·Basic Research·

Influence of bone morphogenetic protein type IA receptor conditional knockout in lens on expression of bone morphogenetic protein 4 in lens

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Abstract

• **AIM: To investigate the influence of bone morphogenetic protein type IA receptor [BMPR-IA (ALK3)] conditional knockout in lens on expression of bone morphogenetic protein 4 (BMP4) in lens 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) 4 embryos, 8 eyes and 50% Cre-negative offspring (wild type, WT) 4 embryos, 8 eyes. The embryos were fixed in 4% paraformaldehyde, embedded in paraffin, and sectioned to a thickness of 4 μm. Removal of paraffin wax and dehydrating for sections, and then the procedure of *in situ* hybridization was processed, BMP4 MK1784-m (BOSTER) was used, and observed the expression of BMP4 in the lens in experimental group and control group. We selected SPSS11.0 software for statistical analysis, P<0.05 showed that the difference was statistically significant.**

• **RESULTS: Four embryos of each genotype were examined, totally we had 8 embryos, 16 eyes. We got the uniform outcomes in all the embryos. We found ALK3 was required during lens growing, but was not essential for the formation of lens. We observed that the expression of BMP4 in the lens was significantly reduced in all 8 ALK3 CKO lens, BMP4 expression was normal in all the 8 WT lens, P<0.01. This phenomenon became increasingly visible in accordance with embryo development. The most apparent alteration was present at stage E15.5.**

• **CONCLUSION: ALK3 is essential for lens growth. The influence of ALK3 on the expression of BMP4 is present during the development of mice lens.**

• **KEYWORDS:** bone morphogenetic protein type IA receptor; bone morphogenetic protein 4; lens
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INTRODUCTION

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. 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[1,2]. The BMP family has been implicated in the control of inductive processes during normal lens development [3]. The eye lens forms from the ectoderm of the head surface. 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. However, the precise genetic mechanisms by which BMP signals regulate these developmental processes are obscured in null mutants.

BMP4 is critical in dorsoventral patterning of the optic vesicle in mice. Both BMP4 and BMP7 cooperate with Pax6, a fundamental eye-development gene, in lens placode formation. Furthermore, BMP4 can replace the activity of Sox2, another gene with a critical role in early ocular

development, therefore, BMP4 is necessary for eye development. BMPs bind to two different membrane-bound Ser/Thr kinase receptors (type I and type II receptors) for signal transduction [4-6] .The signaling specificity is initially determined by the type I receptors. BMPR-IA is expressed in many tissues throughout embryonic development and after birth. Studies have implicated members of the BMP gene family, specifically BMP4, in murine eye development. BMP4 (-/-) null mutants exhibit defects in lens induction[7,8].

To investigate the role of BMPR-IA (ALK3) signaling during later stages of eye development, and to confirm the the influence of ALK3 conditional knockout (CKO) in lens on expression of BMP4 in lens during the embryonic development of vertebrates, we generated ALK3 CKO mice, and focused our attention on BMP4 expression, we consider the BMP4 and ALK3 may have a certain mutual relations in the process of lens development, so we try to demonstrate our idea from this experiment.

MATERIALS AND METHODS

Materials Mice expressing Cre recombinase (Le-Cre), which have been previously described, were used in our experiments [9]. In these experiments, we got 4 embryos of each genotype examined, totally we have 8 embryos. CKO mice were 4 embryos, 8 eyes and wild type (WT) mice were 4 embryos, 8 eyes. Mice carrying the Cre transgene or the floxed alleles were used in this study. Genomic DNA from embryonic tail tissue was extracted using the HotSHOT method. The PCR conditions were selected according to the universal PCR protocol. In this experiment, the mice were provided by China Medical University, and were 11.5-15.5d 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 (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. When *in situ* hybridization was processed, we use BMP4 MK1784-m (BOSTER). In our experiments, the guide for the care and use of laboratory animals follows the directions of china medical university laboratory animals center.

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 4 μm. The sections were processed for *in situ* hybridization.

