·Basic Research·

Mutually inductive interactions between the lens and retina require ALK3 functions during mouse embryonic development

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Abstract

• AIM: To investigate the mutually inductive interactions that occur between the lens and retinal tissue during the development of the vertebrate eye.

• METHODS: Cre-positive mice were mated with Cre-negative mice to generate 50% Cre-positive (conditional knockout, CKO) and 50% Cre-negative offspring (wild type, WT). The embryos were fixed in 4% paraformaldehyde, embedded in paraffin, and sectioned to a thickness of 4μ m. The sections were processed for hematoxylin and eosin staining. The primary antibody used for immunofluorescence detection was sc-9305 bone morphogenetic proteins (bmp7) (Santa Cruz, US). The secondary antibody was IF-0314 aG0IgG/FITC (Santa Cruz) in combination with the primary antibody. Bright-field and fluorescent images were taken.

• RESULTS: Changes in the lens and retina were associated with specific alterations to the expression of type IA BMP receptor [BMPR-IA (ALK3)], which have already been implicated in eye growth. BMPR-IA was required for lens and retinal growth, but was not essential for the formation of lens. We observed that the expression of Bmp7 in the embryonic retina was reduced in the ALK3 lens of CKO mice. This phenomenon became increasingly visible in accordance with embryo development. This apparent alteration was present at stage E15.5.

• CONCLUSION: ALK3 is essential for lens and retinal growth. Mutually inductive interactions between the lens and retina are present in the developing mouse eye.

• KEYWORDS: BMPR-IA; Bmp7; inductive interactions; lens; retina
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INTRODUCTION

Embryonic eye development in vertebrates proceeds through a series of inductive processes that involve multiple tissue components and has been studied as a model system for exploring the general mechanisms that underlie embryonic tissue interactions [1]. Development of the embryonic dorsoventral axis of vertebrates requires signaling via bone morphogenetic proteins (Bmp) and feed-forward regulation that depend on the tight fine-tuning of protein expression and distribution levels [2,3]. The bone morphogenetic protein (Bmp) family has been implicated in the control of inductive processes during normal lens and retina development [4]. The eye lens forms from the ectoderm of the head surface, whereas the retina forms from the neural tube. Lens development begins with the lens placode, which is the thickened surface of the ectoderm that comes into contact with the optic vesicle. The Bmp signals that are critical for lens formation have already been confirmed, and some studies also claim that the inactivation of Bmp4 results in the absence of lens induction and that the targeted inactivation of Bmp7 in the lens placode leads to defective lens placode formation. However, the precise genetic mechanisms by which Bmp signals regulate these developmental processes are obscured in null mutants.

Formation of the neural retina follows a transient period of close contact between the DD region of the optic vesicle and the surface ectoderm/presumptive lens. Early evidence that

suggests that the surface ectoderm might play a role in neural retina specification was provided by observations in tissue experiments, specifically that the neural retina does not develop if the surface ectoderm is removed [5]. More recent experiments have confirmed this observation [6,7]. In addition, it has been shown that if the amphibian optic cup is rotated by 180°, such that the presumptive RPE faces the surface ectoderm, the optic cup develops into a secondary neural retina [8]. These observations indicate that the surface ectoderm plays a very important role in directing neural retina formation. Interestingly, transplantation experiments have indicated that lens-to-retina signaling is important for retinal maintenance in the mature fish eye, and its absence is a step in the evolution of a species of blind cave fish [9].

In 1901, Spemann made the significant observation that ablation of the presumptive retinal region during the neural plate stage in *Rana temporaria* embryos resulted not only in the absence of retinal development but also the loss of lens formation [10]. Because early fate mapping had already shown that the lens is derived from a region outside of the regional region that was ablated, Spemann argued that the development of the lens depends on the presumptive retina. This observation not only illustrated the importance of tissue interactions during eye development, but also provided the first experimental evidence that lead to the idea of embryonic induction [11]. The development of the vertebrate eye requires mutually inductive interactions between the lens and retina tissue.

