RPE and NR specific genes. No apparent transdifferentiation of neural retina into RPE occurs.	Muller et al., 2007; Kalaskar and Lauderdale (Current work)

Table 4.2: List of primers used for in situ probe design and qPCR analysis of expression of genes in ocular tissues.

List of Primers for qPCR and In Situ Probe Preparation		
Name of the Gene	**Forward Primer**	**Reverse Primer**
Rx	**cgacgttcaccacttaccaa**	**tcggttctggaaccatacct**
Six3	**aacttccgcgacctgtacc**	**gccgtgagactccttagtgaa**
Lhx2	**cagcttgcgcaaaagacc**	**taaaaggttgcgcctgaact**
FGF15	**ggcaagatatacgggctgat**	**tccatttcctccctgaaggt**
Tcf4	**ggctcccctccttcactc**	**gaggcctgtcctccatttct**
BMPR2	**agatcctgggccatcaaag**	**ttgtggctgaaagatccagag**
β-Catenin	**atggaccctatgatggagca**	**cccatcaactggatagtcagc**
SFRP1	**cacaacgtgggctacaagaa**	**tcacctctgccatggtctc**
DKK1	**atggaatatgcatgccctct**	**ccaaggttttcaatgatgctt**
FGFR2	**tggctctgttcaatgtgacg**	**gcctgccctatataattggaga**
Pax6	**cccggcagaagatcgtag**	**cagaattcgggaaatgtcg**
α-Tubulin	**aacctaaacaggttgataggtcaaa**	**aacattcagggccccatc**
β-Actin	**agccatgtacgtagccatcc**	**tcacaatgcctgtggtacg**
αA-crystallins	**gcgagggccttttgagt**	**tagtaggggctgatggtgga**
Vsx2	**gcagagccaagtggagga**	**gaccatactcagccatgacg**
Mitf	**tcaacctctgaagagcagca**	**gcgtagcaagatgcgtgat**
Msx1	**aggacgcctttcaccaca**	**tactgcttctggcggaactt**
Sox2	**acggcagctacagcatga**	**gacgtcgtagcggtgcat**
Tbx2	**cctagcactagcctccttacca**	**gactgggctcacggctatt**
Tbx5	**ccactggatgcgacaactt**	**gtccaggtggttgttggtg**
Vax2	**cccagcctcctagcactg**	**ggggtcaaccaaggaggt**
Otx1	**accagtggtgagcgttgg**	**agcgcagggtaggaaagg**
Otx2	**gattgcttggattataaggacca**	**ccaagcagtcagcattgaag**
Tyrosinase	**caccatgcttttgtggacag**	**ggcttctgggtaaacttccaa**
TRP1	**gtaacagcactgagggtggac**	**cactgctggtctccctacattt**
Gli2	**ggtgtctagcaccgtggact**	**atggcatcaaagtcaatctgg**
Smo	**tcagcatgtcaccaagatgg**	**ggggtaacggacacatcct**
SHH	**cactatgagggtcgagcagtg**	**agccagcatgccgtactt**
Sox8	**caagaccctaggcaagctgt**	**ctgggtggtctttcttgtgc**
Tyrosinase In Situ Probe Primers	**T7+Forward Primer: TAATACGACTCACTATAGGG CCTTCTGTGGAGTTTCCAG**	**SP6+Reverse Primer: ATTTAGGTGACACTATAGAA AAGCCAAACAGCTATGGTC**

Chapter 5

Conclusion

Developmental ocular anomalies are one of the major causes of childhood blindness in humans. Mutations affecting the genes involved in early eye development have been implicated to be the primary cause for these developmental defects. These defects are manifested at birth in the form of cataract, glaucoma, retinal dystrophy, which can result in a spectrum of ocular disease ranging from slight visual impairment to complete loss of vision. The genes involved in most of these congenital ocular diseases are not well understood. However, recent screening of individuals with ocular malformations by direct genome sequencing revealed mutations in the Bone morphogenetic protein-4 (BMP4) gene locus. These individuals reported ocular abnormalities such as anterior segment (cornea, lens, iris, ciliary body) malformations, retinal defects, optic nerve aplasia and in some cases showed severe microphthalmia or anophthalmia. Though the molecular mechanisms underlying these developmental ocular defects are not understood, these studies suggest that BMP4 has a role in the formation as well as subsequent development of the ocular tissues. In order to understand these disease processes and to design appropriate therapies it is essential to elucidate the role of BMP4 in the mammalian early eye development.

Studies indicate that the molecular mechanisms of BMP signaling is very complex as different BMP members reveal 70-80% sequence homology and exhibit functional redundancy as analyzed in different tissue systems (Solloway and Robertson, 1999; Wordinger and Clark, 2007). It was shown that members of BMP signaling including BMP2 to BMP7 and their

receptors, BMPR1 and BMPR2 exhibit restricted expression patterns in the ocular tissue during early and later stages of development (Dudley and Robertson, 1997; Furuta and Hogan, 1998; Liu et al., 2003; Wawersik et al., 1999). Studies also revealed that BMP ligands tend to form homodimers and heterodimers and possess differential abilities to activate specific BMP receptor complexes under different physiological conditions (Little and Mullins, 2009). Added to these complexities, BMP4$^{-/-}$ mouse embryos exhibit embryonic lethality at mid-gestation precluding its functional analysis during ocular organogenesis. Considering the expression of multiple BMP members in the ocular tissues with the possibility of functional redundancy and the limitations of genetic knockout approaches, I established an in vitro culture system to determine the effect of gain and loss of function of BMP4 signaling during early stages of mammalian eye development. The goal of this dissertation work was to establish a serum-free culture system that supports ex utero development of ocular tissues comparable to that observed in utero, and to utilize this culture system to externally manipulate the BMP signaling in the ocular region to determine its role during early stages of mammalian eye development.

Establishing a serum-free culture system for ocular development ex utero

Recently utilized defined culture systems to study mammalian embryonic eye development included the use of optic rudiments which consists of the entire optic primordium (Thut et al., 2001; Wawersik et al., 1999). When tested, the optic rudiments in culture exhibited retarded growth and the development of cornea, lens and retina appeared abnormal. The lens fiber cells were highly disorganized and the cornea was observed as a thin epithelial layer with no further development due to the absence of migrating mesenchymal cells. However the tissues showed expression of tissue markers such as, crystallins in the lens fiber cells and Pax6 in the

lens epithelium, corneal epithelium and retina. I concluded that the optic rudiments do not support proper morphological growth of the ocular tissue; however, the tissue survives and expresses some of the tissue specific genes appropriate to the stage at which the optic rudiment was isolated from the embryo. The use of whole heads in the culture resulted in improved ocular development, but still not comparable to that observed in utero.

Currently utilized embryo culture systems for studying ocular development were mainly dependent on serum for proper growth and maintenance of the embryos in the culture (Behesti et al., 2006). The use of serum in culture has many disadvantages as previously mentioned and therefore I utilized a serum-free whole embryo culture system to evaluate the ocular development ex utero (Kalaskar and Lauderdale, 2014; Moore-Scott et al., 2003). I modified the antibiotic concentration in the culture medium to enable long term culture of mouse embryos (Kalaskar and Lauderdale, 2014). I showed that, mid-gestation stage mouse embryos when cultured for 16 to 40 hrs under these defined conditions exhibited progression in morphological development of overall embryonic body and different structures such as the heart, limbs, brain and eyes indicating appropriate levels of cellular proliferation, migration, differentiation and tissue interactions. Molecular analysis of the embryonic development in the culture for one of the complex organ systems such as the eye revealed the ocular development to be consistent with that observed in ocular tissue in embryos developing in utero (*Kalaskar and Lauderdale, in preparation*). I concluded that, this method will be useful for investigators needing to utilize whole embryo culture to study signaling interactions important in embryonic organogenesis.

Analysis of the role of BMP signaling in early eye development

Utilizing the mouse embryo culture system, I evaluated the role of BMP4 signaling in early eye development after lens induction. I showed that significant differences can exist among vertebrate species in the mechanisms that BMP signaling operates to regulate the expression of different transcription factors important in early eye specification and development. In avians, ectopic BMP4 was shown to support RPE development by induction of *Mitf* and other RPE specific genes and downregulation of NR markers like *Vsx2* (Muller et al., 2007). Contrary to these results reported for chickens, I showed that ectopic BMP4 either inhibits or decreases the pigmentation in the mammalian RPE in a stage-dependent manner by down-regulating important genes involved in RPE specification and development such as *Mitf*, *Otx2* and *Pax6*. In NR, ectopic BMP down-regulated most of the early genes but no apparent transdifferentiation into RPE was observed.

This apparent difference between birds and mammals in the role of BMP signaling in eye development could be due to differences in experimental parameters. Several lines of evidence suggest that this may not be the case. First, in chickens *Mitf* is expressed only in the proximal part of the optic vesicle, which forms the future RPE. In mice *Mitf* is initially expressed throughout the early optic vesicle and later becomes restricted to the proximal part of the optic vesicle at the time of RPE specification (Muller et al., 2007; Nguyen and Arnheiter, 2000). This suggests that in chickens the optic vesicle may already be specified into RPE and NR domains before it contacts the surface ectoderm, whereas in mice the optic vesicle is specified into RPE and NR domains only after its contact with the surface ectoderm. Second, consistent with this idea, when the proximal and distal halves of chicken optic vesicle were cultured separately they became RPE and NR structures respectively (Kagiyama et al., 2005). Third, in both chickens and

mice, the surface ectoderm was shown to be the source of FGF signaling which induces neural retinal markers in the optic vesicle (Hyer et al., 1998; Pittack et al., 1997). However, the optic vesicles in these species exhibit different responses to this FGF signaling. When the surface ectoderm or the source of FGF signaling was removed in the mouse embryos, the distal optic vesicle, which is near to the surface ectoderm and usually forms NR, will now form into RPE-like structure with a single epithelium of cuboidal cells (Nguyen and Arnheiter, 2000). In chickens, removal of surface ectoderm resulted in the proximal optic vesicle to still retain its multilayered structure typical of NR differentiation with very few NR-like cells intermingled with pigmented cells (Hyer et al., 1998). Together these results in conjunction with my own observations strongly indicate the existence of species specific differences between birds and mammals for BMP signaling in regulating the genes important in NR and RPE specification and development.

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APPENDIX – A

Additional information on Mouse Optic Rudiment, Head and Embryo Culture Systems

Method

The optic rudiment culture was carried out as previously described (Thut et al., 2001; Wawersik et al., 1999). Briefly, the method involved isolation of entire optic primordium with or without some part of the surrounding periocular mesenchyme from mouse embryos at embryonic day, E10.5 or E11.5 days post coitus (dpc). The optic rudiments were then cultured in defined medium in either 6-well or 12-well plates with Nucleopore filters (Figure A.1). The tissues were cultured for 1 to 5 days with changing the culture medium every day. For whole head culture, the entire head is separated from the embryo and cultured in rolling bottles following our recent protocol and as previously described (Kalaskar and Lauderdale, 2014; Moore-Scott et al., 2003). The whole embryo culture system is described in detail in chapters 2 and 3. The optic rudiments, whole heads and embryos were imaged at specific time points under dissecting microscope using Spot imaging software and the tissues processed for histology and immunohistochemistry as previously described.

