Moxifloxacin Modulated TGF-β1-Related Interleukin-12 Secretion in Corneal Fibroblasts

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REGULATION OF CORNEAL STROMAL CELLS IN CORNEAL WOUND HEALING

Regulation of corneal stromal cells in corneal wound healing is mediated in part by growth factors,1-3 cytokines,4 and extracellular components.5 Corneal keratocytes are normally quiescent and could differentiate into fibroblasts and myofibroblasts in response to corneal epithelium-derived growth factors such as platelet-derived growth factor and transforming growth factor-β1 (TGF-β1).3-7 In addition, exposure to TGF-β1 increases TGF-β1 secretion through an autocrine process in human corneal fibroblasts (HCFs).8 It is thus inspiring to illustrate whether the increased autocrine TGF-β1 in cornea stromal cells further induce other intracellular responses in themselves.

Interleukin-12 (IL-12) is a covalently linked heterodimer with a light chain IL-12A (p35) and a heavy chain IL-12B (p40), which controls its biological activity.9 IL-12 is a proinflammatory cytokine that activates natural killer cells and T lymphocytes.10 It also inhibits apoptosis of T cells.11 However, the production, function, and regulation of IL-12 in noninflammatory cells remain less well understood.

In the eye, IL-12 is present in the tear at 89.8 ± 115.8 pg/mL.12 Its expression increased significantly in the corneas with fungal13 and herpetic stromal keratitis.14 It also increased in the tears of patients following refractive surgery.15 In vitro study on the stimulation of secretory IL-12 in noninflammatory cells is limited. There is only one study illustrating expression of IL-35, which contains IL-12A as a member of the IL-12 family, following TGF-β1 induction in dermal fibroblasts.16 In contrast, interferon-β suppresses the production of IL-12B production in dendritic cells.17 Whether corneal fibroblasts participate in the regulation of IL-12 and/or if there is an interindividual variation in IL-12 secretion remain less well explored.

Topical moxifloxacin (MOX), a four-generation fluoroquinolone, is frequently used prophylactically after refractive surgery,18,19 cataract surgery,20,21 and intravitreous injection.22-25 In addition to its well-known antimicrobial activity, MOX also modifies corneal fibroblast-to-myofibroblast differentiation in HCFs.8 Furthermore, MOX suppresses inflammatory responses by reducing IL-1β and IL-17A in pneumonia mice.24 Therefore, it is inspiring whether MOX regulates the IL-12 secretion in corneal stromal cells. As reducing inflammation is beneficial in corneal infection as well as following surgery, we investigated if corneal fibroblasts produce proinflammatory cytokine IL-12 and its effects on corneal stromal cells proliferation and migration. We also investigated the interindi-
oidal variation of IL-12 and its alteration after MOX application.

**Materials and Methods**

**Primary HCFs**

This study was approved by the Institutional Research Ethics Review Committee (Protocol No. FEMH-100102-F). Human corneal rims were harvested as the source of corneal fibroblasts after the central corneas were removed for transplantation. A total of 27 donors aged between 20 and 70 years were included and all met the inclusion and exclusion criteria according to the Eye Bank Association of America standards for human corneal transplantation. 25 In the laboratory, the residual corneal rims were digested in collagenase-containing (2 mg/mL; Roche Applied Science, Mannheim, Germany) Dulbecco’s modified Eagle’s medium (DMEM; Gibco, Grand Island, NY, USA) with 10% heat-inactive fetal bovine serum (FBS; Biological Industries, Kibbutz Beil Haemek, Israel), 200 unit/mL penicillin G, and 200 µg/mL streptomycin sulfate (Sigma-Aldrich Corp., St. Louis, MO, USA) for 6 to 8 hours. Then, the digested cells were resuspended and maintained in DMEM with 10% heat-inactive FBS, 100 unit/mL penicillin G, and 100 µg/mL streptomycin sulfate as a complete culture medium under a humidified atmosphere containing 5% CO2 at 37°C. The isolated HCFs were used in passages 4 to 10 with complete culture medium, containing 10% FBS.