BMPs are members of the TGF superfamily. Although BMPs have been identified by their ectopic bone-formation activities by overexpression analysis, many reports now indicate that members of the BMP subfamily play critical roles during organogenesis. BMPs bind to two different membrane-bound Ser/Thr kinase receptors (type I and type II receptors) for signal transduction [12]. The signaling specificity is initially determined by the type-I receptors (BMPR-IA or ALK3). BMPR-IA is expressed in many tissues throughout embryonic development and after birth. Studies have implicated members of the Bmp gene family, specifically Bmp7, in murine eye development. Bmp7 (-/-) null mutants exhibit defects in lens induction [13,14]. To investigate the role of BMPR-IA signaling during later stages of eye development, and to confirm the importance of inductive interactions between the lens and retina in the embryonic development of vertebrates, we generated BMPR-IA (ALK3) CKO mice, and focused our attention on Bmp7 expression.

MATERIALS AND METHODS

Materials Mice expressing Cre recombinase (Le-Cre), which have been previously described [15], were used in this experiment. Mice carrying the Cre transgene or the floxed alleles were used in this study (Acvr1fx[exon7]). Genomic DNA from embryonic tail tissue was extracted using the HotSHOT method [16,17]. The PCR conditions were selected according to the universal PCR protocol [18]. In this experiment, the mice were provided by the University of Southern California, USA and were 11.5-15.5 days old in terms of the embryonic gestational age. Mice that were homozygous floxed for BMP receptor type-IA genes, one of which was Cre-positive, were mated to generate 50% Cre-positive (CKO) and 50% Cre-negative offspring (wild type, WT). Cre-positive animals were always mated with Cre-negative animals, assuring that the Cre-positive offspring inherited only one copy of the Cre transgene. The primary antibody used for immunofluorescence analysis was sc-9305 bmp7 (Santa Cruz, USA). The secondary antibody was IF-0314 aG0IgG/FITC (Santa Cruz).

Methods We selected specific pregnant mice. The mice were killed at a specific gestational age, and the embryos were removed and washed with PBS. The embryos were fixed in 4% paraformaldehyde in PBS overnight at room temperature, dehydrated through a graded series of methanol, embedded in paraffin, and sectioned to a thickness of 4μm. The sections were processed for hematoxylin and eosin (HE) staining and immunofluorescence.

The procedure for the HE staining was as follows: 1) roast 30 minutes ahead of schedule; 2) rinse with xylene, 5 minutes; 3) rinse with xylene, 5 minutes; 4) rinse with anhydrous ethanol, 5 minutes; 5) Rinse with 95% alcohol for several seconds; 6) rinse with 90% alcohol for several seconds; 7) Rinse with 80% alcohol for several seconds; 8) flush with water; 9) rinse with hematoxylin, 5-10 minutes; 10) flush with water; 11) rinse with eosin, 2-3minutes; 12) flush with water; 13) rinse with 95% alcohol for several seconds; 14) rinse with 95% alcohol for several seconds; 15) rinse with anhydrous ethanol, 5 minutes; 16) rinse with anhydrous ethanol, 5 minutes; 17) rinse with xylene, 5 minutes; 18) rinse with xylene, 5 minutes; 19) dry and mount; and 20) observe under an optical microscope.

The procedure for immunofluorescence analysis was as follows: 1) conventional paraffin dewaxing: three washes with xylene for 5 minutes each time, two washes with 100% ethanol for 5 minutes each time, rinse with 9% ethanol for 2 minutes, rinse with 70% ethanol for 2 minutes, rinse with 40% ethanol for 2 minutes, rinse with PBS for 5 minutes; 2) wash with citrate buffer solution for microwave antigen retrieval; 3) rinse with distilled water for 3 minutes, rinse with PBS twice, rinse with 4% hydrogen peroxide at room temperature, incubate for 20 minutes to clear the endogenous peroxidase activity, and soak twice in PBS for 3 minutes each time; 4) incubate in normal serum at room temperature for 20 minutes to close nonspecific antigens; 5) suction out sheep serum, then add the primary antibody and incubate overnight; 6) wash three times with PBS, add the

secondary antibody (avoid contact with light), incubate for 1 hour, and wash three times with PBS; 7) mount using glycerol phosphate; and 8) observe using fluorescence microscopy.