Results

Ocular development retarded in Optic Rudiment Culture

The growth of optic rudiments in culture appeared retarded right from start of culture. After day 1 in culture the optic rudiments appeared smaller than the in utero developed eyes. At later time periods the growth with regards to increase in optic rudiment size appeared almost

halted. This type of growth was observed when the culture was started at both time points beginning at E10.5 or E11.5 dpc. However the tissue survived over the whole culture time period of 4 or 5 days (Figure A.2). Histological analysis of these optic rudiments in culture revealed under-development at all tissue levels compared to the in utero developed eyes (Figure A.3). In the eyes developed in utero, the cornea is multilayered with outer epithelial layer followed underneath by migrating mesenchymal cells. The lens shows a single layer of epithelium followed by posterior lens fiber cells which are oriented along anterior-posterior direction with their nuclei aligned along the equatorial region. The optic cup is well differentiated with outer distinct retinal pigmented epithelium and inner multilayered neural retina (Figure A.3B, C). While in the culture, the cornea appeared as a single layer of epithelial cells with no further development (Figure A.3D, E). This could be due to the lack of migrating mesenchymal cells. The lens in the cultured optic rudiments although separated from the surface ectoderm looks like a mass of cells. The lens cells are very much disorganized with no proper orientation along the anterior posterior direction. The optic cup showed slight increase in retinal size but overall appeared morphologically similar after one or two days in culture (Figure A.3D, E). Though these optic rudiments in culture exhibited retarded growth and under-development of the tissues in the eye, they showed the expression of tissue specific differentiation markers. Crystallins are lens specific proteins and indicate differentiation of lens fiber cells. In these cultured optic rudiments αA-Crystallins were specifically detected in the lens and their expression was comparable to the expression levels observed in eyes developed in utero (Figure A.4). These tissues also showed comparable expression of Pax6 proteins in the corneal and lens epithelium and also in retina (data not shown). The expression of these tissue specific markers may be due to already existing pre-determined mechanisms that these cells carry after being specified.

Mouse Whole Head Culture improved Ocular Development

Mouse whole heads in oxygenated culture system improved the ocular development ex utero (Figure A.5). The cornea showed migrating mesenchymal cells which appeared to be loosely packed and disoriented. The lens showed anterior epithelial layer and posterior lens fiber cells and their orientation was partially rescued (Figure A.5E-G). The retina also showed improved development but overall the optic region looked very much smaller than the in utero developed eyes ((Figure A.5A-D).

Cellular Proliferation and Apoptosis in Mouse Embryo Culture was comparable to that observed In Utero

The ocular development in mouse embryo culture system was comparable to that observed in ocular tissues developed in utero both at the morphological level and also at the expression of molecular markers. I further analyzed these tissues for extent of proliferation and apoptosis. For detecting proliferating cells I stained for Ki67 and also for Phospho Histone H3 (PH3) and for detecting apoptotic cells we stained for Cleaved Capase-3 and Cleaved PARP following protocols previously described (Kim and Lauderdale, 2006). I counted cells in at least 5 ocular tissue sections from 3 biological replicates (different eyes) developed in culture or in utero. In the ocular tissues from embryos developed in mouse embryo culture I found cellular proliferation to be comparable to that observed in ocular tissues developed in utero as revealed by the PH3 stained cells (Figure A.6A, B). Also there was no significant increase in cells expressing apoptotic markers as revealed by Cleaved Caspase 3 expression in the embryonic eyes developed in culture compared to in utero developed tissues (Figure A.6C, D).

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Moore-Scott, B. A., Gordon, J., Blackburn, C. C., Condie, B. G. and Manley, N. R. (2003). New serum-free in vitro culture technique for midgestation mouse embryos. *Genesis* **35**, 164-168.

Thut, C. J., Rountree, R. B., Hwa, M. and Kingsley, D. M. (2001). A large-scale in situ screen provides molecular evidence for the induction of eye anterior segment structures by the developing lens. *Developmental biology* **231**, 63-76.

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Figure A.1: Optic rudiment culture. The optic area was surgically excised and cultured on nucleopore filters with culture medium. The white circle covers the entire optic region.

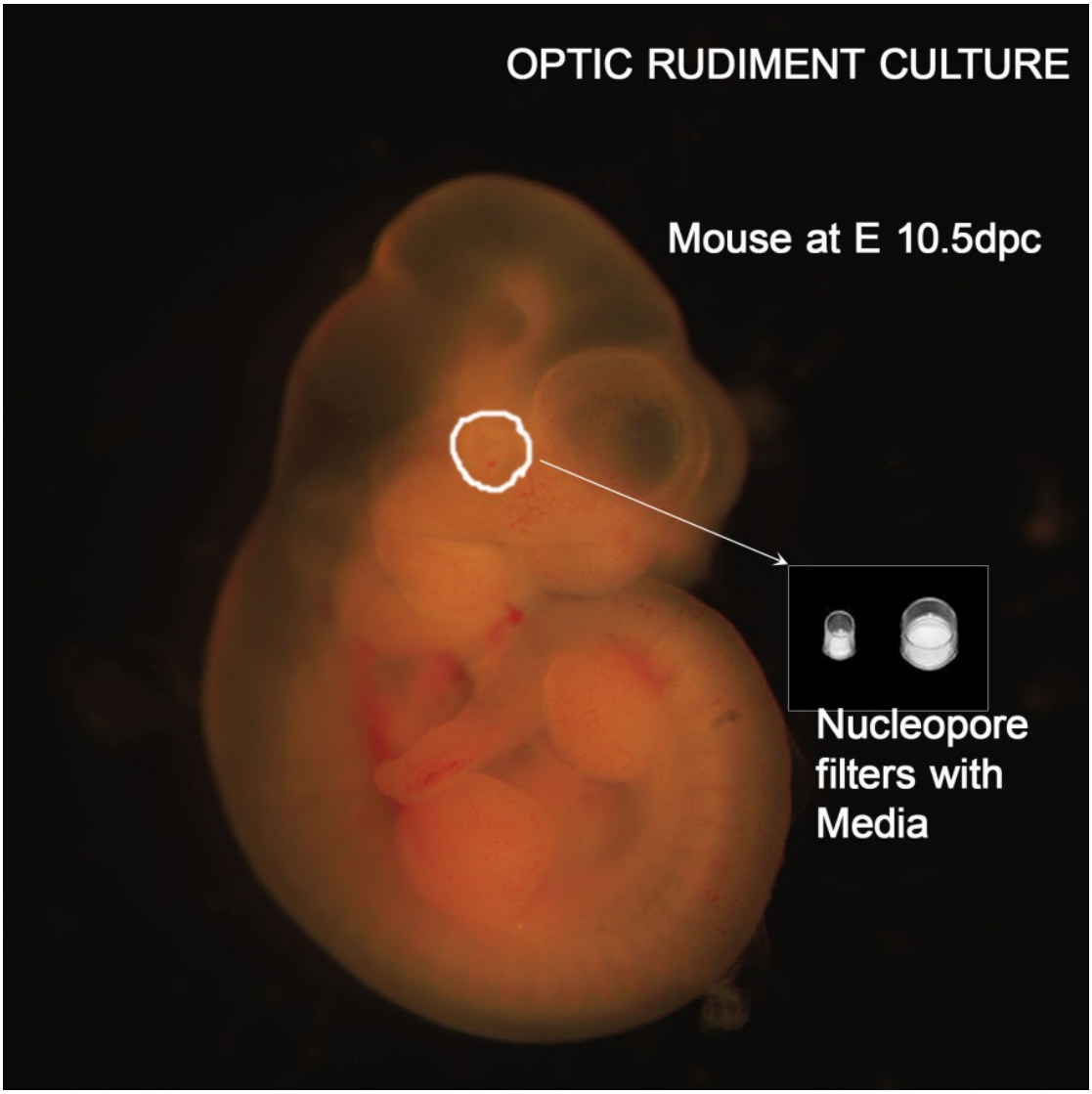

OPTIC RUDIMENT CULTURE
Mouse at E 10.5dpc
Nucleopore
filters with
Media

Figure A.2: Ocular tissue growth retarded in optic rudiment culture. 1st Row: Ocular development in utero from E10.5 to E15.5. 2nd Row: Ocular development in optic rudiment culture for 5 days starting from E10.5. 3rd Row: Ocular development in optic rudiment culture for 4 days starting from E11.5.

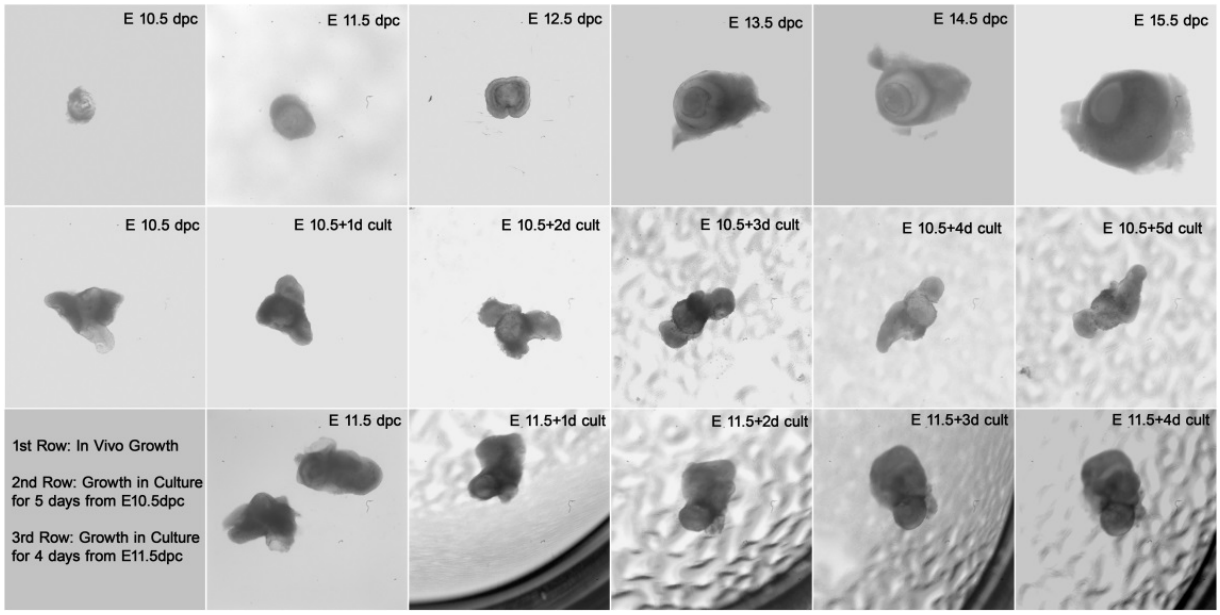

E 10.5 dpc
E 11.5 dpc
E 12.5 dpc
E 13.5 dpc
E 14.5 dpc
E 15.5 dpc
E 10.5 dpc
E 10.5+1d cult
E 10.5+2d cult
E 10.5+3d cult
E 10.5+4d cult
E 10.5+5d cult
1st Row: In Vivo Growth
2nd Row: Growth in Culture for 5 days from E10.5dpc
3rd Row: Growth in Culture for 4 days from E11.5dpc
E 11.5 dpc
E 11.5+1d cult
E 11.5+2d cult
E 11.5+3d cult
E 11.5+4d cult

Figure A.3: Histological analysis of optic rudiments in culture. (A-C) Ocular development in utero at E10.5 (A), E11.5 (B), E12.5 (C). (D-E) Ocular development in optic rudiment culture after day one (D) and day two (E). Scale bar in (E) applies to all panels.