**IL-12 Silencing**

To reduce IL-12 secretion, the lentiviral short hairpin RNAi (shRNA) construct shIL12B obtained from the National RNAi Core Facility in Academia Sinica, Taiwan, were used to infect HCFs as the procedures with minor modification. 1 Briefly, HCFs were infected continuously for 3 days with lentivirus-based clone pLKO.1-shIL12B (TRCN0000058760, 5'-GTCTGA-GAATTGGATTGTGTGATX-3') using X-treme HP Transfection Reagent (Roche, Indianapolis, IN, USA).

**Treatment of TGF-β1 and MOX**

To analyze the effect of TGF-β1 and MOX on IL-12 secretion, 2.0 × 10^5 cells were seeded into a 6-cm culture dish and treated on the next day with 3 mL serum-less culture medium containing 0.1% FBS/DMEM, 2 ng/mL recombinant human TGF-β1 (rH/TGF-β1; R&D Systems, Minneapolis, MN, USA), and/or MOX (VIGAMOX; Alcon Laboratory, Inc., Fort Worth, TX, USA) at 0, 10, 50, or 100 µg/mL.

**Collection of Conditioned Medium**

To analyze the effects of conditioned media on cell proliferation and migration, HCFs with/without IL-12 silencing were infected continuously for 3 days with lentivirus-based clone pLKO.1-shIL12B (TRCN0000058760, 5'-GTCTGA-GAATTGGATTGTGTGATX-3') using X-treme HP Transfection Reagent (Roche, Indianapolis, IN, USA). The number of migratory cells was obtained from the National RNAi Core Facility in Academia Sinica, Taiwan, were used to infect HCFs as the procedures with minor modification. 1 Briefly, HCFs were infected continuously for 3 days with lentivirus-based construct shIL12B pseudovirion at 10 multiplicity of infection, which was produced from pseudovirion-packaging 293T cells with the clone pLKO.1-shIL12B (TRCN0000058760, 5'-GTCTGA-GAATTGGATTGTGTGATX-3') using X-treme HP Transfection Reagent (Roche, Indianapolis, IN, USA).

To analyze the effect of TGF-β1 and MOX on IL-12 secretion, 2.0 × 10^5 cells were seeded into a 6-cm culture dish and treated on the next day with 3 mL serum-less culture medium containing 0.1% FBS/DMEM, 2 ng/mL recombinant human TGF-β1 (rH/ TGF-β1; R&D Systems, Minneapolis, MN, USA), and/or MOX (VIGAMOX; Alcon Laboratory, Inc., Fort Worth, TX, USA) at 0, 10, 50, or 100 µg/mL.

**Cell Proliferation**

To evaluate the effect of exogenous IL-12 on HCF proliferation, HCFs were cultivated in 100 µL serum-less DMEM containing recombinant human IL-12 (rH/IL-12; ANTEC Bioscience, Inc., Taipei, Taiwan) and/or MOX for 3 days. In addition, to evaluate the effect of conditioned medium on HCFs, 100 µL ACM collected from the nonsilenced or IL-12-silenced HCFs were used to culture HCFs. HCF cell growth was evaluated by the formazan-based Cell Proliferation Reagent WST-1 assay (Roche Applied Science) according to manufacturer’s instructions. Briefly, 5.0 × 10^3 cells were cultivated individually in each well in a 96-well plate, and the incubation medium was refreshed on the next day according to the above-mentioned conditions. For the above assay conditions, 10 µL WST-1 reagent were directly added into each well at the detection times and subsequently incubated at 37°C for 2 hours. Absorbance of the samples was measured at 450 nm against a reference at 690 nm using a μQuant microplate spectrophotometer with KC Junior software (BioTek Instruments, Inc., Winooski, VT, USA).

