Mitomycin C Retardation of Corneal Fibroblast Migration via Sustained Dephosphorylation of Paxillin at Tyrosine 118

Tsan-Chi Chen,1 Chien-Hsueb Lai,1 Jie-Ling Chang,2 and Shu-Wen Chang1,3

PURPOSE. To investigate how mitomycin C (MMC) modulates corneal fibroblast migration and its molecular mechanisms in the wound healing process.

METHODS. After treatment with 0 and 0.2 μg·mL−1 MMC for 5 minutes, effect of MMC on cell migration of human corneal fibroblasts (HCFs) was examined with a cell migration assay. Both focal adhesion kinase (FAK) and paxillin (PXN) expressions in HCFs were analyzed by semiquantitative real-time PCR, immunoblotting, and immunofluorescence confocal microscopy. Using gene silencing or gene overexpression with lentiviral-based pseudovirion infection, the phosphorylation level of FAK, PXN, and mutated PXNs at tyrosine sites 31 (Y31F-EGFP) and 118 (Y118F-EGFP) were verified in HCFs.

RESULTS. MMC retarded HCF migration at 1 and 2 days post-treatment (dpt). MMC reduced levels of FAK transcript and FAK protein, but increased both transcript and protein expression of PXN at 1 and 2 dpt. Furthermore, MMC upregulated FAK-pY397, which subsequently enhanced PXN-pY31 in a dose-dependent manner at 1 dpt. Concurrently, MMC downregulated PXN-pY118 at 1 dpt. However, MMC treatment resulted in dephosphorylation of FAK-pY397, PXN-pY31, and PXN-pY118 at 2 dpt. The FAK/PXN complex in MMC-treated HCFs was detected at focal adhesion sites more than at the leading edge at 1 and 2 dpt, contributing to retardation of HCF migration. Y118F-EGFP-expressing HCFs exhibited lower mobility than that of PXN-EGFP- or Y31F-EGFP-expressing HCFs.

CONCLUSIONS. The sustained PXN-pY118 dephosphorylation resulted in steadfastness of an incompletely active FAK/PXN complex at focal adhesion sites and played a pivotal role in MMC-retarded HCF migration. (Invest Ophthalmol Vis Sci. 2012;53:1539–1547) DOI:10.1167/iovs.11-9203

Wavefront-guided photorefractive keratectomy (PRK), a laser-based surgical method to correct irregular astigmatism and to improve the optical quality of laser treatment, was first introduced in 2001.1 PRK induces less high order aberra-
tion, less corneal ectasia, and theoretically preserves corneal biomechanical properties better than laser-assisted in situ keratomileusis (LASIK).2-4 Consequently, although visual acuity recovery is slower after PRK,5 it has been reported to have long-term benefits over stromal ablation surgery.6-8 However, because PRK may cause corneal haze in high-risk patients, medical and surgical approaches to prevent postoperative corneal haze are highly desired.6

Mitomycin C (MMC), an antibiotic isolated from Streptomyces caesipes, has been commonly used to prevent corneal scar formation9,10 and recurrence of subepithelial fibrosis after PRK.11 MMC is also used to prevent corneal haze formation during high-risk forms of surface ablation such as PRK after cases with LASIK flap complications,12-14 radial keratotomy, penetrating keratoplasty,15 and extended prophylactic use in regular cases.16 In rabbits, MMC application results in a sustained subnormal keratocyte density up to 3 months postoperatively.17,18 This subnormal keratocyte density could result from increased apoptosis,17 decreased repopulation because of inhibited cell proliferation,19 and possibly slower keratocyte migration from the adjacent stroma.20 Our previous study revealed that MMC application indirectly modifies corneal epithelial cell migration.21 Therefore, it is interesting to understand whether MMC directly interferes with corneal fibroblast migration.