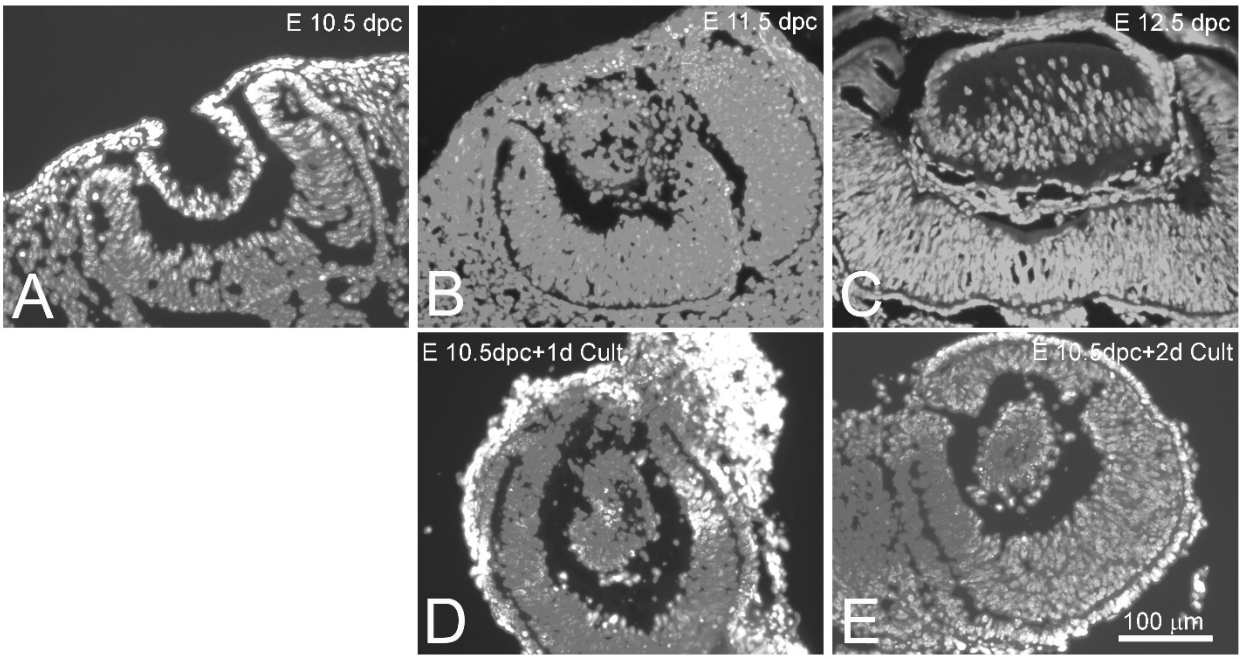

E 10.5 dpc
A
E 11.5 dpc
B
E 12.5 dpc
C
E 10.5dpc+1d Cult
D
E 10.5dpc+2d Cult
E
100 μm

Figure A.4: Tissue specific markers expressed in optic rudiments in culture. (A-B) αA-Crystallins expression in lens tissue in eyes developed in utero at E11.5 (A), E12.5 (B). (C-D) αA-Crystallins expression in lens tissue in optic rudiments grown in culture after day one (C) and day two (D).

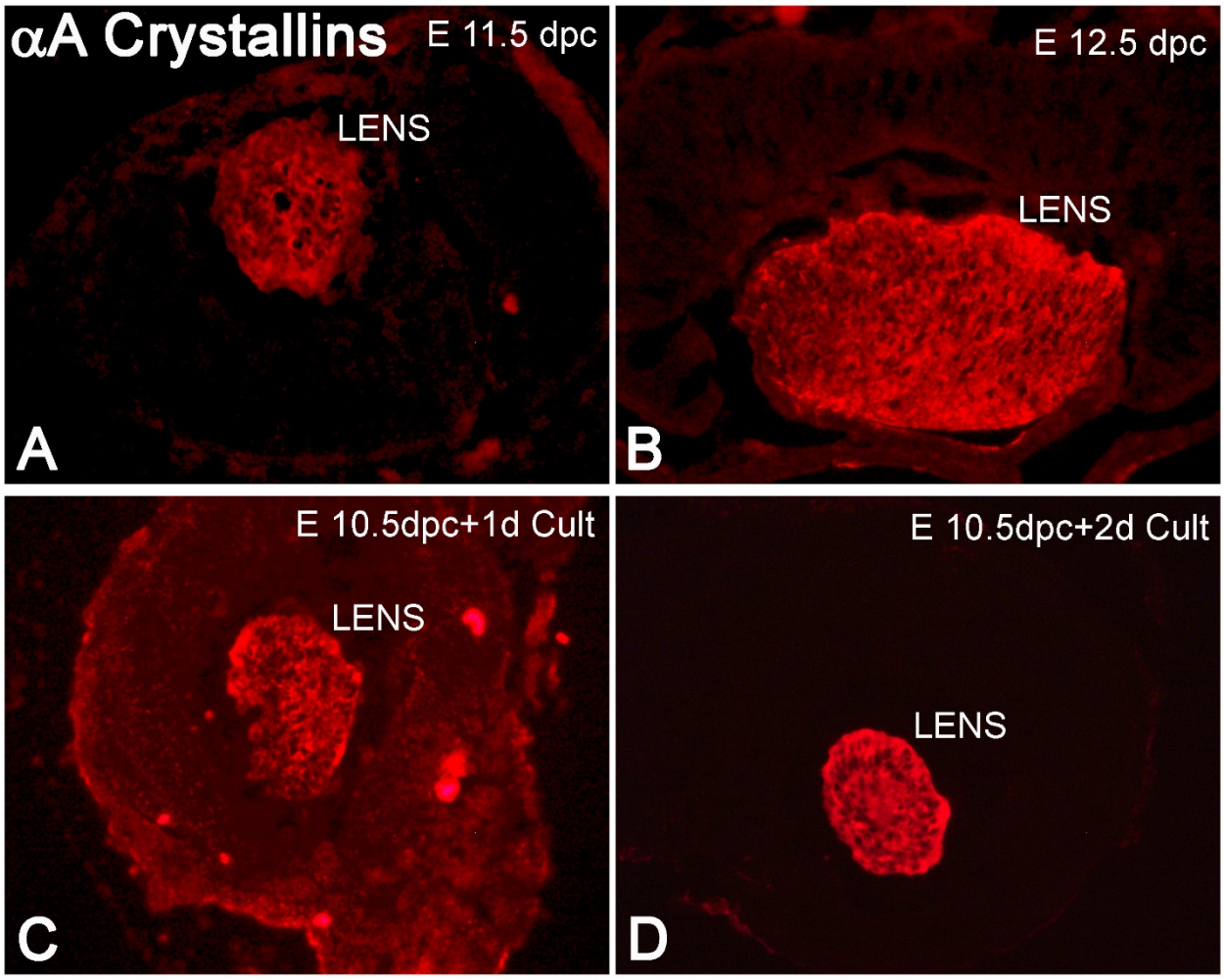

αA Crystallins
E 11.5 dpc
LENS
A
E 12.5 dpc
LENS
B
E 10.5dpc+1d Cult
LENS
C
E 10.5dpc+2d Cult
LENS
D

Figure A.5: Histological analysis of ocular tissue in mouse whole head culture. (A-D) Ocular development in utero at E10.5 (A), E11.5 (B), E12.5 (C), E13.5 (D). (E-G) Ocular development in mouse whole head culture after day one (E), day two (F) and day three (G).

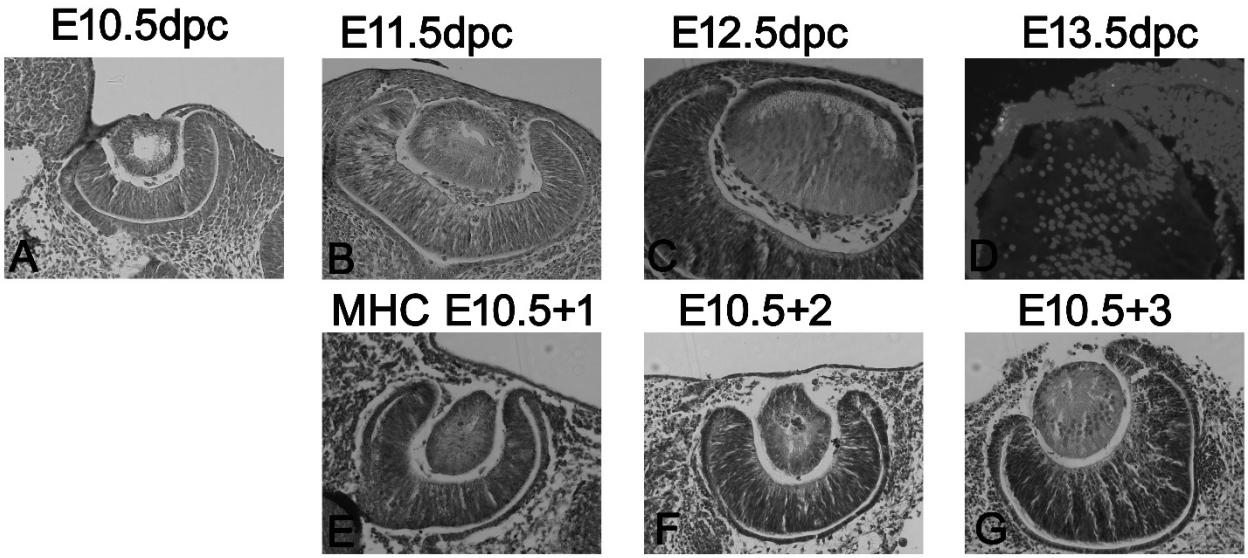
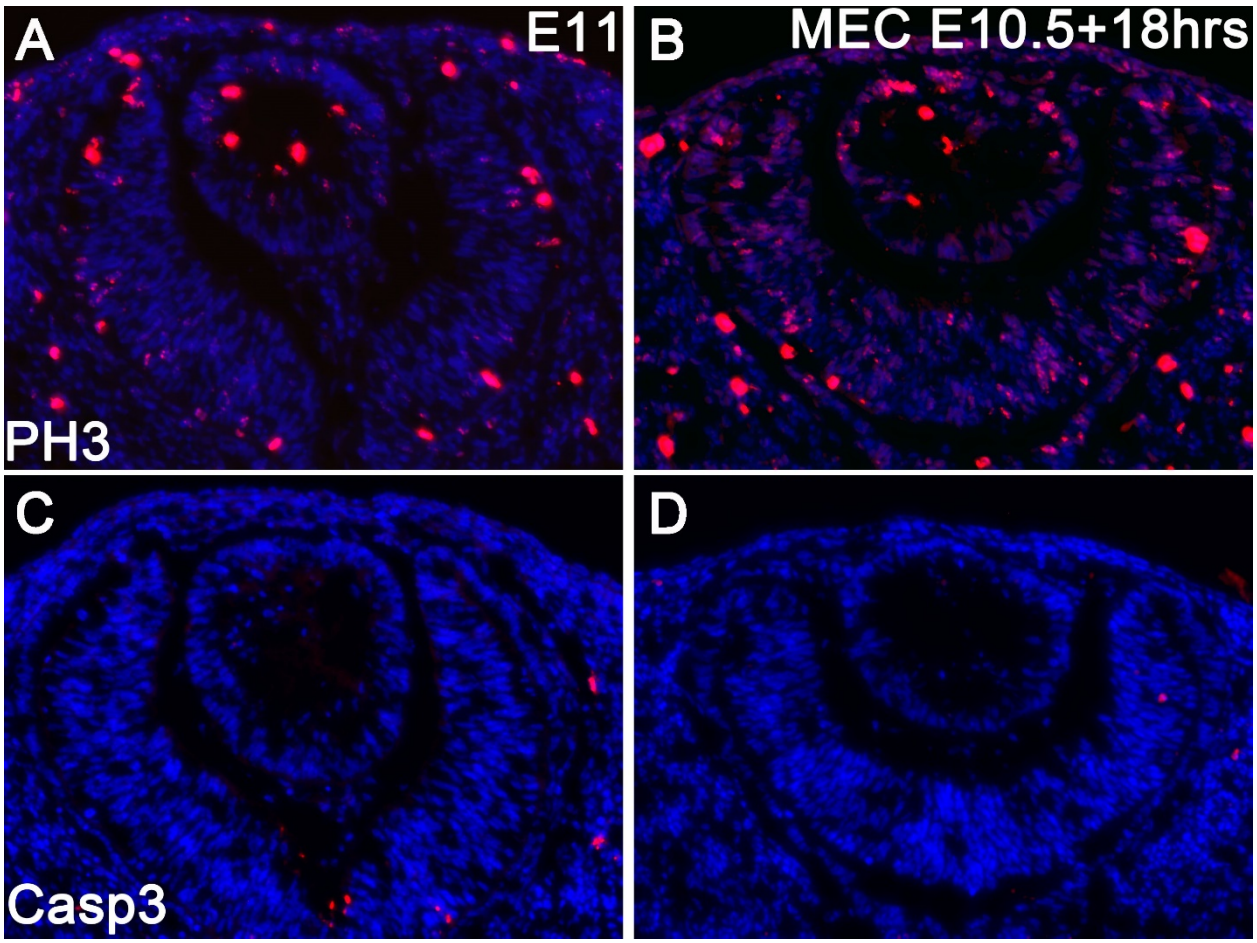

