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Deletion of the *FHL2* Gene Attenuating Neovascularization after Corneal Injury

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Abstract

PURPOSE—The four-and-one-half LIM domain-containing protein2 (FHL2) is a member of the four-and-a-half LIM domain-only (*FHL*) gene family. Although *FHL2* is expressed in the cornea, its role in angiogenesis is unclear. The aim of this study was to investigate the role of the *FHL2* gene in corneal angiogenesis after chemical injury.

METHODS—*FHL2-LacZ* knock-in mice were used to trace *FHL2* gene expression before and after corneal injury. Corneal angiogenesis between *FHL2*-null mice and wild-type mice that underwent chemical and mechanical denudation of corneal and limbal epithelium were compared. New growth vessel density was assessed by CD31 staining and was analyzed using image analysis software. Levels of vascular endothelial growth factor (VEGF) and cyclooxygenase (COX)-2 proteins were determined by Western blot assay.

RESULTS—β-Galactosidase staining of corneal tissue in *FHL2-LacZ* knock-in mice revealed that *FHL2* gene expression is upregulated in the corneal epithelium after corneal injury. Ten days after injury, corneal neovascularization was observed in control and *FHL2*-null mice. New corneal vessel density was found to be lower in the *FHL2*-null mice injury group than in the wild-type mice injury group. Western blot analysis showed that VEGF and COX-2 protein levels were higher after chemical injury in *FHL2*-null mice and wild-type mice. However, the upregulated VEGF protein was significantly lower in the *FHL2*-null mice than in the wild-type mice.

CONCLUSIONS—The decreased chemical-induced corneal angiogenesis found in the *FHL2*-null mice in this study indicated that FHL2 protein plays a role in inhibiting inflammatory angiogenesis.

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The four-and-a-half LIM domain (FHL) protein family is a newly identified group of proteins containing four-and-half LIM domains. This family consists of six members—FHL1, FHL2, FHL3, FHL4, FHL5, and ACT (Activator of CREM in Testis).[1–4] The LIM domain is a zinc-binding, cysteine-rich motif consisting of two tandemly repeated zinc fingers that are thought primarily to mediate protein-protein interactions. [1] Johannessen et al.[5] showed that human four-and-a-half-LIM-only protein family members, including FHL2, are expressed in a cell- and tissue-specific manner and participate in various cellular processes such as regulation of gene expression, cytoarchitecture, cell adhesion, cell survival, cell mobility, transcription, and signal transduction. Our previous work has demonstrated that FHL2 is abundantly expressed in the vascular system, including blood vessels,[3] suggesting that it might play an important physiological or pathologic role in the regulation of the circulatory system.[6] Therefore, we have investigated the role of FHL2 protein during corneal angiogenesis induced by chemical and mechanical denudation of corneal and limbal epithelium.

Corneal transparency and avascularity are important for maintaining the proper optical performance of the cornea. Corneal neovascularization (CNV) involves the development of new vascular structures in areas that were previously avascular and is a sight-threatening condition associated with cloudy cornea.[7] CNV may be induced by infection, inflammation, degeneration, or delayed wound-healing disorders in ocular surfaces. CNV could result from a disrupted balance between the upregulation of angiogenic factors and the downregulation of antiangiogenic factors.[7]

The extent of CNV was quantified and analyzed in different experimental groups. Vascular endothelial growth factor (VEGF), a key mediator of vasculogenic and angiogenic events, controls pathologic angiogenesis and increased vascular permeability in diseased cornea.[7–10] Additionally, the prostaglandin-cyclooxygenase pathway has been reported to influence new blood vessel growth in a variety of tissues. [11–15] The isoenzymes cyclooxygenase (COX)-1 and COX-2 are involved in prostaglandin biosynthesis. Generally, COX-1 is expressed in most tissues and cells, whereas COX-2 is upregulated by cytokines, inflammatory mediators, and tumor progression.[16] Overexpression of COX-2 in colon cancer cells and endothelium increases the production of prostaglandins and angiogenic cytokines, migration of endothelial cells, and tube formation.[11,16] Therefore, in the present study, the levels of VEGF and COX-2 have been measured in neovascularized cornea and compared among different groups after sodium hydroxide (NaOH) injury.

