Am J Transl Res 2019;11(6):3620-3628
www.ajtr.org /ISSN:1943-8141/AJTR0090235

Original Article

Role of surface ectoderm-specific mitofusin 2 in the corneal morphologic development of mice

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Received December 21, 2018; Accepted March 6, 2019; Epub June 15, 2019; Published June 30, 2019

Abstract: Mitofusin 2 (*Mfn2*) mediates the mitochondrial fusion in dynamic balance between mitochondrial fission and fusion. This study aimed to investigate the role of *Mfn2* in mice corneal dysplasia with conditional knockout (CKO) technique. The *Mfn2* CKO mice model was established with the *Cre-loxP* system. Each offspring of *Le-Cre*$^{+/-}$; *Mfn2*$^{fl/fl}$ (*Mfn2* CKO) mice and Mfn2$^{fl/fl}$ (*Mfn2* WT) mice was identified by polymerase chain reaction (PCR). Macroscopic observation, immunohistostaining and HE staining were used to evaluate the corneal morphologic development in *Mfn2* CKO mice and *Mfn2* WT mice. The cells proliferation and apoptosis were detected by BrdU labeling and TUNEL assay. Real-time PCR was used to detect mRNA expression of corneal markers (K12, Col1α1, Pax6, keratocan and NSE). Results showed that *Mfn2* CKO mice showed increased corneal thickness, small eyeball from E15.5 to P60 and small eye crack after birth. The corneal stromal thickness significantly increased in *Mfn2* CKO mice, and the random arrangement fibers of the corneal stroma increased in *Mfn2* CKO mice. The proliferative cells in the cornea of *Mfn2* CKO mice were less than in *Mfn2* WT mice while the apoptotic cells in the cornea of *Mfn2* CKO mice were increased. K12 and Pax6 expression decreased in the cornea and the Col1α1 expression increased in *Mfn2* CKO mice as compared to *Mfn2* WT mice. The expression of corneal stromal marker Col1α1 in the *Mfn2* CKO mice was significantly higher than that in the *Mfn2* WT mice. Corneal thickness was mainly caused by corneal stroma collagen proliferation. In conclusion, *Mfn2* deletion affects corneal development, especially because of collagen hyperplasia in the corneal stroma.

Keywords: *Mfn2,* corneal development, mitochondria, proliferation, apoptosis

Introduction

Mitochondria are present in most eukaryotic cells. As the most important energy source, mitochondria play an important role in many cellular functions, such as the responses to cell stress, nonapoptotic cell death and physiological metabolism [1]. In addition, mitochondria are involved in the pathogenesis of many aging related degenerative diseases, such as Alzheimer's disease [2], Huntington's disease [3], Parkinson's disease [4, 5], and cerebellar degeneration [6]. In these diseases, the progressive mitochondrial destruction may lead to energy damage [7]. Accumulated mitochondrial damage is an important cause of age-related eye diseases such as cataract and macular degeneration [8, 9]. Moreover, mitochondria may maintain the normal reticular formation through continuous fusion and division. Mitofusin 2 (*Mfn2*) is one of the proteins mediating mitochondrial fusion [10, 11] and a critical player in the regulation of mitochondrial motility, positioning, respiratory activity, quality control and mitophagy and, notably, in the modulation of contacts with ER [12]. *Mfn2* mediates the mitochondrial fusion and participates in the dynamic balance between mitochondrial morphology and fusion [13].

In mouse, the corneal morphologic development involves the differentiation of surface ectoderm and the migration of mesenchymal cells of neural crest origin [14]. Corneal development is strongly affected by the lens epithelium which derives from surface ectoderm [15]. The differentiation of surface ectoderm forms corneal epithelium at about embryonic day 11 (E11). The corneal epithelium can synthesize extracellular matrix (ECM) for the formation of

primary stroma when the lens detaches from the ectoderm at E12 [15]. From E12 to E14, the future cornea consists of two-layer epithelium from surface ectoderm. With the development of cornea, the mesenchymal cells of neural crest origin begin to invade the original corneal stroma, which is synthesized by the corneal epithelium [16]. The mesenchymal cells of neural crest origin gives rise to corneal endothelial cells and keratocytes. In this stage, corneal endothelial cells beneath the stroma are unobservable. By E15, the corneal endothelium appears, the anterior chamber forms, the corneal stroma becomes thicker, and the stromal cells become more regularly arranged [14]. In E17 to E19, the cornea gradually matures [17].

