Wnt-7a Up-regulates Matrix Metalloproteinase-12 Expression and Promotes Cell Proliferation in Corneal Epithelial Cells during Wound Healing*

Jungmook Lyu and Choun-Ki Joo‡

From the Department of Ophthalmology and Visual Science, College of Medicine, Catholic University of Korea, Seoul 137-040, Korea

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Corneal wound repair involves the rapid coverage of a denuded area by residual epithelial cells. During wound healing, there are different cell behaviors in different regions of the epithelium: cell proliferation in the peripheral epithelium and cell migration in the central epithelium. We found that Wnt-7a was rapidly induced in the wounded cornea, promoted the proliferation of corneal epithelial cells, and enhanced wound closure. Matrix metalloproteinase-12 (MMP-12) was detected in the peripheral epithelium, where cell proliferation was enhanced, but was diminished in the migrating central epithelium. Wnt-7a induced the accumulation of β-catenin and the activation of Rac and β-catenin, and Rac synergistically induced the transcription of MMP-12. Blocking the function of MMP-12 delayed wound closure induced by Wnt-7a. Our results also suggest that, in addition to the β-catenin pathway, Wnt-7a might induce a β-catenin-independent pathway. By regulating the proliferation of corneal epithelial cells, Wnt-7a and MMP-12 appear to contribute to corneal wound healing.

Wound repair involves the coordination of complex processes to cover the area of the defect and to quickly re-establish barrier function (1). These processes include the migration, proliferation, and differentiation of epithelial cells. During corneal wound healing, epithelial cells migrate without proliferating until the wound closes. After wound closure, proliferation and upward movement of the cells from the basal layer act in concert to form a multilayered structure (2). Recent studies have shown that a wound enhances the rate of proliferation in the peripheral epithelium, whereas the more central cells that migrate to cover the wound do not progress through the cell cycle. This indicates that the migratory and proliferative responses are regulated separately (3, 4). Cell migration and proliferation are believed to be regulated by several cues, including epidermal growth factors, hepatocyte growth factor, and keratinocyte growth factor secreted from epithelial or stromal cells (4, 5). However, the extracellular cues that induce the compartmentalized responses to these events during wound healing are poorly understood.

Wnt genes encode secreted glycoproteins that control cell proliferation, motility, differentiation, and morphology by pathways that are termed "canonical" or "non-canonical" (6, 7). Binding of Wnt proteins to members of the Frizzled (Fz) family of receptors stimulates these different signaling pathways via a mediator called Disheveled (Dvl) (8). In the canonical pathway, β-catenin primarily regulates gene expression. Activation of this pathway causes β-catenin to be stabilized by the inactivation of glycogen synthase kinase-3β. Stabilized β-catenin binds and activates the TCF/LEF transcription factors, stimulating transcription of the target genes (8). The non-canonical pathway primarily affects cell shape and movement. Wnt-5a and Wnt-11 regulate convergent extension movements during vertebrate gastrulation (9) by activating Rho and Rac GTPases (10). The Rho and Rac pathways regulate cytoskeletal rearrangement, cell adhesion, and nuclear events through JNK (11, 12). Wnt-1 also activates JNK via Rac during gastrulation (11), and Wnt-1 and Wnt-3a activate Rho kinase to induce neurite retraction in PC12 and N1E-115 cells (13).

A number of Wnt target genes have been identified, including c-myc, cyclin D1, MMP-7, and membrane type 1 MMP. These target genes have important implications in understanding the role of Wnt proteins in cell motility and proliferation. c-Myc and cyclin D1 are well known regulators of cell proliferation. MMPs are proteinases that can degrade almost all of the components of the extracellular matrix, including collagen, fibronectin, and elastin. They play important roles in many physiological and pathological processes, including angiogenesis, wound healing, and inflammatory diseases (14).

