Stem Cell Research (2012) 9, 9–23

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PI3-K/Akt/JNK/NF-κB is essential for MMP-9 expression and outgrowth in human limbal epithelial cells on intact amniotic membrane

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Received 2 June 2011; received in revised form 29 February 2012; accepted 29 February 2012
Available online 8 March 2012

Abstract Matrix metalloproteinase-9 (MMP-9) plays an important role in the outgrowth of expanded human limbal epithelial cells on intact amniotic membranes (AM). The mechanisms of MMP-9 expression and cell outgrowth remain unknown. Here, we demonstrated that MMP-9 is preferentially expressed at the leading edge of limbal epithelial outgrowth. Treatment with the inhibitors of PI3-K (LY294002), Akt (SH-5), MEK1/2 (U0126), and JNK1/2 (SP600125) attenuated the outgrowth area, indicating that PI3-K/Akt, p42/p44 MAPK, and JNK1/2 are involved in the outgrowth of intact AM-expanded limbal epithelial cells. However, MMP-9 expression at both transcriptional and translational levels was attenuated by treatment with SP600125, LY294002, or SH-5, not by U0126 and SB202190, suggesting that JNK1/2 and PI3-K/Akt participate in MMP-9 expression. Moreover, NF-κB phosphorylation and nuclear translocation was especially noted at the leading edge, which was attenuated by treatment with SP600125 or LY294002. Helenalin, a selective NF-κB inhibitor, reduced both the limbal epithelial outgrowth and MMP-9 expression. Finally, the data reveal that PI3-K/Akt is an upstream component of the JNK1/2 pathway in MMP-9 expression. Thus, both MAPKs and PI3-K/Akt are required for limbal epithelial outgrowth on intact AM, only the PI3-K/Akt/JNK is essential for MMP-9 expression mediated through activation of transcriptional factor NF-κB in this model.

Introduction

An integrated ocular surface is composed of a healthy pre-corneal tear film and the intact corneal, conjunctival as well as the limbal epithelia. The homeostasis of the corneal epithelial cells is maintained by their stem cells (SCs), which are located at the limbal zone (Schermer et al., 1986). Limbal stem cell deficiency (LSCD), which may be caused by chemical and thermal burns, Stevens-Johnson syndrome,

Abbreviations: MMP-9, matrix metalloproteinase-9; SCs, stem cells; LSCD, limbal stem cell deficiency; ECM, extracellular matrix; PI3-K, phosphatidylinositol 3-kinase; ERK1/2, extracellular signal-regulated kinase 1/2; JNKs, c-Jun N-terminal kinases

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doi:10.1016/j.scr.2012.02.005

repetitive ocular surgeries or other conditions, is manifested clinically by chronic inflammation, corneal neovascularization, and loss of vision (Holland and Schwartz, 1996). Therefore, restoration of the SC population is a prerequisite to obtain a stable ocular surface and is required for a subsequently successful corneal transplantation (Sangwan et al., 2005). By the concepts and applications of limbal epithelial SCs, such patients, especially those with total LSCD, were treated either by transplantation of one or more segments of limbal tissue (Kenyon and Tseng, 1989) or by transplantation of *ex vivo* cultivation of limbus-derived epithelial cells on amniotic membranes (AM) (Tsai et al., 2000). Currently, the latter is thought to be superior to the former, which may escape from the incidence of allograft rejection and the potential risk of LSCD to the donor eye (Lavker et al., 2004). To date, such *ex vivo*-expanded limbal epithelial cells on AM have been successfully transplanted for reconstructing the corneal surface in patients suffering from total LSCD (Tsai et al., 2000).

Several culture techniques have been developed for *ex vivo* expansion of limbal epithelial cells, including those that used intact or denuded AM as a supporting matrix; adopted an airlifting technique to promote epithelial differentiation; and prepared 3T3 fibroblasts as feeder layers (Sun et al., 2005). Previous studies have shown that limbal epithelial cells expanded on denuded AM grow faster than those on intact AM, and distinctly morphologic differences are also noted between these two culture conditions (Sun et al., 2005). Recent studies indicate that intact AM preserves the limbal epithelial phenotype, whereas denuded AM promotes corneal phenotype (Grueterich et al., 2002). Taken together, these facts reflect that the different status of microenvironments of the AM may modulate the outgrowth of limbal epithelial cells cultured on AM. The interaction between expanded limbal epithelial cells and underlying AM is complex yet interesting. Since the amniotic basement membrane contains several extracellular matrix (ECM) components such as type IV collagen and laminin-5 (Endo et al., 2004; Kurpakus-Wheater, 2001), the significance and involvement of matrix degradation enzymes in this *in vitro* culture model should deserve further investigation. To overcome ECM barriers (the basement membrane of AM), advancing limbal epithelial cells may produce proteases such as matrix metalloproteases (MMPs) (Endo et al., 2004; Li et al., 2006) or protease activators such as urokinase (Cheng et al., 2009) at their leading edge, where complex proteolysis can direct migration and preserve ECM attachment.