Cell migration generally requires motion in the actin cytoskeleton be signaled to the extracellular environment via disassembly of the existential focal adhesions at the trailing edge and formation of the new focal complexes at the leading edge.22,23 Focal adhesions may act as a molecular clutch, providing the traction that pulls the cell body forward at the leading edge and mediates retraction at the rear of the cell. However, too much cell-substratum focal adhesion prevents movement, whereas too little results in insufficient traction.24 Consequently, an optimal balance of focal adhesion assembly and disassembly must occur to allow maximum cell spreading, elongation, and migration.

Focal adhesions consist of multimeric proteins that provide a structural and adhesive link between cell surface integrins that are bidirectionally ligated to extracellular matrix and the intracellular cytoskeleton complex.26 Proper function of this complex results in modulation of cell spreading, proliferation, and survival.27,28 Paxillin (PXN), a key focal adhesion adaptor in the intracellular focal adhesions, switches cell-substratum dynamics and cell migration by recruiting numerous intracellular proteins to the leading edge29—including focal adhesion kinase (FAK)-Src,30 Crk-associated substrate proteins (Cas),31 and PTEN32—to complete a dynamic multilayered signaling and structural complex between the cytoskeleton and extracellular matrix. PXN has two major tyrosine phosphorylation sites located at tyrosine 31 (PXN-Y31) and tyrosine 118 (PXN-Y118).27,33,34 Phosphorylation of PXN-Y31 and PXN-Y118 is necessary for cell membrane spreading and ruffling during cell adhesion and migration.27,32,35 FAK, a protein tyrosine kinase that is located at intracellular focal adhesions and protrusive lamellipodia at
the leading edge, is mainly involved in a diverse range of critical cellular events including spreading, proliferation, migration, and invasion.\(^5\) When bound to focal complexes, active FAK directly induces tyrosine phosphorylation of PXN as an effector of FAK to generate the downstream signals that regulate motility, survival, and proliferation.\(^3\) Phosphorylation of FAK at tyrosine 397 (FAK-Y397) is associated with integrin-mediated focal adhesions and with tyrosine phosphorylation of PXN, thereby regulating both assembly and turnover of adhesion sites.\(^3,4,5\) In addition, activation of FAK-Y397 induces PXN phosphorylation and dissociation of the old focal adhesion complexes from focal adhesions to stimulate turnover of focal adhesions and allow cell movement.\(^3\)

Although intraoperative MMC application is accepted to prevent corneal haze formation, little information is available about the molecular mechanism by which MMC regulates corneal fibroblast migration. In this study, we provide insight into the regulation of FAK/PXN-dependent migration of corneal fibroblasts by MMC.

**MATERIALS AND METHODS**

**Culture of Human Corneal Fibroblasts**

Human corneal rims were obtained from residual corneas after penetrating keratoplasty surgery. After scraping epithelium and endothelium with a surgical blade, the denuded corneal tissue was cut into small fragments (\(1 \times 1 \times 1\) mm in size) and incubated in Dulbecco’s modified Eagle’s medium (DMEM; Gibco, Grand Island, NY) with 10% heat-inactivated fetal bovine serum (FBS; Biological Industries, Kibbutz Beit Haemek, Israel) and 2 mg \(\cdot\) mL\(^{-1}\) collagenase A (Roche Applied Science, Mannheim, Germany) at 37°C for approximately 6 hours. After washing twice with antibiotic-free 10% FBS/DMEM, human corneal fibroblasts (HCFs) were isolated and seeded in antibiotic-free 10% FBS/DMEM in a humidified atmosphere containing 5% \(\text{CO}_2\) at 37°C. To analyze protein expression and phosphorylation level of FAK and PXN, HCFs were washed with PBS and disrupted in RIPA lysis buffer (150 mmol \(\cdot\) L\(^{-1}\) NaCl; 1% NP-40; 0.2% SDS; 0.5% sodium deoxycholate; 50 mmol \(\cdot\) L\(^{-1}\) Tris-HCl, pH 6.8) containing complete EDTA-free inhibitor cocktail tablet (Cat. No. 04693132001) and phosphatase inhibitor cocktail tablet (PhosSTOP; Cat. No. 0900687001) following the manufacturer’s instructions (Roche Applied Science). After protein quantification with a protein assay kit (BCA Protein Assay Kit; Pierce, Rockford, IL), 3 \(\mu\)g of each sample were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes. After blocking nonspecific binding with 5% bovine serum albumin (BSA)/TBST (20 mmol \(\cdot\) L\(^{-1}\) Tris-HCl, pH 7.5, 0.5 mol \(\cdot\) L\(^{-1}\) NaCl, 0.1% Tween-20) for 1 hour, the membranes were incubated at 4°C overnight in 5% BSA/TBST with the appropriate primary antibodies (1:1000) as follows: FAK (A-17), PXN (H-114), PXN-pY31, PXN-pY118 (Santa Cruz Biotechnology, Santa Cruz, CA), pAS2.PXN-Y118F-EGFP, pAS2.PXN-Y31F-EGFP, and pAS2.PXN-Y31F-EGFP.

