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 is rapidly induced in the wounded cornea and that Wnt 7a promotes the proliferation of corneal epithelial cells and enhances the wound closure. Matrix metalloproteinase (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 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, while 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 epithermal 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, which control cellular proliferation, motility, differentiation, and morphology by pathways that are termed “canonical” or “non-canonical” (6, 7). Binding of Wnts 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 (GSK)-3β. Stabilized β-catenin binds and activates the TCF/LEF transcription factors, stimulating the transcription of the target genes (8). The non-canonical pathway primarily affects cell shape and movement. Wnt 5a and Wnt 11 regulate convergent extension (CE) movements during vertebrate gastrulation (9) by activating Rho and Rac GTPases (10). The Rho and Rac pathway 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 the PC12 and N1E-115 cells (13).

A number of Wnt target genes have been identified, including c-myc, cyclin D1, MMP-7, and MT1-MMP. These target genes have important implications in understanding the role of Wnts 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 the components of the extracellular matrix (ECM), 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, since 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.

**EXPERIMENTAL PROCEDURES**

*Antibodies—* The polyclonal MMP-12 (N-terminus) antibody was from Chemicon and the monoclonal MMP-12 antibody (C-terminus) was from Sigma. The monoclonal antibodies against β-catenin and Rac were...
from Transduction Laboratories. The monoclonal antibodies against HA (F-7), Dvl2 (10B5), Dvl3 (4D3), Myc (9E10) and P-c-jun (KM-1) and the polyclonal antibody against c-jun (H-79) was purchased from Santa Cruz Biotechnology.

**Cells and cell culture**—Corneoscleral rims taken from human donors provided a 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 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 Molecular Biochemicals), which is similar to the basement membrane of corneal epithelium, and incubated in serum-free for 24 hrs. To generate conditioned medium that contained control, Wnt7a or a Wnt antagonist, pcDNA3-EGFP, pcDNA3-Wnt 7a-myc, or pcDNA3-sFRP-1-myc plasmids were transfected into HEK-293 cells.

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

**RT-PCR and Real-Time PCR**—Total RNA was isolated using TRIzol Reagent (Invitrogen). 2μg of total RNA was reverse transcribed using the Superscript II kit (Invitrogen). PCR amplification was performed using appropriate primer pairs. The primer sequences corresponding to each mRNA are available on request. Real-Time PCR was carried out using SYBR green I fluorescence (BD Biosciences). GAPDH 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 the following primers: 5’-GTAAAGTTCAGTTTCCCTC-3’ and 5’-TAAACATTCTAACCCTGATCAAT-3’. The PCR products were inserted into the pGL2 basic-vector (Promega). In order to generate 1208/+39 (P2) and 631/+39 (P3) promoter plasmid, the pGL2/MMP-12 promoter (-1830+39) 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
pCDNA 3.1-His/Myc vector (Invitrogen) to Myc tag the C-terminal. For the construction of the retroviral plasmid expressing Wnt 7a, β-catenin, TCF4, ΔNTCF4, Rac17, RacG12, or EGFP, their fragments were restriction digested and subcloned into either pLNCX II, pQCXIHI (BD Biosciences), or pQXCIP (BD Biosciences). The sequences of all the plasmids were confirmed by sequencing.

**Retroviral particle production and infection**—The GP2-293 cell line (BD Biosciences) used to generate the retroviral supernatant was cotransfected with retroviral plasmid and the pVSV-G plasmid. For infection, 1.5 × 10^5 HCET cells were mixed with supernatant containing the virions in the presence of 8 μg/ml Polybrene (Sigma). The transduced cells were subcultured and selected with either 4 μg/ml puromycin or 60 μg/ml hygromycin.