MMPs, a family of Ca^{2+}- and Zn^{2+}-dependent endopeptidases, are able to degrade most of the ECM components and to regulate several physiological processes such as cell migration, proliferation and apoptosis through cleavage and release of ECM microenvironment molecules and modulation of gene expression (Nelson et al., 2000; Vu and Werb, 2000). Both MMP-2 and MMP-9 are important members of the MMP family which are involved in the cleavage of denatured collagens and native basement membrane components and the primary matrix-degrading enzymes produced by the corneal epithelium (Fini and Girard, 1990). Furthermore, MMPs have been shown to play a role in epithelial proliferation and wound healing (Fini et al., 1992). Several lines of evidence suggest that MMP-9 expression is induced in cells at the front of the migrating epithelial sheet as it begins to resurface the wounded area following injury (Legrand et al., 1999). Previously, we have demonstrated that both latent and active forms of MMP-9 are up-regulated during the outgrowth of human limbal explants cultured on intact AM (with devitalized amniotic epithelial cells preserved; H/A^{intact} group) and denuded AM. In contrast to MMP-9, MMP-2 is usually constitutively expressed throughout the culture period, but the activity is higher on denuded AM than that on intact AM (Sun et al., 2005; Li et al., 2006). We also used a specific MMP-2 antibody in the H/A^{intact} group and found that it was not as effective as MMP-9 antibody for inhibiting the outgrowth of limbal explants. In addition, the results from histologic staining showed that the devitalized amniotic epithelial cells under expanded limbal epithelial cells are barely discernible compared with those on naive intact AM (Sun et al., 2005), implying a key role for MMP-9 in limbal epithelial migration and ECM remodeling in the H/A^{intact} group. However, the molecular mechanisms by which MMP-9 is regulated in the context of cell-cell matrix interactions remain unclear.

Post-translational modification of MMP has been reported to remove amino acids from the propeptide of MMP or to make structural changes. It has been reported that pro-MMP-9 can be modified and activated *in vitro* by many proteases such as plasmin, MMP-7, MMP-3, and MMP-13 (Cheng et al., 2009). However, plasmin was reported to be generated from plasminogen by the endogenous uPA and it was recognized as a direct activator of pro-MMP-9 (Saunders et al., 2005). In previous study, pro-MMP-9 has been shown to be post-translationally activated by uPA/plasmin, which is necessary for ECM remodeling and facilitating limbal epithelial cell migration in this culture model of human limbal explants on intact AM (Cheng et al., 2009). In addition, MMP activity is primarily controlled at the level of transcription (Fini et al., 1998). Transcription can be enhanced or inhibited through regulatory proteins that bind to response elements in the promoters of each MMP gene. The gene structure and promoter of MMP-9 have been characterized (Sato and Seiki, 1993) and binding sites for several transcriptional factors, including AP-1, SP-1, Ets and NF-κB are found in MMP-9 promoter region. Several studies have also indicated that NF-κB activation is required for maintaining cancer cell invasion and promoting epithelial wound healing (Liang et al., 2009; Egan et al., 2003). However, the role of NF-κB in the regulation of MMP-9 expression in the current model remains elusive.

Cell adhesion to the ECM appears to trigger various intracellular signaling pathways. In addition to proteolytic degradation of amniotic basement membrane components by proteases such as MMP-9, intracellular signaling cascades stimulated by growth factors or cytokines released from cell-cell matrix interactions might participate in migration and proliferation of intact AM-expanded limbal outgrowth. It has been reported that epidermal growth factor (EGF) and nerve growth factor (NGF) are required for limbal explant culture on AM (Yokoo et al., 2008). These factors may, when coupling with their respective receptors, activate intracellular signaling pathways, primarily through the mitogen-activated protein kinases (MAPKs) and phosphatidylinositol 3-kinase (PI3-K)/Akt cascades (He et al., 2006). There are three major superfamilies of MAPKs in several

cell types, including extracellular signal-regulated kinase 1/2 (ERK1/2), p38 MAPK, and c-Jun N-terminal kinases (JNKs, also termed stress-activated protein kinases) (Johnson and Lapadat, 2002). Activation of MAPKs exerts distinct cellular responses such as cell proliferation and migration by phosphorylation of specific target proteins (Johnson and Lapadat, 2002). PI3-K is an important enzyme that promotes cell survival and growth (Datta et al., 1999) by phosphorylating the downstream core component Akt/protein kinase B (PKB), first identified as the cellular homologue of the transforming oncogene (Staal, 1987).

Accumulating evidences demonstrate the involvement of both MAPK and PI3-K/Akt pathways in regulation of MMP-9 production (Lin et al., 2009; Ellerbroek et al., 2001). However, the association between the activation of these intracellular signaling pathways and intact AM-expanded limbal epithelial cells is not completely understood. Therefore, the purpose of the current study is to investigate the signaling cascades involved in the outgrowth of limbal explants and the regulatory mechanisms of MMP-9 expression in this model. Our results demonstrate that both MAPKs and PI3-K/Akt are required for limbal epithelial outgrowth on intact AM, only the PI3-K/Akt/JNK is essential for MMP-9 expression mediated through activation of transcriptional factor NF-κB in this model.

Results

In situ casein zymographic activity of MMP-9 in limbal epithelial cells expanded on intact AM

MMP-9, a protease responsible for ECM degradation, has been implicated in migration and basement membrane remodeling of corneal epithelial cells (Fini and Girard, 1990). In our previous study, we have demonstrated that MMP-9 activity in its latent or active form is over-expressed in limbal epithelial cells cultivated on intact AM as compared to that on denuded AM or plastic dish (Sun et al., 2005). To examine the regional distribution of MMP-9 activity in intact AM-expanded limbal epithelial cells, we performed dye-quenched *in situ* casein zymography on frozen-sectioned specimens obtained 3 weeks after culture. To prove that casein could be digested by MMP-9, we performed casein zymography using conditioned medium and demonstrated the caseinolytic activity of MMP-9 in a time-dependent manner (Fig. 1A). Furthermore, the *in situ* MMP-9 activity was localized on expanded limbal epithelial cells rather than those on AM as shown in Fig. 1B (a→f; g→l served as a negative control without dye-quenched gelatin substrate). We also found the fluorescence intensity in limbal epithelial cells cultured on intact AM (a→c) was more intense than that on denuded AM (d→f), consistent with our previous study (Sun et al., 2005).