Cell proliferation and migration are key events in re-epithelialization during corneal wound healing. These processes may be mediated by the activation of MMPs because several MMPs are differentially expressed during corneal wound repair and wound closure delayed by MMP inhibitors (15, 16). Wnt proteins have been implicated in cell proliferation in a variety of tissues during development and tumorigenesis. However, the role of Wnt signaling in re-epithelialization of corneal wounds has not been studied. Therefore, we asked whether Wnt signals might play roles in corneal epithelial wound repair process, including proliferation and migration. We demonstrate here that, during corneal wound healing, Wnt-7a activates Rac GTPase and β-catenin and may control cell proliferation via the induction of MMP-12.

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‡ To whom correspondence should be addressed: Dept. of Ophthalmology and Visual Science, College of Medicine, Catholic University of Korea, 505 Banpo-dong, Seocho-ku, Seoul 137-040, Korea. Tel.: 822-590-2613; Fax: 822-533-3801; E-mail: cjjo@catholic.ac.kr.

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The abbreviations used are: TCF, T-cell factor; LEF, lymphoid enhancer factor; JNK, c-Jun N-terminal kinase; MMP, matrix metalloproteinase; HCE, human corneal epithelial; THCE, SV40-immortalized (transfected) human corneal epithelial; CM, conditioned medium; RT, reverse transcription; PIPES, 1,4-piperazinediethanesulfonic acid; AP-1, activator protein-1; sFRP-1, secreted Frizzled-related protein-1; PAK1, p21-activated kinase-1; DN, dominant-negative.
**EXPERIMENTAL PROCEDURES**

**Antibodies**—The polyclonal antibody against the N terminus of MMP-12 was from Chemicon International, Inc., and the monoclonal antibody against the C terminus of MMP-12 was from Sigma. The monoclonal antibodies against β-catenin and Rac were from Transduction Laboratories. The polyclonal antibodies against hemagglutinin (F-7), Dvl-2 (10B5), Dvl-3 (4D3), Myc (9E10), and phospho-c-Jun (KM-1) and the polyclonal antibody against c-Jun (H-79) were purchased from Santa Cruz Biotechnology, Inc.

**Cells and Cell Culture**—Corneoscleral rims taken from human donors provided the source of primary human corneal epithelial (HCE) cells. Each scleral rim, with the endothelial layer removed, was treated with Dispase II for 15 min, and epithelial cells were then isolated. SV40-immortalized (transfected) human corneal epithelial (THCE) cells were kindly provided by Kaoru Araki-Sasaki (Osaka University, School of Medicine, Osaka, Japan). For the assays used in this study, the cells were plated on a diluted Matrigel matrix (Roche Applied Science), which is similar to the basement membrane of the corneal epithelium, and incubated in serum-free medium for 24 h. To generate control medium or conditioned medium (CM) that contained Wnt-7a or a Wnt antagonist, plasmid pcDNA3-EGFP, pcDNA3-Wnt-7a-Myc, or pcDNA3-sFRP-1-Myc, respectively, was transfected into human embryonic kidney 293 cells.

**Rat Corneal Wound and Organ Culture**—The central regions of Sprague-Dawley rat corneas were demarcated with a 4-mm trephine, and epithelial cells within the circle were removed using a soft brush. For extraction of RNA, the central epithelium was first removed with a 2.5-mm trephine, and the peripheral epithelium was isolated. For organ culture, the wounded corneas were dissected from the eyes. The anterior chamber beneath the endothelium was filled with 1% agarose made up in minimal essential medium and 1 mg/ml rat tail collagen (Sigma). Minimal essential medium was then added to cover the periphery of the cornea. Fluorescein staining was used to monitor closure of the epithelial defect. The extent of healing was determined by the ratio of the difference between the original and the remaining wound areas after 48 h as described previously (16).

**RT-PCR and Real-time PCR**—Total RNA was isolated using TRIzol reagent (Invitrogen). 2 μg of total RNA was reverse-transcribed using the Superscript II kit (Invitrogen). PCR amplification was performed using appropriate primer pairs. Real-time PCR was carried out using SYBR Green 1 fluorescence (BD Biosciences). Glyceraldehyde-3-phosphate dehydrogenase transcript levels were used to normalize the samples. Each experiment was performed at least three times.