In our previous study, results showed *Mfn2* directly affected the morphological, metabolic and intracellular distribution of mitochondria, leading to the abnormal lens development, such as smaller lens, congenital cataract and other eye abnormalities [18]. However, the mechanism by which *Mfn2* regulates mouse corneal development remains unclear. The present study aimed to investigate the role of surface ectoderm-specifc *Mfn2* in the corneal development with conditional knockout technique (*Mfn2* CKO mice). In this study, results showed the corneal thickening was mainly caused by the thickening of corneal stroma in *Mfn2* CKO mice.

Materials and methods

Experimental mice and genotyping

All animal experiments were approved by the Ethics Committee of China Medical University (16005M) [18] and the animal procedures were conducted according to the guideline for the Animal Use and Care in Experiments. *Le-Cre* transgenic mice [19] and *Mfn2* allele mice [20] were provided by the Experimental Animal Center of China Medical University. All the animals were housed in a specific-pathogen-free (SPF) environment with 12-h light/dark cycle and temperature-controlling. The transgenic mice expressing *Le-Cre* recombinase under the control of Pax6 promoter [19] were mated with *Mfn2* transgenic mice. The male mice with *Le-Cre*$^{+/-}$; *Mfn2*$^{fl/fl}$ genotype were selected to mate with *Mfn2*$^{fl/fl}$ female mice. The genotype of each offspring was identified by polymerase chain reaction (PCR) with Premix Taq™ (TaKaRa Taq™ Version 2.0 plus dye) (Takara, Dalian, China). According to the results, mice were divided into *Le-Cre*$^{+/-}$; *Mfn2*$^{fl/+}$, *Le-Cre*$^{+/-}$; *Mfn2*$^{fl/fl}$; *Mfn2*$^{fl/+}$ and *Mfn2*$^{fl/fl}$. Then, *Le-Cre*$^{+/-}$*Mfn2*$^{fl/fl}$ (*Mfn2* CKO) and *Mfn2*$^{fl/fl}$ (*Mfn2* WT) were used as experimental group and control group, respectively. The primers used for *Mfn2* genotyping by PCR were 5'-GAA GTA GGC AGT CTC CAT CG-3' and 5'-AAC ATC GCT CAG CCT GAA CC-3', and those for the PCR of *Le-Cre* were 5'-CTC TGG TGT AGC TGA TGA TC-3' and 5'-TAA TCG CCA TCT TCC AGC AG-3' [18]. The *Le-Cre* is 350 bp in length, the mutant *Mfn2* is 180 bp in length and the wild-type *Mfn2* is 145 bp in length.

Histology

Embryonic head or postnatal eyes of control and mutant mice were fixed in 4% paraformaldehyde (PFA) overnight, dehydrated in graded alcohols (50%, 75%, 85%, 95% and 100%) for 2 h at 4°C, respectively, embedded in paraffin and then sectioned at 4 μm for use. Sections were processed for Hematoxylin and Eosin (H&E) staining and images were captured with the Olympus light microscope. The central corneal thickness of *Mfn2* CKO and *Mfn2* WT mice was measured with Image pro plus.

BrdU labeling and TUNEL staining

The pregnant mice were injected intraperitoneally with BrdU (100 μg/g body weight) (Abcam, Cambridge, MA) at 1 h or 2 h at after birth. Then, animals were sacrificed, and tissues were divided in ice-cold PBS. Embryos or eyeballs were removed and immersed in 4% PFA overnight at 4°C, dehydrated, embedded in paraffin and stained with BrdU Immunohistochemistry Kit (ab125306, Abcam) according to the manufacturer's protocol.

The corneal apoptotic cells were detected with In Situ Cell Death Detection Kit (Roche Applied Science, Indianapolis, IN) according to the manufacturer's protocol. Sections were finally incubated with TUNEL reaction mixture for 1 hour at 37°C, followed by DAPI (1:1000) counterstaining of cell nuclei. Photographs were obtained with a Olympus fluorescence microscope.

RNA extraction and quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from the cornea of 2-month-old mouse. Then, RNA was further exa-

Table 1. Primers Used for RT-PCR

Gene Name	FP (5'-3')	RP (5'-3')
keratin 12	CCATGGCTGAGCAAAATCGG	AGACAGTTGGCAGCAGTACC
Pax6	AGGGGGAGAGAACACCAACT	CATTTGGCCCTTCGATTAGA
Keratocan	ACAGAGTGTGAGACAGGCCT	TGGGTGGCATTCTCAAAGGG
NSE	GCTGCCTCTGAGTTTTACCG	GTCAGGTCATCGCCCACTAT
Col1α1	CACCCTCAAGAGCCTGAGTC	CAGACGGCTGAGTAGGGAAC

mined by reverse-transcription PCR (RT-PCR) with PrimeScript™RT reagent Kit containing gDNA Eraser (Takara, Dalian, China) and SYBR Premix Ex Taq™ II (Tli RNaseH Plus) (Takara, Dalian, China). The sequences of primers used in this study are listed in **Table 1**. β-actin (Invitrogen) was used as the endogenous control. After 3 independent experiments, data were analyzed using $2^{-\Delta\Delta CT}$ method.