**Cell Migration**

To compare the effect of IL-12, TGF-β1, or conditioned medium on the motility of HCFs, each area of Culture Inserts (ibidi GmbH, Martinsried, Germany) was seeded with 1.0 × 10^5 cells in complete DMEM and cultured for 1 day in a six-well culture dish. The area surrounding the Culture Inserts remained cell-free. After removing the Culture Inserts, the cells were further cultured in 2 mL serum-less DMEM containing 10 ng/mL rH/IL-12, 10 ng/mL rH/TGF-β1, or ACM of the treated HCFs. The HCFs were allowed to migrate, and the images were directly captured every 1 hour for 3 days using a Real-Time Cultured Monitoring System with Multiple-Point Imaging Capture (CCM-MULTI; ASTEC Co., Ltd., Fukuo, Japan). The number of migratory cells was counted and the difference among the conditions analyzed.

**Immunoblotting**

To analyze the expression of secretory IL-12 in HCFs, 10 µL of each ACM sample was mixed in 5 µL 3× sample buffer (180 mM Tris-HCl, pH 6.8, 30% glycerol, 0.1% bromophenol blue) without β-mercaptoethanol and then analyzed by 10% SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA, USA). Following blocking nonspecific binding with 5% bovine serum albumin (BSA) in Tris-buffered saline with Tween (TBST; 20 mM Tris-HCl, pH 7.5, 0.5 M NaCl, and 0.1% Tween-20) for 1 hour, the membranes were incubated at 4°C overnight in 5% BSA/TBST with the primary IL-12B-specific antibody (#5924-1; 1:1000; rabbit monoclonal; Epitomics, Inc., Burlingame, CA, USA). The membranes were subsequently incubated at room temperature with horseradish peroxidase (HRP)-conjugated antirabbit secondary antibodies for 1 hour. Finally, the blots were developed by chemiluminescence (Millipore) and analyzed by FUJIFILM LAS-4000 Imaging System (FUJIFILM, Tokyo, Japan).

**Equation for IL-12 Concentration Estimation**

To estimate the secretory IL-12 concentration, a twofold serial dilutions of rH/IL-12 ranging from 0.625 to 20 ng/mL were mixed with 3× sample buffer in a final volume of 15 µL and analyzed by immunoblot for each experiment. The density of
the blots was digitized and an equation of the IL-12 standard curve by linear regression was generated for every experiment. All the equivalent secretory IL-12 concentrations of the individual experiment conditions were then calculated accordingly.

**Semiquantitative Real-Time PCR**

To determine the silence efficiency, total RNA in nonsilenced and IL-12-silenced HCFs was extracted by RNeasy Mini Extraction kit (QIAGEN, Valencia, CA, USA) according to the manufacturer’s recommendations. The first-strand cDNA was synthesized with the reaction containing all extract of total RNA in a total volume of 30 μL containing 20 pmol of oligo(dT)15, 10 pmol of dNTP mix, and 200 units of MMLV Reverse Transcriptase (Promega, Madison, WI, USA). A fluorescein quantitative PCR detection system (Clontech, Mountain View, CA, USA) was used to evaluate the expressions in transcription levels using the specific primer sets, namely 5'-TCAGTGTCAAAAGCAGCAGA-3' and 5'-TGATGATGTCCCTGATGAAG-3' for IL-12; 5'-CACCACCAACTGCTTAG-3' and 5'-CTTCACCACCTTCTTGATG-3' for GAPDH; 5'-TCCTGTGGCATCCAGAA-3' and 5'-ATGATCTTGATCTTCATT-3' for β-actin; 5'-CAGAAGGTGACCAGCCTAAC and 5'-GTTCTGGGAGGAGATGGGTG-3' for Thy-1; 5'-CCACCAGAGGAGATGGTG-3' and 5'-CCAGGTACAGTTCAATAG-3' for FAK. The amount of IL-12 mRNA was normalized to mRNA of GAPDH, β-actin, Thy-1, and FAK, respectively. The fluorescence results were analyzed with the LightCycler Software version 3.5 (Roche Applied Science).