To determine the role of FHL2 in angiogenesis, we assessed the effects of corneal injury in the *FHL2*-null mice model that we previously generated through homologous recombination.[17] Our results suggest that deletion of the *FHL2* gene attenuates the process of corneal angiogenesis.

MATERIALS AND METHODS

Generation and Maintenance of *FHL2*-Deficient Mice

FHL2-null mice were generated as previously described.[17,18] Briefly, the endogenous ATG start codon of FHL2 was replaced by a cDNA encoding LacZ and a pGKneo cassette. In this manner, the lacZ cDNA was brought under the control of the endogenous FHL2 promoter while it ablated the endogenous *FHL2* gene. All animals used in this study (*FHL2*-null and wild-type littermates) were on a C57BL/6 genetic background and were genotyped by PCR. All procedures for handling mice were approved by the Institutional Animal Care and Use Committee of Chang Gung Memorial Hospital and were performed in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

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β-Gal Staining for LacZ Reporter Gene in *FHL2*-Null Mice

For LacZ expression analysis, wild-type and *FHL2*-null corneal sections were fixed and stained using reported procedures.[19] The duration of staining was dependent on color development and ranged from 4 to 12 hours at room temperature, or 30°C. Samples were photographed and analyzed under a dissecting microscope.

Murine Models of Chemical-Induced Angiogenesis

Heterozygous interbreeding generated a cohort of 180 male *FHL2*-null and wild-type mice that were divided into four groups, as follows: *FHL2*-null control, *FHL2*-null injury, wild-type control, and wild-type injury. At 6 to 8 weeks of age, 50% of the wild-type and *FHL2*-null mice were anesthetized with tribromoethanol (Avertin; Sigma-Aldrich, St. Louis, MO). The model of angiogenesis in mouse cornea was established according to a previously established protocol.[20,21] Briefly, filter paper cut by a trephine of 2-mm diameter was placed on the central cornea, and 2 μL of 0.15 M NaOH was applied to the right cornea of each mouse for 1 minute after topical application of proparacaine (Alcaine; Falcon Pharmaceuticals, Fort Worth, TX) to each eye. Corneal and limbal epithelia were removed with a corneal knife (Tooke; Katena Products, Denville, NJ) using a rotary motion applied parallel to the limbus and rinsed with normal saline. Erythromycin ophthalmic ointment was applied immediately after epithelial denudation. The most prominent neovascularization was observed on the 10th day after injury.

Labeling of Corneal Neovascularization

Immunohistochemical staining of vascular endothelial cells was performed on corneal flat mounts. Briefly, fresh corneas were dissected, rinsed with PBS for 30 minutes, and fixed in 100% acetone (Sigma) for 20 minutes. After the corneas were washed in PBS three times, nonspecific binding was blocked with 0.1 M PBS and 2% albumin (Sigma) for 1 hour at room temperature. Corneal flat mounts were incubated with FITC-coupled monoclonal anti–mouse CD31 antibody (PharMingen, San Diego, CA) at a concentration of 1:500 in 0.1 M PBS and 2% albumin at 4°C overnight and then washed in PBS three times at room temperature. Samples were mounted with an antifading agent (Gel-mount; Biomeda, San Francisco, CA) and visualized with a fluorescence microscope (Leica, Wetzlar, Germany).[20,22]

Quantification Corneal Neovascularization and Statistical Analysis

For CNV quantification, image analysis software (Image-Pro Plus; Media Cybernetics, Bethesda, MD) was used to delineate CD31-stained vessels on the corneal surface. The entire mounted cornea was analyzed to minimize sampling bias. Documentation and calculations of CNV length were performed in a masked fashion. Each experiment was performed at least three times with similar results. For quantification of the differences in CNV in various groups, one-way ANOVA was used, followed by the Tukey-Kramer test for multiple comparisons. $P < 0.05$ was considered significant.