**Chromatin immunoprecipitation analysis**—ChIP analysis was performed as described elsewhere (17). Briefly, THCE cells were cross-linked with 1% formaldehyde for 20 min at room temperature, incubated with 125 mM 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, protease inhibitors) for 30 min on ice, centrifuged (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, protease inhibitors). The lysates were sonicated to yield 200-1000-bp DNA fragments. After centrifugation (13,000 rpm), the lysates were diluted 1:5 in ChIP dilution buffer (15 mM Tris, pH 8.0, 1% Triton X-100, 0.01% SDS, 1 mM EDTA, 150 mM NaCl, protease inhibitors), and 0.1 volume of the lysate was used for input control. Antibodies to TCF4, β-catenin, c-jun, or rabbit IgG were added to the pre-cleared samples and incubated overnight at 4 °C with gentle agitation followed by the addition of the protein A G-Sepharose beads for 1h 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, 1% SDS). Formaldehyde-linked complexes were dissociated at 65 °C overnight and DNA was purified using a PCR purification kit (Qiagen). The sequences of the promoter-specific primer for amplifying the TCF/LEF binding site were: forward, 5'-TACAGAGGTTTCTC-3'; reverse, 5'-AGGCTACTCCATAAGATGT-3'. The primer sequences for amplifying the AP-1 binding site were: forward, 5'-GCTAACTCCATGATGT-3'; reverse, 5'-CTAGCCCTAGTCC-3'. Purified DNA was amplified for 40 cycles with an annealing temperature of 58 °C.

**Western blot analysis**—The cells were lysed with a RIPA buffer containing a protease inhibitor mixture (Roche Molecular Biochemicals). The lysates were subjected to electrophoresis in 10% or 12% SDS-PAGE and immunoblotted with the appropriate antibodies. Protein expression levels from infected cDNA plasmids were monitored by quantifying the anti-myc or anti-HA tags. To
detect cytoplasmic β-catenin, cells were lysed using a chilled Potter-Elvehjem homogenizer (Wheaton) in buffer containing 10mMTris-HCl (pH7.4), 140mM NaCl, 5mM EDTA, 2mM DTT, 0.5mM PMSF, 2ug/ml aprotinin, 1ug/ml leupeptin. Cleared lysates were subjected to ultracentrifugation at 100,000 X g for 90 min at 4 ℃. Supernatants (cytoplasmic fraction) were collected and resolved by 10% SDS-PAGE, blotted and probed with anti-β-catenin.

Rac activity assays— The cells were incubated in serum-free medium for 24hr, then lysed in lysis buffer. Rac activity was measured using a Rac Activation Kit (Pierce), according to the manufacturer’s recommendations.

Proliferation assays— A single cell suspension containing $2 \times 10^2$ cells was seeded in 96-well plates coated with diluted Matrigel, incubated in serum-free medium for 24 hrs, and further incubated at the indicated conditions for 36 hrs. The level of cell growth was determined using Cell Proliferation Reagent WST-1 (Roche Molecular Biochemicals). For the colony-forming efficiency assay, the epithelial cells were isolated from the peripheral region including the limbal conjunctiva, and cultivated using a method described elsewhere (18).

Luciferase assay— HCET cells were transfected by Lipofectamine 2000 (Life Technologies) with the reporter plasmid and the internal control pRL-TK. Luciferase assays were performed 24 hours after transfection using a dual luciferase assay system (Promega).

RESULTS

Expression of Wnt 7a in corneal epithelial cells increases re-epithelialization— In order to determine the expression of Wnts and their receptors during wound healing, transcripts isolated from the central and the peripheral regions of the epithelium were analyzed by RT-PCR. mRNAs encoding several Wnts (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). Levels of Wnt 7a transcripts increased significantly in the central and peripheral epithelium of the wounded cornea (Fig. 1A), returning to a basal level by 3 days (data not shown).