**Semiquantitative Real-Time PCR**

To analyze protein expression and phosphorylation level of FAK and PXN, HCFs were washed with PBS and disrupted in RIPA lysis buffer (150 mmol \(\cdot\) L\(^{-1}\) NaCl; 1% NP-40; 0.2% SDS; 0.5% sodium deoxycholate; 50 mmol \(\cdot\) L\(^{-1}\) Tris-HCl, pH 6.8) containing complete EDTA-free inhibitor cocktail tablet (Cat. No. 04693132001) and phosphatase inhibitor cocktail tablet (PhosSTOP; Cat. No. 0900687001) following the manufacturer’s instructions (Roche Applied Science). After protein quantification with a protein assay kit (BCA Protein Assay Kit; Pierce, Rockford, IL), 3 \(\mu\)g of each sample were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes. After blocking nonspecific binding with 5% bovine serum albumin (BSA)/TBST (20 mmol \(\cdot\) L\(^{-1}\) Tris-HCl, pH 7.5, 0.5 mol \(\cdot\) L\(^{-1}\) NaCl, 0.1% Tween-20) for 1 hour, the membranes were incubated at 4°C overnight in 5% BSA/TBST with the appropriate primary antibodies (1:1000) as follows: FAK (A-17), PXN (H-114), PXN-pY118 (Santa Cruz Biotechnology, Santa Cruz, CA), FAK-Y397 (Millipore, Billerica, MA), and \(\beta\)–actin (Sigma-Aldrich). The membranes were incubated at room temperature.

**Table 1. Oligonucleotides Used in This Study**

<table>
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<th>Gene</th>
<th>Direction</th>
<th>Sequence (5′-3″)</th>
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<td>(\beta)-Actin</td>
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<tr>
<td>Y118F</td>
<td>Reverse</td>
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* Abbreviations for genes: FAK, focal adhesion kinase; PXN, paxillin.
† Abbreviation for plasmid: PXN-EGFP, pAS2.PXN-EGFP.
‡ The restriction cutting site in each primer is represented by characters in italics with underline.
§ Abbreviations for mutated plasmids: Y31F, pAS2.PXN-Y31F-EGFP; Y118F, pAS2.PXN-Y118F-EGFP.
|| The mutation site in each oligonucleotide is represented by character in italics.
peritoneum with horseradish peroxidase–conjugated anti-mouse or antirabbit antibodies (1:10,000) for 1 hour. Finally, the blots were developed with chemiluminescence (Millipore) and analyzed (Fujifilm LAS-4000 Imaging System; Fujifilm, Tokyo, Japan). The density of each band was quantified using commercial multigauge software (Fujifilm MultiGauge, Version 3.0; Fujifilm).