Western Blot Analysis

Corneal tissues from eight mice per group (*FHL2*-null control, *FHL2*-null injury, wild-type control, and wild-type injury) were collected for Western blot analysis. The cornea was dissected and homogenized with 2 mL lysis 250 (50 mM Tris-HCl [pH 7.4], 250 mM NaCl, 5 mM EDTA, 0.1% NP-40, 25 mM NaF, and 1× protease inhibitor cocktail [Sigma]). The lysis 250 soluble fraction was separated by centrifugation at 13,000 rpm for 5 minutes at 4°C. Protein concentrations from lysis 250 soluble fractions were measured by spectrophotometry at OD 280 nm. An equal volume of 2× SDS sample buffer was added to each sample. Each sample was boiled for 10 minutes and subjected to 10% SDS-PAGE, followed by Western blotting with anti–VEGF (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA), COX-2 (1:1000; Novus Biologicals, Littleton, CO), or actin (1:15,000; Chemicon, Temecula, CA) antibody. The

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immunocomplex was visualized with horseradish peroxidase-conjugated secondary goat anti–rabbit IgG (1:1000; Elmer) and stabilized substrate (Western Blue; Promega).

RESULTS

FHL2 Gene Expression in Corneal Tissue after Chemical Injury

FHL2-null mice were generated by targeted replacement of the *FHL2* gene with a LacZ reporter gene fused in-frame downstream of the FHL2 regulatory region. Therefore, *cis*-element-reporter constructs linking LacZ were used to trace the temporal and spatial expressions of specific gene. β-Gal staining of the *FHL2-LacZ* knock-in mice revealed that sporadic FHL2 expression is found in corneal epithelial cells but is upregulated in swelling epithelial cells after corneal injury (Fig. 1).

Reduction of Corneal Neovascularization in *FHL2*-Null Mice after Corneal Injury

One hundred twelve *FHL2*-null mice and 68 wild-type mice were used in this study. No significant differences in phenotype, such as eye size or corneal clarity, were found between *FHL2*-null mice and wild-type mice before the chemical-induced angiogenesis study. Corneal neovascularization was recorded over time after injury (1 day, 2 days, 5 days, 7 days, 10 days, and 20 days). The timing of new blood vessel appearance in the cornea was similar in wild-type and *FHL2*-null mice. The most prominent neovascularization was observed on the 10th day after injury in both groups. As shown in Figure 2, increased CNV after chemical injury was demonstrated by CD31 (Fig. 2A, right side) staining in wild-type and *FHL2*-null mice. However, the density of new blood vessel growth was significantly higher in wild-type mice than in *FHL2*-null mice ($P < 0.001$).

Western Blots of VEGF and COX-2 Expression in Wild-Type and *FHL2*-Null Mice after Corneal Injury

Protein levels of VEGF and COX-2 were measured by Western blot analysis using protein extracts from different groups. VEGF and COX2 expression levels were increased after chemical injury in wild-type and *FHL2*-null mice (Fig. 3), whereas COX-2 levels were upregulated in the *FHL2*-null injury group and the wild-type injury group. COX-2 levels were significantly different between wild-type control and wild-type injured cornea and between *FHL2*-null control and *FHL2*-null injured cornea ($P = 0.029$ and $P = 0.014$, respectively; Fig. 3B), but there was no significant difference in COX-2 expression levels between the *FHL2*-null mice injury group and the wild-type mice injury group ($P = 0.167$ and $P > 0.05$, respectively; Fig. 3B). In contrast, there was a significant difference in VEGF expression between the *FHL2*-null mice injury group and the wild-type mice injury group. VEGF levels were not significantly different between the *FHL2*-null control and the *FHL2*-null injured corneas ($P = 0.051$), whereas VEGF levels were significantly different between the wild-type control and the wild-type injured corneas and between the wild-type injured and the *FHL2*-null injured corneas ($P = 0.030$ and $P = 0.041$, respectively; Fig. 3B)

DISCUSSION

In the present study we demonstrate that the pathologic progression of corneal injury-induced neovascular lesions is reduced in *FHL2*-deficient mice. Chemical and mechanical denudation of the corneal and limbal epithelia induce angiogenesis by tilting the balance between angiogenic and antiangiogenic factors toward an angiogenic response.[23,24] Previous studies have shown that FHL2, a member of the four-and-a-half LIM domain-only protein family, is expressed in heart, blood vessels, and skeletal muscle during development. Additionally, it has been reported that some four-and-a-half LIM domain-only proteins participate in cell lineage determination and pattern formation during development.[25] To investigate the expression and

