·Basic Research·

Anterior segment dysgenesis correlation with epithelial-mesenchymal transition in Smad4 knockout mice

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Abstract

• **AIM: To explore the molecular mechanisms in lens development and the pathogenesis of Peters anomaly in Smad4 defective mice.**

• **METHODS: Le-Cre transgenic mouse line was employed to inactivate Smad4 in the surface ectoderm selectively. Pathological techniques were used to reveal the morphological changes of the anterior segment in Smad4 defective eye. Immunohistochemical staining was employed to observe the expression of E-cadherin, N-cadherin and α-SMA in anterior segment of Smad4 defective mice and control mice at embryonic (E) day 16.5. Real-time quantitative polymerase chain reaction (qPCR) was performed to detect the expression of Snail, Zeb1, Zeb2 and Twist2 in lens of Smad4 defective mice and control mice at E16.5.**

• **RESULTS: Conditional deletion of Smad4 on eye surface ectoderm resulted in corneal dysplasia, iridocorneal angle closure, corneolenticular adhesions and cataract resembling Peters anomaly. Loss of Smad4 function inhibited E-cadherin expression in the lens epithelium cells and corneal epithelium cells in Smad4 defective eye. Expression of N-cadherin was up-regulated in corneal epithelium and corneal stroma. Both E-cadherin and N-cadherin were down-regulated at the future trabecular meshwork region in mutant eye. The qPCR results showed that the expression of Twist2 was increased significantly in the mutant lens (P<0.01).**

• **CONCLUSION: Smad4 is essential to eye development and likely a candidate pathogenic gene to Peters anomaly by regulating epithelial-mesenchymal transition. Twist2 can be regulated by Smad4 and plays an essential role in lens development.**

• **KEYWORDS:** Peters anomaly; anterior segment dysgenesis; Smad4; N-cadherin; Twist2
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INTRODUCTION

Peters anomaly is referred to a range of a congenital abnormality of the anterior segment of the eye, such as corneal opacity, shallow anterior chamber, corneolenticular adhesions, cataract and so on [1-2]. Over 50% of Peters anomaly cases have glaucoma and more than 15% of cases accompanied lenticular malformations[1-2]. Right now there is no effective treatment for the disease, and visual loss is inevitable. Although surgical techniques have been constantly improved to cure the disease, the rate of surgical success still remains low.

Organogenesis of the eye is a complicated process. The surface ectoderm becomes thickened and invaginates to form the lens vesicle. The lens vesicle gradually develops into the mature lens, while the remained surface ectoderm develops into corneal epithelium. The cranial paraxial mesoderm and mesenchymal cells of neural crest origin migrate into the space between the lens vesicle and the remained surface ectoderm, and give rise to corneal stroma, corneal endothelium, ciliary muscle as well as the trabecular meshwork. It has been proposed that abnormal development of surface ectoderm and disturbed neural crest cells migration during eye development are responsible for Peters anomaly, but the precise pathogenesis still remains unknown[1-2].

Smad4 is a key intracellular effector of the transforming growth factor β (TGF-β) superfamily of secreted ligands, which plays an essential role in organogenesis and tissue homeostasis during developmental process. Previous studies have shown that Smad4 is expressed in both the lens vesicle

and presumptive corneal ectoderm, and conditional deletion of Smad4 in the eye surface ectoderm leads to severe abnormality in the anterior segment [3-5]. However, the precise role of Smad4 in anterior segment development and the underling mechanism are still unclear. Here we present data that Smad4 in the ocular surface ectoderm is required for cornea, lens and anterior chamber angle development. Conditional deletion of Smad4 on eye surface ectoderm resulted in corneal dysplasia, iridocorneal angle closure, corneolenticular adhesions and cataract resembling Peters anomaly. Mechanistically, Smad4 in the eye surface ectoderm affected the epithelial-mesenchymal transition, and regulated the expression of Twist2.

MATERIALS AND METHODS

Animals All animal experiments followed the guidelines of the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research. Le-Cre transgenic mice [6] and mice carrying floxed Smad4 alleles (Smad4$^{fl/fl}$)[7] were kindly gifted from Dr. Yi-Hsin Liu (University of Southern California, Los Angeles, USA). The Le-Cre; Smad4$^{fl/fl}$ mice were acquired as the mating picture shown (Figure 1A), and littermate mice carrying Smad4$^{fl/fl}$ or Smad4$^{fl/+}$ were used as controls. Polymerase chain reaction (PCR) was performed to establish the genotype with the primers as previous described (Figure 1B, 1C) [6-7].