For all of the experiments described here, 10 or more embryos of each genotype were examined. For immunofluorescence analysis, the primary antibody used for immunofluorescence detection was sc-9305 bmp7 (Santa Cruz). The secondary antibody, IF-0314 aG0IgG/FITC (Santa Cruz), was used at an appropriate ratio with the primary antibody (1:200). All of the bright-field images were taken using an Olympus BX60 microscope and Spot camera. The fluorescent images were taken using the Olympus BX51 with Spot camera with an absorption peak of 492 nm and an emission peak of 510 nm.

RESULTS

ALK3 is Essential for Lens and Retinal Growth Earlier studies have shown that the BMP ligands (specifically, BMP7) are required for lens formation [19-21]. To determine the roles of different BMP receptors in the formation of the lens, we deleted the genes for type-I BMP receptors, specifically BMPR-IA, at the surface of the ectoderm/ presumptive lens using a Cre recombinase that was driven by the Pax6 P0 promoter/enhancer (Le-Cre). In transgenic mice, Cre is expressed in the prospective lens placode by stage E9.0 [22]. The loss of BMPR-IA did not prevent lens formation, however BMPR-IA CKO lenses were reduced in size with notable defects in fiber cell differentiation [23]. Although BMPR-IA maintains normal levels of cell proliferation and survival during the formation of the lens placode, it is not essential for lens formation (Figure 1,2).

The mutants appeared indistinguishable from the normal littermates until stage E11.5. At birth, however, these mutants exhibited mic-anophthalmia. Gross morphological abnormalities were apparent by stage E13.5, with the mutant embryos showing smaller eyes with a rough margin at the retinal pigment epithelium. Histological examination of the mutants revealed that the abnormal thickness of the retinal neuroectoderm began at stage E13.5, and the abnormal alterations become apparent by stage E15.5 (Figure 3, 4). In Figures 3 and 4, we show the changes that occurred in the retina; in these sections, our aim was to protect retinal morphology, so sometimes the lens may be incomplete or missing.

Changes in the lens and retina are associated with specific alterations to the expression of regulatory genes that are necessary for eye growth. BMPR-IA (ALK3) is a key regulator in this process; however, although it is required for lens and retinal growth, it is not essential for the formation of lens.

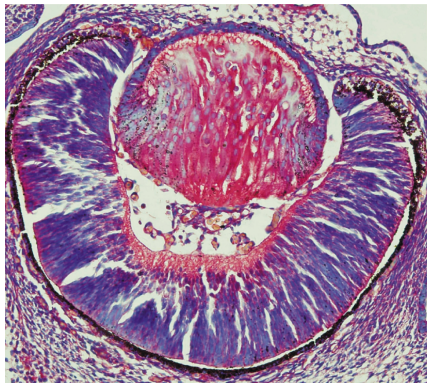

Figure 1 BMPR –IA (ALK3) CKO lens. Section of the indicated embryo at stage E13.5 that was stained with HE.

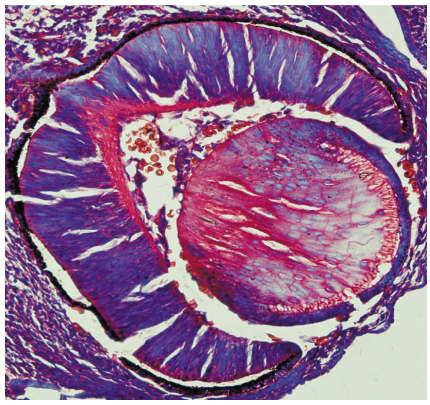

Figure 2 WT mice eye. Section of the indicated embryo at stage E13.5 that was stained with HE.