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function of the *FHL2* gene, we used homologous recombination to target a LacZ cDNA into the endogenous FHL2 locus. β-Galactosidase staining of *FHL2-LacZ* knock-in corneal sections shows that LacZ expression increases significantly in the corneal epithelium layer after chemical injury. Except for corneal epithelial cells, there is no LacZ expression in the corneal stroma or endothelium layer of *FHL2-LacZ* knock-in mice. Our previous studies have shown a significant increase in FHL2 expression in the developing vasculature, indicating that FHL2 plays an important role in the development of the circulatory system.[25] In this study, we demonstrate that deletion of the *FHL2* gene attenuates the neovascularization response to corneal injury. These results clearly point toward an important pathophysiological role for FHL2 during angiogenesis.

VEGF, a potent angiogenic stimulator, has been reported to promote proliferation, migration, proteolytic activity, and capillary tube formation in endothelial cells.[26–28] During CNV, VEGF is highly expressed in the vascular endothelial cells of limbal vessels and in newly formed vessels in the stroma but is weakly expressed in keratocytes.[29] Correspondingly, VEGF was significantly increased in vascularized cornea compared with normal cornea.[29] Although the level of VEGF protein was upregulated in wild-type mice and *FHL2*-null mice after injury, the increase in CNV in the wild-type mice injury group was much greater than in the *FHL2*-null injury group (Figs. 2A, 2B; $P < 0.05$) Moreover, the protein level of VEGF was significantly lower in the *FHL2*-null injury group than in the wild-type mice injury group (Fig. 3). Based on our data, therefore, it can be inferred that FHL2 protein potentially has an antiangiogenic effect by preventing CNV. Further investigation is needed to clarify the mechanism of action of the FHL2 protein.

COX-2, a catalyst in prostaglandin synthesis from arachidonic acid, contributes to the regulation of angiogenesis. At inflammation sites, COX-2 is the rate-limiting enzyme during the synthesis of proinflammatory and angiogenic prostaglandin, such as PGE2, which induces metalloproteinase and VEGF.[30,31] COX-2 plays an important role in the progression of carcinogenesis, tumor invasiveness, and angiogenesis. Lim et al.[30] reported a significant positive correlation between the expression of VEGF and COX-2 in head and neck squamous cell carcinoma. Marrogi et al.[31] also showed that COX-2 levels were found to correlate positively with VEGF status in the angiogenesis of non–small cell lung carcinoma. In our study, significant overexpression of VEGF and COX-2 were found in *FHL2*-null and wild-type mice after chemical injury (Fig. 3). We found no significant difference in levels of COX-2 expression between wild-type and *FHL2*-null mice after chemical injury, though a significant difference was observed in VEGF expression between wild-type and *FHL2*-null mice after injury, indicating that VEGF might play a more important role during chemical-induced angiogenesis. Furthermore, our data show that deletion of the *FHL2* gene attenuates CNV by lowering the expression of VEGF. The role of FHL2 protein on corneal angiogenesis is related to the COX-2 pathway.

Our corneal angiogenesis model was accomplished by performing chemical and mechanical denudation of the corneal and limbal epithelia. Our CNV model involved corneal wound healing and chemical inflammatory reaction. Because FHL2 is a multifunctional protein that resides in different subcellular compartments, FHL2 can interact with integrin, presenilin-2, and voltage-gated K^+-channel receptors.[5,32] Moreover, FHL2 interacts with signal transduction pathways, including β-catenin/TCF-mediated transcription and ERK-2–mediated induction of transcription.[33–35] Future work will focus on elucidating the molecular mechanisms that mediate FHL2-regulated inhibition of CNV.

In conclusion, FHL2 is expressed in the cornea, and its expression is significantly increased after corneal injury. Deletion of FHL2 is associated with relative resistance to angiogenesis, including a decrease in VEGF expression and inflammation. FHL2 protein in the ocular surface

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may be partly responsible for CNV after chemical-induced inflammatory angiogenesis. These results suggest that FHL2 is intimately associated with the regulation of inflammation function and the process of angiogenesis. To our knowledge, this is the first report documenting the potential involvement of FHL2 in the attenuation of CNV after alkali injury. Further work is needed to delineate the complex interactions between angiogenic factors and antiangiogenic factors and will increase our understanding of the molecular mechanism of regulation of angiogenesis in the corneas of *FHL2*-null mice.