**Construction of Plasmids**—The MMP-12 promoter (−1830/+39) was amplified from human genomic DNA using primers 5'-TTTATCTATTGCAACTCT-3' (forward) and 5'-AAAGGACTTTACAACCAGAAT-3' (reverse). The PCR products were inserted into the pGL2-Basic vector (Promega). To generate the −1208/+39 (P2) and −631/+39 (P3) promoter plasmids, the pGL2/MMP-12 promoter (−1830/+39) plasmid was digested with restriction enzyme. Full-length human Wnt-7a was generated from human mRNA by RT-PCR. The cloned cDNA was inserted into the pcDNA1.1/His/Myc vector (Invitrogen) to tag the C terminus with Myc. To construct a retroviral plasmid expressing Wnt-7a, β-catenin, TCF4, ΔNTCF4, RacN17, RacG12, or enhanced green fluorescent protein, their fragments were restriction-digested and subcloned into pLNCXII, pQCXIH, or pQXCIP (BD Biosciences). The sequences of all plasmids were confirmed by sequencing.

**Retroviral Particle Production and Infection**—The GP2-293 cell line (BD Biosciences) used to generate the retroviral supernatant was co-transfected with a retroviral plasmid and the pVSV-G plasmid. For infection, 1.5 × 10⁶ THCE cells were mixed with supernatant containing the virions in the presence of 8 μg/ml Polybrene (Sigma). The transfected cells were subcultured and selected with either 4 μg/ml puromycin or 60 μg/ml hygromycin.

**Chromatin Immunoprecipitation Analysis**—Chromatin immunoprecipitation analysis was performed as described (17). Briefly, THCE cells were cross-linked with 1% formaldehyde for 20 min at room temperature, incubated with 125 μM glycine for 10 min, and washed with ice-cold phosphate-buffered saline. The cells were lysed in lysis buffer (5 mM PIPES (pH 8.0), 85 mM KCl, 0.5% Nonidet P-40, and protease inhibitors) for 30 min on ice and centrifuged at 5000 rpm, and nuclei were resuspended in nuclear lysis buffer (50 mM Tris (pH 8.0), 1% Triton X-100, 0.01% SDS, 1 mM EDTA, 150 mM NaCl, and protease inhibitors). The lysates were sonicated to yield 200–1000-bp DNA fragments. After centrifugation at 13,000 rpm, the lysates were diluted 1:5 in chromatin immunoprecipitation dilution buffer (15 mM Tris (pH 8.0), 1% Triton X-100, 0.01% SDS, 1 mM EDTA, 150 mM NaCl, and protease inhibitors), and 0.1 volume of the lysate was used for input control. Antibody to TCF4, β-catenin, or c-Jun or rabbit IgG was added to the precleared samples and incubated overnight at 4 °C with gentle agitation, followed by the addition of protein A/G-Sepharose beads for 1 h at 4 °C. The beads were washed, and immune complexes were dissociated from the beads by heating at 65 °C for 15 min in elution buffer (50 mM Tris (pH 8.0), 10 mM EDTA, and 1% SDS). Formaldehyde-linked complexes were dissociated overnight at 65 °C, and DNA was purified using a PCR purification kit (Qiagen Inc.). The sequences of the promoter-specific primers used to amplify the TCF/LEF-binding site were 5′-TACATAGATGACAGGTCTC-3′ (forward) and 5′-AGGCTACCTCCATTAAAGT-3′ (reverse). The sequences of the primers used to amplify the AP-1-binding site were 5′-GCTAATTGATCCATTGT-3′ (forward) and 5′-TCTAAGCTTAAGTCC-3′ (reverse). Purified DNA was amplified for 40 cycles at an annealing temperature of 58 °C.