**Dot Blotting**

To determine the concentration of TGF-β1 in the media, 100 μL ACM from the treated HCFs in a 6-cm dish was loaded on PVDF membrane (Millipore). After blocking nonspecific binding with 5% BSA (Sigma-Aldrich Corp.) in TBST for 1 hour, membranes were incubated at 4°C overnight in 5% BSA containing TBST with the TGF-β1-specific primary antibody (#5249; 1:1000, rabbit monoclonal; Epitomics, Inc.). The PVDF membranes were then incubated at room temperature with HRP-conjugated antirabbit (1:10,000) secondary antibody for 1 hour. Finally, the blots were developed with ECL chemiluminescence (Millipore) and analyzed with a FUJIFILM LAS-4000 Imaging System (FUJIFILM). The density of the blots was digitized and folds of TGF-β1 concentration change estimated.

**Statistical Analysis**

Each triplicate experiment was performed independently for at least three times as described in figure legends. The means of each triplicates of the indicated parameters were used for further analysis to investigate whether there was difference among different groups in each experiment. The differences in IL-12 concentration, folds of IL-12 concentration change, cell viability, and number of migratory cells among groups were assessed using the Student’s t-test, one-way ANOVA, or two-way ANOVA as appropriate. A p-value of less than 0.05 was considered statistically significant.
evaluated using 1-way ANOVA followed by Tukey's honestly significant difference (HSD) post hoc test according to the experimental design as described in figure legends. The difference in donors’ age between the secretory and nonsecretory groups was examined using Student’s t-test. The correlation between the folds of increase in TGF-β1 and IL-12 concentration in the conditioned media was examined using Pearson’s two-tailed correlation. A P value less than 0.05 was considered statistically significant. All the statistics were conducted by SPSS version 16.0 software (SPSS, Inc., Chicago, IL, USA).

RESULTS

TGF-β1 Stimulated IL-12 Secretion

The secretory IL-12 concentrations in the media were estimated using an IL-12 equivalent equation (Fig. 1A). The 27 isolated HCFs were first classified as nonsecretory (n = 7) and secretory groups (n = 20) according to the signal intensity of the baseline IL-12 on immunoblots (Fig. 1B). The secretory group was further subdivided into three subgroups according to the estimated baseline IL-12 concentrations on immunoblots; namely low secretory group, medium secretory group, and high secretory group. The donors averaged 55.6 ± 18.6 and 56.5 ± 16.5 years old in the IL-12 secretory and nonsecretory group, respectively. There was no significant difference between these two groups (P = 0.92). To mimic the IL-12 expression during corneal wound healing, exogenous rhTGF-β1 was added to HCFs. Two of the nonsecretory HCFs responded to rhTGF-β1 to secrete IL-12, while five of them remained nonsecretory. One representative rhTGF-β1 stimulated IL-12 response in each secretory HCFs group is shown in Figure 1C. There was more IL-12 secretion in the high secretory group. However, there was no significant difference in the folds of TGF-β1-stimulated secretory IL-12 among the three subgroups (P = 0.870), which averaged to 1.90 ± 0.84 folds. We also found a 1.54 ± 0.67-fold increase in TGF-β1 concentration in the conditioned media of TGF-β1 treated cells at 3 days. The folds of increase in TGF-β1 concentration correlated significantly with the folds of increase in IL-12 concentration (correlation coefficient r = 0.454, P = 0.044).

Figure 2. TGF-β1 upregulated IL-12 secretion in HCFs. Cells were incubated in serum-less medium with rhTGF-β1 at 2 ng/mL, and the conditioned media were harvested at the indicated incubation time points. Secretory IL-12 in conditioned media was analyzed by immunoblotting with IL-12–specific antibody and the folds of IL-12 induction calculated. The quantitative data in the histograms represent the mean ± SD of the individual experiments performed in different cell sources (n = 3). Differences in the histograms were analyzed by 1-way ANOVA followed by Tukey’s HSD. Numbers represent P values between the two indicated time periods. u.d., undetectable.