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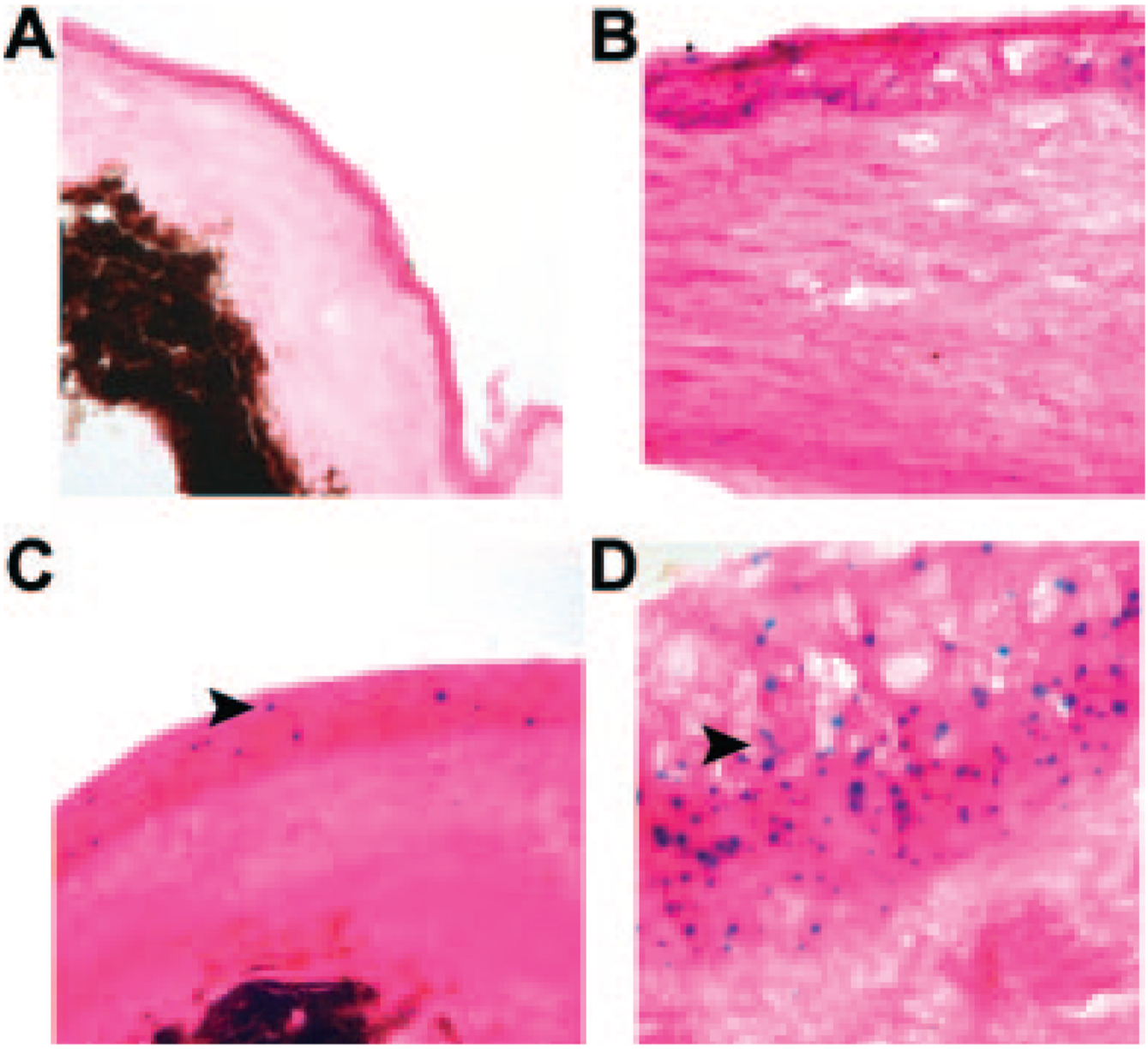

Figure 1.
Localization of *FHL2* gene expression in corneal tissue. β-Gal staining of *FHL2-LacZ* knock-in mice reveals FHL2 expression in the different layers of epithelium (**A**, 200×; **C**, 600×, *arrowhead*, *blue spot*); *FHL2-LacZ* overexpression in swelling epithelial cells after corneal injury (**B**, 200×; **D**, 600×, *arrowhead*, *blue spot*).