Immunofluorescence staining

After deparaffinization and rehydration, the mouse eye sections were boiled for 10 min in citrate solution (Maxim, China) for antigen retrieval, and then permeabilized with 0.1% Triton X-100. These sections were blocked with 5% BSA for 1 h and then incubated with anti-Keratin 12 antibody (ab185627, Abcam, USA, 1: 50), anti-Collagen I antibody (ab34710, Abcam, USA, 1:500), and anti-NSE antibody (ab79757, Abcam, USA, 1:100) for 12 h at 4°C. Then, sections were incubated with secondary antibodies (anti-rabbit antibody 488 [Invitrogen, USA, 1:500] and anti-rabbit antibody 594 [Invitrogen, USA, 1:500]) at room temperature for 2 h. Cell nuclei were counterstained with DAPI (1:1000).

Statistical analysis

All the data were expressed as mean ± standard deviation (SD), and statistical analysis was performed with SPSS for Windows version 16.0 (SPSS Inc. IL, USA). Comparisons were done with unpaired Student's t-test. A value of two-tailed $P < 0.05$ was considered statistically significant.

Results

Construction and identification of Mfn2 CKO mice

In this study, *Mfn2* conditional knockout mice based on the loxp system were constructed using *Le-Cre* transgenic mice. The *Le-Cre*$^{+/-}$ transgenic mouse expressing Cre recombinase under the control of Pax6 P0 promoter were mated with *Mfn2*$^{fl/fl}$ mice. The mice whose offspring genotype was *Le-Cre*$^{+/-}$; *Mfn2*$^{fl/+}$ were selected to mate with *Mfn2*$^{fl/fl}$ mice, and the *Le-Cre*$^{+/-}$; *Mfn2*$^{fl/fl}$ mice served as the experimental group and the *Mfn2*$^{fl/fl}$ mice as the control group (**Figure 1A**). The *Mfn2* gene was knocked out selectively in the head surface ectoderm, and PCR was performed to determine the genotypes (**Figure 1B**). Small eye crack and eyeball were observed in *Mfn2* CKO mice by macroscopic examination (**Figure 1C**).

Corneal development in Mfn2 CKO mice

To observe corneal development of *Mfn2* CKO mice, time-mated embryos at E15.5, 16.5, 17.5, P4 and P60 were subjected to HE staining and sections were observed under an Olympus microscope (**Figure 2Aa-j**). At E15.5, the morphologic development of the cornea in *Mfn2* WT mice was consistent with normal mice, but the anterior chamber of *Mfn2* CKO mice collapsed and the cornea did not separate from the lens surface as compared to *Mfn2* WT mice. From E16.5 to E17.5, the corneal thickness of *Mfn2* CKO mice was significantly thicker than that of *Mfn2* WT mice. The thickness of corneal epithelium, corneal stroma and corneal endothelium of *Mfn2* CKO mice and WT mice were measured from E15.5 and P60 (**Figure 2B-E**). No obvious abnormalities of corneal epithelium and corneal endothelium were identified in *Mfn2* CKO mice as compared to their wild type littermates, but there was significant difference in the corneal stroma--corneal stroma thickness significantly increased in *Mfn2* CKO mice. At P60, the *Mfn2* WT mice had a well-defined endothelial layer, and the cornea stroma was arranged in a parallel manner. In *Mfn2* CKO mice, the random arrangement fibers in the corneal stroma increased in the thickness (**Figure 2Ak**, **2Al**). This may be ascribed to the proliferation of corneal cells and collagen fibers in the corneal stroma.