Gene Silencing
To silence FAK and PXN expression, lentiviral short hairpin RNAi (shRNA) constructs of FAK (shFAK) and PXN (shPXN), obtained from the National RNAi Core Facility in Academia Sinica, Taiwan, were used to infect HCFs as in a previous study with minor modification. In brief, before MMC treatment, HCFs were infected continuously for 3 days with lentivirus-based shFAK or shPXN pseudovirions at >5 multiplicity of infection. The pseudovirions were produced from pseudovirion-packaging 293T cells with the hairpin-pLKO.1-shFAK clone (TRCN0000121318, 5′-CCG ATT GGA AAC CAA CAT ATA-3′) or the hairpin-pLKO.1-shPXN clone (TRCN0000123138, 5′-ACC CAA CTG GAA ACC ACA CAT-3′) using a commercial transfection reagent (Fgene HD Transfection Reagent; Roche, Indianapolis, IN).

Immunofluorescence Staining
To observe intracellular distribution of the target proteins, 1.0 × 10^4 cells were grown on a 12-mm round cover glass placed in a 24-well culture plate before MMC treatment. MMC-treated or untreated cells were fixed with 4% paraformaldehyde in PBS (pH 7.4) for 10 minutes. The fixed HCFs were washed three times with PBS and incubated in permeabilization solution (0.1% Triton X-100, 10 mg·mL⁻¹ BSA in PBS) for 30 minutes. The slides were incubated with appropriate primary antibody as follows: FAK (A-17), paxillin (H-114), paxillin-pY118 (Santa Cruz Biotechnology), paxillin, paxillin-pY31, and FAK-pY397 (Millipore). Subsequently, the slides were incubated with secondary antibody conjugated to Alexa Fluor 488 or 594 (Invitrogen, Paisley, UK). The nuclei were stained with DAPI, and the stained cells were mounted with aqueous mounting medium (DAKO, Carpinteria, CA) and analyzed using a confocal microscopy system (Zeiss LSM510; Zeiss, Jena, Germany).

Expression of Fluorescent Paxillins
To verify the role of PXN during HCF migration, we constructed PXN-expressed plasmid pAS2.PXN-EGFP and its mutation plasmids (pAS2.PXN-Y31F-EGFP and pAS2.PXN-Y118F-EGFP). Briefly, we amplified and cloned the full-length PXN fragment (NM_001080855; the primer sequences are listed in Table 1) into the vector systems (pGEM-T Easy Vector Systems; Promega) as an intermediate plasmid pGEM-T.PXN, with the restriction cutting sites for Nhel at two ends. After isolation of the PXN fragment from pGEM-T.PXN with Nhel digestion, the PXN fragment was subligated into an original plasmid pAS2.EGFP.puro as a final construct pAS2.PXN-EGFP. Subsequently, pAS2.PXN-Y31F-EGFP and pAS2.PXN-Y118F-EGFP plasmids were produced by ultra high-fidelity DNA polymerase (KOD; Toyobo, Osaka, Japan) with their specific single-mutated oligonucleotides (Table 1). All the PXN-expressed constructs were confirmed by direct sequencing and indirectly introduced into HCFs using the pseudovirion infection system similar to the earlier description.

Statistical Analysis
Migration assay, immunoblotting, and immunofluorescent staining were performed in triplicate with similar results. Quantitative data in gene transcription and the relative expressions of phospho-FAK and phospho-PXN were expressed as the mean ± SEM of three independent experiments with similar trends. Differences among the various conditions were evaluated by predictive analytic software (SPSS version 13.0; SPSS Inc., Chicago, IL) using Student’s t-test. Values of P < 0.05 were considered statistically significant.

RESULTS

Mitomycin C Retarded Human Corneal Fibroblast Migration
MMC has been shown to induce significant keratocyte loss and retard repopulation. Therefore, we investigated whether MMC also affects HCF migration in addition to the well-known effects on keratocyte loss. Using phase-contrast microscopy, we observed migration patterns in an in vitro migration assay using silicone inserts (Culture-Inserts; ibidi) (Fig. 1). At 1 and 2 dpt, the density of migratory MMC-treated HCFs in the cell-free space between the two cell areas was significantly lower compared with untreated cells, and the migration distance was shorter. Furthermore, both effects were MMC concentration dependent. This result suggests that MMC directly interfered with HCF migration.