Regional distribution of *in situ* MMP-9 activity in intact AM-expanded limbal epithelial cells

The migrating limbal epithelial cells, especially those at the leading edge, may produce proteases to breach biological barriers such as basement membranes. Therefore, we investigated if there was differential distribution of MMP-9 activity during *ex vivo* expansion. As shown in Fig. 2, the *in situ* casein zymographic activity was noted along the expanded limbal epithelial cells, the activity of which was especially prominent at the leading edge of expanded limbal epithelial progenitor cells (Fig. 2A and B). The *in situ* protease activity was consistent with the distribution pattern of MMP-9 expression, implying that more proteolytic enzymes were necessary at the migrating front to carve out a path for facilitating subsequent epithelial proliferation.

Roles of MAPK and PI3-K/Akt in the outgrowth of intact AM-expanded limbal epithelial cells and MMP-9 expression

Although we have identified that MMP-9 dissolves underlying ECM to facilitate subsequent migration of limbal epithelial cells in this *ex vivo* expansion model, the upstream signaling mechanisms regulating the expression of MMP-9 was not elucidated yet. Several lines of evidence suggest that MAPKs and PI3-K/Akt pathways are important intracellular signaling molecules mediating cell migration and proliferation (Johnson and Lapadat, 2002; Datta et al., 1999). Therefore, we investigated whether these pathways were involved in limbal outgrowth and MMP-9 expression in this culture system. Cultures at the end of the second week with similar sizes of limbal epithelial outgrowth were treated with or without pharmacological inhibitors. One week after the addition of pharmacological inhibitors, the limbal epithelial outgrowth in both the control group and the pharmacological inhibitors-treated group increased in size. We show that the area of limbal epithelial outgrowth was significantly reduced by the inhibitor of ERK1/2 (U0126), JNK (SP600125), PI3-K (LY294002), or Akt (SH5), but was not significantly inhibited by a p38 MAPK inhibitor, SB202190 (Fig. 3A and B). To further delineate which signaling pathways participated in regulating MMP-9 activity and expression, we utilized gelatin zymography and RT-PCR to analyze the effects of these signaling components on MMP-9 expression. As demonstrated in Fig. 3C, the MMP-9 activity was only attenuated by SP600125, LY294002, or SH5, but not by SB202190 (upper panel). Moreover, the RT-PCR showed that MMP-9 transcripts were selectively attenuated by SP600125, LY294002 or SH5. In contrast, the MMP-9 activity and transcripts were slightly enhanced by U0126 (Fig. 3C). These findings prompt us to investigate whether an endogenous inhibitor of MMP-9 (*e.g.* TIMP-1) was regulated by ERK1/2. As shown in Fig. 3D, TIMP-1 transcripts were inhibited by U0126, determined by RT-PCR (upper panel) and quantitative real-time PCR (lower panel), supporting the finding that U0126 had no significant attenuation on MMP-9 expression. Taken together, these results indicate that MAPKs and PI3-K/Akt are required for proliferation of expanded limbal epithelial cells on intact AM, however, only the JNK and PI3-K/Akt related signaling pathways play a part in the regulation of MMP-9 expression.

Role of transcription factor NF-κB in outgrowth and MMP-9 expression of intact AM-expanded limbal epithelial cells

The fact that there are NF-κB binding sites on human MMP-9 promoter region prompted us to investigate the role of NF-