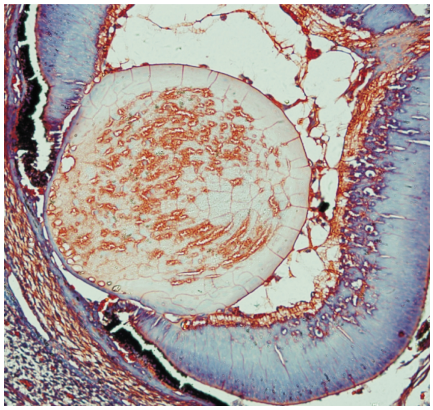

Figure 3 BMPR –IA (ALK3) CKO lens. Section of the indicated embryo at stage E15.5 that was stained with HE.

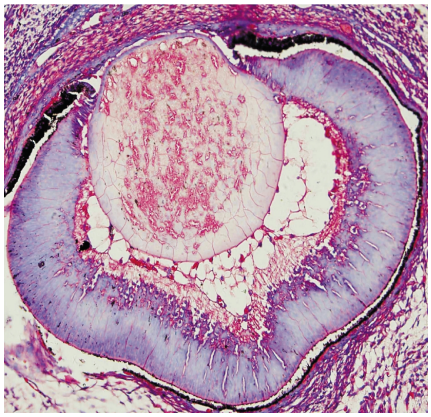

Figure 4 WT murine eye. Section of the indicated embryo at stage E15.5 that was stained with HE.

Mutually inductive interactions between the lens and retina during embryonic development Spemann deleted the retinal region during the neural plate stage in *R. temporaria* embryos, which resulted not only in the absence of retinal development but also defects in lens formation. Spemann thought that the development of the lens depended on the presumptive retina. Previous evidence that the surface ectoderm might play a role in neural retina differentiation was provided by observations in tissue explantation experiments that the neural retina would not develop if the surface ectoderm was removed. More recent experiments have confirmed this observation. This observation not only illustrates the importance of tissue interactions during eye development, but also provides the first experimental evidence that resulted in the idea of embryonic induction. The development of the vertebrate eye requires mutually inductive interactions between lens and retina tissue

The expression of BMP7, an important ligand in the Bmp family, was reduced in the lens and retina of the embryos in which we conditionally deleted the gene that encodes BMPR-IA in the developing mouse lens (Figure 5, 6). We observed that BMP7 expression in the lens was gradually reduced in the ALK3 mutants, which resulted in the abnormal lens formation, as verified by immunofluorescence experiments; this specific phenomenon was apparent by stage E15.5, however, BMP7 levels were normal in the WT lens. The most important thing that we observed is that the expression of BMP7 in the embryonic retina was reduced in the ALK3 CKO mice. This phenomenon was increasingly visible in accordance with embryo development. This apparent alteration was present by stage E15.5 (Figure 7, 8). These results suggest that mutually inductive interactions are present between the lens and retina in the developing mouse eye.

DISCUSSION

Murine BMPR-IA CKO has demonstrated that BMP signaling is essential for the development of the lens and retina, although these studies did not identify the cellular mechanisms underlying this process [24, 25]. In addition, the use of murine BMPR-IA CKO has uncovered the indispensable role of Bmp signaling for maintaining growth and differentiation in the developing lens and retina of mice. Here, we provide evidence that BMPR-IA acts redundantly to mediate lens and retina formation and confirm the notion that embryonic induction is present.

BMPR-IA plays redundant roles in embryonic eye development. We identified direct genetic evidence of the functional redundancy of BMPR-IA. The receptor is capable of binding to Bmp2, Bmp4, and Bmp7 ligands, albeit with varying affinities [26]. A study that utilized the forced expression of constitutively active BMPR-IA, both in transgenic mice and neural stem cells, suggested that

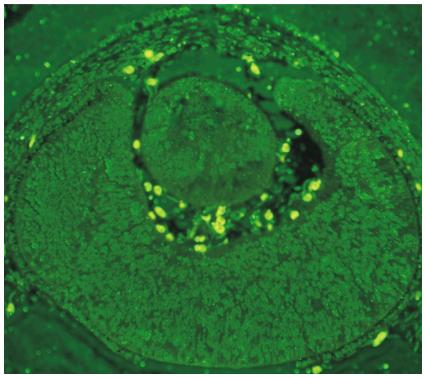

Figure 5 BMPR –IA (ALK3) CKO lens. Section of the indicated embryo at stage E13.5, showing the immunofl – uorescence detection of BMP7 expression.