**Western Blot Analysis**—The cells were lysed with radiolabeled immunoprecipitation assay buffer containing a protease inhibitor mixture (Roche Applied Science). The lysates were subjected to 10 or 12% SDS-PAGE and immunoblotted with the appropriate antibodies. Protein expression levels from infected cDNA plasmids were monitored by quantifying the antigen specific signals using an ImageMaster VDS system (Amersham Biosciences). When Western blot signals were weak, quantification was done using AlphaImager (Alpha Innotech Corporation).

**Proliferation Assays**—A single cell suspension containing 2 × 10⁵ cells was seeded in 96-well plates coated with diluted Matrigel, incubated for 24 h, and further incubated for the indicated conditions for 36 h. The level of cell growth was determined using the cell proliferation reagent WST-1 (Roche Applied Science). For the colony-forming efficiency assay, the epithelial cells were isolated from the peripheral region, including the limbal conjunctiva, and cultivated as described previously (18).

**Luciferase Assays**—HCE cells were transfected by Lipofectamine 2000 (Invitrogen) with the reporter plasmid and the internal control plasmid pRL-TK. Luciferase assays were performed 24 h after transfection using the Dual-Luciferase assay system (Promega).

**RESULTS**

**Expression of Wnt-7a in Corneal Epithelial Cells Increases Re-epithelialization**—To determine the expression of Wnt proteins and their receptors during wound healing, transcripts isolated from the central and peripheral regions of the epithelium were analyzed by RT-PCR. mRNAs encoding several Wnt proteins (Wnt-2, Wnt-4, and Wnt-5a) and Fz receptors (Fz-1, Fz-3, and Fz-4) were strongly expressed in the corneal epithelium, but their levels were not altered during wound healing. No PCR product was obtained with primers for Wnt-1. As positive controls, these products were amplified from embryonic brain mRNA (data not shown). The levels of Wnt-7a transcripts increased significantly in the central and peripheral regions of the epithelium of the wounded cornea (Fig. 1A), returning to a basal level by 3 days (data not shown).

We next tested the ability of Wnt-7a to promote wound healing (Fig. 1B). Injured corneas were cultured in control medium, Wnt-7a CM, sFRP-1 CM, or vehicle for 48 h. In control medium, 76% of the wound surface was covered. In wounded corneas cultured in Wnt-7a CM, the epithelial cells completely covered the wound. Incubation with sFRP-1 (a Wnt antagonist) (19) CM delayed epithelial wound closure (Fig. 1B). Similarly, scratch-wounded THCE and HCE cells cultured in Wnt-7a CM migrated more rapidly compared with control cells. The increased migration induced by Wnt-7a was inhibited by sFRP-1 CM (Fig. 1C).
Wnt-7a also increased the growth of primary HCE and THCE cells. As shown in Fig. 1D, the accumulation of primary HCE cells cultured in Wnt-7a CM increased significantly to ~3 times that of the control cells, and Wnt-7a-promoted growth was more than 3 times that of the control cells.}

**Fig. 1.** Wnt-7a responses in wounded epithelial cells of the cornea. A, gene expression was determined in the peripheral epithelium (PE) and in the central epithelium (CE) by RT-PCR and is represented as the level of Wnt-7a compared with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a control. uw, unwounded; w, wounded; r, rat. B, the extent of wound healing in cultured corneas was calculated. The wounded corneas were incubated for 48 h in control medium, Wnt-7a CM, or Wnt-7a CM combined with sFRP-1 CM and stained with fluorescein (0.25%). The healing rate in the presence of Wnt-7a CM increased relative to that in control medium and decreased in the presence of Wnt-7a CM combined with sFRP-1 CM. C, a wound was introduced into primary HCE multilayers (upper panels) and THCE monolayers (lower panels) with a micropipette tip. Completion of the scratch wound was determined in cultures incubated with control medium (M), Wnt-7a CM, or Wnt-7a CM combined with sFRP-1 CM and in cells transduced with virus expressing the control vector, Wnt-7a, or Wnt-7a and sFRP-1. D, primary HCE and THCE cells were grown under the indicated conditions for 36 h. The cells were seeded in triplicate in 96-well plates coated with diluted Matrigel at 2 x 10^4 cells/well and starved for 24 h. Cell growth was assayed using the cell proliferation reagent WST-1. The absorbance values of the samples are shown on the y axis. The error bars indicate the mean ± S.D. of triplicate tests.