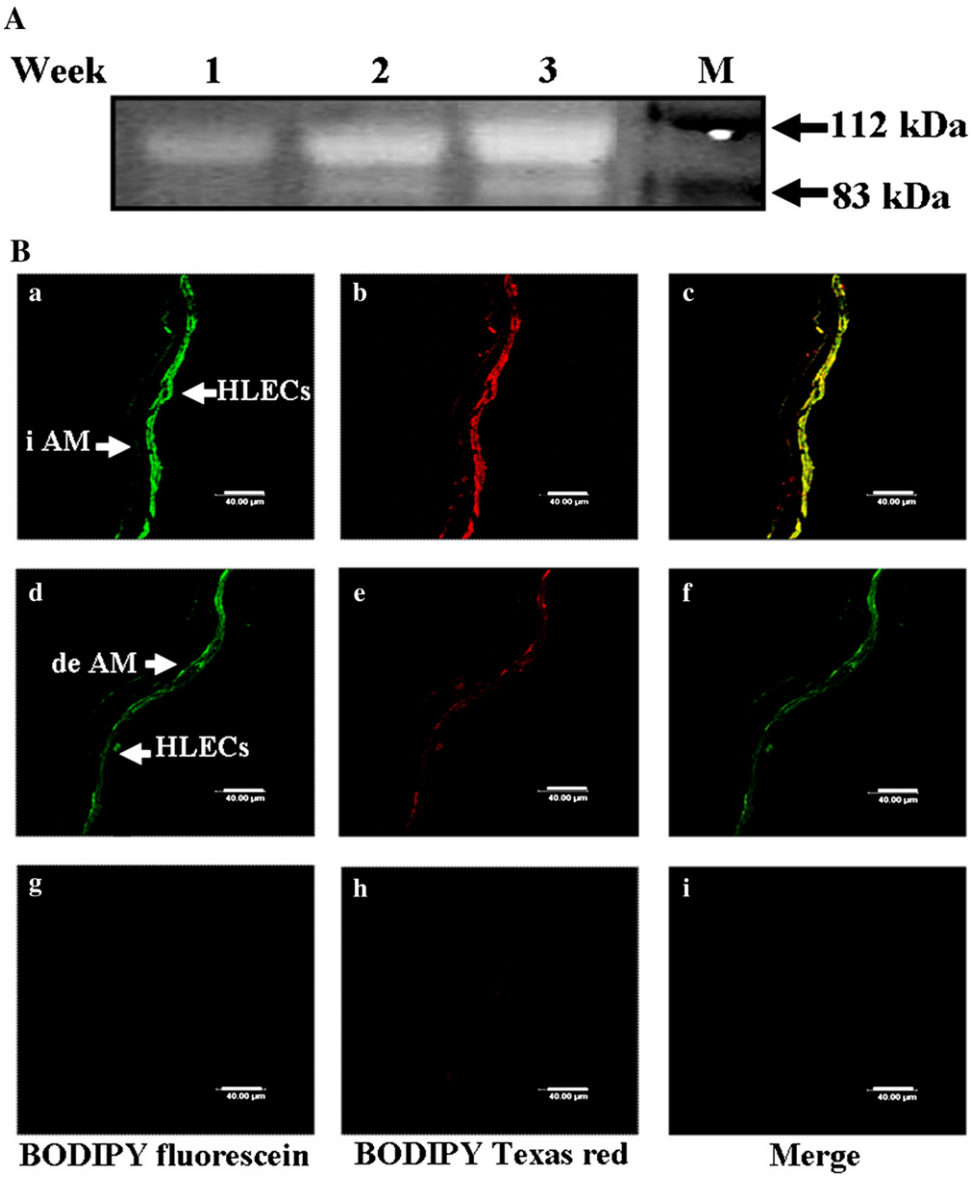


Figure 1 *In situ* casein zymography of human limbal epithelial cells (HLECs) cultured on intact AM (iAM) versus on denuded AM (deAM). (A) Casein zymography by conditioned medium from explant cultures showed that MMP-9 was capable of digesting casein in a time-dependent manner. (B) Dye quenched (DQ)-*in situ* casein zymography demonstrated that protease activity was over-expressed in limbal epithelial cells expanded on iAM (a, b, and c) as compared to those on deAM (d, e, and f). Figs. g, h, and i served as DQ-casein negative controls. M: molecular weight marker. Scale bar, 40 μm.

κB in these responses. Helenalin, a specific NF-κB inhibitor, exerts its effect by alkylating the p65 subunit (Lyss et al., 1998), was utilized to determine the role of NF-κB on limbal outgrowth and MMP-9 activity. As shown in Fig. 4A, the area of limbal epithelial outgrowth was significantly attenuated by Helenalin in a concentration-dependent manner. In addition, MMP-9 zymographic activity was decreased in a time-dependent fashion by addition of Helenalin from day 14 through day 21 (Fig. 4B). Moreover, the protein expression of MMP-9 in expanded limbal epithelial cells was substantially abrogated by Helenalin, revealed by immunofluorescence staining and western blotting (Fig. 4C). In addition, the transcriptional levels of MMP-9 gene were also down-regulated by Helenalin (Fig. 4D). These findings imply that NF-κB might directly modulate limbal epithelial proliferation through MMP-9 expression.

Effects of PI3-K and JNK signaling on NF-κB (p65) nuclear translocation and phosphorylation in ex vivo expanded limbal epithelial cells on intact AM

Knowing that the *in situ* MMP-9 activity was preferentially distributed at the leading edge of expanded limbal epithelial cells and was regulated by NF-κB, we wondered if there was a similar expression pattern with NF-κB revealed by an immunofluorescence staining. As demonstrated in Fig. 5A, the number of cells with NF-κB nuclear translocation increased

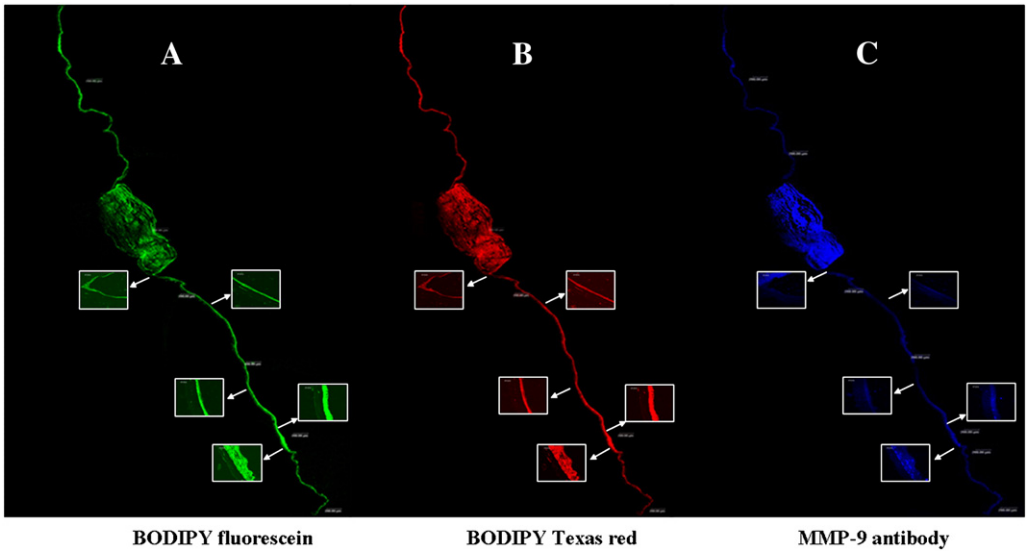


Figure 2 Regional distribution of *in situ* casein zymography and MMP-9 immunofluorescence staining in *ex vivo* expansion of human limbal epithelial cells on intact AM. (A, B) DQ-*in situ* casein zymography showed the preferential localization of protease activity at the migrating leading edge (see inlets). (C) The regional distribution of MMP-9 immunofluorescence staining performed on the same specimen was well correlated with the pattern in (A) and (B). Magnification: ×100 (×400 in inlets).

when the limbal epithelial cells expanded toward the front of migration. To investigate the association between NF-κB and relevant signaling pathways (*i.e.* the PI3-K and JNK pathways) in mediating MMP-9 activity, LY294002 and SP600125 were used to detect their effects on NF-κB (p65) translocation and phosphorylation. We found that LY294002 completely abolished NF-κB (p65) nuclear translocation and phosphorylation (Fig. 5B and C). In addition, SP600125 also reduced NF-κB (p65) phosphorylation in limbal epithelial cells (Fig. 5D). These results indicated that both PI3-K and JNK are involved in regulating the transcriptional activity of NF-κB in this model.