Abnormal cell proliferation and apoptosis in the cornea of Mfn2 CKO mice

In order to determine whether corneal thickness is related to the proliferation of keratocan,

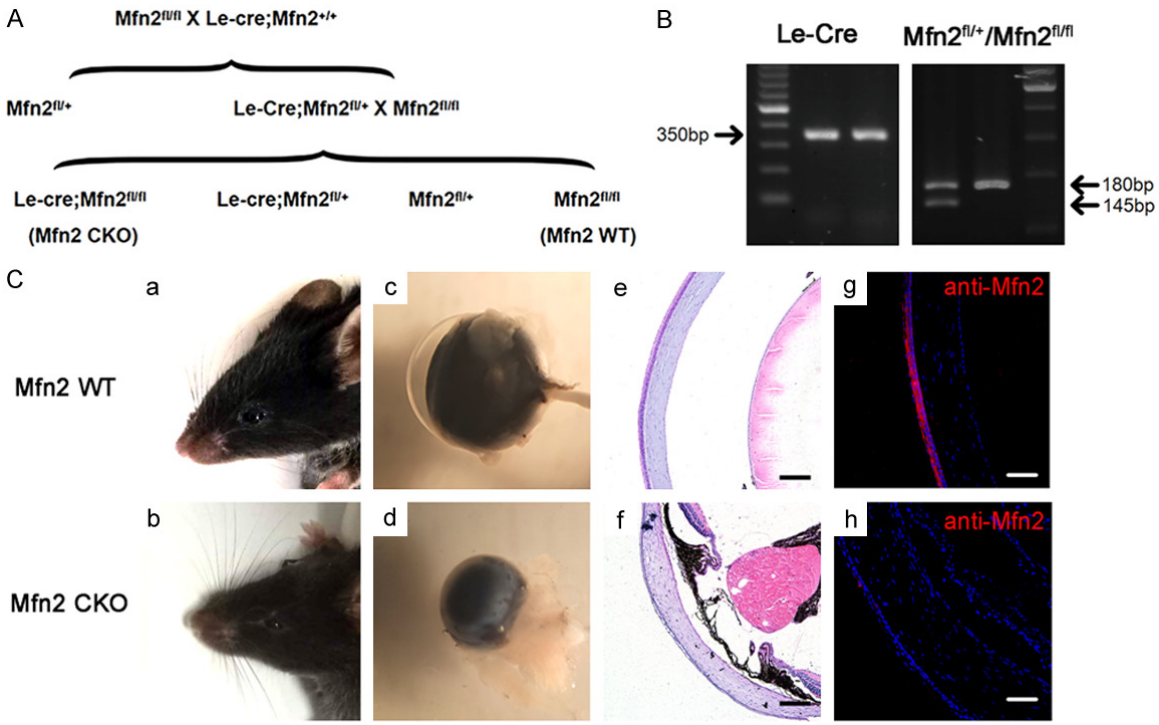

Figure 1. Construction and identification of *Mfn2* conditional knockout mice. (A) *Mfn2* CKO mice were screened from the offspring after *Le-Cre* mice mated with *Mfn2*$^{fl/fl}$ mice. (B) Genotyping by PCR. *Le-Cre* is 350 bp in length, mutant *Mfn2* is 180 bp in length and wild-type *Mfn2* is 145 bp in length. (C) Phenotypes of *Mfn2* WT and *Mfn2* CKO mice. Compared with *Mfn2* CKO mice, the eye crack and eyeball volume reduced, and the iris was adherent to the cornea. Scale bars: 100 μm (e, f), 50 μm (g, h).

mice were injected with BrdU at E16.5, E17.5 and E18.5. TUNEL staining was performed to investigate the effect of *Mfn2* knockout on the corneal apoptosis. Results showed cell proliferation was mainly observed at E16.5 and E17.5, and the BrdU(+) cells in *Mfn2* CKO mice were less than in *Mfn2* WT mice (**Figure 3A**, **3B**). Compared with control group, the apoptotic cells increased significantly in *Mfn2* CKO mice. Moreover, the apoptotic cells were mainly concentrated in the corneal endothelium (**Figure 3C**, **3D**).

Expression of corneal markers in the Mfn2 CKO mice

In order to determine the effect of *Mfn2* on the cornea, the expression of corneal epithelium marker K12, corneal endothelial marker Col-1α1, Keratocan and Pax6 was detected in the *Mfn2* CKO mice and *Mfn2* WT mice. Results showed that the increase in the corneal thickness was mainly due to the corneal stroma collagen hyperplasia (**Figure 4A**). The corneal endothelial marker NSE was not detected in the present study because of its low expression. The expression of corneal stromal marker Col1α1 in *Mfn2* CKO mice was significantly higher than in the *Mfn2* WT mice. Results showed that the increase in the corneal thickness was mainly attributed to the corneal stroma collagen hyperplasia.