**Fig. 2.** MMP gene expression in primary HCE cells. MMP mRNA levels were determined by real-time PCR. The levels in the HCE cells incubated with Wnt-7a CM (A) or 10 μM SB216763 (B), an inhibitor of glycogen synthase kinase-3β, are given as the -fold mRNA induction relative to the levels in the control cells incubated with control medium or Me2SO. The error bars represent the mean ± S.D. of five experiments.

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was decreased by sFRP-1. Similar results were also observed in THCE cells infected with retrovirus expressing the empty vector, Wnt-7a, or Wnt-7a and sFRP-1. These results suggest that Wnt-7a-induced proliferation can induce the wound closure of HCE cells.

**MMP-12 Expression and Wnt-7a Signaling in Corneal Epithelial Cells**—The expression levels of the various MMPs are higher in tumors that overexpress Wnt-7a (20) or that show elevated β-catenin protein levels (21). In addition, MMP-1, MMP-3, and MMP-7 have a putative TCF/LEF-binding site in their promoters (22). For this reason, we hypothesized that Wnt signaling might induce some of the MMPs that are involved in re-epithelialization during corneal wound healing (15, 23).

In accord with this hypothesis, the expression of the mRNAs for several MMPs increased in primary HCE cells after treatment with Wnt-7a (Fig. 2A). MMP mRNA levels were similarly increased in HCE cells treated SB216763 (Fig. 2B), a drug that inhibits glycogen synthase kinase-3β. This suggests that the transcriptional activation of MMPs is mediated by β-catenin (24).

Interestingly, MMP-12 (macrophage elastase) mRNA and protein increased in primary HCE cells stimulated by Wnt-7a (Fig. 2) and in the peripheral epithelium of wounded corneas (Fig. 3A). Incubation with Wnt-7a CM also induced the accumulation of cytosolic β-catenin (Fig. 3B), suggesting that MMP-12 expression might be regulated by the Wnt/β-catenin pathway. To test this possibility, we examined MMP-12 levels in THCE cells transduced with retrovirus expressing Wnt-7a, β-catenin, or TCF4. Cells that overexpressed β-catenin or that coexpressed β-catenin with TCF4 showed increased levels of MMP-12 mRNA and protein compared with cells that expressed the control vector (Fig. 3C). There was no significant difference between the levels of MMP-12 expression in cells transduced with β-catenin alone or with β-catenin and TCF4. Consistent with the increased MMP-12 mRNA levels, casein zymography assays showed that Wnt-7a increased the level of MMP-12 enzyme activity (data not shown).

Based on the sequence data available from the Human Genome Project and the published DNA sequences, we found that the MMP-12 promoter contains two putative TCF/LEF-binding sequences (Fig. 4A). A 1.8-kb fragment of the MMP-12 promoter was cloned by genomic PCR with HCE cell DNA. When THCE cells were transduced with the β-catenin construct, luciferase activity increased by 7–10-fold (Fig. 4B). The region containing the TCF-binding sites was required for this stimulation, and mutation of both TCF-binding sites blocked stimulation by β-catenin. Stimulation was also inhibited by cotransfecting a dominant-negative form of TCF4 (β-catenin 4NTCF4) (Fig. 4B). These results show that the Wnt-7a/β-catenin signaling pathway increases the transcription of MMP-12.

In THCE cells transfected with the luciferase reporter plasmid containing the MMP-12 promoter, Wnt-7a CM increased the levels of luciferase activity by 4.5-fold (Fig. 5A). We used chromatin immunoprecipitation to show that the region containing the TCF/LEF-binding sequences was required for this stimulation, and mutation of both TCF-binding sites blocked stimulation by β-catenin. Stimulation was also inhibited by cotransfecting a dominant-negative form of TCF4 (ΔNTCF4) (Fig. 5B). These results show that the Wnt-7a/β-catenin signaling pathway increases the transcription of MMP-12.