Mitomycin C Increased Phosphorylation of FAK-Y397 and Paxillin-Y31, but Induced Dephosphorylation of Paxillin-pY118 at 1 dpt
FAK and PXN are two key regulators during cell migration, and their phosphorylation sites, including serine and tyrosine residues, play important roles in regulating cell migration. Because MMC impeded HCF migration (Fig. 1), we evaluated whether MMC acted via regulating phosphorylation of FAK and PXN. Using a qPCR assay, we quantitated the transcript levels of FAK and PXN in HCFs. Our results showed that MMC slightly downregulated FAK transcript levels (10% decrease with 0.2 mg·mL⁻¹ MMC), but upregulated PXN transcript levels (18% increase with 0.2 mg·mL⁻¹ MMC) (Fig. 2A). Next, we determined the expression levels of FAK, FAK-pY397, PXN, PXN-pY31, and PXN-pY118 at 1 dpt using immunoblotting assay with protein- and phospho-specific antibodies (Fig. 2B). Relative to the change of endogenous FAK and PXN proteins, the immunoblotting results indicated that MMC enhanced both FAK-pY397 (Fig. 2C) and PXN-pY31 (Fig. 2D, blank columns)

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in a dose-dependent manner. In contrast, MMC also reduced PXN-pY118 dose-dependently (Fig. 2D, gray columns). These results suggest that MMC converted PXN into a less active form via dephosphorylation of PXN-pY118, which subsequently retarded HCF migration.

FAK-pY397 Was Required for Phosphorylation of Paxillin-Y31 and Paxillin-Y118 in Mitomycin C–Treated Corneal Fibroblasts at 1 dpt

Phosphorylated FAK is required for PXN phosphorylation on tyrosine 31 and 118, which are prerequisite for cell migration.27,52,54 To examine whether MMC modulated the phosphorylation of PXN-Y31 and PXN-Y118 via FAK-Y397 phosphorylation, we individually silenced FAK and PXN expressions using the short hairpin RNAi (shRNA) approach and analyzed the FAK and PXN expressions after MMC treatment.

Infection of HCFs with pseudovirions of shRNA-FAK (shFAK) or shRNA-PNX (shPXN) efficiently reduced the respective transcript and protein levels with a consistent knockdown efficiency > 80% was verified by qPCR and immunoblotting assay (Fig. 3). Pseudovirion infection with shFAK markedly downregulated the expression of total FAK and FAK-pY397, and reduced the phosphorylation level of PXN-pY31 and PXNpY118 in (B) and PXNpY118 in (C) were calculated and normalized to the present endogenous FAK and PXN, respectively. Quantitative data were expressed as the mean ± SEM of three independent experiments with a similar trend. The relative changes of FAK and PXN in gene transcription and protein phosphorylation were compared with the untreated groups (*P < 0.05; **P < 0.01).

FIGURE 2. MMC increases phosphorylation of FAK-Y397 and PXN-Y31, but leads to dephosphorylation of PXN-pY118. HCFs were treated with the indicated concentration of MMC, harvested at 1 dpt, and analyzed by (A) qPCR with FAK- or PXN-specific primer sets and (B) immunoblotting with the indicated target-specific primary antibodies, respectively. The numbers below images of endogenous FAK and PXN were calculated relative to β-actin expression and normalized to those in the untreated cells. Relative expressions of (C) FAK-pY397, (D) PXN-pY31, and PXN-pY118 in (B) were calculated and normalized to the present endogenous FAK and PXN, respectively. Quantitative data were expressed as the mean ± SEM of three independent experiments with a similar trend. The relative changes of FAK and PXN in gene transcription and protein phosphorylation were compared with the untreated groups (*P < 0.05; **P < 0.01).
contrary, whereas shPXN pseudovirion infection did not interfere with FAK at transcripts (Fig. 3A, blank columns 5 and 6) or protein levels (Fig. 3B, lanes 5 and 6), MMC still increased FAK-pY397 in the PXN-silenced HCFs (Fig. 3C, columns 5 and 6). However, the residual PXN proteins might still be phosphorylated at PXN-Y31 but not at PXN-Y118 by MMC-activated FAK-pY397 (Fig. 3B, lanes 5 and 6; Fig. 3D, columns 5 and 6). These confirm that FAK was an upstream regulator of PXN activation during MMC treatment in HCFs, even though MMC led to dephosphorylation of PXN-pY118.