E10.5dpc
E11.5dpc
E12.5dpc
E13.5dpc
A
B
C
D
MHC E10.5+1
E10.5+2
E10.5+3
E
F
G

Figure A.6: Analysis of cellular proliferation and apoptosis levels in ocular tissues developed in mouse embryo culture (MEC) system. (A, B) Cellular proliferation as assessed by Phospho Histone H3 expression in ocular tissue developed in utero (A) and in MEC (B). (C, D) Cellular apoptosis as assessed by Cleaved Caspase 3 expression in ocular tissue developed in utero (C) and in MEC (D).


A
E11
PH3
B
MEC E10.5+18hrs
C
Casp3
D

APPENDIX – B

Analysis of the Role of BMP Signaling in Lens Cell Survival using Mouse Embryos Over-Expressing Pairedless Pax6 in a Mouse Embryo Culture System

Introduction

Individuals with mutations in Bone morphogenetic protein-4 (BMP4) gene locus revealed ocular abnormalities such as anterior segment (cornea, lens, iris, ciliary body) malformations, retinal defects, optic nerve aplasia and in some cases showed severe microphthalmia or anophthalmia. Though the molecular mechanisms underlying these developmental ocular defects are not understood, these studies suggest that BMP4 has a role in the formation as well as subsequent development of the ocular tissues. Recent studies demonstrated that over-expression of an isoform of Pax6 gene (called Pairedless Pax6) in mouse embryos resulted in upregulation of BMP4 expression in the optic cup with concomitant apoptotic degeneration of lens cells (Kim and Lauderdale, 2008). Pairedless Pax6 is an isoform of Pax6 gene that is formed from an internal promoter of Pax6 gene and contains only one DNA binding domain, the pairedlike homeodomain and lacks the paired domain while the canonical Pax6 has both the DNA binding domains. The pairedless isoform of Pax6 is expressed in the distal optic cup and in some subset of cells in the inner nuclear layer of retina (Kim and Lauderdale, 2008). These studies suggest that Bmp4 gene levels in the dorsal optic cup are critical and any alteration in their expression levels can affect the lens development and survival. In this study, I have analyzed the role of BMP4 signaling in lens cell survival using

these pairedless Pax6 over-expressing embryos in a mouse embryo culture system. I showed that, although BMP4 levels were upregulated in the distal optic cup, BMP signaling does not play a role in lens cell survival.

Method

Mouse embryos over-expressing Pairedless Pax6 were obtained as previously described (Kim and Lauderdale, 2008). Mouse embryo culture and implantation of BMP4, Noggin and BSA protein treated beads and subsequent tissue analysis were performed as previously described in Chapter 2 and 3.

Results

Analysis of Pairedless Pax6 over-expressing embryos reveals upregulation of BMP signaling in optic cup and lens

I first determined the time point at which the lens starts undergoing degeneration in these Pairedless Pax6 over-expressing embryos. The lens appeared to be normal at E10.5 dpc and starts to degenerate between E10.5 to E10.75 dpc (~35-37 ss) and shows partial lens degeneration by E11 dpc (Figure B.1D) which further degenerates by E12 dpc (Figure B.1E) while the wild type lens looks well developed by these stages (Figure B.1B, C). The lens was shown to degenerate completely by E15 dpc and at postnatal stages the eye looks microphthalmic with no lens compared to wild type eyes (Figure B.1F, F', G) (Kim and Lauderdale, 2008). Analysis of these eyes for BMP signaling activity revealed upregulation of Phosphorylated Smads 1/5/8 (PSmad 1/5/8) in the opic cup and also in the lens tissue compared to that observed in wild type eyes (Figure B.1H, I). Previous studies also revealed upregulation

of BMP4 gene expression in the distal optic vesicle (Kim and Lauderdale, 2008). These results strongly indicate a possible role for BMP4 signaling from optic cup in lens cell survival.

Mouse embryo culture replicates lens degeneration and expression of molecular markers in the eyes in Pairedless Pax6 over-expressing embryos

To determine the role of BMP4 signaling in lens cell survival I utilized an embryo culture system and evaluated this culture system for supporting ocular development ex utero (chapters 2 and 3) and tested whether these pairedless Pax6 over-expressing embryos exhibit similar development in culture. When pairedless Pax6 over-expressing embryos were cultured starting at E10.5 dpc, they exhibited progressive degeneration of the lens starting shortly after culture which was consistent with that observed in these embryos developing in utero (Figure B.2A-D). To rule out the possibility that this degeneration is caused by culture conditions, I tested for the expression of tissue specific molecular markers in these cultured embryonic eyes. In the cultured embryos the ocular tissues exhibited comparable development to that observed in these embryos developing in utero. The expression of tissue specific genes such as Pax6 and αA Crystallins and the level of cellular proliferation in the cultured ocular tissues was comparable to the in utero developed embryonic eyes (Figure B.3A-F). These results suggest that the mouse embryo culture conditions can replicate the lens degeneration phenotype in these pairedless Pax6 over-expressing embryos in culture.

BMP4 signaling may not have a role in lens cell survival

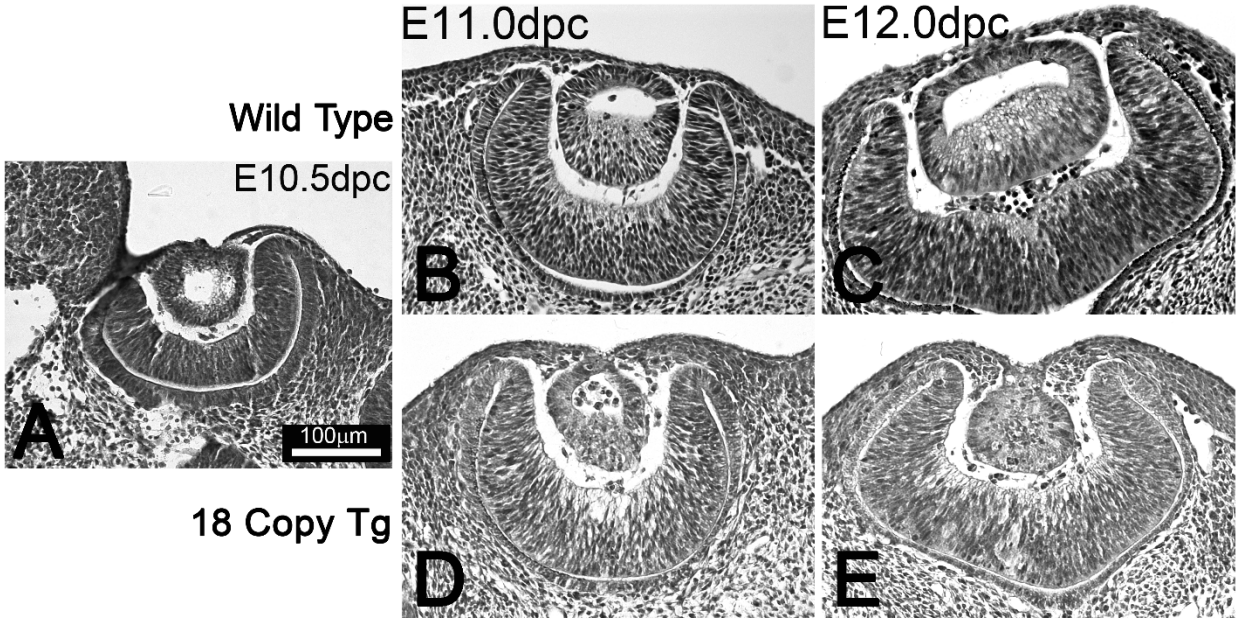
I utilized the mouse embryo culture system to further test the role of BMP4 signaling in lens cell survival using this pairedless Pax6 over-expressing embryos. I cultured wild type

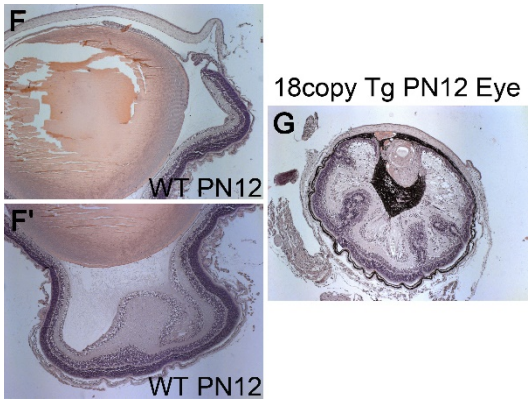
embryos and transgenic embryos carrying 8 or 18 copies (over-expressed) of the pairedless Pax6 gene. Before starting the culture, I implanted the wild type embryos and those carrying 8 copies of pairedless Pax6 transgene with affigel agarose beads treated with different doses of BMP4 protein in the left eye and with BSA treated beads in the right eye which act as control. For the embryos carrying 18 copies of pairedless Pax6 transgene, I implanted with beads treated with different doses of Noggin protein in the left eye and the right eye with BSA treated beads (control). I cultured these embryos for 16 to 24 hours and evaluated the ocular tissues for the role of BMP4. If the upregulated BMP4 signaling in pairedless Pax6 over-expressing embryonic eyes resulted in lens degeneration, then the BMP4 treated eyes in the cultured embryos should also exhibit lens degeneration. However, these BMP4 treated eyes in the cultured embryos appeared to be normal and comparable to the control eyes (Figure B.4A-B). Similar results were obtained when beads were implanted in the dorsal optic cup or even in the space between lens and retina. On the other hand, Noggin beads implanted in the pairedless Pax6 over-expressing embryonic eyes did not rescue the lens from degeneration (Figure B.3C-D). These results indicate that although there was upregulation of BMP4 signaling in optic cup and lens tissue in these pairedless Pax6 over-expressing embryonic eyes, the lens degeneration was mediated by some other signaling mechanism emanating from the optic cup or surface ectoderm as the surface ectoderm was also abnormal in these embryos. From these results, I concluded that the BMP4 signaling levels in the optic cup may not play a significant role in lens cell survival and over-expression of pairedless Pax6 might have altered some other factors that resulted in lens cell degeneration.