PI3-K/Akt acts upstream of JNK pathway in regulating MMP-9 activity through NF-κB (p65) nuclear translocation in *ex vivo* expanded limbal epithelial cells on intact AM

Akt, one of the major downstream targets of PI3-K, plays an important role in facilitating growth factor-mediated cell survival and blocking apoptotic cell death (Datta et al., 1999). By utilization of a selective Akt inhibitor, SH5, we have identified a critical role of Akt in mediating limbal outgrowth and MMP-9 expression (Fig. 3). Here, we further confirmed these results by using immunofluorescence staining, indicating that the *in situ* zymographic activity and Akt phosphorylation were attenuated by both LY294002 and SH5 (Fig. 6A and B). These results imply that PI3-K phosphorylates Akt, which in turn, activates MMP-9 expression in expanded limbal epithelial cells. Besides, NF-κB (p65) phosphorylation was also decreased by SH5, indicating the involvement of Akt in NF-κB activation (Fig. 6C). Finally, explant cultures were treated with the inhibitor for PI3-K or JNK to investigate their effects on Akt and JNK1/2 protein expression. It was noted that phosphorylation of JNK1 was significantly diminished by LY294002, whereas phosphorylation of Akt was not affected by SP600125 (Fig. 6D and E), illustrating that PI3-K/Akt signaling is upstream of the JNK pathway in this culture model.

Discussion

MMPs are proteolytic enzymes that degrade almost all components of the ECM and basement membrane proteins which are associated with physiological and pathological processes under various situations. In fact, several lines of evidence suggest that MMP-9 is implicated in migration and axonal outgrowth of neural SCs (Lin et al., 2009; Tonti et al., 2009), as well as in transmigration of hematopoietic progenitor cells (Rao et al., 2004). Like the SCs in other tissues, limbal epithelial progenitor cells are in continuous contact and interact with their surrounding environment, termed the stem cell niche, which is a network composed of several ECM components, cytokines and growth factors (Grueterich et al., 2003). Previous reports have demonstrated that intact AM-expanded limbal epithelial cells behave as a limbal epithelial phenotype (Grueterich et al., 2002) and maintain in a less apoptotic state (Sun et al., 2006). Therefore, the intact AM may serve as an ideal "niche" to support *ex vivo* expansion of human limbal epithelial progenitor cells (Grueterich et al., 2003), this is the reason why we used an intact AM as the culture carrier in this study.

In the present study, we demonstrate that *in situ* MMP-9 zymographic activity and protein expression were co-localized and accumulated at the leading edge of expanded human limbal epithelial cells on intact AM (Fig. 2). The focally active MMP-9 location at the front of migration could be explained, at least in part, by the fact that much more ECM components were left on intact AM, necessitating more proteases to degrade them. This notion was supported by the observation that *in situ* MMP-9 activity was more prominently distributed in intact AM-expanded limbal