To illustrate the temporal IL-12 expression during corneal wound healing, exogenous rhTGF-β1 was added to three of the secretory HCFs and the secreted IL-12 was detected by immunoblotting. We illustrated that continuous rhTGF-β1 incubation significantly induced IL-12 secretion in a time-dependent manner; that is, 5.02 ± 0.53 folds at 2 dpi (P < 0.001) and 6.92 ± 0.87 folds at 3 dpi (P < 0.001) (Fig. 2).

Figure 3. MOX suppressed IL-12 secretion in HCFs. Cells were incubated in serum-less medium with rhTGF-β1 at 2 ng/mL and/or MOX at the indicated concentration for 3 days. The conditioned media were harvested at 3 dpi. The secretory IL-12 was analyzed by immunoblotting with IL-12–specific antibody and the folds of IL-12 induction calculated. The quantitative data in the histograms represent the mean ± SD of the individual experiments performed in different cell sources (n = 5). Differences in the histograms were analyzed by 1-way ANOVA followed by Tukey’s HSD. Numbers represent P values between the two indicated groups.

MOX Suppressed IL-12 Secretion

In the absence of rhTGF-β1, MOX inhibited IL-12 secretion concentration-dependently (Fig. 3, lanes 1–4, ANOVA, P = 0.001). The difference was statistically significant at 50 and 100 µg/mL (lanes 5 and 4 versus lane 1, P = 0.007 and < 0.001, respectively). While rhTGF-β1 significantly induced IL-12 secretion by approximate 1.67 folds (Fig. 3, lane 5 versus lane 1, P < 0.001), the increase was also diminished by MOX concentration-dependently (Fig. 3, lanes 5–8, ANOVA, P < 0.001). The difference was significant at concentration of higher than 10 µg/mL and concentration-dependently.

MOX Suppressed IL-12-Enhanced HCF Proliferation

To verify the effect of secretory IL-12 on corneal stromal cell proliferation, rhlL-12 at 0.1, 1, and 10 ng/mL were added to incubate HCFs in serum-less culture medium for 3 days. We illustrated that rhlL-12 incubation significantly enhanced HCFs proliferation (Fig. 4A), suggesting that IL-12 promoted corneal stromal cell proliferation. The difference for 1 ng/mL IL-12 was significant since day 2, while the difference for 10 ng/ml IL-12 was significant since day 1. We also illustrated that MOX incubation significantly diminished the rhlL-12 enhanced HCF proliferation (Fig. 4B).
IL-12 Promoted HCF Migration

We also investigated whether secretory IL-12 affects HCF migration. Using a migration assay, HCFs were added to the center of an ibidi culture inserts and incubated in 6-cm culture dishes. Either 10 ng/mL rhIL-12 or 10 ng/mL rhTGF-β1 was added to the serum-less medium (Fig. 5). The results revealed that rhIL-12, but not rhTGF-β1, enhanced HCF migration at dpi 3 (Fig. 5C, 141.8 ± 32.9 vs. 93.3 ± 18.4, P = 0.034 and 65.0 ± 19.7 vs. 93.3 ± 18.4, P = 0.520, for rhIL-12 and rhTGF-β1, respectively). These suggest that increased IL-12 secretion in HCF could promote HCF migration.

Reduced IL-12 Secretion Interfered HCF Migration

Using the gene silence method, we had generated shIL12 pseudovirions efficiently to decrease the IL-12 expression in HCFs by approximately 80% at transcription level (Fig. 6A). The HCFs, with or without IL-12 silencing, were treated with MOX and/or rhTGF-β1 in a serum-less medium (0.1% FBS) for 3 days. The secretory IL-12 and TGF-β1 in the conditioned medium were determined by immunoblotting (Fig. 6B) and dot blotting (Fig. 6C), respectively. TGF-β1 significantly enhanced IL-12 secretion while MOX significantly inhibited TGF-β1 related IL-12 secretion (Fig. 6B, lanes 1–4). There was almost no detectable secretory IL-12 in the conditioned medium of IL-12-silenced HCFs (Fig. 6B, lanes 5–8). Addition of TGF-β1 resulted in significantly higher TGF-β1 concentration in the conditioned media (Fig. 6C, dot 3 vs. dot 9). In addition, there was similar TGF-β1 concentration in the medium between nonsilenced and silenced HCFs (Fig. 6C, dot 3 vs. dot 7, P = 0.656). This indicates that silencing IL-12 did not interfere with the auto-regulation of TGF-β1 secretion in HCFs.