References

Kim, J. and Lauderdale, J. D. (2008). Overexpression of pairedless Pax6 in the retina disrupts corneal development and affects lens cell survival. *Developmental biology* **313**, 434-454.

Figure B.1: Analysis of Pairedless Pax6 over-expressing mouse embryonic eyes. (A-C, F, F') Wild type mouse embryonic eyes at E10.5 dpc (A), E11 dpc (B), E12 dpc (C) and PN12 (Postnatal day 12) (F, F'). (D-E, G) Pairedless Pax6 over-expressing mouse embryonic eyes showing lens degeneration at E11 dpc (D), E12 dpc (E) and PN12 (G). (H-I) PSmad 1/5/8 expression upregulated in Pairedless Pax6 over-expressing mouse embryonic eye (I) compared to wild type eye (H), at E11 dpc. Scale bar in (A) applies to (A-E).


E11.0dpc
E12.0dpc
Wild Type
E10.5dpc
A
100µm
B
C
18 Copy Tg
D
E


F
WT PN12
F'
WT PN12
18copy Tg PN12 Eye
G

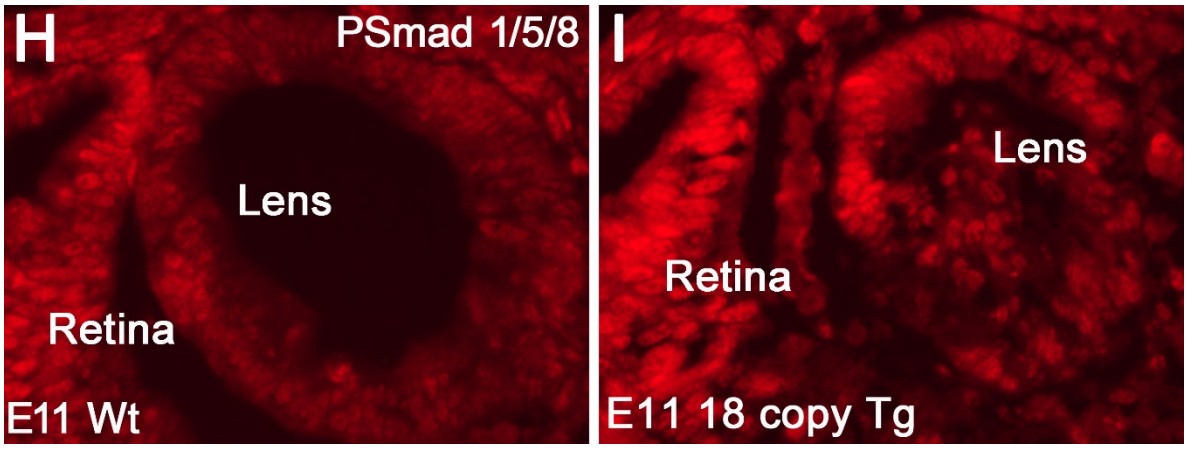

H
PSmad 1/5/8
Lens
Retina
E11 Wt
I
Lens
Retina
E11 18 copy Tg

Figure B.2: Mouse embryo culture replicates lens degeneration in Pairedless Pax6 over-expressing embryos. (A-D) Eyes from transgenic mouse embryos carrying 8 copies (A, C) and 18 copies (over-expression) (B, D) of Pairedless Pax6 at E12 dpc and developed in utero (A, B) and in mouse embryo culture (C, D).

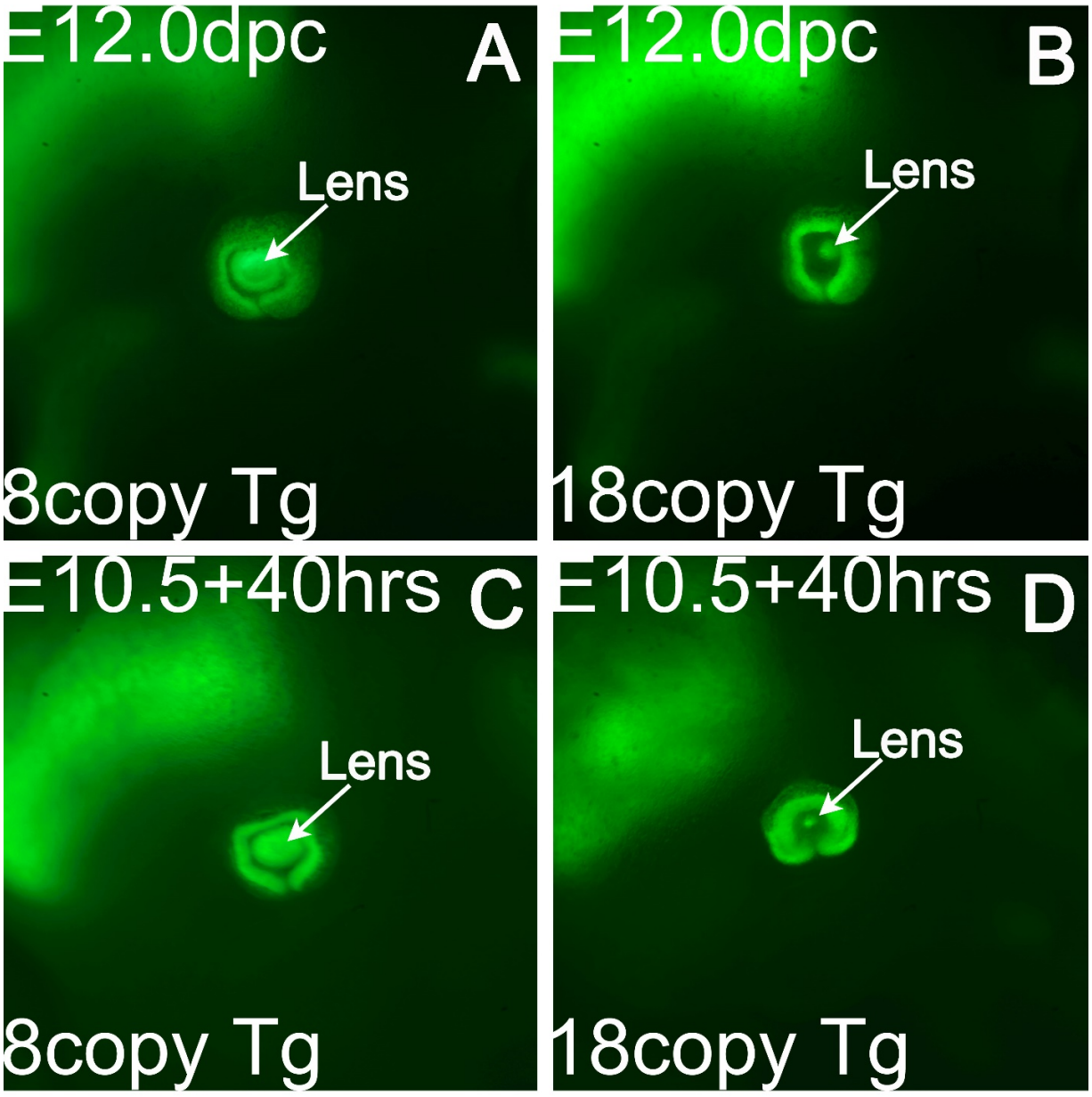

E12.0dpc
A
Lens
8copy Tg
E12.0dpc
B
Lens
18copy Tg
E10.5+40hrs
C
Lens
8copy Tg
E10.5+40hrs
D
Lens
18copy Tg

Figure B.3: Expression of tissue specific markers retained in the eyes in Pairedless Pax6 over-expressing embryos developed in mouse embryo culture. (A, C, E) Eyes from in utero developed embryos. Eyes from embryos developed in culture (B, D, F). Expression of Pax6 (A, B), αA Crystallins (C, D) and Phospho Histone H3 (PH3) (E, F). Scale bar in (F) applies to all panels.