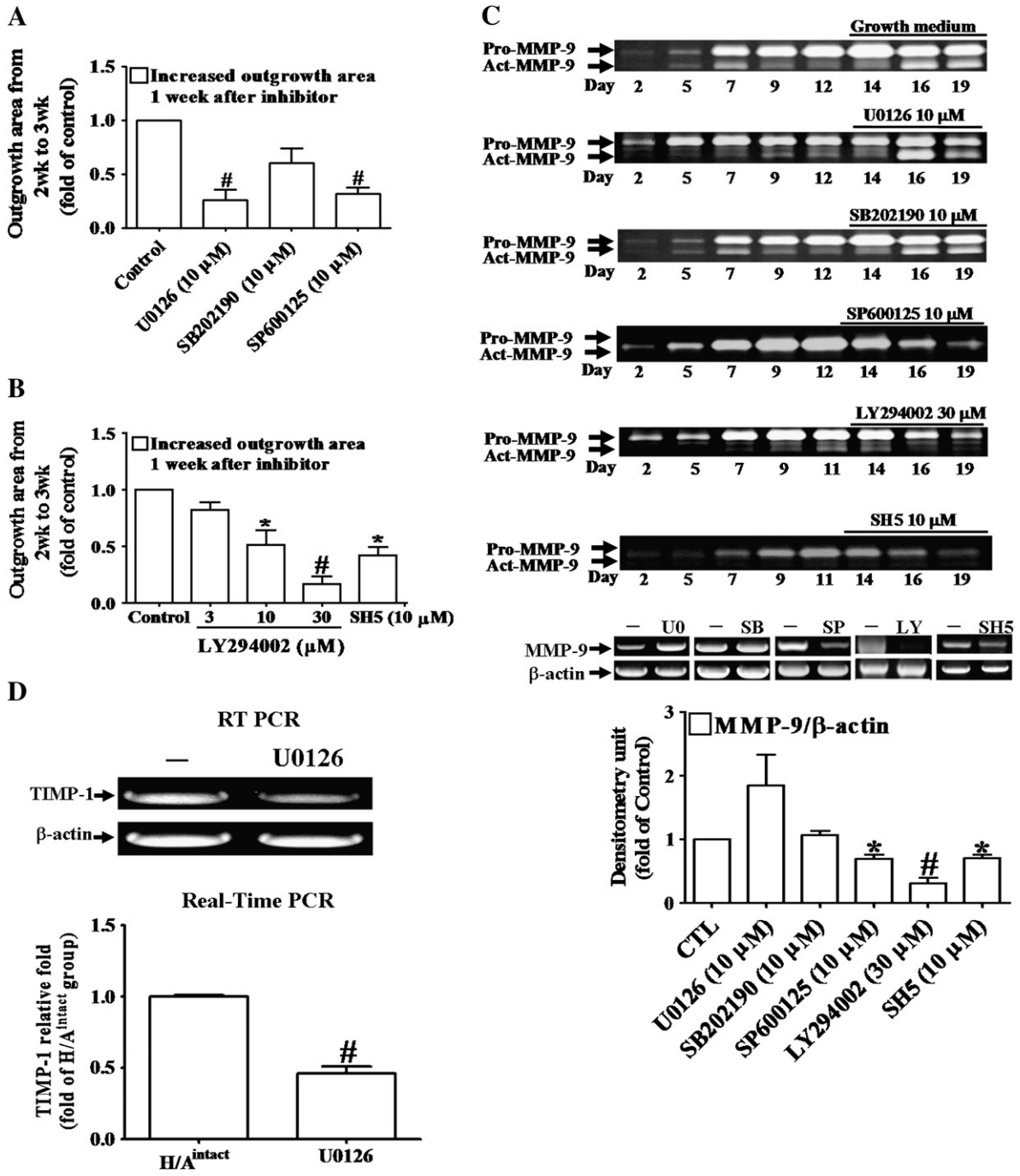


Figure 3 Involvement of MAPK and PI3-K/Akt pathways in the outgrowth and MMP-9 activity of human limbal epithelial cells on intact AM. **(A)** Limbal epithelial outgrowth on intact AM were inhibited by these MAPK inhibitors, including U0126, SB202190 and SP600125 at 10 μM. **(B)** Intact AM-expanded limbal epithelial outgrowths were significantly attenuated by the inhibitors of PI3-K (LY294002, 10 and 30 μM) and Akt (SH5, 10 μM). **(C)** The MMP-9 activity was only attenuated by SP600125, LY294002 and SH5, but not by U0126 and SB202190, as determined by gelatin zymography (upper panels). The MMP-9 RNA transcripts were down-regulated by SP600125, LY294002 and SH5, but not by U0126 and SB202190, as determined by RT-PCR (bottom panel). **(D)** The TIMP-1 RNA transcripts were down-regulated by U0126, as determined by RT-PCR (upper panel) and QRT-PCR (lower panel). U0: U0126. SB: SB202190. SP: SP600125. LY: LY294002. CTL: control. Data were summarized and expressed as the mean ± SEM of six independent experiments. $^{*}P<0.05$, $^{\#}P<0.01$, as compared with the control.

epithelial cells than on denuded AM (Fig. 1). On the other hand, accumulating evidence demonstrates that protein fragments cleaved by MMPs could gain activities that are inactive in the intact molecule. For instance, MMP-9 was involved in the release and activation of ECM-sequestered transforming growth factor−β (TGF-β) (Yu and

Stamenkovic, 2000). Another example is laminin-5, which is cleaved by MMP-2 to expose a cryptic site that stimulates mammary epithelial cell migration (Giannelli et al., 1997). These results were in accordance with our recent work which indicates that MMP-9, together with urokinase plasminogen activator (uPA), could process human laminin-5 γ2-chain into several protein fragments implicated in limbal epithelial outgrowth on intact AM (Cheng et al., 2009). However, the structural characteristics and functional significance of these protein fragments derived from laminin-5 remain to be clarified.

The interaction between intact AM and limbal explants does not only degrade the matrix of the AM but also activate intracellular signaling cascades triggered by matrix components. By use of selective pharmacological inhibitors, we investigated the downstream effectors critical for intact AM-induced limbal epithelial outgrowth and secretion of MMP-9 in *ex vivo* expanded limbal epithelial cells. We found that ERK1/2, JNK1/2, and PI3-K/Akt signaling pathways were required for limbal explant outgrowth on intact AM (Fig. 3A and B). However, the MMP-9 activity and RNA transcription were only up-regulated by JNK1/2 and PI3-K/Akt in this model (Fig. 3C and D). The involvement of MAPKs and PI3-K/Akt in such explant culture model has also been demonstrated by others (He et al., 2006). In contrast to their results, our data show that JNK1/2 was required for limbal epithelial outgrowth on intact AM. A possible explanation might be that different JNK inhibitors were used between these two studies. SP600125 (anthrapyrazolone), a selective JNK 1, 2 and 3 inhibitor (Bennett et al., 2001), was used in this work, whereas JNK Inhibitor 1 (L-stereoisomer) was utilized by others (He et al., 2006).