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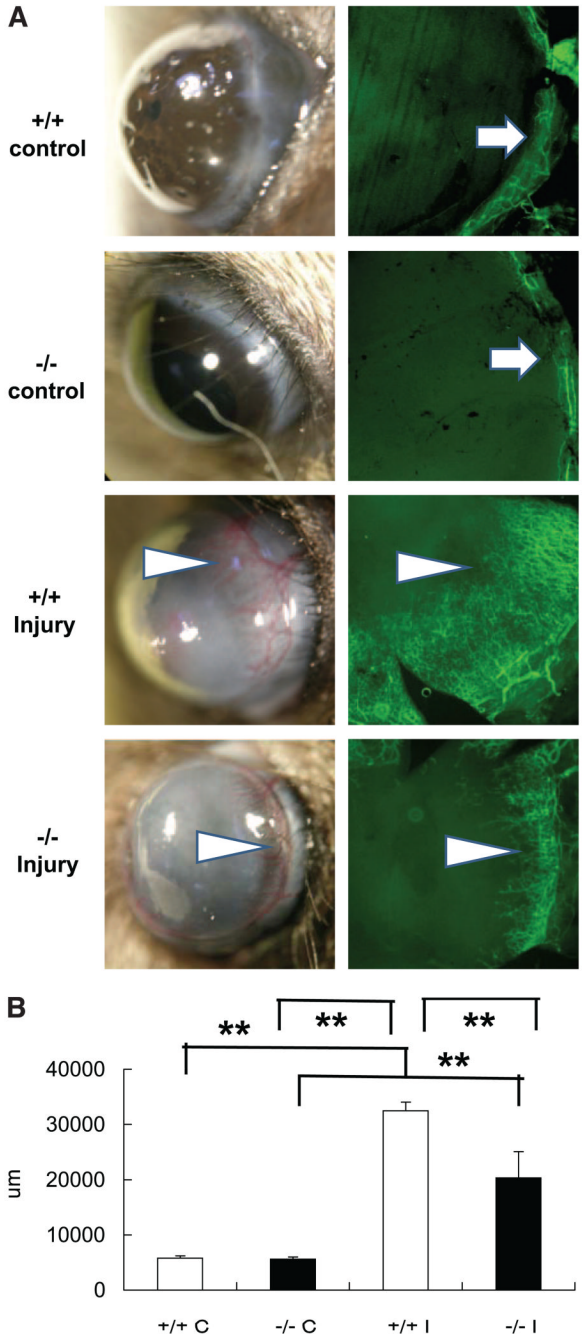

Figure 2.

Comparison of CNV in *FHL2*-null mice and wild-type mice after alkali injury. (**A**) CNV after 10th day of injury was observed in wild-type (+/+ injury) and *FHL2*-null mice (−/− injury). Wild-type mice (+/+) show more CNV after corneal injury (+/+ injury), whereas *FHL2*-null mice show less CNV (−/− injury). CD31 immunostaining was shown on the *right* side. (**B**) The extent of neovascularization was much lower in *FHL2*-null mice (−/− I. $^{**}P < 0.001$) compared with wild-type mice (+/+ I) demonstrated by CD-31 stains and semiquantitative analytical methods.