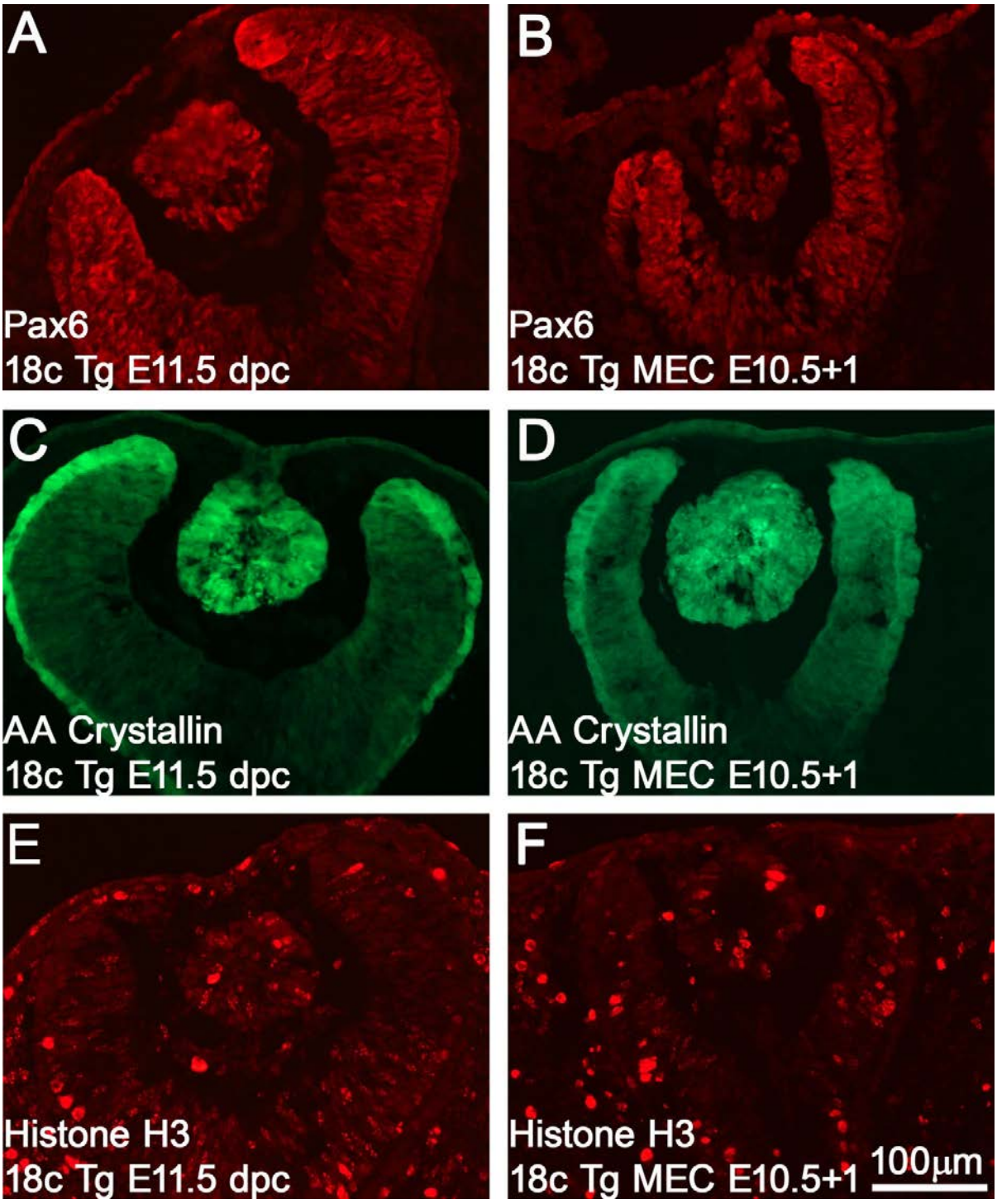
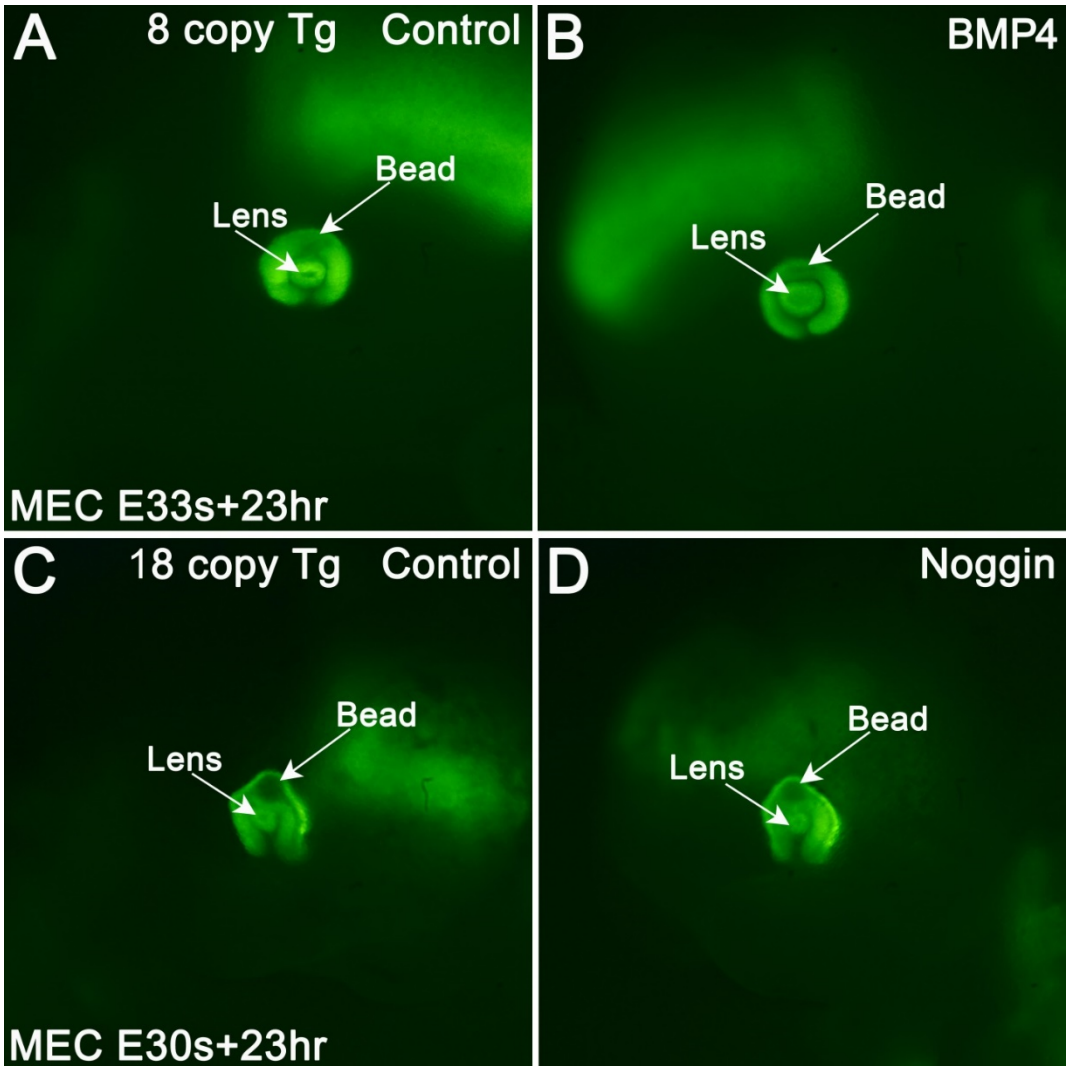

A
Pax6
18c Tg E11.5 dpc
B
Pax6
18c Tg MEC E10.5+1
C
AA Crystallin
18c Tg E11.5 dpc
D
AA Crystallin
18c Tg MEC E10.5+1
E
Histone H3
18c Tg E11.5 dpc
F
Histone H3
18c Tg MEC E10.5+1
100μm

Figure B.4: BMP4 signaling may not be required for lens cell survival. (A-D) Eyes from transgenic mouse embryos carrying 8 copies of Pairedless Pax6 gene (A, B) and implanted with BSA treated bead on right eye (Control) (A) and with BMP4 treated bead on left eye (B). The lens did not degenerate in BMP4 treated eyes and both eyes showed comparable lens development. Eyes from transgenic mouse embryos carrying 18 copies (over-expression) of Pairedless Pax6 gene (C, D) and implanted with BSA treated bead on right eye (Control) (C) and with Noggin treated bead on left eye (D). The Noggin treatment did not rescue the lens degeneration in these Pairedless Pax6 over-expressing embryonic eyes.


A
8 copy Tg
Control
Bead
Lens
MEC E33s+23hr
B
BMP4
Bead
Lens
C
18 copy Tg
Control
Bead
Lens
MEC E30s+23hr
D
Noggin
Bead
Lens

APPENDIX – C

Rabbit model of Limbal Stem Cell Deficiency: Surgical Reconstruction by Transplantation of Single Human Amniotic Membrane with Cultured Limbal Epithelial Progenitor Cells

Abstract

Objective: To create a rabbit model of limbal stem cell deficiency (LSCD) and functionally reconstruct the limbal region by the surgical transplantation of limbal epithelial progenitor cells (LEPC's) cultured on human amniotic membrane (AM).

Animal Studied: 29 New Zealand White rabbits.

Procedures: A limbal stem cell deficiency (LSCD) in right eye of normal New Zealand white rabbits was created by performing a 360 degree limbal peritomy and limbalectomy. Intact human amniotic membranes (AM), with (n=22) or without (n=9) cultured LEPC's were secured to the cornea. The LEPC's were cultured on the stromal surface of the AM. The AM with or without LEPC's were sutured with the stromal surface facing the cornea. Genetically modified LEPC's transfected with EGFP gene were used to test for successful incorporation and expression of foreign transgenes in the rabbit eyes. The rabbits were euthanized at 45 or 135 days after surgery and eyes were collected for histopathology, immunohistochemistry, and immunofluorescence. The left eye of each rabbit served as a normal control.

Results: Rabbit eyes transplanted with only the AM had thin corneal epithelium with only 3 – 4 cell layers consisting of morphologically normal basal epithelial cells and 2 – 3 layers of polygonal cells presumed to be wing cells. The corneas of eyes with AM and LEPC's were

morphologically similar to normal rabbit corneas. Eyes with AM and LEPC's had 6 – 7 layers of epithelial cells consisting of normal basal and wing cells that blended into the adjacent, unalteredcorneal epithelium. This single AM transplant technique is effective in functionally reconstructing the corneal epithelium for at least 135 days post transplantation.

Conclusions: The single membrane transplantation technique is an effective method to transfer cultured LEPC's to functionally reconstruct the limbal region and maintain the corneal epithelium in a rabbit model of LSCD.

Introduction

The cornea is the transparent front part of the eye developed from the surface ectoderm. It covers and protects the inner ocular structures and as well focusses the entry of light into the eye. Structurally the cornea is comprised of five layers including outermost epithelium followed by Bowman's layer, Stroma, Descemet's membrane and the innermost neural crest derived endothelium. The corneal epithelium consists of 6 to 7 layers of cells with innermost basal layer of columnar cells with prominent nucleus. Above the basal layer the cells begin to flatten and form 4 – 5 layers of suprabasal wing-like cells. The cells in the superficial layers are more flattened and contain tight junctions. These superficial layers are replenished constantly over time from the underlying cellular layers.

The corneal epithelium is maintained by a population of stem cells located in the basal epithelium at the corneoscleral limbus (Lavker et al., 2004; Schlotzer-Schrehardt and Kruse, 2005). These limbal stem cells (LSCs) cycle slowly to give rise to fast-dividing progenitor cells, termed transit amplifying (TA) cells, which make up the majority of the proliferative cell population in the basal epithelia of limbus and peripheral cornea. TA cells migrate centripetally

along the basement membrane of the corneal epithelium, proliferate and add cells to the basal layer of the corneal epithelium. These cells move apically contributing to the superficial layers of the corneal epithelium. The LSCs are characterized by expression of progenitor cell markers such as ABCG2, p63, vimentin and integrin α9 (Schlotzer-Schrehardt and Kruse, 2005). When the LSCs are damaged or dysfunctional, a disease state termed limbal stem cell deficiency (LSCD) develops, which is characterized by impaired corneal wound healing and loss of corneal integrity. LSC dysfunction or deficiency may arise from congenital or acquired conditions and has been implicated in the pathogenesis of ocular disorders, such as Stevens-Johnson syndrome, chemical injuries, and aniridia-related keratopathy (ARK) (Puangsricharern and Tseng, 1995).

ARK is a leading cause of blindness in patients with aniridia caused by heterozygous null mutations within the *Pax6* gene locus resulting in the failure of the limbal stem cells (LSC) to maintain the corneal integrity. ARK is typically treated by transplantation of limbal tissue, which is thought to renew the patient's LSCs population; however, this treatment carries a high risk of immunorejection (Ang et al., 2007). The cells in the aniridic eye are physiologically different than those of a normal individual and often respond uniquely to surgical treatment. For example, penetrating or lamellar keratoplasty is usually successful when used to treat non-*Pax6* deficient individuals, but fails when used to treat aniridic keratopathy (Gomes et al., 1996). One of the option for successful treatment of ARK may be to modify the LSCs in these patients by introducing a functional copy of Pax6 gene so that these LSCs can properly maintain the corneal integrity and prevent it from developing keratopathy. In the present study, we created a rabbit model of limbal stem cell deficiency by performing a 360^{o} limbalectomy and transplanted it with limbal epithelial/stem cells cultured on a single human amniotic membrane (AM). We also transfected the cells in culture with EGFP gene construct to test their incorporation into the

corneal tissue upon transplantation. We showed that limbal epithelial cells cultured on AM can functionally reconstruct the limbal region and maintain the corneal epithelium with no signs of corneal keratopathy. We also showed that the cultured LECs also incorporated the transfected EGFP gene into the limbal and corneal regions of the rabbit upon transplantation.