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reporter plasmid were incubated with control medium (M) or Wnt-7a CM, and the luciferase activity was determined. B, chromatin immunoprecipitation (IP) analysis with anti-TCF4 (α-Tcf) and anti-β-catenin (α-β-catenin) antibodies showed that only in nuclear extracts from primary HCE cells expressing Wnt-7a was a product amplified for the TCF/LEF-binding site, indicating that the TCF/β-catenin complex is recruited to the MMP-12 promoter in response to Wnt-7a. Input DNA was used as a positive control, and rabbit IgG was used as a negative control for antibodies. A schematic representation of the TCF/LEF-binding sites in the MMP-12 promoter is shown with the primer sites used for PCR (arrows). C, THCE cells were transfected with the MMP-12 promoter-reporter plasmid containing the TCF/LEF sites (P0) or with these sites removed (P2 and P3) and further incubated with control medium or Wnt-7a CM. D, THCE cells expressing the empty vector or ΔNTCF4 were incubated with MeSO (DMSO), SB216763, control medium, or Wnt-7a CM. The levels of MMP-12 mRNA were determined by real-time PCR. Note that the transcription of MMP-12 activated by Wnt-7a was independent of the TCF/LEF-binding sites and β-catenin. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

5C). In addition, coexpression of a dominant-negative form of TCF4 did not block this stimulation (Fig. 5D). In contrast, the increased expression of the MMP-12 promoter caused by treatment with SB216763 was blocked by ΔNTCF4. These results suggest that Wnt-7a induces MMP-12 expression via β-catenin-dependent and β-catenin-independent pathways.

To identify how Wnt-7a activates MMP-12 expression using a β-catenin-independent pathway, we investigated the function of the small GTPase Rac and the transcription factor c-Jun in THCE cells infected with retrovirus expressing Wnt-7a. As shown by its ability to bind to PAK1, Rac was activated in THCE cells expressing Wnt-7a (Fig. 6A). Immunoprecipitation of the endogenous Rac protein showed that Wnt-7a promoted the association of Rac with the Dvl-2 protein. In contrast, no increased association between Rac and Dvl-3 was detected (Fig. 6B). c-Jun was also more active and more highly phosphorylated in THCE cells expressing Wnt-7a than in control cells (Fig. 7A). The activation and phosphorylation of c-Jun were inhibited by transduction of a dominant-negative form of Rac (DN RacN17). Because c-Jun binds to AP-1 sites (13, 25), we tested whether the transcriptional activation of MMP-12 is associated with increased binding of c-Jun to the AP-1 site in the MMP-12 promoter. THCE cells expressing Wnt-7a showed strong binding of c-Jun to the region of the MMP-12 promoter containing the AP-1 site. This binding was strongly inhibited by coexpression of DN RacN17 (Fig. 7B). To further assess the effect of Wnt-7a on AP-1 binding, AP-1 activity was determined using an AP-1-reporter plasmid. Wnt-7a or a constitutively active form of Rac (RacG12) significantly increased AP-1 activity. Conversely, DN RacN17 inhibited the ability of Wnt-7a to increase the activation of AP-1 (Fig. 7C). As expected, a dominant-negative form of TCF4 had no effect on AP-1 activity stimulated by Wnt-7a.

We then investigated the transcriptional activation of the MMP-12, MMP-1, and P0 promoter (a synthetic 1.8-kb fragment of the MMP-12 promoter) in cells that expressed Wnt-7a. All three promoter constructs contain TCF- and AP-1-binding sites (22, 26). Real-time PCR analysis revealed that either DN RacN17 or ΔNTCF4 reduced the transcriptional activity of the promoters. Inhibition was even greater when these constructs were coexpressed (Fig. 8, A and B). Taken together, these
Determined. The error bars on RacN17, active RacG12, or plasmid was cotransfected with the empty vector or Wnt-7a, DN to PCR. Input DNA was used as a positive control. Lysates with rabbit IgG or anti-c-Jun antibody, the DNA was subjected were fixed and lysed. After immunoprecipitation (IP) of the cross-linked lysates with rabbit IgG or anti-c-Jun antibody, the DNA was subjected to PCR. Input DNA was used as a positive control. C, the AP-1-reporter plasmid was cotransfected with the empty vector or Wnt-7a, DN RacN17, active RacG12, or ΔNTCF4, and the luciferase activity was determined. The error bars represent the mean ± S.D. of five experiments.