Hematoxylin and Eosin Staining and Immunohistochemical Staining Pregnant mice were sacrificed to get the embryos. The embryos were fixed in 4% paraformaldehyde overnight at 4℃, then dehydrated through graded alcohols and embedded in paraffin. The 4 μm sections were cut for hematoxylin and eosin (HE) staining and immunohistochemical staining. Immunohistochemical staining was performed as previously described [8]. Primary antibodies were used as follows: rabbit polyclonal anti-E-cadherin (Santa Cruz, CA, USA), rabbit polyclonal anti-N-cadherin (Santa Cruz, CA, USA), rabbit polyclonal anti-α-SMA (Santa Cruz, CA, USA). Nine sections, obtained from three independent animals of different litters, were employed for each antibody staining.

Real-time Quantitative Polymerase Chain Reaction of Lens Tissue Three RNA specimens were extracted from control and Le-Cre; Smad4$^{fl/fl}$ lens of three independent litters respectively at embryonic (E) day 16.5 using RNeasy micro kit (Cat#74004, QIAGEN, GmBH, Germany). Quantitative PCR (qPCR) was carried out using SYBR Premix Ex TaqTM II (Takara, Dalian, China) and repeated thrice with each independent specimen. The qPCR procedure was 95℃ for 30s, followed by 40 cycles at 95℃ for 5s and at 60℃ for 34s. The results were analyzed based on the equation RQ= $2^{-\Delta\Delta CT}$.

The sequences of qPCR primers were listed as follows: snail: forward 5'-ATTTGTCCTGGTGACACCTGTTT-3', reverse 5'-ACTTGGCCCCTAACAAGTGATG-3'; Zeb1: forward 5'-TCCCTTTCCCCAGTTTTTAATAGGA-3', reverse 5'-GTTATGGCTGGGCCAACTCT-3'; Zeb2: forward 5'-CATGCCCAACCATGAGTCCT-3', reverse 5'-TTGCAGAATCTCGCCACTGT-3'; Twist2: forward 5'-CTGCTCAGCTAGCCGTGTTT-3', reverse 5'-TCCTGGGTGTGGAGCGTTAT-3'.

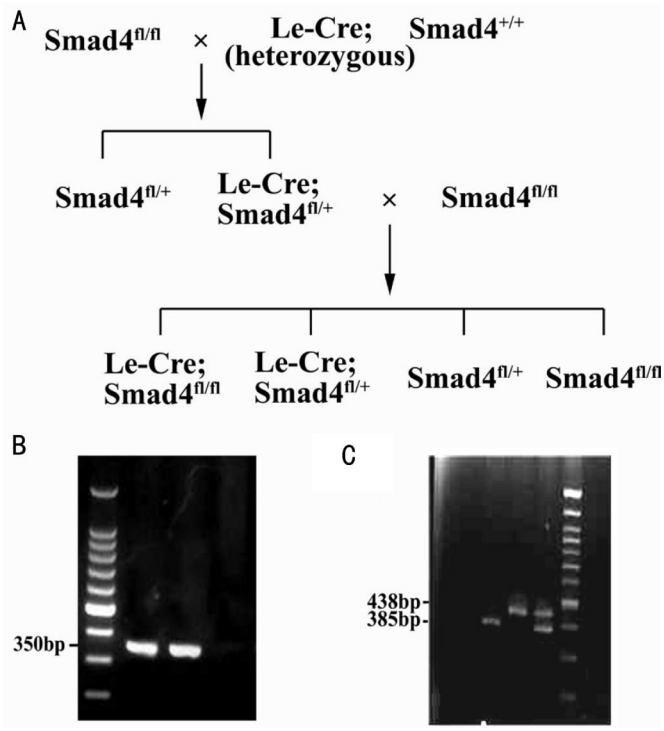

Figure 1 Generation of conditional deleted of the Smad4 gene in surface ectoderm A: Mice mating procedure to acquire Le-Cre; Smad4$^{fl/fl}$ mice; B, C: The detection of Le-Cre (B) and Smad4 (C) allele by PCR. A fragment of 350 bp indicated the existence of Cre gene. The fragment of 438 bp indicated the Smad4 floxed allele and 385 bp of wild-type Smad4 gene.

Statistical Analysis Statistical evaluations between control and mutant samples were performed using the unpaired Student's t-test (two-tailed).