Immunofluorescence staining of corneal tissues in Mfn2 CKO mice

The expression of corneal tissue markers with abnormal expression shown in qRT-PCR was further detected in *Mfn2* CKO mice by immunofluoresence staining. Results showed the expression of Col1α1 in *Mfn2* CKO mice was significantly higher than that in *Mfn2* WT mice, and the corneal stroma was thickened (**Figure 4C**, **4D**). This was consistent with that from qRT-PCR. In *Mfn2* WT mice, the corneal epithelial marker K12 was stained uniformly, but its expression in *Mfn2* CKO mice significantly reduced. Results indicated that *Mfn2* conditional knockout affected the corneal epithelial and stroma development.

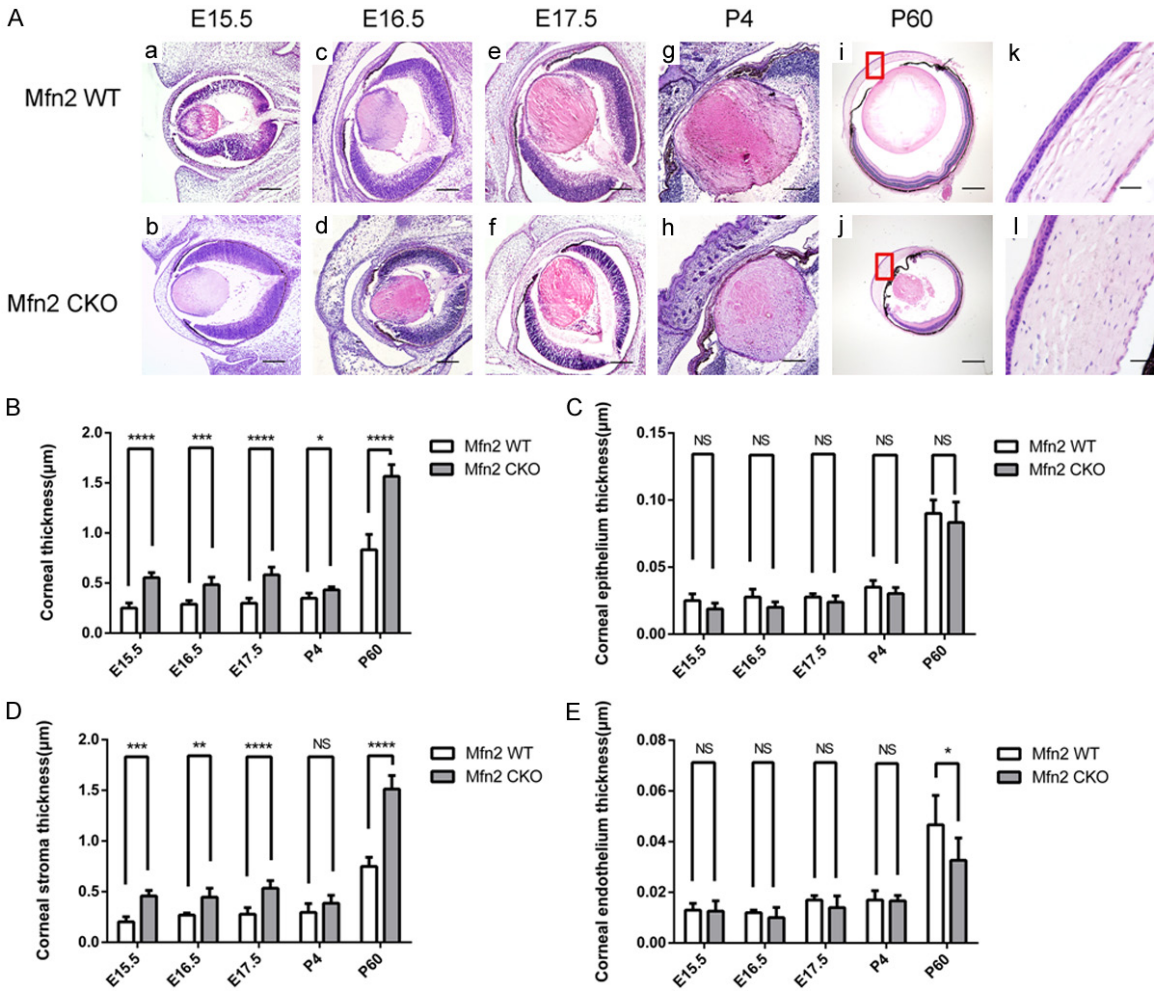

Figure 2. Corneal dysplasia in *Mfn2* CKO mice. (A) Compared with *Mfn2* WT mice, the corneal thickness of *Mfn2* CKO mice increased at E15.5. (B) Increased corneal thickness was mainly due to the increased thickness of corneal stroma in postnatal mice. (C) The central corneal thickness of *Mfn2* CKO and *Mfn2* WT mice. The central cornea of *Mfn2* CKO mice was thicker than that of *Mfn2* WT mice during embryonic period and after birth. (D, E) The central thickness of corneal epithelium, stroma and endothelium. Corneal stroma thickness was significantly different from the other two layers in two groups at P18 and P60. (n = 3, *P < 0.05, **P < 0.001, ***P < 0.0001, ****P < 0.00001). Scale bars: (A) 100 μm (a-h), 500 μm (i, j), 20 μm (k, l).

Discussion

Mitochondria are present in most eukaryotic cells, but their structures varies in different cells. Mitochondria maintain their normal reticular formation through continuous fusion and division. *Mfn2* was first identified in the rat vascular smooth muscle cells [20], and their further study confirmed that it could promote mitochondria fusion. In addition to its role in mitochondrial fusion, *Mfn2* is indispensable for the embryonic development [13]. Studies showed that *Mfn2* mutations are closely related to the neurodegenerative diseases [21, 22], diabetes [23], and cardiovascular diseases [24-26]. In addition, *Mfn2* conditional knockout on the surface ectodermal layer may lead to congenital cataract in mice [18]. However, these studies do not investigate the thickness of cornea. Our results showed that the cornea became thicker in *Mfn2* CKO mice than in wild-type ones, and thus, we speculate that *Mfn2* may affect corneal development.

During eye development, morphogenesis of ocular surface tissues mainly include the differentiation of surface ectoderm and the migration of periocular mesenchymal cells from neural crest [17]. During the embryonic development, the ocular surface ectoderm can usually