MAPKs phosphorylate specific serines and threonines of target proteins and regulate cellular activities including gene expression, cell proliferation, migration, metabolism, and programmed death (Johnson and Lapadat, 2002). Recent evidence suggests that MMP-9 is induced by TGF-β via activation of ERK1/2, p38 MAPK, and JNK1/2 pathways in corneal epithelial cells (Kim et al., 2005). However, it was intriguing to note that only the JNK1/2 pathway was involved in mediating MMP-9 expression in our study despite the essential roles of ERK1/2 and JNK1/2 in mediating limbal epithelial outgrowth (Fig. 3). The roles of individual MAPKs in MMPs expression vary with cell types and stimuli. For example, activation of p38 MAPK might play an important role in TGF-β-induced corneal epithelial migration in C57BL/6 J mice (Saika et al., 2004). While JNK1/2 and ERK1/2, rather than p38 MAPK have been implicated in the regulation of MMPs expression induced by IL-1β in corneal fibroblasts (Lu et al., 2004). It is also interesting to note that MMP-9 expression was enhanced by U0126, a selective MEK1/2 inhibitor, in this model (Fig. 3C). This phenomenon let us to further delineate the role of ERK1/2 signaling and

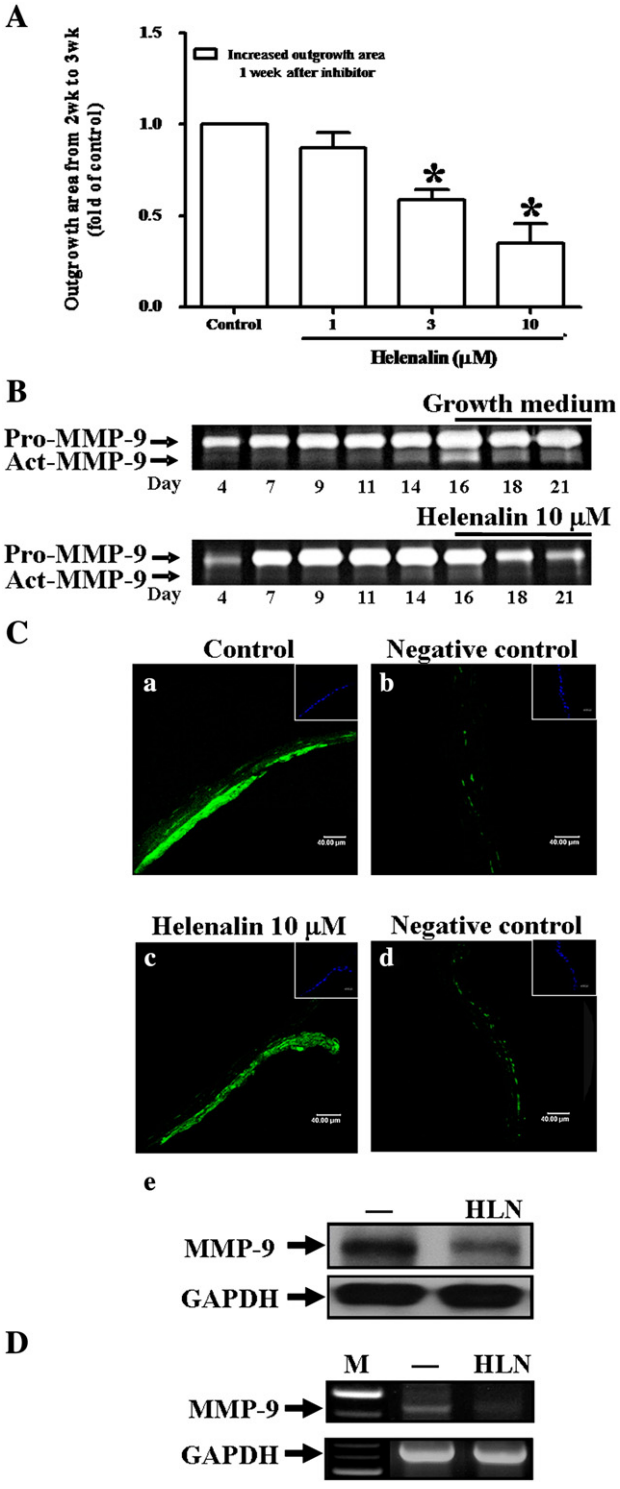
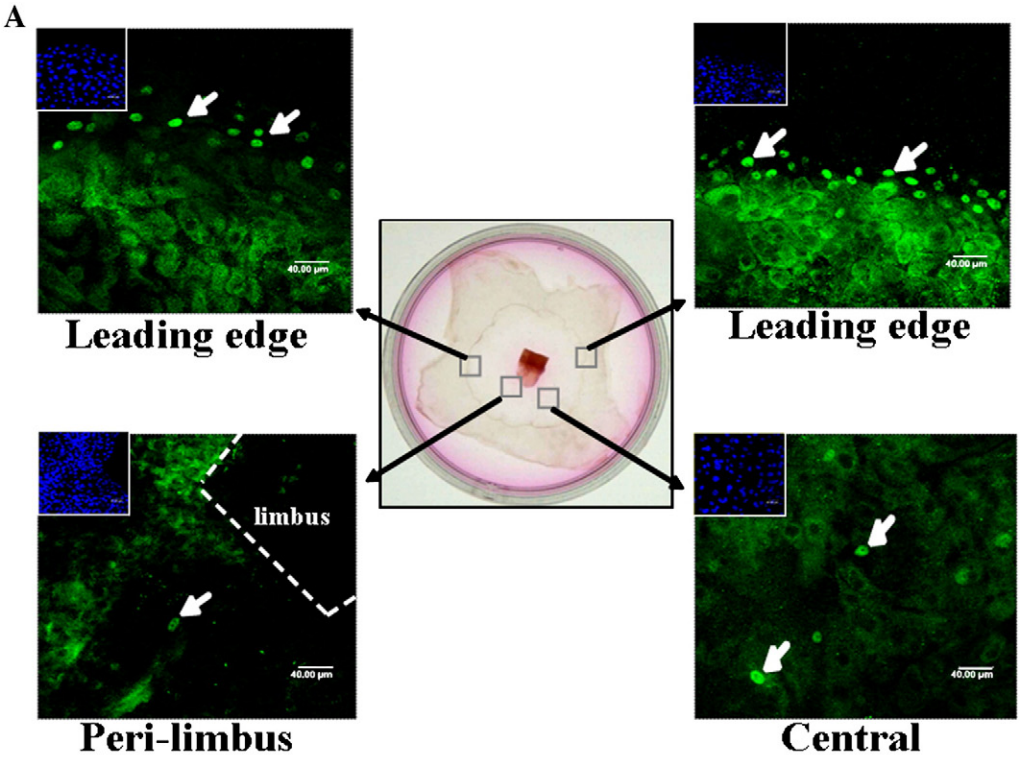
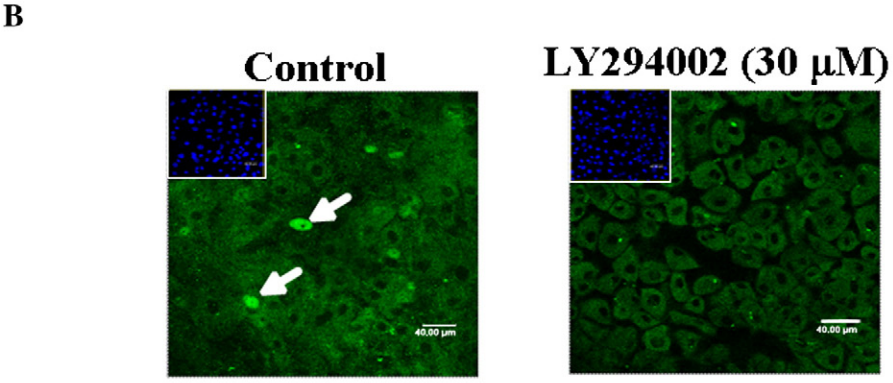
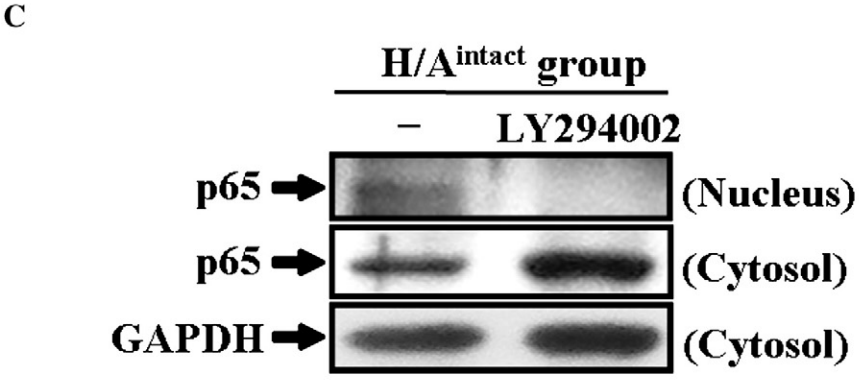