RESULTS

Targeted Disruption of Smad4 on Eye Surface Ectoderm Results in Anterior Segment Dysgenesis Resembling Peters Anomaly We used the Le-Cre transgenic mouse line to inactivate Smad4 in the surface ectoderm selectively. The mutant mice showed abnormal development of anterior segment. At E12.5 mass of neural crest cells migrated into the space between the lens vesicle and the remained surface ectoderm, leading to thickened corneal stroma and excessive neural crest cells around lens in Smad4 defective mice (Figure 2A, 2E). At E16.5, the corneal stromal cells appeared loosely arranged and displayed highly variable shapes and sizes in Smad4 deficient mice (Figure 2B, 2F). The mutant eye showed congenital cataract with lens epithelium cells lost its polarity and distributed diffusely in the lens capsule, and numerous vacuole appeared (Figure 2C, 2G). By E16.5, the anterior chamber angle had not formed with failed separation between cornea and iris stroma, and the cornea attached severely to the lens (Figure 2C, 2D, 2G, 2H).

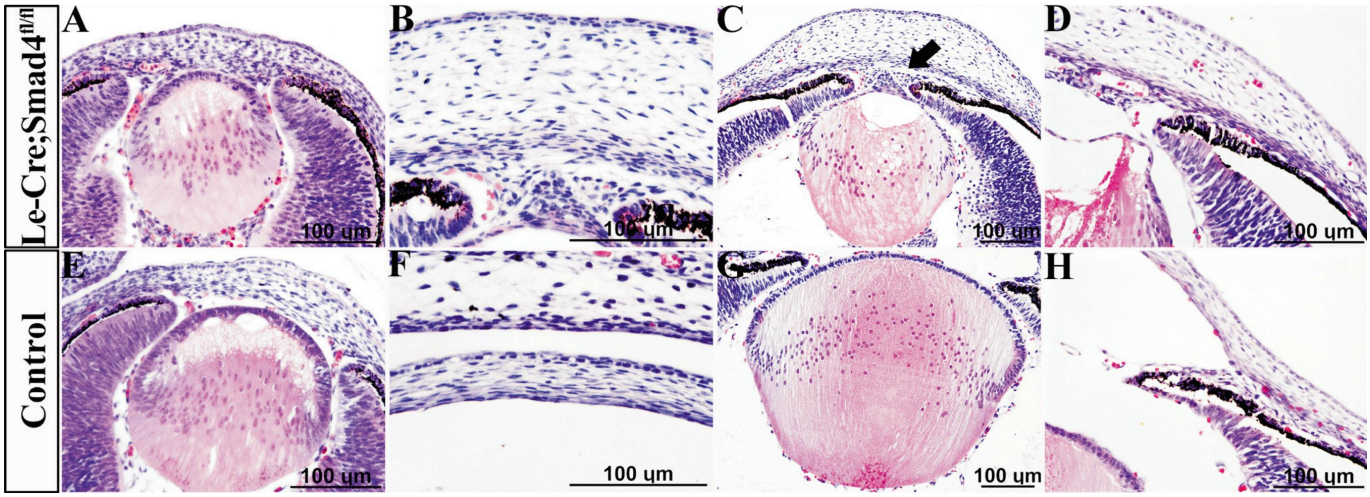

Figure 2 Loss of Smad4 on eye surface ectoderm leading to anterior segment dysgenesis resembling Peters anomaly A, E: Photographs of the mutant and control eye section at E12.5 by HE staining. B-D, F-H: Photographs of the mutant and control eye section at E16.5 by HE staining to show the developmental changes in cornea (B, F), lens (C, G) and the anterior chamber angle (D, H) between the Smad4 defective mice and control mice. Corneolenticular adhesions appeared (arrow).

Loss of Smad4 Function Down-regulates E-cadherin Expression and Up-regulates N-cadherin Expression Immunostaining was performed to detect the proteins related to cell-cell adhesion, namely E-cadherin and N-cadherin. The expression of E-cadherin was down-regulated in the lens epithelium cells and corneal epithelium cells in mutant eye, and the expression of N-cadherin was up-regulated in corneal epithelium cells (Figure 3A, 3B, 3D, 3E, 3G, 3J). The lens epithelium cells showed lower expression of N-cadherin probably due to massive loss of lens epithelium cells in the mutant eye (Figure 3H, 3K). In addition, the expression of N-cadherin was up-regulated in corneal stroma in Smad4 defective eye, while the expression of E-cadherin was absent in corneal stroma which showed no difference between the two groups (Figure 3A, 3B, 3D, 3E). Both E-cadherin and N-cadherin expression were down-regulated at the future trabecular meshwork region in the mutant eye (Figure 3M, 3N, 3P, 3Q).The expression of α-SMA was also detected which showed no difference between the two groups (Figure 3C, 3F, 3I, 3L, 3O, 3R).