Results indicate that either Rac activation or β-catenin accumulation can induce MMP-12 expression, but full induction of MMP-12 transcription by Wnt-7a requires both Rac and β-catenin.

MMP-12 Is Required for Wnt-7a-mediated Cell Proliferation—Because the expression of Wnt-7a induces wound closure and MMP-12 expression, we tested the importance of MMP-12 expression in the behavior of THCE cells. THCE cells were infected with retrovirus expressing Wnt-7a-Myc or the control vector, and stable transfectants were isolated using hygromycin resistance. When monolayers of THCE cells overexpressing Wnt-7a were wounded artificially, cells incubated with a function-blocking antibody to MMP-12 (27) were delayed in wound closure compared with cells incubated with IgG (Fig. 9A). Part of the effect on wound closure might be related to cell proliferation because Fig. 9B shows that antibody to MMP-12 antagonized the Wnt-7a-induced growth of HCE cells. Coexpression of ΔNTCF4 and DN RacN17 also inhibited the ability of Wnt-7a-expressing cells to close a scratch wound (Fig. 9C). Similarly, THCE cells expressing either DN RacN17 or ΔNTCF4 showed decreased Wnt-7a-promoted proliferation, which decreased further when the two constructs were co-infected (Fig. 9D).

We next used colony-forming efficiency assays to evaluate the proliferation of HCE cells isolated from the peripheral region of human corneas. Epithelial cells were co-cultured with 3T3 feeder cells expressing the control vector, Wnt-7a, or Wnt-7a and sFRP-1 in the presence of IgG or neutralizing antibody to MMP-12. Proliferation was evaluated by colony size and colony-forming efficiency (Fig. 9, E and F) (28). The number of cells/colony was measured by counting 20 randomly selected colonies for each treatment, and the total number of colonies was counted. Wnt-7a CM increased the colony size by 8.1-fold (Fig. 9E) and the colony-forming efficiency by 2.5-fold compared with control medium (Fig. 9F). As expected, sFRP-1 antagonized the effects of Wnt-7a. Treatment of Wnt-7a-stimulated cells with antibody to MMP-12 also decreased the size and number of colonies compared with cells cultured with IgG (~52 and 33% reduction, respectively). Taken together, these results demonstrate that the increase in MMP-12 expression stimulated by Wnt-7a increases the migration and proliferation of corneal epithelial cells.

**DISCUSSION**

We have shown that both Wnt-7a and MMP-12 are expressed in the corneal epithelium during wound healing and that MMP-12 expression is significantly increased in Wnt-7a-expressing THCE cells. Reporter assays showed that the MMP-12 promoter is regulated by β-catenin-dependent and β-catenin-dependent and...
The β-catenin-independent pathway depends on the activation of Rac and c-Jun. Previous studies have shown that the Wnt-7a pathway signals through the canonical β-catenin pathway (29) and via a β-catenin-independent pathway in some tissues (30). Recent studies have shown that Wnt-1 and Fz-1, which activate β-catenin signaling, also activate Rac (7, 11). In this case, Wnt signals activate JNK through a Dvl-Rac complex. Consistent with this result, we have shown that Wnt-7a induced the formation of a complex between Dvl-2 and Rac and that Rac increased c-Jun activity. Interestingly, Wnt-7a activated an AP-1-reporter plasmid through Rac. It is known that AP-1 plays an important role in regulating MMP-12 expression. For example, phorbol 12-myristate 13-acetate and insulin, which are known activators of AP-1, promote MMP-12 expression (31). Furthermore, a reporter plasmid with a mutation in the AP-1 site has reduced ability to activate MMP-12 transcription (32). Therefore, these results imply that Wnt-7a-mediated Rac activation might induce nuclear binding to the AP-1 site by c-Jun, thereby increasing the transcriptional activation of MMP-12 (33). We have also shown that DN RacN19 inhibited the transcriptional activity of MMP-12 and that ΔNTCF4 enhanced the response to DN RacN19. These results suggest that both c-Jun and β-catenin contribute to the Wnt-7a-induced transcription of MMP-12. Similarly, binding of β-catenin and AP-1 increased the activity of the MMP-1 promoter as described previously (26, 34). In addition, a recent study reported that the β-catenin cofactor LEF-1 acts synergistically with c-Jun through the AP-1 motif via the formation of a physical complex, suggesting a role for the coordinated function of the β-catenin and AP-1 pathways in the transcription of several MMP genes (35).