We then tested the ability of Wnt 7a to promote wound healing (Fig. 1B). Injured corneas were cultured with control medium, Wnt 7a CM, sFRP-1 CM, or vehicle for 48 hr. In control medium, 76% of the wound surface was covered. In wounded corneas cultured with Wnt 7a CM, the epithelial cells completely covered the wound. Treatment with sFRP-1 CM, a Wnt antagonist (19), delayed epithelial wound closure (Fig. 1B). Similarly, scratch wounded THCE and HCE cells cultured in the presence of Wnt 7a CM migrated more rapidly than 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, cell accumulation increased significantly to approximately 3 times that of the control cells in primary HCE cells treated with Wnt 7a CM, and Wnt 7a-promoting growth was decreased by sFRP-1. Similar results in the THCE cells infected with the retrovirus expressing empty vectors, or expressing Wnt 7a, or Wnt 7a and sFRP-1 were also observed. 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 over express Wnt 7a (20), or show elevated β-catenin protein levels (21). In addition, MMP-1, -3, and 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 GSK-3β. This suggests that 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). Treatment 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 retroviruses expressing Wnt 7a, β-catenin, or TCF4. Cells that over expressed β-catenin or co-expressed 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 not 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 using genomic PCR of HCE DNA. When THCE cells were transduced with the β-catenin construct, luciferase activity increased 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 co-transfecting a dominant-negative form of TCF4 (ΔNTCF4) (Fig. 4B). These results show that the Wnt 7α/β-catenin signal pathway increases the transcription of MMP-12.

In THCE cells transfected with the luciferase reporter plasmid containing the MMP-12 promoter, Wnt 7α CM increased the levels of luciferase activity 4.5 fold (Fig. 5A).

We then used ChIP to show that the region containing the TCF/LEF binding sites in the MMP-12 promoter is bound to the TCF/β-catenin complex in THCE cells that expressed Wnt7α (Fig. 5B). Although the portion of the MMP-12 promoter that lacked TCF binding sites (constructs P2 or P3) were not stimulated by β-catenin (Fig. 4B), these constructs were expressed at higher levels in cells exposed to Wnt 7α CM (Fig. 5C). In addition, co-expression 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 7α induces MMP-12 expression via β-catenin-dependent and β-catenin-independent pathways.

To identify how Wnt 7α 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 7α, Rac. As shown by its ability to bind to PAK1, Rac was activated in THCE cells expressing Wnt 7α (Fig. 6A).

Immunoprecipitation of the endogenous Rac protein showed that Wnt 7α 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 7α than in controls (Fig. 7A). The activation and phosphorylation of c-jun were inhibited by transduction of a dominant-negative form of Rac (DN RacN17). Since c-jun binds to AP1 sites, we tested whether the transcriptional activation of MMP-12 was associated with increased binding of c-jun to the AP-1 site in the MMP-12 promoter (13, 25). THCE cells expression Wnt 7α 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 co-expression of DN RacN17 (Fig. 7B). To further assess the effect of Wnt 7α on AP-1 binding, AP-1 activity was determined using an AP-1-reporter plasmid. Wnt 7α or a constitutively active form of Rac (RacG12) significantly increased AP-1 activity. Conversely, DN RacN17 inhibited the ability of Wnt 7α to increase the activation of AP-1 (Fig. 7C). As expected, a dominant-negative form of TCF4 had no effect on the AP-1 activity stimulated by Wnt 7α.

We then investigated the transcriptional activation of the MMP-12 (full length and P0) and MMP-1 promoters in cells that expressed Wnt 7α. All three promoter constructs contained TCF and AP1 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 co-expressed (Fig. 8A, 8B). Taken together, these results indicate that either Rac activation or the accumulation of β-catenin 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 the Wnt 7a-mediated cell proliferation—Since expression of Wnt 7a induces wound closure and MMP-12 expression, we tested the importance of MMP-12 expression on the behavior of THCE cells. THCE cells were infected with a retrovirus expressing Wnt 7a-myc or control vector, and stable transfectants were isolated using a hygromycin resistance. When monolayers of THCE cells over expressing Wnt 7a were wounded artificially, cells incubated with a function-blocking antibody to MMP-12 (27) were delayed in wound closure compared to cells incubated with IgG (Fig. 9A). Part of the effect on wound closure might be related to cell proliferation, since Figure 9B shows that the antibody to MMP-12 antagonized the Wnt 7a-induced growth of HCE cells. Co-expression 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 then used colony-forming 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 control vector, Wnt 7a, or Wnt 7a and sFRP-1 in the presence of IgG or a neutralizing antibody to MMP-12. Proliferation was evaluated by colony size and colony-forming efficiency (28, Fig. 9E, F). The number of cells per 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 to control cells not expressing Wnt 7a (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 to cells cultured with IgG (approximately 52% and 33% reduction, respectively). Taken together, these results demonstrate that the increase in MMP-12 expression that is 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 MMP-12 promoter is regulated by β-catenin and β-catenin-independent...
pathways. The β-catenin-independent pathway depended on the activation of Rac activation and c-jun. Previous studies have shown that Wnt 7a pathway signals through the canonical β-catenin pathway (29) and via a β-catenin-independent pathway in some tissue (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 showed 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, PMA 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 had 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 also showed that DN RacN19 inhibited the transcriptional activity of MMP-12, and 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 co-factor, 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, cell motility, cytoskeletal rearrangement, and cell adhesion. For example, DN Rac overexpression suppressed the growth of NIH 3T3 and PC-3 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, fibronectin, laminin, vitronectin, collagen type IV, and collagen type IV. Indeed, this enzyme was recently reported to play a role in the cell motility and cell fate, including macrophage infiltration after emphysema (38) and the morphological differentiation of oligodendrocytes (27). Although MMP-12 is expressed after injury in corneal tissue (23), 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 constantly in the peripheral epithelium during wound healing, but 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 the 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 involve in the regulation of MMP-12 transcription.