Loss of Smad4 Function Up-regulates Twist2 Expression We performed real-time qPCR to detect the expression of E-cadherin repressors, namely Snail, Zeb1, Zeb2 and Twist2, with lens isolated from control and Smad4 defective mice, respectively. The results showed that the expression of Twist2 was increased significantly in the mutant lens, but the expression of Snail, Zeb1 and Zeb2 showed no difference between the two groups (Figure 4).

DISCUSSION

Peters anomaly is a congenital and developmental eye disease, which can lead to severe visual impairment. Until now there is no efficient treatment and the pathogenesis of Peters anomaly still remains ambiguously. In the present work, we found that conditional deletion of Smad4 on surface ectoderm led to corneal dysplasia, failed development of anterior chamber, corneolenticular adhesions and cataract resembling Peters anomaly, which suggested that Smad4 was essential to eye development and likely a candidate pathogenic gene to Peters anomaly.

The TGF-β signaling can induce epithelial-mesenchymal transitions (EMT) in lens epithelial cells. The EMT plays an important role in the pathogenesis of anterior subcapsular cataract and posterior capsular opacification, characterized by inducing expression of numerous extracellular matrix proteins, such as α-SMA [9-13]. On the other hand, EMT also plays a vital role in embryogenesis [14-15]. The transitions process includes changes in cellular morphology and a loss of cell polarity. Loss of E-cadherin expression is a hallmark of EMT, and several E-cadherin repressors have been reported such as Snail, Zeb1, Zeb2, Twist2 and so on[14-18]. In our present work, loss of Smad4 resulted in congenital cataract, and lens epithelium cells lost its polarity and distributed diffusely in the lens capsule. The mutant lens showed loss of E-cadherin expression in lens epithelial cells and up-regulation of Twist2 expression, which indicated that the EMT process might be activated. But the expression of α-SMA showed no difference in Smad4 defective lens compared to the control. These results are opposite to the TGF-β -induced EMT process in the anterior subcapsular cataract and posterior capsular opacification. Therefore, it can be demonstrated that different mechanisms of EMT may be existed in TGF-β inducing cataract and lens development, which requires further exploration. Moreover, immensely up-regulation of Twist2 expression in Smad4 defective mice manifests that Twist2 plays an essential role in the lens development and pathogenesis of congenital cataract. It has been reported that Twist2 can directly act on the promoter of E-cadherin and silence E-cadherin genes [18], thus the inhibition of E-cadherin expression in Smad4 defective eye is probably due to increase of Twist2 expression.