The above-mentioned conditioned media were further used to incubate HCFs to verify their effects on cell motility and cell proliferation (Fig. 7). The conditioned medium from the nonsilenced, rhTGF-β1-treated HCFs significantly enhanced motility (Figs. 7B, 7C, panel c) and cell proliferation (Fig. 8, 1.91 ± 0.06 vs. 1.54 ± 0.04 folds at 3 days, P < 0.001). In contrast, the conditioned medium from IL-12-silenced HCFs completely retarded HCF migration (Figs. 7B, 7C, panels e–b) but did not suppress HCF proliferation (Fig. 8, 1.36 ± 0.08 vs.
The number of migratory HCFs that passed the dotted line, which indicates original ibidi insert margin, was counted. The quantitative data represent mean ± SD of three individual experiments performed in different cell sources with similar trend. The difference among the three groups were analyzed by 1-way ANOVA followed by Tukey’s HSD. Numbers represent P values between the two indicated groups.

Among the 25.9% (7/27) of the isolated corneal cells that did not secrete IL-12 at baseline, 7.4% (2/27) of them responded to TGF-β1 stimulation (Fig. 1B). In contrast, among the 74.1% (20/27) of the isolated corneal cells that secreted IL-12, 25.9% (7/27) were low-, 37.0% (10/27) were medium-, and 11.1% (3/27) were high-IL-12 secretory HCFs. The autocrine TGF-β1 autoregulation resulted in an averaged 1.54 ± 0.04-fold increase in TGF-β1 concentration and an averaged 1.90 ± 0.84-fold of TGF-β1 related IL-12 upregulation. These could be confirmed by the positive correlation between the increase in folds of TGF-β1 concentration and the increase in folds of IL-12 concentration. It is possible that the interindividual variability in IL-12 secretion both at baseline condition and their response to TGF-β1 contributed to the interindividual difference following LASIK15 even though the surgery is quite standardized. In spite of the IL-12 concentrations after TGF-β1, incubation was higher in the high secretion subgroup (Fig. 1C).
FIGURE 6. Secretory IL-12 and TGF-β1 expression in the IL-12-silenced HCFs. (A) IL-12 gene in HCFs was reduced by infection system with the lentivirus-based shIL12 pseudovirion and cultured in complete culture medium with 10% FBS. Silence efficiency on IL-12 expression was analyzed by semi-quantitative real-time PCR with IL-12-specific primers. Differences in the relative changes in silence efficiency were analyzed by the Student’s t-test. (B) Conditioned media of nonsilenced or IL-12-silenced HCFs were harvested after 3-day incubation in the serum-less DMEM with 2 ng/mL rhTGF-β1 and/or 100 μg/mL MOX. The amount of IL-12 secretion in each ACM were determined by immunoblotting with IL-12-specific antibody and the folds of IL-12 induction calculated. Differences among groups were analyzed by 1-way ANOVA followed by Tukey’s HSD. (C) The amount of TGF-β1 in the same ACM were determined by dot blotting with TGF-β1-specific antibody. The standard indicates 2 ng/mL TGF-β1 used for HCF culture. The quantitative data in the histograms represent mean ± SD of three individual experiments performed in different cell source (n = 3). Differences among groups were analyzed by 1-way ANOVA followed by Tukey’s HSD. Numbers represent P values between the two indicated groups.
and there was no difference in the folds of induction among the three subgroups ($P = 0.870$). This indicates that corneal cells that secrete more IL-12 at baseline will secrete more IL-12 in the presence of TGF-$\beta_1$. This echoes the reports that the tear concentration of IL-12 is low but with great variability. It increases following surgery since there is a TGF-$\beta_1$ surge following corneal wounding and/or infection.