Method

Animal handling and maintenance

All the animals used in this study were maintained and handled in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and was approved by Institutional Animal Care and Use Committee at the University of Georgia.

Limbal epithelial explant culture

A 3.5 x 3.5 cm^2 human amniotic membrane (Bio-Tissue South Miami, FL, USA) was rinsed three times in sterile PBS and placed on a culture dish with the stromal surface facing upward. A sterile glass ring was placed on top of the AM to prevent rolling of the corners and fold formation.

Following a limbalectomy of normal rabbit eyes, limbal epithelial explants were placed in sterile phosphate buffered saline (PBS) with Gentamicin antibiotic. The limbal explants were then cut into 1 – 1.5 mm^2 size sections and 6 – 8 limbal sections were placed on the AM with a drop of fetal bovine serum (FBS) and incubated at 37ºC / 5% CO_2 for 1 hour. The FBS was replaced with culture medium that consisted of supplemented hormonal epidermal medium (SHEM) containing equal amounts (1:1 mixture) of DMEM/F12 medium supplemented with 5% FBS, 5 ng/ml EGF, 5 µg/ml Insulin, 5 µg/ml Transferrin, 5 ng/ml sodium selenite, 0.5 µg/ml Hydrocortisone, 30 ng/ml Cholera toxin A, 0.5% DMSO, 50 µg/ml Gentamicin and 1.25 µg/ml

Amphotericin B. 5 ml of culture medium was placed in the culture dish to cover the inside and outside of the glass ring. The AM and limbal explants were incubated at 37ºC under 5% CO_2 and 95% humidity for 12 – 14 days. The media was changed every 3 – 4 days. Immediately before transplantation, the explant tissue was removed from the AM leaving the cultured limbal epithelial cells on the AM.

LEC transfection in culture

The transfection was performed on 4 – 7 day old AM-LEPC cultures using an Enhanced Green Fluorescent Protein (EGFP) pA reporter construct (Kim and Lauderdale, 2006). Roche X-treme GENE HP transfection reagent was used to transfect the LEPC's in culture following the manufacturer's protocol. Briefly, a transfection mixture of 5 □g DNA, 500 transfection reagent was incubation at room temperature for 30 min. Before transfection, the culture medium was removed and the transfection mixture was added drop wise onto the cells and the AM-LEPC culture was incubated for 30 seconds with gentle swirling. 5 ml of culture medium was then added to the culture dish and incubated overnight at 37ºC under 5% CO_2 and 95% humidity. Transfection efficiency was evaluated the following day and re-transfection performed if required.

Surgical technique

A total of 29 New Zealand White Rabbits were used in the study. All rabbits were determined to be normal following a complete physical and ophthalmic examination including, Schirmer tear test (Eye Supply USA, Inc., Tampa, Florida, USA), rose bengal (Eye Supply USA, Inc., Tampa, Florida, USA) and fluorescein (Amcon Laboratories, St. Louis, Missouri, USA) staining, applanation tonometry (Tonopen XL; Medtronic Solan, Jacksonville, Florida, USA), slit- lamp biomicroscopy (SL-14; Kowa Company Ltd., Japan), and indirect

ophthalmoscopy. The rabbits were anesthetized with subcutaneous (SC) ketatmine hydrochloride (40 mg/kg) and xylazine (7 mg/kg), and maintained with isoflurane (Abbott Laboratories, North Chicago, Illinois, USA) and oxygen. A 360° limbal peritomy and limbalectomy was performed on the right eye of each rabbit. The surgical procedure was modified from previously published procedures to create a total LSCD (Kenyon and Tseng, 1989; Ti et al., 2002; Tsai and Tseng, 1994). Briefly, the bulbar conjunctiva was removed 5 mm posterior to the limbus, and a 360° 3 mm wide lamellar limbal ring was removed in a double ring fashion with the use of a 64-beaver blade, Martinez corneal dissector, and Wesscot corneal scissors. Following the limbal peritomy and limbalectomy, an AM, with or without LEPC's was placed stromal side down over the cornea. The AM was trimmed to cover the entire cornea and limbalectomy site. The AM's were secured to the ocular surface with 20 to 22, 9-0 nylon sutures (Focus Ophthalmics, Ontario, California, USA) to prevent retraction of the membrane.

Post-operative animal treatment, evaluation and euthanasia

Both groups of rabbits (AM and AM-LEPC) were treated post-operative with the same treatment that was modified from previously established protocols (Du et al., 2003; Koizumi et al., 2000a; Ti et al., 2002). Post-operative treatment consisted of butorphanol (Fort Dodge Animal Health, Inc., Overland Park, Kansas, USA) 0.4 mg/kg SC q 4 – 6 hrs for 5 days, topical Ofloxacin 0.3% ophthalmic solution (Alcon Laboratories, Forth Worth, Texas, USA) q 6 hours for 21 days, dexamethasone sodium phosphate 0.1% (Alcon Laboratories, Forth Worth, Texas, USA) q 6 hours for 21 days, and cyclosporine A 0.5% q 6 hours for 45 days then q 12 hours until euthanasia. Systemic post-operative treatment consisted of Baytril® (Bayer Animal Health, LLC, Animal Health Division, Shawnee Mission, Kansas, USA) 7.5 mg/kg SC q 12 hours for 7 days, and cyclosporine A, (DRAXIS Specialty Pharmaceuticals Inc., Kirkland, Quebec, Canada) IM or

oral (Neoral;Novartis Pharmaceuticals Corporation, Plantation, Florida, USA) 15 mg/kg/day for 7 days, then half-dose once a day for 14 days.

A complete ophthalmic examination was performed on all rabbits on post-operative day 1, 4, 7, then every 7 days until euthanasia at day 42 – 45. Additional ophthalmic examinations were performed on the rabbits transplanted with the AM-LEPC at day 60, 90, 120 for those that were euthanasia at 130 – 135 days. The eyes were photographed at the time of each ophthalmic examination. All rabbits were euthanized by intravenous administration of euthanasia solution (Beuthanasia-D Special) 1 ml/5 – 10 kg (Schering Plough Animal Health Corporation, Summit, New Jersey, USA). Immediately post-euthanasia, all eyes were enucleated, fixed in Davidson solution. After 48 hours, the eyes were placed in 10% formalin and stored for later evaluation.

Histopathology

The eyes were washed in 1x PBS, dehydrated in a series of alcohols and xylene, and embedded in paraffin. 3 – 4 µm sections were stained in hematoxylin and eosin.

Results

Human amniotic membrane supports propagation of limbal explant cells in culture

Limbal explant tissues obtained from surgical limbalectomy of limbal region in rabbits were cultured on single human amniotic membrane (AM) for 12 – 14 days before transplantation onto the rabbit eye (Figure C.1A). Proliferation of limbal epithelial progenitor cells (LEPC's) from the limbal explant tissues was observed starting day one in culture with cells extending in all directions around the explant tissue. Robust proliferation and expansion of the LEPC's was observed in the next 6 – 7 days covering 80 – 90% of the AM in 10 – 12 days of culture. To determine the integration and contribution of the cultured cells in corneal reconstruction when

transplanted onto rabbit eyes, the cells in the culture were transfected with an EGFP reporter construct after 4 – 7 days of start of culture. The transfection was repeated if required after 24 – 48 hours to achieve a transfection efficiency of 60 – 70% before transplantation. The LEPC's growing on AM incorporated the transfected EGFP construct and exhibited continued proliferation in the culture (Figure C.1B).

Surgical transplantation of single AM effectively integrates cultured LEPC's onto rabbit cornea

A 360° limbal peritomy and limbalectomy was performed on rabbit eyes to create limbal stem cell deficiency and the eyes were transplanted with a single AM with or without the cultured LEPC's (Figure C.1C). The AM was transplanted with LEPC's facing and covering cornea and limbal region of the eye and tightly secured to the cornea with twenty 9-0 nylon sutures. The sutured AM aligns close to the corneal epithelium and limbus region (Figure C.1D, C.1E). The LEPC's eventually gets integrated into the limbal region while the AM undergoes degradation in 2 - 3 weeks following surgery. The eye on left in figure C.1F (eye on right is non-transfected control) shows the integration of the EGFP transfected LEPC's into the limbal region and their contribution to the maintenance of the corneal epithelial as revealed by the green fluorescence in the limbus and corneal regions of the rabbit eye.

Cultured LEPC's prevent clinical manifestation of corneal breakdown in limbalectomized rabbit eyes

The superficial layer of corneal epithelium undergoes constant turnover and replacement with the cells from underlying layers. These cells are derived from the basal layer of corneal

epithelium which is constantly replenished by the proliferating cells from the corneal periphery and the limbal regions. The limbal region in the eye has been shown to harbor a niche of stem cells which proliferate and contribute the cells for maintenance of cornea. Surgical removal of this limbal region was supposed to result in clinical signs of corneal breakdown ultimately leading to corneal keratopathy and loss of vision. We tested this by creating a rabbit model of limbal stem cell deficiency by performing surgical limbalectomy of the entire limbus and transplantation of an AM with no cells or with cultured LEPC's. The LEPC's on AM are expected to functionally reconstruct the limbal region and maintain corneal integrity in these limbalectomized rabbits. Compared to the normal rabbit eyes (Figure C.2A), the eyes that have undergone limbalectomy and received transplantation of AM with no cells exhibited clinical signs of corneal breakdown by 45 days of surgery. These eyes revealed corneal neovascularization with new blood vessels growing onto the cornea from surrounding conjunctival region extending up to 3 – 4 mm into the peripheral cornea. Some of the rabbit eyes developed corneal conjunctivalization while some developed corneal ulceration as revealed by the uptake of the fluorescein stain (Figure C.2B). Contrary to this, the rabbit eyes that have undergone limbalectomy and received transplantation of AM with LEPC's exhibited no visible signs of corneal breakdown. These rabbit eyes showed normal cornea's even after 45 or 135 days of surgery with no evidence of neovascularization or other signs (Figure C.2C).

Cultured LEPC's on single AM reconstruct limbal region and maintain corneal epithelium in limbalectomized rabbit eyes

Histologically, rabbit corneal epithelium consists of 6 – 7 layers of cells with innermost single layer of basal cells having prominent nucleus followed by 4 – 5 layers of wing-like cells.