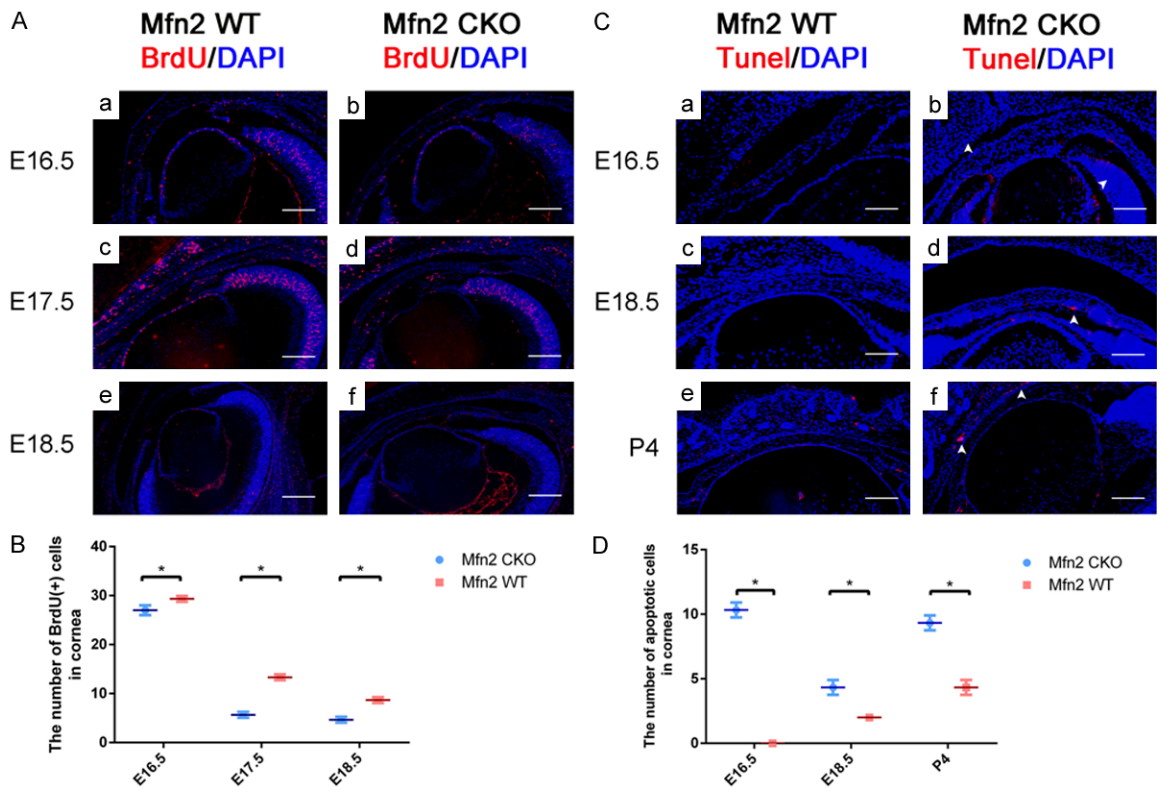

Figure 3. BrdU staining of the cornea in *Mfn2* CKO mice and *Mfn2* WT mice. (A) Cell proliferation of the cornea was mainly concentrated at E16.5 and E17.5. (B) The number of BrdU+ cells in *Mfn2* CKO mice was less than in *Mfn2* WT mice. Cell apoptosis of the cornea in *Mfn2* CKO mice and *Mfn2* WT mice. (C) In *Mfn2* CKO mice, cell apoptosis of the cornea was observed from E16.5 and it mainly found in the peripheral corneal endothelium (arrow). (D) Cell apoptosis of postnatal cornea was mainly found in the corneal stroma. However, the corneal cell apoptosis was not significantly different between two groups. (n = 3, *P < 0.05). Scale bars: (A) 100 μm (a-f); (C) 50 μm (a-d), 100 μm (e, f).

differentiate into lens, cornea, conjunctiva, eyelid epidermis, lacrimal gland, and Meibomian gland [27]. The present study investigated the effects of surface ectoderm-specifc *Mfn2* conditional knockout on the corneal morphologic development in mice.

In this study, surface ectoderm-specifc *Mfn2* conditional knockout was employed in mice, and the corneal development of *Mfn2* CKO mice was compared with that of *Mfn2* WT mice. In addition, cells proliferation, apoptosis and expression of corneal markers were further investigated in *Mfn2* conditional deletion.

Results showed that *Mfn2* expression was mainly located in the corneal epithelium during embryonic period and after birth. But there was no significant difference on cotneal epithelium between *Mfn2* CKO mice and controls. On the contrary, the increased corneal thickness was mainly attributed to the thickening of the corneal stroma. The corneal stroma accounts for approximately 90% of the entire corneal thickness. Corneal stroma is composed of ECM and keratocytes. Densely and regularly arranged collagen fibers play an important role in maintaining the transparency of the corneal stroma [28]. The corneal stroma consists of mainly Col1α1 and a lesser amount of collagen type V [29] and four proteoglycans: lumican, keratocan, osteoglycin and decorin. Our results showed the expression of Col1α1 in *Mfn2* CKO mice increased significantly which may be due to epithelial mesenchymal transformation (EMT). Neural crest cells invade the space between corneal epithelium and corneal endothelium to form keratoblasts. These cells can also become corneal endothelium. The keratoblasts proliferate to form an embryonic corneal stroma ECM and then differentiate into keratocytes [28]. The number of keratocytes between