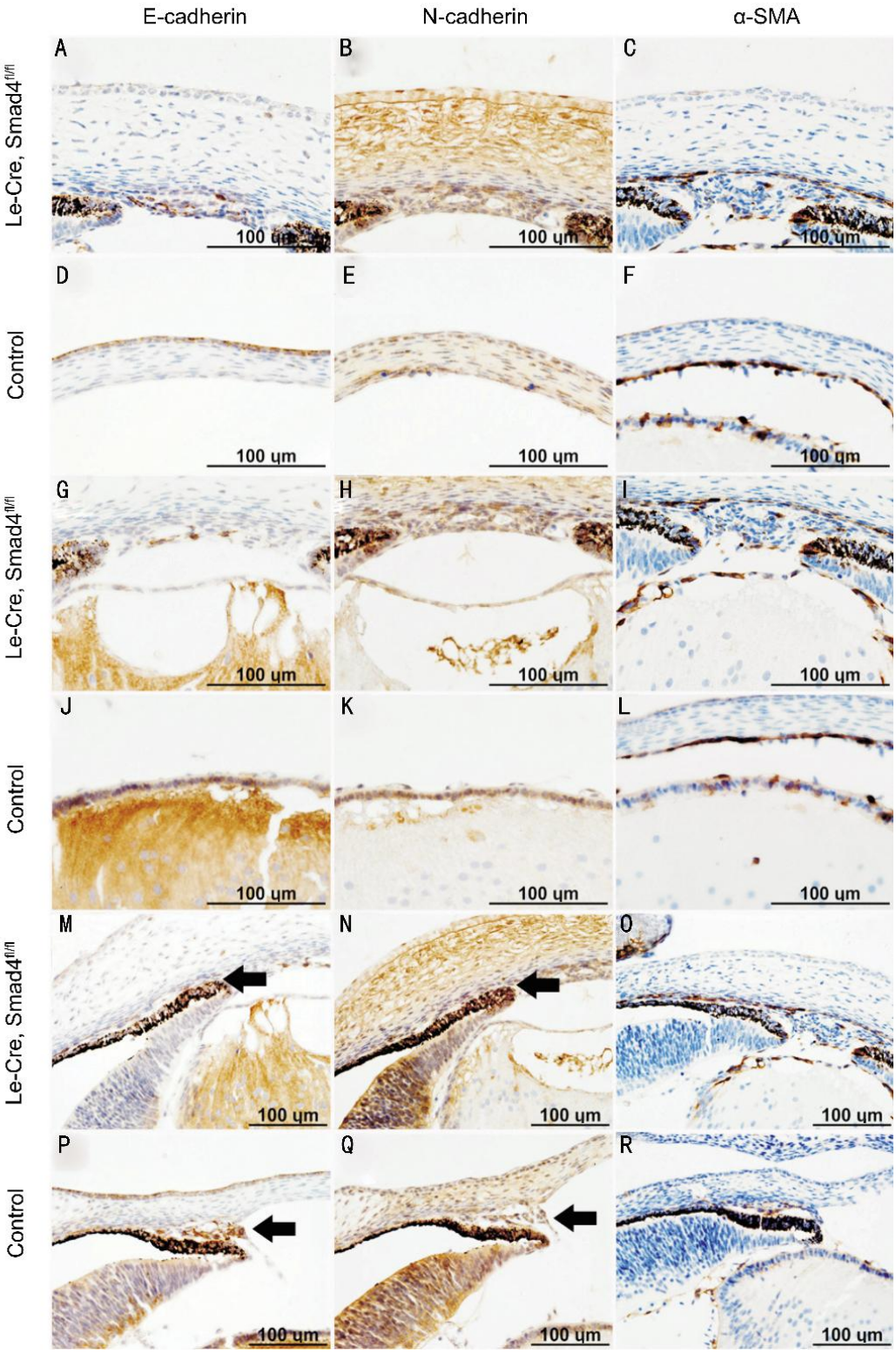

Figure 3 Detect of E–cadherin, N–cadherin and α–SMA on Smad4 defective eye and control eye at E16.5 A-F: Detect of E-cadherin, N-cadherin and α-SMA on Smad4 defective cornea and control cornea. G-L: Detect of E-cadherin, N-cadherin and α-SMA on Smad4 defective lens and control lens. M-R: Detect of E-cadherin, N-cadherin and α-SMA on Smad4 defective anterior chamber angle and control anterior chamber angle. Both E-cadherin and N-cadherin were down-regulated at the future trabecular meshwork region in mutant eye (arrows).

Proper development of the eye relies on coordinated interactions of the neuroepithelium, overlying surface ectoderm, and neural crest mesenchyme. Lens has been reported crucial in the development of the cornea and anterior chamber[8]. In our present work, conditional deletion of Smad4 on surface ectoderm led to corneal dysplasia and failed development of anterior chamber, further demonstrating that the lens can influence the development of surrounding tissues. In the Smad4 defective mice, the expression of N-cadherin was up-regulated in corneal stroma

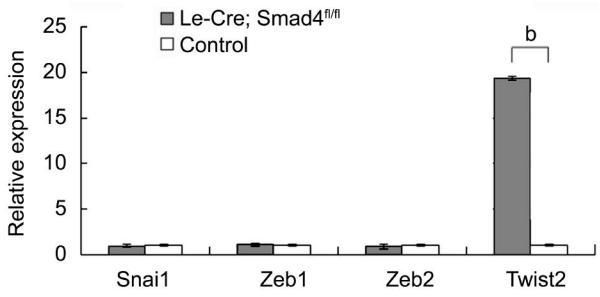

Figure 4 Real-time qPCR to detect the expression of Snail, Zeb1, Zeb2 and Twist2 with lens isolated from control and Smad4 defective mice at E16.5 n=9, ^{b}P<0.01.

and expression of both E-cadherin and N-cadherin were down-regulated at the future trabecular meshwork region. Moreover, TGF-β signaling and ablation of p120 catenin can induce iridocorneal angle closure and corneal dysplasia in mice and rat, and affect the expression of cadherins [19-21]. Therefore, it can be deduced that conditional deletion of Smad4 on surface ectoderm led to corneal dysplasia and failed development of anterior chamber by interrupting TGF-β signaling pathway and cadherins expression in surrounding tissues.

In conclusion, our works elaborate the possible pathogenesis of Peters anomaly in Smad4 defective mice and demonstrate the role of E-cadherin and N-cadherin in this process. We also propose for the first time that the Twist2 is a downstream target of Smad4 and plays an essential role in the lens development. These results may well pave the way to improve the clinical diagnosis and therapeutic schedule of Peters anomaly.

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Conflicts of Interest: Li J, None; **Qin Y,** None; **Zhao FK,** None; **Wu D,** None; **He XF,** None; **Liu J,** None; **Zhao JY,** None; **Zhang JS,** None.

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