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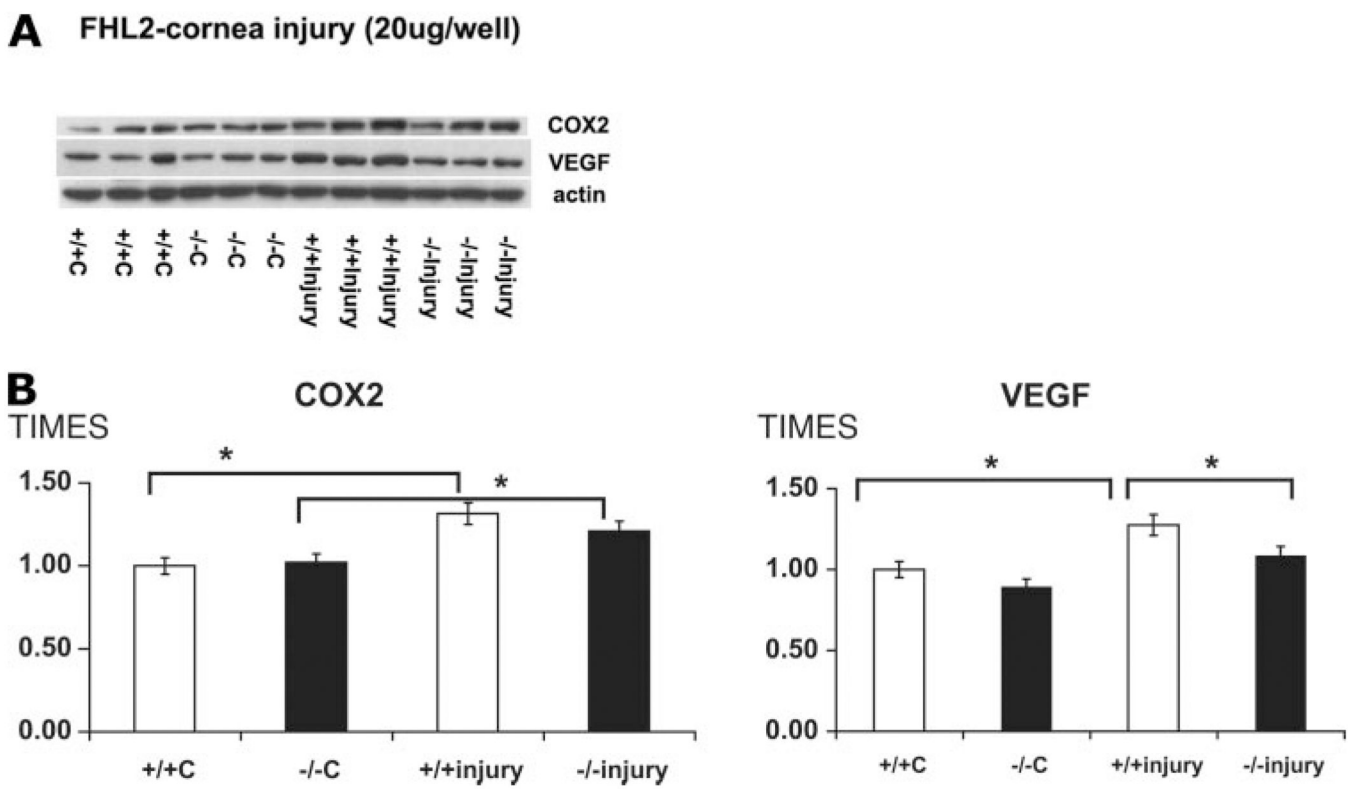

Figure 3.

VEGF and COX-2 immunoblotting in wild-type and *FHL2*-null mice. (**A**) Corneal samples were harvested from each group on the 10th day after injury, extracted with lysis buffer, and subjected to 10% SDS-PAGE followed by Western blotting. 20 μg protein was loaded into each well and probed with primary antibody raised against COX-2, VEGF, or actin. Vascular endothelial growth factor (VEGF) and cycloxygenase-2 isoforms (COX-2) were up-regulated after corneal injury in wild-type and *FHL2*-null mice. (**B**) The level of VEGF expression was significantly increased in wild-type mice after injury (bar 3 vs. 1; *right*), whereas VEGF expression levels were not significantly increased in null mice after injury (bar 4 vs. 2; *right*). Levels of VEGF expression were significantly increased after injury in wild-type versus null mice (bar 4 vs. 3; *right*). The level of COX-2 expression was significantly increased in both groups after corneal injury, though levels were not significantly decreased after injury in the null mice when compared with wild-type controls (bar 3 vs. 4; *left*). (*$P < 0.05$).