Mitomycin C Led to Dephosphorylation of Both FAK and Paxillin at 2 dpt

To further illustrate the duration of effect after transient MMC application, we analyzed the levels of protein and transcripts of FAK and PXN at 2 dpt using qPCR and immunoblotting assay, respectively (Fig. 4). The results indicated that MMC strongly reduced FAK transcript levels (Fig. 4A, 38% decrease with 0.2 mg · mL⁻¹ MMC) and endogenous FAK protein expression (Fig. 4B) at 2 dpt in a dose-dependent manner. In addition, even though total FAK-pY397 was concurrently decreased in a dose-dependent manner, the ratio of residual FAK-pY397 to FAK increased (Figs. 4B, 4C). On the other hand, both PXN transcripts (Fig. 4A) and endogenous PXN protein (Fig. 4B) were still noticeably increased at 2 dpt, but phosphorylation of PXN-Y31 and PXN-Y118 was oppositely decreased in a dose-dependent manner (Figs. 4B, 4D). These data suggest that depletion of FAK-pY397, PXN-pY31, and PXN-pY118 at 2 dpt might contribute to the sustained retardation of HCF migration (Fig. 1).

Mitomycin C Restrained the FAK/Paxillin Complexes at the Focal Adhesion Sites

Activated (phosphorylated) FAK combines and activates PXN to form newly synthesized active FAK/PXN complexes at the cell periphery to promote cell migration. Using immunofluorescence confocal microscopy, we monitored the distribution of FAK, PXN, and their phosphorylated forms in HCFs after MMC treatment at 1 and 2 dpt (Fig. 5). The results revealed that FAK/PXN complexes were mostly localized at the leading edge of untreated HCFs in nontreated cells (Fig. 5A, arrowheads in top panels). In contrast, more complexes were located at the focal adhesion sites and fewer complexes were located at the leading edges at both 1 and 2 dpt after MMC.
treatment (Fig. 5A, arrows in middle and bottom panels). At 1 dpt, both FAK-pY397 and PXN-pY31 colocalized at the leading edge with total FAK and total PXN in both treated and untreated cells (Figs. 5B, 5C, arrowheads in middle panels). However, PXN-pY118 was barely detectable at sites of PXN localization in MMC-treated cells (Fig. 5D, middle panel). At 2 dpt, FAK was barely detectable and FAK-pY397 was not detectable (Figs. 5A, 5B, bottom panels). Similarly, both PXN-pY31 and PXN-pY118 were undetectable (Figs. 5C, 5D, bottom panels).

As MMC retarded HCF migration (Fig. 1), we hypothesized that the sustained dephosphorylation of endogenous PXN-pY118 is the critical factor hindering formation of active FAK/PXN complexes in MMC-treated HCFs. Therefore, we expressed fluorescent PXN (PXN-EGFP) and its mutated forms (Y31F-EGFP and Y118F-EGFP) in HCFs (Fig. 6). Using immunoblotting assay, we did verify dephosphorylation of the fluorescent PXN mutations and found that expressions of PXN-EGFP and Y31F-EGFP could increase the phosphorylation level of FAK-pY397 (Figs. 6A, 6B). Under fluorescent microscope, our results revealed that PXN-EGFP and Y31F-EGFP distributed mostly at the leading edge. In contrast, Y118F-EGFP mainly distributed in the ventral region of HCFs (Fig. 6C), similar to the distribution of endogenous dephosphorylated PXN-pY118 in MMC-treated HCFs (Fig. 5D). Compared with EGFP-expressing HCFs, expressions of PXN-EGFP and Y31F-EGFP promoted the HCF migration, but Y118F-EGFP expression noticeably retarded HCF migration (Fig. 6D). These data suggest that the retarded HCF migration could be attributed to the stationary adhesion of the FAK/PXN complexes at the focal adhesion sites due to sustained PXN-pY118 dephosphorylation.