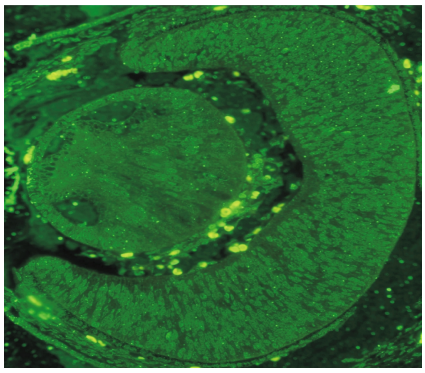

Figure 6 WT murine eye. Section of the indicated embryo at stage E13.5, showing the immunofluorescence detection of BMP7 expression.

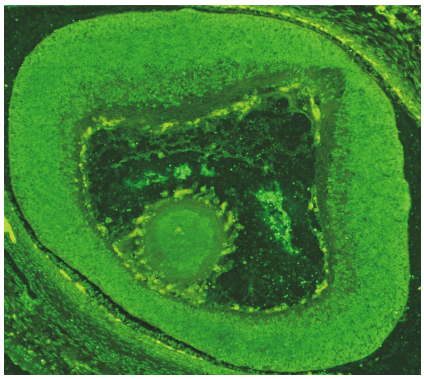

Figure 7 BMPR –IA (ALK3) CKO lens. Section of the indicated embryo at stage E15.5, showing the immunofluorescence detection of BMP7 expression.

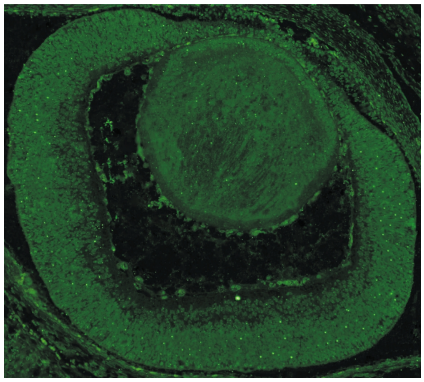

Figure 8 WT murine eye. Section of the indicated embryo at stage E15.5, showing the immunofluorescence detection of BMP7 expression.

BMPR-IA regulates proliferation in the developing central nervous system [27]. Similarly, in the limb bud development of chicks, ectopic expression of constitutively active and dominant-negative forms of the receptor revealed that BMPR-IA plays a role in controlling the latter differentiation processes [28]. The apparent distinction between these reports may be attributed, at least in part, to differences in the experimental models, tissues, and cell types that were examined. One wide implication of the current study is that BMPR-IA may also play significant roles in other tissues. For instance, the identification of BMPR-IA functions in the embryonic telencephalon reveal that its effects are active only at the dorsal midline, unlike the more lateral telencephalic regions where the expression of BMPR-IA and BMPR-IB overlap [29]. Therefore, it is likely that the disruption of receptors in the telencephalon may reveal a more extensive role for Bmp signaling. In addition, this general principle may be extended to the receptors of other subgroups in the TGF-β superfamily. Some differences in expression could result from varying temporal/spatial expression domains, levels of expression, and/or biochemical properties.

Inductive interactions between the lens and retina in the developing mouse eye are necessary. The development of the vertebrate eye, similar to that of many other organs, is the result of complex, mutual interactions between tissues of different embryological origins. The lens, for instance, is derived from the surface ectoderm that overlies the optic vesicle; nevertheless, without the juxtaposition of a normal optic vesicle, lens development is disturbed [30-33]. In contrast, without a proper lens placode, the optic vesicle remains rudimentary and an optic cup with a well-defined neuroretinal layer and pigmented layer does not develop. BMP cross-talk also plays a necessary role in the very early stages of lens and retinal development [34].