Our results indicate an interindividual variation in the production and TGF-$\beta_1$ regulation of IL-12 in noninflammatory cell; that is, corneal fibroblast. IL-12 is involved in antigen presentation and corneal inflammation. As IL-12 is also

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<th>Source of Conditioned Medium</th>
<th>Non-Silenced HCFs</th>
<th>IL-12-Silenced HCFs</th>
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<tr>
<td>Untreated</td>
<td>MOX</td>
<td>rhTGF-$\beta_1$</td>
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FIGURE 7. Effect of conditioned medium from IL-12-silenced HCFs on cell migration. (A) A scheme depicting the location of image capture in a culture insert in a six-well culture dish and the area surrounding culture inserts remained cell-free. After removing the culture insert, 2 mL ACM from the HCFs with different treatments as described in Figure 6 were used to incubate HCFs. (B) HCF migration incubated with the conditioned media was monitored, and the images were captured at 3 dpi. HCF migration was performed in different cell sources ($n = 3$). (C) The number of migratory HCFs that passed the dotted line in (B), which indicates original ibidi insert margin, was counted. The quantitative data represent mean ± SD of the individual experiments performed in different cell sources ($n = 3$). The difference among the eight groups (panels a-h) were analyzed by 1-way ANOVA followed by Tukey’s HSD. Numbers represent $P$ values between the two indicated groups.
used rhTGF-β1 mL, in cell migration assay. For parallel comparison, we hence used higher IL-12 concentration; that is, 10 ng/mL medium compared with the same cells in complete culture medium. However, HCFs migrated very slowly in serum-less culture media (0.1% FBS/DMEM) in the migration experiment. This proliferation would confound cell migration as HCF proliferation in complete culture medium (10% FBS/DMEM). This proliferation would confound cell migration as HCF proliferation in complete culture medium (10% FBS/DMEM).

Promotion of cell migration indirectly by increasing IL-12 secretion, which subsequently enhanced HCF migration (Fig. 5). TGF-β1 promoted cell migration indirectly by increasing IL-12 secretion. However, if the HCFs could secrete IL-12 to promote HCF migration, there should have been an enhanced HCF migration front when rhTGF-β1 was added to the culture media, no matter how many cells were seeded in the culture dish. However, there was no enhanced HCF migration front when HCFs were seeded only in the center of the ibidi culture insert (Fig. 5), although the culture media did contain sufficient rhTGF-β1; that is, 10 ng/mL. This confirmed that rhTGF-β1 alone could not promote HCF migration. In addition, if the HCFs could secrete IL-12 to promote HCF migration indirectly, sufficient number of HCFs would be needed to secrete enough amount of IL-12. This assumption is also confirmed when rhTGF-β1 was added to the 6-cm HCF culture dish in our experiment. The amount of the rhTGF-β1-stimulated IL-12 secretion from the large number of HCFs was sufficient to advance the HCF migration front (Fig. 7B). We thus confirmed that TGF-β1 could indirectly enhance HCF migration via increasing the IL-12 secretion.

In addition to demonstrating that MOX modifies corneal fibroblast-to-myofibroblast differentiation, we further illustrated for the first time that MOX reduced IL-12 expression in HCFs concentration-dependently. Our results are clinically relevant and indicate a potentially beneficial anti-inflammatory effect of the topically applied MOX for two reasons. First, the suppression of IL-12 secretion was significant at MOX concentration of greater than 10 µg/mL and higher in the presence of rhTGF-β1. MOX concentration in the cornea is 18.6 ± 9.66 µg/g at 10 minutes after one drop of topical application and achieves 2.1 times higher concentrations with repeated topical instillation. Since there is a TGF-β1 surge following corneal wounding and/or infection, the difference in IL-12 inhibition at MOX concentration of greater than 10 µg/mL in the presence of rhTGF-β1 reflect true clinical scenario. Second, HCFs secrete IL-12 in the presence of lipopolysaccharide stimulation and following refractive surgery. MOX is normally applied after corneal surgery and/or infection, indicating the current practice of MOX application in a high IL-12 concentration environment. The result of MOX inhibiting IL-12 secretion echoes previous study demonstrating the immunosuppressive effects of ciprofloxacin during human immunosenescence is associated with alteration in inflammation and natural killer cells function in the elderly and that IL-12 is associated with natural killer cells regulation, it is inspiring to clarify whether there is an age-related difference in IL-12 secretion. We found that there was no significant difference in the age between the IL-12 secreting and nonsecreting group. This indicates that age factor is less important than interindividual genetic variation in the management of corneal infection and/or wound healing regarding IL-12.