The superficial cellular layers consist of flattened cells that undergo regular turnover and are replaced by cells from underlying layers (Figure C.3A). Limbalectomized rabbit eyes that received only AM transplantation showed corneas that were thin with only 3 - 4 cell layers consisting of morphologically normal basal epithelial cells and 2 - 3 layers of polygonal cells that did not have specifically identifiable features but were presumed to be wing cells (Figure C.3B). However, rabbit eyes that received AM with cultured LEPC's exhibited corneas that were morphologically similar to normal rabbit corneas. These eyes showed 6 – 7 layers of epithelial cells consisting of normal basal and wing cells that blended into the adjacent, unaltered corneal epithelium (Figure C.3C). The limbus region in these rabbit eyes was also reconstructed and histologically appeared normal comparable to the normal rabbit limbus region (Figure C.3D). The area adjacent to the limbus was characterized by areas of fibroplasia with mild to absent inflammation in both surgical groups.

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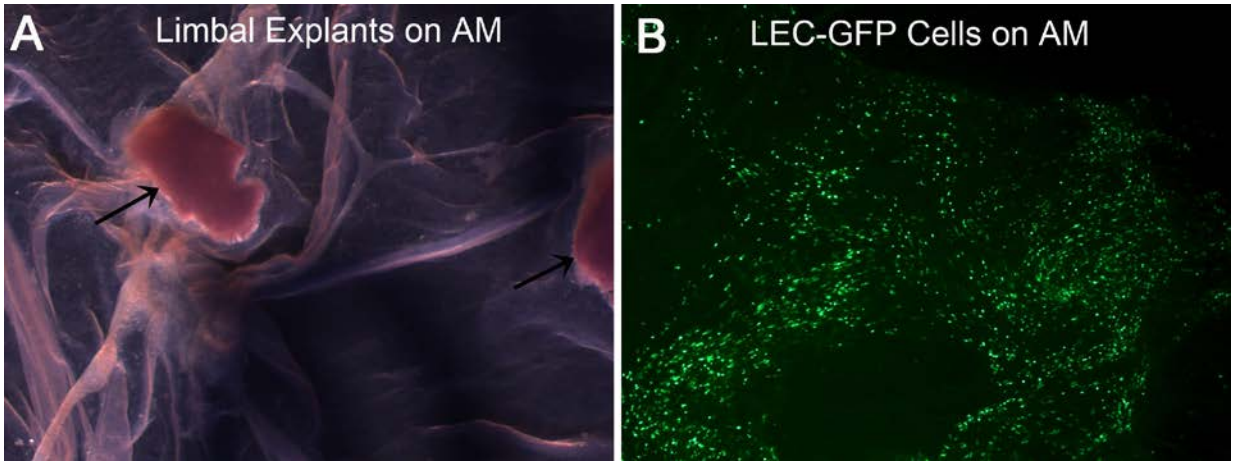
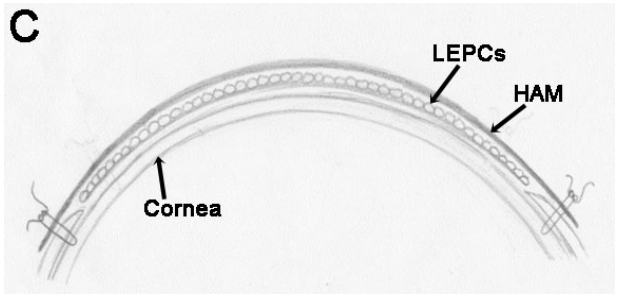
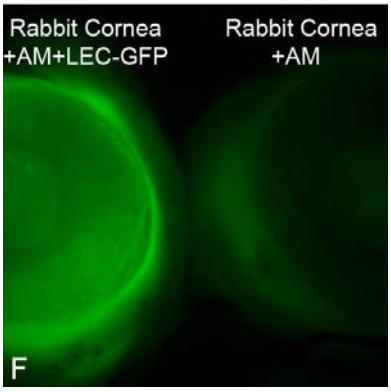
Figure C.1: Human amniotic membrane (HAM) supports LECs in culture. (A) Limbal explant on AM, (B) GFP transfected LECs on HAM in culture, (C) Surgical transplantation of HAM with LECs, (D-E) HAM upon transplantation on cornea (D) and Limbalectomized site (E), (F) Integration of LECs transfected with GFP into rabbit limbus and cornea.


A
Limbal Explants on AM
B
LEC-GFP Cells on AM


C
LEPCs
HAM
Cornea


Rabbit Cornea
+AM+LEC-GFP
Rabbit Cornea
+AM
F

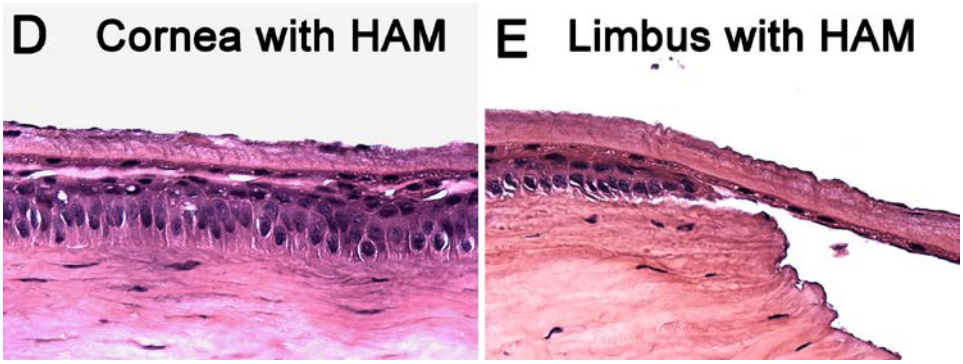
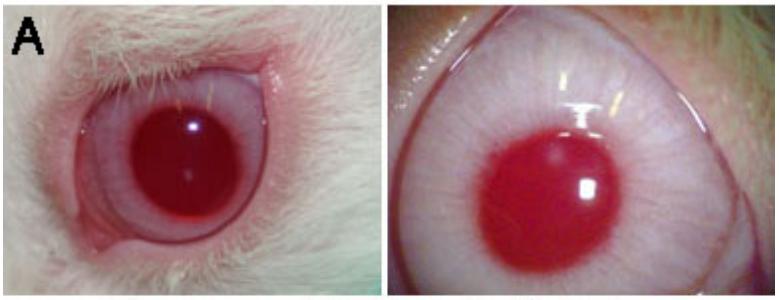
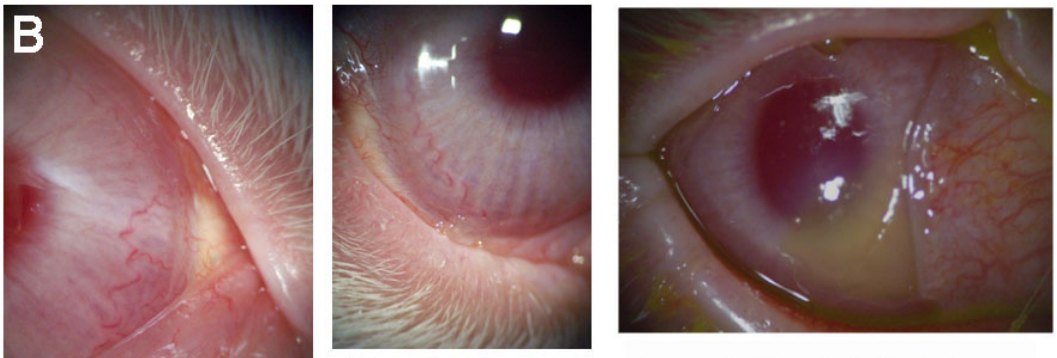

D
Cornea with HAM
E
Limbus with HAM

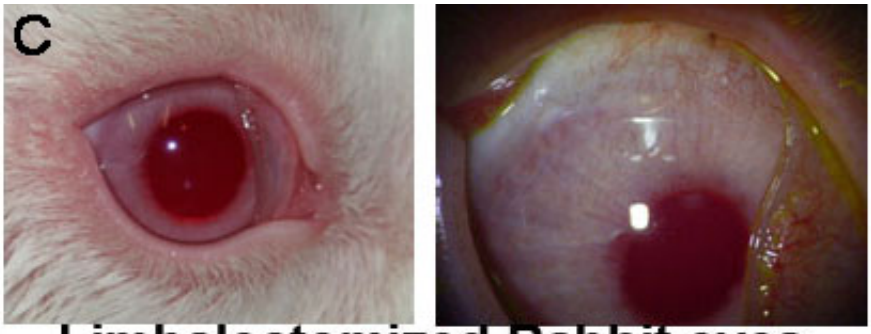
Figure C.2: Transplanted LECs prevents clinical manifestation of corneal breakdown in limbalectomized rabbits. (A) Normal rabbit eyes, (B) Limbalectomized rabbit eyes that received HAM with no LECs showing corneal vascularization and fluorescein staining, (C) Limbalectomized rabbit eyes that received HAM with LECs with no signs of corneal breakdown.



Normal Rabbit Eyes

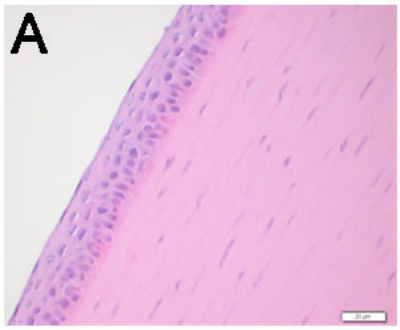


Limbalectomized Rabbit Eyes
+ HAM + No LEC's

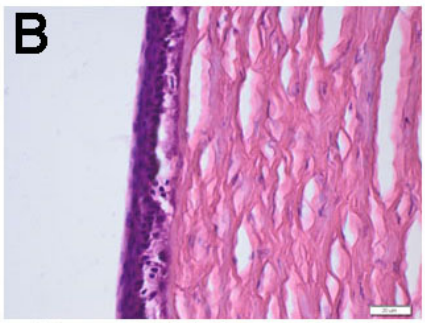


Limbalectomized Rabbit eyes
+ HAM + LEC's

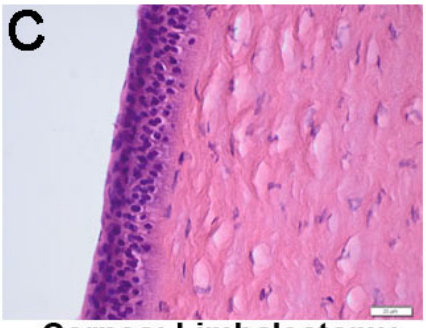
Figure C.3: Transplanted LECs maintain corneal integrity. (A) Normal rabbit cornea, (B) Limbalectomized rabbit eye cornea that received HAM with no LECs showing abnormal corneal epithelium, (C) Limbalectomized rabbit cornea that received HAM with LECs showing normal corneal epithelium, (D) Limbalectomized rabbit corneal-limbus region that received HAM with LECs showing reconstruction of rabbit limbus.



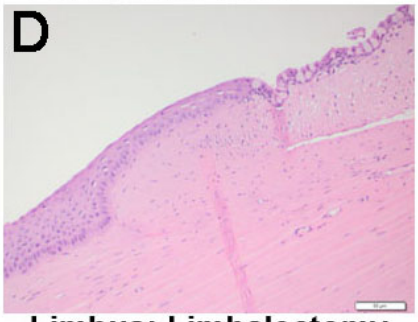
Normal Rabbit Cornea



Cornea: Limbalectomy
+ HAM (No LEC's)



Cornea: Limbalectomy
+ HAM + LEC's



Limbus: Limbalectomy
+ HAM + LEC's