TGF-β1 suppresses the proliferation of the epithelial cell types through the induction of p15 and p21 (39, 40). It seems possible that TGF-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 TGF-β1 (data not shown). Therefore, it is likely that the suppression of cell proliferation through the deregulation of MMP-12 expression is mediated by signals other than TGF-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 demonstrates 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 corneal epithelia, 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.

**REFERENCES**


**FOOTNOTES**
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**FIGURE LEGENDS**
Fig. 1. **Wnt 7a responses in the wounded epithelial cells of the cornea.** *A*, Gene expression was determined in the peripheral epithelium (PE) and central epithelium (CE) by RT-PCR, represented as the level of Wnt 7a compared to GAPDH as a control. *B*, The extent of wound healing in cultured cornea was calculated. The wounded corneas were incubated for 48 hrs in control medium, Wnt 7a CM, or Wn7a 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 Wn7a CM combined with sFRP-1. *C*, A wound was introduced into primary HCE multilayers (top) and THCE monolayers (bottom) with a micropipette tip. Closure of the scratch wound was determined in the cultures incubated with control medium, Wnt CM, or Wn7a CM combined with sFRP-1, and in cells transduced with viruses expressing control vector, Wnt 7a, or Wnt 7a and sFRP-1. *D*, Primary HCE cells and THCE cells were grown at the indicated condition for 36 hrs. The cells were seeded in triplicate in 96-well coated with diluted Matrigel at $2 \times 10^2$ cells per well, and starved for 24 hrs. Cell growth was assayed using a Cell Proliferation Reagent WST-1. The absorbance values of the samples are shown on the Y-axis. The error bars indicate the mean ± SD of triplicate tests.

Fig. 2. **MMP genes expression in the 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 GSK-3β are given as the fold mRNA induction relative to the levels in the control cells incubated with control medium or DMSO. The error bars represent the mean ± SD of five experiments.

Fig. 3. **MMP-12 gene expression in the THCE cells and rat corneal epithelium.** *A*, At 24 hrs after injury, the peripheral epithelium (PE) and central epithelium (CE) were isolated from rat corneas. The expression of MMP transcripts was determined by RT-PCR. Analyses of at least three different RNA preparations from the same tissues provided similar results. *B*, In the primary HCE cells incubated with control medium, Wnt 7a CM, or Wn7a CM combined with sFRP-1 CM, MMP-12 expression was determined by RT-PCR (*top*) and western blotting (*bottom*), and the accumulation of β-catenin was determined in the cytoplasmic fraction (*bottom*). *C*, THCE cells were infected with retrovirus expressing the control vector, Wnt 7a-myc, or HA-β-catenin in the presence or absence of retrovirus expressing Myc-TCF4. Cell lysates were subjected to western blot analysis and RT-PCR.