Our results demonstrated that 2 ng/mL rhTGF-β1 promoted HCF proliferation in complete culture medium (10% FBS/DMEM). This proliferation would confound cell migration as proliferation could force cell migration. We thus used serumless media (0.1% FBS/DMEM) in the migration experiment. However, HCFs migrated very slowly in serum-less culture medium compared with the same cells in complete culture medium, we hence used higher IL-12 concentration; that is, 10 ng/mL, in cell migration assay. For parallel comparison, we used rhTGF-β1 10 ng/mL. We found that both IL-12 and TGF-β1 enhanced HCF proliferation (Figs. 4, 8). However, only IL-12, but not TGF-β1, promoted HCF migration (Fig. 5). TGF-β1 promoted cell migration indirectly by increasing IL-12 secretion, which subsequently enhanced HCF migration (Fig. 7C). This is different from previous studies that showed TGF-β1 promoted cell migration in HCFs and dermal fibroblasts. The difference could be evidenced by our experiments using a high concentration of rhTGF-β1 to incubate the isolated HCFs.

Our results revealed that 2 ng/mL rhTGF-β1 treatment increased the number of viable HCFs (Fig. 8). If rhTGF-β1 could also promote HCF migration, there should have been an enhanced HCF migration front when rhTGF-β1 was added to the culture media, no matter how many cells were seeded in the culture dish. However, there was no enhanced HCF migration front when HCFs were seeded only in the center of the ibidi culture insert (Fig. 5), although the culture media did contain sufficient rhTGF-β1; that is, 10 ng/mL. This confirmed that rhTGF-β1 alone could not promote HCF migration. In addition, if the HCFs could secrete IL-12 to promote HCF migration indirectly, sufficient number of HCFs would be needed to secrete enough amount of IL-12. This assumption is also confirmed when rhTGF-β1 was added to the 6-cm HCF culture dish in our experiment. The amount of the rhTGF-β1-stimulated IL-12 secretion from the large number of HCFs was sufficient to advance the HCF migration front (Fig. 7B). We thus confirmed that TGF-β1 could indirectly enhance HCF migration via increasing the IL-12 secretion.
mixed lymphocyte reaction via suppressing the IL-12 produc-
tion in monocytes.6 Our results thus facilitate understanding of
current MOX anti-inflammatory mechanism, in addition to
previous antimicrobial and antibiostatic mechanisms.8 However,
the beneficial anti-inflammatory effect should be more
noticeable in individuals with high baseline IL-12 secretion.
MOX might potentially benefit corneal wound healing by
prohibiting myofibroblast differentiation8 and by decreasing
inflammation via inhibiting TGF-β-induced IL-12 secretion as
shown in this study. On the other hand, MOX inhibited HCF
migration, which is necessary for wound healing. MOX also
diminished HCF proliferation, which is also necessary in
stromal repopulation when there is cell depletion. The
advantages and disadvantage in wound repair should thus be
balanced in clinical application.

Another limitation of our study is that we estimated
secretory IL-12 via equation calculation from digitized immu-
noblot instead of direct ELISA measurement due to the lack of
availability of appropriate antibody. However, we generated an
equation of the IL-12 standard curve for every experiment in
our experiment design to reduce the variation in IL-12
concentration estimation. We suggest that our results of TGF-
β1 and MOX related IL-12 alteration revealed a reliable
conclusion. Confirming the moxifloxacin-related inhibition of
IL-12 secretion using other fluoroquinolones is expected
before generalization this wound healing modulation phenom-
emon.

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