The procedure was as follows: 1) Roast 30min ahead of schedule; 2) Rinse with xylene, 5min; 3) Rinse with xylene, 5min; 4) Rinse with anhydrous ethanol, 5min; 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) Add 3% H_2O_2 on tissue sections for inactivation of endogenous enzymes, 10min at room temperature, wash 3 times with distilled water; 10) Drop pepsin which is diluted with 3% citric acid, 15min at room temperature, wash 3×5min with PBS, wash 1 time with distilled water; 11) Add 1% paraformaldehyde to fix at room temperature for 10min, wash 3 times with distilled water; 12) At each section, add 20 μL pre-hybrid liquid , 2-4h at 42℃ in wet box, discard excess liquid, do not wash; 13) Add 20 μL hybrid liquid, cover slice, 16-20h at 42℃ in wet box; 14) After the hybrid, remove the cover glass, wash 3×5min with 2×SSC (room temperature), wash 1×15min with 0.5×SSC (around 40℃ water temperature), wash 1×15min with 0.2× SSC (around 40℃ water temperature); 15) Drop blocking solution, 37℃ for 30min, discard excess liquid, do not wash; 16) Drop biotinylated anti-mouse digoxin, 37℃ for 60min, wash 4×5min with PBS; 17) Drop SABC, 37℃ for 20min, wash 5×3min with PBS; 18) Drop biotinylated peroxidase, 37℃ for 20min,wash 4×5min with PBS; 19) DAB display colors, 20-30min, fully wash; 20) Hematoxylin stain, if necessary, fully wash; 21) 80% ethanol dehydration for 3-5min, 95% ethanol dehydration for 3-5min, 100% ethanol dehydration for 10min, soak 3 ×15min with xylene; 22) Mount neutral gum.

For all of the experiments described here, 4 embryos of each genotype were examined, totally we have 8 embryos. The embryos with ALK3 CKO in lens were 4, 8 eyes; The embryos in WT were 4, 8 eyes. When in situ hybridization was processed, we used BMP4 MK1784-m (BOSTER), and observed the expression of BMP4 in the lens in experimental group and control group. We selected SPSS 11.0 software for statistical analysis, $P<0.05$ showed that the difference was statistically significant.

RESULTS

ALK3 is required for lens growth, but is not essential for the formation of lens. The loss of ALK3 does not prevent lens formation, but it may result in the abnormal lens formation. During the early stage of embryonic eye development, BMP4 plays a key role in the process of embryo development inducement.

In our experiments, We observed that BMP4 expression in the lens was significantly reduced in all 8 ALK3 CKO lens (Figure 1), this specific phenomenon was apparent from stage E15.5, however, BMP4 levels were normal in all the 8 WT lens (Figure 2), statistical analysis indicated $P<0.01$, and the difference was statistically significant. These results suggest that the influence of ALK3 CKO in lens on expression of BMP4 in lens is present in the developing mouse eye.

DISCUSSION

BMP is one of the members of the TGF-beta family, in addition to playing an important role in the process of the

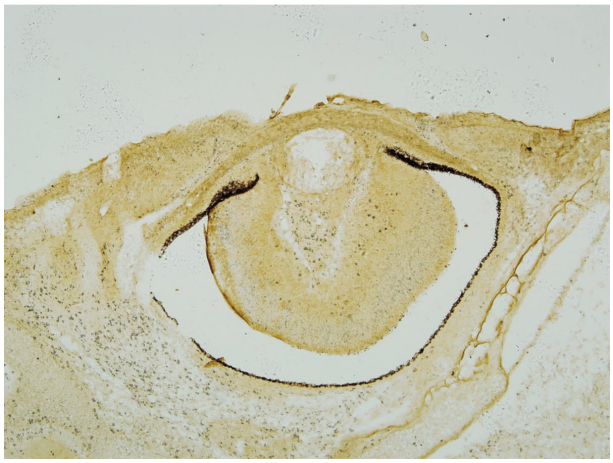

Figure 1 Expression of BMP4 in BMPR-IA (ALK3) CKO lens Section of the indicated embryo at stage E15.5 that was stained with *in situ* hybridization.