Fig. 4. **The human MMP-12 promoter sequence contains potential TCF/LEF binding sites.** *A*, Schematic presentation of TCF/LEF binding sites in the human MMP-12 promoter. Two TCF/LEF binding sites (underlined and designated T1 and T2) were identified. The synthetic 1.8-
Kb MMP-12 promoter constructs contain different TCF/LEF sites. The mutations in site T1 and T2 are shown in site T1m and T2m (underline) and are indicated by X. The TCF/LEF sites are indicated by O. B, The activities were determined in THCE cells co-transfected with β-catenin, or with the dominant negative TCF4 (ΔNTCF4). Each error bar represents the mean ± SD of five independent experiments; each assay was performed in duplicate.

Fig. 5. Wnt 7a and β-catenin induce the transcriptional activation of MMP-12. A, THCE cells transfected with an MMP-12 promoter reporter plasmid were incubated with control medium or Wnt 7a CM, and the luciferase activity was determined. B, ChIP analysis with anti-TCF4 and anti-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 the negative control for antibodies. A schematic representation of the TCF/LEF binding sites of the MMP-12 promoter is shown with the primer sites used for PCR (arrows). C, THCE cells were transfected with an MMP-12 promoter reporter plasmid containing the TCF/LEF sites (P0) or with these sites removed (P2, P3), and further incubated with control medium or Wnt 7a CM. D, THCE cells expressing empty vector or ΔNTCF4 were incubated with DMSO, SB216763, control medium, or Wnt 7a CM. Levels of MMP-12 mRNA were determined by real-time PCR. Note that the transcription of MMP-12 activated by Wnt 7a is independent of the TCF/LEF binding sites and of β-catenin.

Fig. 6. Wnt 7a activates Rac. A, The activity of Rac 1 in THCE cells infected with retrovirus expressing empty vector or Wnt 7a was detected by a GST-PAK1 pull-down assay. Cell lysates were incubated with the agarose-immobilized GST-PAK1 and the co-precipitates were subjected to western blot analysis with anti-Rac1 to reveal the amount of GTP-bound Rac 1 protein. B, The formation of a complex between Dvl-2 and Rac but not of the Dvl-3-Rac complex. Endogenous Rac was immunoprecipitated from the cells expressing empty vector or Wnt 7a, and the immune complexes subjected to western blot analysis.

Fig. 7. Wnt 7a stimulates AP-1 activity through Rac activation. A, In THCE cells infected with retrovirus expressing empty vector, Wnt 7a, or DN RacN17, c-jun activation was determined by a c-jun trans-reporting system (left) and by western blot analysis with phospho-c-jun antibody (right). B, THCE cells expressing control vector, Wnt 7a, or Wnt 7a and DN RacN17 were fixed and lysed. After immunoprecipitation of the cross-linked lysates with rabbit IgG or c-jun antibody, the DNA was subjected to PCR. Input DNA was used as a positive control. C, AP-1 reporter plasmid was cotransfected with empty vector or Wnt 7a and with DN RacN17, active RacG12, or
ΔNTCF4, and the luciferase activity determined. The error bars represent the mean ± SD of five experiments.

Fig. 8. Activation of Rac by Wnt 7a induces MMP-12 expression. A, P0 reporter plasmid was cotransfected with Wnt 7a and with DN RacN17. The luciferase activity was significantly reduced. However, when the HCET cells were cotransfected with ΔNTCF4, the reduction in activity was weak. B, THCE cells expressing empty vector, ΔNTCF4, or RacN17 were infected with retrovirus expressing empty vector or Wnt 7a, and the transcriptional activation of MMP-12 was determined by real-time PCR analysis. Note the significant effect of RacN17 and the weak capacity of ΔNTCF4 to inhibit the expression of MMP-12 induced by Wnt 7a, and the significant effect of ΔNTCF4 or DN RacN17 on MMP-1 expression. GAPDH transcript levels were used for normalization. Protein expression levels from the infected plasmids were monitored by anti-myc. The error bars represent the mean ± SD of six experiments.