**DISCUSSION**

Our study provides the first demonstration of the molecular mechanism underlying MMC-induced retardation of HCF migration. We illustrate that MMC deactivated PXN by inducing dephosphorylation of PXN-pY118 at 2 dpt. HCFs were treated with the indicated concentrations of MMC, harvested at 2 dpt, and analyzed by (A) qPCR with FAK- or PXN-specific primer sets and (B) immunoblotting with the indicated target-specific primary antibodies, respectively. The numbers below images of endogenous FAK and PXN were calculated relative to β-actin expression and normalized to those in the untreated cells. Relative expressions of (C) FAK-pY397, (D) PXN-pY31, and PXN-pY118 in (B) were calculated and normalized to the present endogenous FAK and PXN, respectively. Quantitative data were expressed as the mean ± SEM of three independent experiments with a similar trend. The relative changes of FAK and PXN in gene transcription and protein phosphorylation were compared with the untreated groups (*P < 0.05; **P < 0.01; ***P < 0.001).
though MMC concurrently enhanced PXN-Y31 phosphorylation at 1 dpt (Figs. 2B, 2D) and FAK activation by increasing phosphorylation of FAK-Y397 at 1 dpt (Figs. 2B, 2C) and 2 dpt (Figs. 4B, 4C), they were insufficient to compensate for active FAK/PXN complex formation needed in HCF migration when PXN-Y118 phosphorylation was blocked by MMC (Figs. 2B, 2D, 4B, 4D). In addition, expressions of PXN-EGFP and Y31F-EGFP significantly enhanced FAK-Y397 phosphorylation (Figs. 6A, 6B). These indicate that PXN-pY118 dephosphorylation in MMC-treated HCFs was pivotal in the steadfastness of FAK/PXN complexes and contributed to HCF migration retardation.

Immunostaining revealed that FAK/PXN complexes distributed mainly at focal adhesion sites of the MMC-treated cells at 1 and 2 dpt; furthermore, FAK-pY397, PXN-pY31, and PXN-pY118 staining had almost disappeared at 2 dpt (Fig. 5). In general, formation of newly synthesized active focal complexes is essential for forward cell movement.25,27,32–34 We demonstrated that MMC application led to dephosphorylation of PXN-pY118 at both 1 and 2 dpt, although it concurrently upregulated total PXN expression at both mRNA and protein levels (Figs. 2 and 4). Moreover, Y118F-EGFP expression did not change FAK-Y397 phosphorylation level (Figs. 6A, 6B). It is possible that the MMC-enhanced PXN expression in the early phase (Fig. 2) feedback to reduce the upregulation of FAK-pY397 and PXN-pY31, which further resulted in a dephosphorylation of FAK-pY397 and PXN-pY31 in the late phase (Fig. 4). This explains why MMC enhanced FAK phosphorylation in an early phase but decreased FAK phosphorylation in a later phase. Further investigation to examine this possible feedback mechanism may facilitate our understanding of this molecular alteration.

Moreover, both fluorescent Y118F-EGFP–expressing cells and endogenous PXN in MMC-treated cells were distributed in the ventral region, and the migration of the Y118F-EGFP-expressing cells was slower than that of the PXN-EGFP–expressing cells (Fig. 6D). In contrast, these alterations were not found in the Y31F-EGFP–expressing cells. Moreover, excessive focal adhesions are the stable constructs and display a slower turnover than focal complexes.24 Therefore, inactivation of

**Figure 5.** MMC leads to steadfastness of the FAK/PXN complexes via dephosphorylation of PXN-pY118. HCFs were treated with 0.2 mg/mL MMC for 5 minutes and coimmunostained at 1 and 2 dpt with target-specific antibodies, including FAK (A, B), FAK-pY397 (B), PXN (A, C, D), PXN-pY31 (C), and PXN-pY118 (D). Arrowheads indicate focal complexes at the leading edge; arrows indicate focal adhesion sites. Scale bars, 50 μm.