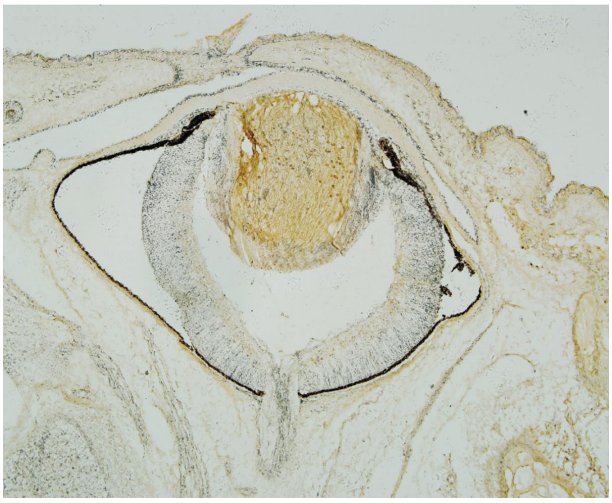

Figure 2 Expression of BMP4 in WT lens Section of the indicated embryo at stage E15.5 that was stained with *in situ* hybridization.

development of bone and cartilage and inducing ectopic bone and cartilage, they also have many biological effects, for example it can regulate cell proliferation, differentiation, apoptosis and so on. BMP plays a key role in the differentiation and formation of almost all developmental tissue and organs. Eye tissue derived from neuroectoderm, epidermal ectoderm and mesoderm, development process is complex, and there is interaction between the various organizations, a variety of BMP members and cytokines are involved in its development, which ALK3 is extremely important members.

The bone morphogenetic protein family plays an important role in the development and differentiation of the eye tissue. Knockout studies have shown that, in the early stage of eye morphogenesis, bone morphogenetic protein is essential [10]. Some members of the bone morphogenetic protein family expressed in mouse development of ocular tissue, bone morphogenetic protein ligand and bone morphogenetic protein receptor play key roles in the development of the lens [11,12]. Some reports indicate that bone morphogenetic protein contribute to the differentiation of lens fiber cells. For the development of the lens, the gene encoding of bone morphogenetic protein is essential[13].

ALK3 is an extremely important member in the BMP family, ALK3 gene knockout may result in the activation of p38MAPK-mediated apoptosis pathway through positively regulating. During embryonic development, apoptosis is to ensure the normal development of the individual which required, if the procedure of apoptosis disorders, ontogeny will also appear abnormal and even cause death. ALK3 is a important receptor on BMP/SMAD signaling pathway has the highest affinity with BMP. ALK3 CKO in lens can lead to increased apoptosis of lens cells, and cause abnormal lens development[14].

The expression pattern of BMP4 in the development of the eye is indispensable, BMP4 plays an important role in the early stage of lens development. In homozygous mice, BMP4 gene mapping results is highly variable, phenotype range from small eye malformations to anophthalmos [15]. In invalid animals severely affected, the lens plate is not formed, this shows that during the process of the lens plate formation, BMP4 plays an important role[16].

ALK3 takes an importantly regulatory role in the developmental process of the lens, but the loss of the ALK3 does not result in the absence of the lens growth [17]. In the early stage of eye development, during the development induction of the embryo lens, BMP also has a key role[18,19]. In chicks and mice, BMP signals have been shown to play important roles during lens induction. Furthermore, in lens epithelial cell assay, inhibition of BMP activity suppresses the FGF-induced secondary fiber differentiation [20,21]. Bone morphogenetic protein signals have been suggested to play important roles in lens development. At gastrula stages, lens placodal progenitors situated at the rostral neural plate border are exposed to BMP activity [22]. In mice, chicks, and zebrafish, the generation of lens placodal cells and up-regulation of early lens markers depends on BMP signals. Lens progenitors require sustained exposure of BMP signals to maintain lens character, in part by inhibiting the generation of olfactory placodal cells [23]. A previous study showed that BMP4 induces p27, indicative of cell cycle exit and osteoblast differentiation in osteoblast-like cell lines, further supporting that BMP activity plays an important role in cell cycle exit[24].

Therefore, we consider that ALK3 and BMP4 take a certainly mutual relationship during the inducing process of the development of the embryo lens, our experiments indicate that due to ALK3 CKO in lens, the expression of BMP4 in embryo lens reduce. The results of *in situ* hybridization can display the differences of BMP4 expression between the lens

of experimental group and control group. The expression differences can be obviously identified from E15.5. When we want to further study the interaction between the bone morphogenetic protein family members, this result might provide another path and construct some experimental basis. The correlation between the two eyes of an individual in the two groups, especially the affected group, could be a source of bias as there are pile of genes that act exactly the same in the formation and development of ocular components in the same person that can be different between either individuals and these genes may be confounding factor, so we should know that the two eyes of an individual are not the two independent eye. In our experiments, we do not especially consider the difference between the two eyes of an individual, which could be a source of bias.

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