Fig. 9. MMP-12 is required for the proliferation of corneal epithelial cells. A, THCE cells were infected with retrovirus expressing Wnt 7a or control retrovirus. Confluent cell monolayers in serum-free medium were wounded with a micropipette tip. After wounding, 10 μg/ml MMP-12 antibody or rabbit IgG was added, and wound closure was monitored after 24 hrs. B, THCE cells were grown in the indicated condition, and cell growth was determined by using Cell Proliferation Reagent WST-1 reagents. The error bars represent the mean ± S.D. of five experiments. C, THCE cells expressing control vector or ΔNTCF4 and DN RacN17 were wounded, and incubated in the presence of control medium or Wnt 7a CM. D, cell growth was determined by using Cell Proliferation Reagent WST-1 reagents under the indicated conditions. The error bars represent the mean ± SD of three experiments. E-F, Cell growth was determined by a colony-forming efficiency assay. Suspended single cells (2×10^3/cm^2) were plated on a 3T3 feeder cells layer expressing control vector, Wnt 7a, or Wnt 7a and sFRP-1, and cultured in the presence of IgG or a neutralizing antibody to MMP-12 for 6 days. The number of cells per colony (E) and the number of colonies (F) were counted. The error bars represent the mean ± SD of three experiments.
Fig. 1

A

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rWnt 2
rWnt 4
rWnt 5a
rWnt 7a
rFrizzled 1
rFrizzled 3
rFrizzled 4
rGAPDH

B

Extent of healing (%)

C

Control M
Wnt7a CM
Wnt7a + sFRP-1 CM

vector
Wnt7a-myc
Wnt7a-myc+sFRP-1-myc

D

THCE
HCE

OD 450
Fig. 2

A

Wnt7a CM

B

SB216763
Fig. 3

A

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rMMP-1
rMMP-7
rMMP-12
rGAPDH

B

Control M
Wnt CM (5X)
Wnt CM (10X)
Wnt + TFP CM

RT-PCR

MMP-12
GAPDH

WB

proMMP-12
cytosolic β-catenin
actin

C

+ - - -
- + + +
- - + +
- - - +
vector
Wnt7a-myc
HA-β-catenin
Myc-TCF4

WB

Myc
HA
Myc
cytosolic β-catenin
proMMP-12
actin

RT-PCR

MMP-12
GAPDH
Fig. 4

Core Tcf/LEF binding sites in human MMP-12 promoter region (-1830 +39)

-1669
AGAGGCTTTGTAAGCAC: T1

-1549
TTTGCCTTTGATGACCA: T2

-1830
+39
P0
P1
P2
P3
-1502
-1208
-651

GCTTTGCGG:T1m

CCCTTTGCGG:T2m

T1m
T2m
T1.T2m

B

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Fold induction
Fig. 5

A

B

C

D

promoter
(-1300 - +39)

fold induction

Control M  Wnt7a CM

fold induction

Control M  Wnt7a CM

fold induction

Control M  Wnt7a CM

fold induction

Input  

α-Tcf  

α-β-catenin  

IgG

-1711

-1469

vector Wnt7a

T1

T2

-1711/-1469

MMP-12/GAPDH

fold induction

MycΔNTCF4

DMSO  

ES2163  

Control M  

Wnt7a CM

Downloaded from http://www.jbc.org/ by guest on September 1, 2017
Fig. 6
Fig. 8

A  

P0 promoter

fold induction

Wnt7a-myc = + + + +
Myc-ΔNTCF4 = - + - +
Myc-RacNI7 = - - + +

B  

MMP-12 / GAPDH

fold induction

MMP-1 / GAPDH

fold induction

Wnt7a-myc = + + + +
Myc-ΔNTCF4 = - + - +
Myc-RacNI7 = - - + +
α-Myc
Fig. 9

A

B

C

D

E

F

- vector

- IgG

- \( \alpha \)-MMP-12

- Wnt7a-myc

- Wnt7a CM

- Myc-ANTCF4

- Myc-RacN17

- vector

- ANTCF4+RacN17

- Wnt7a CM

- Wnt7a CM

- Myc-ANTCF4

- Myc-RacN17

relative colony size

relative C.F.E.
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

J. Biol. Chem. published online March 31, 2005

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