- **A** FAK Merge PXN
  - Untreated
  - 1 dpt
  - 2 dpt
- **B** FAK Merge FAK-pY397
  - Untreated
  - 1 dpt
  - 2 dpt
- **C** PXN Merge PXN-pY31
  - Untreated
  - 1 dpt
  - 2 dpt
- **D** PXN Merge PXN-pY118
  - Untreated
  - 1 dpt
  - 2 dpt
PXN-Y118 but not PXN-Y31 might be critical in blocking synthesis of the new focal complexes to the lamellipodial region in MMC-treated cells.

There are two major clinical concerns regarding MMC-related retardation of corneal fibroblast migration (i.e., corneal haze prevention and long-term stromal cell depletion). It has been documented that TGF-β in the tear increases after PRK. Our previous study revealed that MMC at 0.2 mg/mL indirectly modifies corneal epithelial cell migration, which might contribute to delayed reepithelialization. Furthermore, we also observed that MMC treatment upregulated IL-8 and MCP-1 secretion that might contribute to polymorphonuclear cell and macrophage recruitment. This could allow more access of TGF-β and inflammatory cells into the corneal stroma and subsequently result in more myofibroblast transformation and haze formation, which might seemingly contradict previous observations that MMC application reduced corneal haze formation. In this study, we disclosed that MMC retarded corneal fibroblast migration and suggested that slower repopulation beneficially prohibited corneal fibroblasts from contacting the egressed TGF-β and beneficially prevented myofibroblast transformation. Nevertheless, if the retardation of corneal fibroblast migration were tremendous, it might result in permanently reduced stromal cell density long after

**Figure 6.** Effect of mutated PXNs in HCFs. HCFs expressed EGFP or EGFP-fused PXNs through lentivirus-based pseudovirion infection. (A) Expressions of endogenous FAK and fluorescent PXNs were analyzed by immunoblotting with the indicated target-specific primary antibodies. The numbers below image of endogenous FAK were calculated relative to β-actin expression and normalized to that in the EGFP-expressing cells. (B) Relative expressions of FAK-pY397 in (A) were calculated and normalized to the present endogenous FAK. Quantitative data were expressed as the mean ± SEM of three independent experiments with a similar trend. The relative changes of FAK in protein phosphorylation were compared with the EGFP-expressing group (*P < 0.05). (C) Distribution of the fluorescent PXNs was observed under a confocal microscope. Cell nucleus was stained with a nuclear dye DAPI (blue). Scale bars, 50 μm. (D) Effect of the mutated PXN expressions on HCF migration was monitored by a cell migration assay (ibidi). The phase images were displayed at Day 0 (top row) and Day 1 (second row), and the fluorescence images were displayed only at Day 1 (bottom row). The double-headed arrows (400 μm) indicate the cell-free gap between the two regions of HCFs. Plasmid labels: EGFP, pAS2.EGFP.puro; PXN-EGFP, pAS2.PXN-EGFP; Y31F-EGFP, pAS2.PXN-Y31F-EGFP; Y118F-EGFP, pAS2.PXN-Y118F-EGFP.
MMC application, leading to a long-term safety concern. Our results also disclosed that MMC-treated cells could still migrate. This echoes the clinical results that the initial subnormal corneal cellularity tends to normalize over time.\(^4\) Since superficial stroma hypercellularity is associated with corneal haze formation,\(^4\) we believe that this MMC temporal retardation of corneal fibroblast migration contributes at least partially to the clinically observed less haze formation after MMC application. In conclusion, we demonstrate for the first time that MMC modulates HCF migration by leading to dephosphorylation of PXN-Parkin and subsequent decreased migration of HCFs. Cornea. 2009;28:765–769.

References