A key finding of this study is that Wnt-7a and MMP-12 can control the proliferation of corneal epithelial cells. Wnt-7a controls proliferation through temporal expression (36). Rac activity is also important for cell growth, motility, and adhesion and cytoskeletal rearrangement. For example, dominant-negative Rac overexpression suppresses the growth of NIH 3T3 and PC3 cells (37). Although we cannot rule out the possibility that Wnt-7a signaling has additional targets, it is clear that Wnt-7a promotes the growth of corneal epithelial cells through β-catenin and the Rac pathway. MMP-12 has broad substrate specificity for extracellular matrix components such as elastin, fibronec tin, laminin, vitronectin, and collagen type IV. Indeed, this enzyme was recently reported to play a role in cell motility and fate, including macrophage infiltration after emphysema (38) and the morphological differentiation of oligodendrocytes (27). Although MMP-12 is expressed after injury in corneal tissue (28), no study has yet addressed the functional importance of MMP-12 in corneal wound healing. In this study, blocking MMP-12 enzyme activity suppressed the increase in proliferation that was induced by Wnt-7a. Therefore, the effect of Wnt-7a on HCE cell proliferation requires MMP-12. Interestingly, the expression profile of MMP-12 during wound healing is consistent with the role of MMP-12 in the growth-promoting effects of Wnt-7a. MMP-12 expression was constant in the peripheral epithelium during wound healing, but was diminished in the central epithelium, showing a consistent expression pattern with the region of proliferation in the corneal epithelium in vivo (3, 4). Overall, these observations raise the
possibility that MMP-12 may function in vivo to mediate Wnt-7a-induced proliferation to cover the defect area during corneal wound healing. These results also suggest the presence of a mechanism counteracting the Wnt-7a-induced effect on cell proliferation in the central epithelium. We speculate that this mechanism might be involved in the regulation of MMP-12 transcription.

Transforming growth factor-β1 suppresses the proliferation of the epithelial cell types through the induction of p15 and p21 (39, 40). It seems possible that transforming growth factor-β may interfere with MMP-12 expression through a Smad-dependent pathway (41). However, we did not observe a reduction of MMP-12 expression in corneal epithelial cells stimulated by transforming growth factor-β1 (data not shown). Therefore, it is likely that the suppression of cell proliferation through the de-regulation of MMP-12 expression is mediated by signals other than transforming growth factor-β1. Further experiments will be required to clarify the molecular mechanisms by which MMP-12 expression is regulated in the central epithelium.

In conclusion, this study has demonstrated the importance of Wnt-7a signaling in the behavior of HCE cells during wound healing. Wnt-7a is expressed in the periphery of the wounded epithelium, where it contributes to the proliferation of HCE cells, at least partly through its effects on MMP-12 expression. Wnt-7a signaling appears to play a role in compartmentalized processes in the peripheral and central regions of the corneal epithelium by its effects on the regulation of MMP-12 expression. These findings identify the Wnt pathway as a potential target for therapy when corneal epithelial wounds are difficult to repair.

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