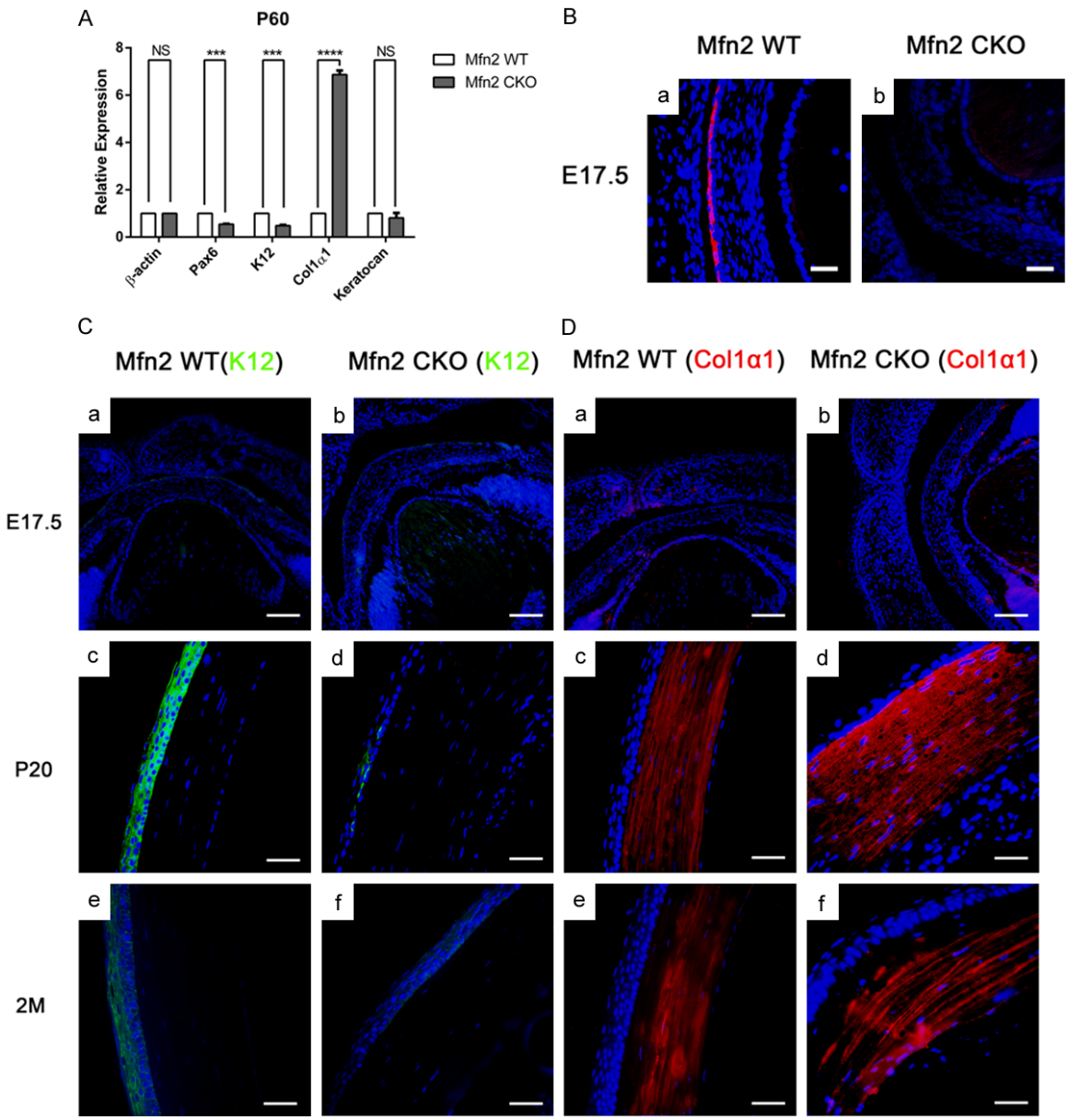

Figure 4. mRNA and protein expression of cornea related genes in the *Mfn2* conditional knockout cornea. (A) Pax6 and K12 mRNA expression was slightly lower in *Mfn2* CKO mice than in *Mfn2* WT mice. Col1α1 mRNA expression was significantly higher in *Mfn2* CKO mice than in *Mfn2* WT mice. The expression of K12 and Keratocan was comparable between *Mfn2* CKO mice and *Mfn2* WT mice. Data are expressed as means ± SD of 3 separate experiments. The expression of K12 and Col1α1 was almost undetectable during embryonic period. (B) *Mfn2* expression was mainly located in the corneal epithelium compared to the *Mfn2* CKO mice. (C) After birth, the expression of corneal epithelial marker K12 was stained uniformly in Mfn2 WT mice, but the expression of corneal epithelium K12 in Mfn2 CKO mice significantly reduced (c-f). (D) The expression of Col1α1 in Mfn2 CKO mice was significantly higher than in Mfn2 WT mice, and the corneal stroma was thickened (c-f). (n = 3, *P < 0.05, **P < 0.001, ***P < 0.0001, ****P < 0.00001). Scale bars: (B) 20 μm (a, b); (C) 50 μm (a-f); (D) 50 μm (a-f).

Mfn2 CKO and *Mfn2* WT mice had no significant difference in the embryonic period and after birth. Pax6 is a transcription factor that plays a pivotal role in modulating vertebrate eye development [30-32]. It has been found that the paired-box gene Pax6 is affected by various alleles of mouse and rat small eye mutants [33]. The Pax6 expression decreased slightly in *Mfn2* CKO mice as compared to *Mfn2* WT mice. This indicates that *Mfn2* has a certain

influence during the mouse eye development. We speculate that *Mfn2* deletion in the eye interacts with Pax6 to cause small eyeballs, but the specific mechanism still needs to be further studied.

These findings revealed that *Mfn2* may affect corneal development. This is the first study to investigate the spacial and temporal effects of *Mfn2* on the corneal development. However, the specific mechanism of corneal stroma thickening caused by *Mfn2* conditional knockout remains unclear. Further study should focus on the relationship between *Mfn2* and thickened corneal stroma resulted from increased collagen fibers.

Acknowledgements

This work was supported by the National Natural Science Foundation of China [No. 8137-1003].

Disclosure of conflict of interest

None.

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