Previous evidence that the surface ectoderm/presumptive lens plays a role in neuroretinal specification was provided by observations in tissue explantation experiments, specifically that the neural retina would not develop if the surface ectoderm was removed. More recent experiments have confirmed this observation. It has been shown that if the amphibian optic cup is rotated by 180°, such that the presumptive RPE would face the surface ectoderm, the optic cup would develop into a secondary neural retina [35,36]. These observations indicate the very important role the surface ectoderm plays in directing neural retina formation. Lens-to-retina signaling is also important for retinal development and maintenance.

Spemann made the observation that ablation of the presumptive retinal region during the neural plate stage in *R. temporaria* embryos resulted in not only the absence of retinal development but also the loss of lens formation. Early fate mapping had already shown that the lens is derived from a region outside of the one that Spemann had ablated, but Spemann thought that the development of the lens depended on the presumptive retina. This observation not only illustrated the importance of tissue interactions during eye development, but also provided the first experimental evidence that lead to the notion of embryonic induction. Though the severity of morphological abnormalities in ALK3 CKO mice may be variable, through our experiments we can concluded that ALK3 is essential for lens and retinal growth when mutually inductive interactions between the lens and retina are present during embryonic development.

REFERENCES

1 Jacobson AG, Sater AK. Features of embryonic induction. *Development* 1988;104(3):341-359

2 Little SC, Mullins MC. Extracellular modulation of BMP activity in patterning the dorsoventral axis. *Birth Defects Res C Embryo Today* 2006; 78:224-242

3 Little SC, Mullins MC. Bone morphogenetic protein heterodimers assemble heteromeric type I receptor complexes to pattern the dorsoventral axis. *Nat Cell Biol* 2009;11:637-643

4 Chow RL, Lang RA. Early eye development in vertebrates. *Annu Rev Cell Dev Biol* 2001;17(2):255-296

5 Holtfreter J. Gewebeaffinitat, ein Mittel der embryonalen Formbildung. *Arch Exp Zellforsch* 1939;23:169-209

6 Hyer J, Mima T, Mikawa T. FGF1 patterns the optic vesicle by directing the placement of the neural retina domain. *Development* 1998;125 (5): 869-877

7 Nguyen M, Arnheiter H. Signaling and transcriptional regulation in early mammalian eye development: a link between FGF and MITF. *Development* 2000;127(16):3581-3591

8 Mikami Y. Reciprocal transformation of the parts in the developing eye-vesicle, with special reference to the inductive influence of the lens-ectoderm on the retinal determination. *Zool Mag* 1939;51:253-256

9 Yamamoto Y, Jeffery WR. Central role for the lens in cave fish eye degeneration. *Science* 2000; 289(5479):631-633

10 Spemann H. Uber Correlationen in der Entwickelung des Auges. *Ver Anat Ges* 1901;15:61-79

11 Hamburger V. The heritage of experimental embryology: Hans Spemann and the organizer. New York: Oxford Univ Press; 1988

12 Morcillo J, Martinez-Morales JR, Trousse F, Fermin Y, Sowden JC, Bovolenta P. Proper patterning of the optic fissure requires the sequential activity of BMP7 and SHH. *Development* 2006;133(16):3179-3190

13 Massague J, Blain SW, Lo RS. TGFbeta signaling in growth control, cancer, and heritable disorders. *Cell* 2000;103(2):295-309

14 Shi Y, Massague J. Mechanisms of TGF-beta signaling from cell membrane to the nucleus. *Cell* 2003;113(6):685-700

15 Ashery-Padan R, Marquardt T, Zhou X, Gruss P. Pax6 activity in the lens primordium is required for lens formation and for correct placement of a single retina in the eye. *Genes Dev* 2000;14(21):2701-2711

16 Dudas M, Sridurongrit S, Nagy A, Okazaki K, Kaartinen V. Craniofacial defects in mice lacking BMP type I receptor Alk2 in neural crest cells. *Mech Dev* 2004;121(2):173–182

17 Truett GE, Heeger P, Mynatt RL, Truett AA, Walker JA, Warman ML. Preparation of PCR–quality mouse genomic DNA with hot sodium hydroxide and tris (HotSHOT). *Biotechniques* 2000;29(1):52–54

18 Stratman JL, Barnes WM, Simon TC. Universal PCR genotyping assay that achieves single copy sensitivity with any primer pair. *Transgenic Res* 2003;12(4):521–522

19 Wawersik S, Purcell P, Rauchman M, Dudley AT, Robertson EJ, Maas R. BMP7 acts in murine lens placode development. *Dev Biol* 1999;207(1): 176–188

20 Furuta Y, Hogan BLM. BMP4 is essential for lens induction in the mouse embryo. *Genes Dev* 1998;12(23):3764–3775

21 Jena N, Martin–Seisdedos C, McCue P, Croce CM. BMP7 null mutation in mice: developmental defects in skeleton, kidney, and eye. *Exp Cell Res* 1997;230(1):28–37

22 Ashery–Padan R, Marquardt T, Zhou X, Gruss P. Pax6 activity in the lens primordium is required for lens formation and for correct placement of a single retina in the eye. *Genes Dev* 2000;14(21):2701–2711

23 Beebe D, Garcia C, Wang X, Rajagopal R, Feldmeier M, Kim JY, Chytil A, Moses H, Ashery–Padan R, Rauchman M. Contributions by members of the TGFbeta superfamily to lens development. *Int J Dev Biol* 2004;48 (8–9):845–856

24 Dudley AT, Robertson EJ. Overlapping expression domains of bone morphogenetic protein family members potentially account for limited tissue defects in BMP7 deficient embryos. *Dev Dyn* 1997;208(3):349–362

25 Luo G, Hofmann C, Bronckers AL, Sohocki M, Bradley A, Karsenty G. BMP–7 is an inducer of nephrogenesis, and is also required for eye development and skeletal patterning. *Genes Dev* 1995;9(22):2808–2820

26 Mishina Y. Function of bone morphogenetic protein signaling during mouse development. *Front Biosci* 2003;1(8):855–869

27 Panchision DM, Pickel JM, Studer L, Lee SH, Turner PA, Hazel TG, McKay RD. Sequential actions of BMP receptors control neural precursor cell production and fate. *Genes Dev* 2001;15(16):2094–2110

28 Zou H, Wieser R, Massague J, Niswander L. Distinct roles of type I bone morphogenetic protein receptors in the formation and differentiation of cartilage. *Genes Dev* 1997;11(17):2191–2203

29 Hebert JM, Mishina Y, McConnell SK. BMP signaling is required locally to pattern the dorsal telencephalic midline. *Neuron* 2002;35 (6): 1029–1041

30 Lewis WH. Experimental studies on the development of the eye in amphibia. I. On the origin of the lens, Rana palustris. *Am J Anat* 1904;3: 505–536

31 Li HS, Yang JM, Jacobson RD, Pasko D, Sundin O. Pax– 6 is first expressed in a region of ectoderm anterior to the early neural plate: implications for stepwise determination of the lens. *Dev Biol* 1994;162(1): 181–194

32 Porter FD, Drago J, Xu Y, Cheema SS, Wassif C, Huang SP, Lee E, Grinberg A, Massalas JS, Bodine D, et al. Lhx2, a LIM homeobox gene, is required for eye, forebrain, and definitive erythrocyte development. *Development* 1997;124(15):2935–2944

33 Kamachi Y, Uchikawa M, Collignon J, Lovell–Badge R, Kondoh H. Involvement of Sox1, 2 and 3 in the early and subsequent molecular events of lens induction. *Development* 1998;125(13):2521–2532

34 Boswell BA, Overbeek PA, Musi LS. Essential role of BMPs in FGF–induced secondary lens fiber differentiation. *Developmental Biology* 2008;324:202–212

35 Detwiler SR, van Dyke RH. The induction of the neural retina from the pigment epithelial layer of the eye. *J Exp Zool* 1953;128:367–383

36 Dragomirov N. The influence of neighboring ectoderm on the organization of eye rudiment. *Dokl AN SSSR* 1937;15:61–64