Figure 4 Involvement of NF-κB in the outgrowth, MMP-9 expression and activity of human limbal epithelial cells expanded on intact AM. (A) Limbal epithelial outgrowths on intact AM were retarded by Helenalin in a concentration-dependent manner. (B) The MMP-9 activity was apparently attenuated by Henelanin at 10 μM as determined by gelatin zymography. (C) The MMP-9 expression at day 21 was inhibited by Helenalin (HLN, 10 μM) as demonstrated by immunofluorescence staining (a and c) and western blotting analysis (e). b. and d. served as negative controls and inlets represented nuclear counterstained by DAPI. (D) Moreover, the MMP-9 RNA expression at day 21 was down-regulated by HLN (10 μM) as determined by RT-PCR. "-": control. M: molecular weight markers. GAPDH was used as an internal control. Data were summarized and expressed as the mean ± SEM of six independent experiments. *$P<0.05$ compared with the control. Scale bar, 40 μm.


A
Leading edge
Leading edge
limbus
Peri-limbus
Central


B
Control
LY294002 (30 μM)


C
H/A^intact group
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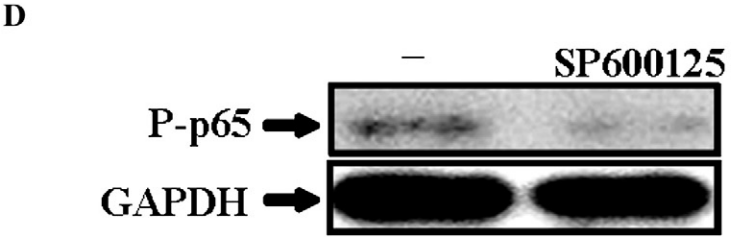
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SP600125
P-p65
GAPDH

its downstream effectors in these responses. TIMP-1, an endogenous inhibitor of MMP-9, was shown to be attenuated by U0126 treatment (Fig. 3D), implying that a negative feedback regulation of gene expression and activity related to MMP-9 is mediated through ERK1/2 signaling in this study.

By phosphorylation of its downstream target Akt, the PI3-K signaling pathway plays an important role in a wide array of cellular functions, including mitogenic signaling, cytoskeletal remodeling, metabolic control, and cell survival (Wymann and Pirola, 1998). In this report, inhibition of PI3-K or Akt activity by LY294002 or SH5 impaired expansion of limbal epithelial cells on intact AM as well as MMP-9 production (Fig. 3). It has been indicated that hepatocyte growth factor (HGF) protects corneal epithelial cells from apoptosis through the PI-3 K/Akt-1 but not the ERK1/2-mediated signaling pathway (Kakazu et al., 2004). Recent study has also demonstrated that translational control mediated by PI3K/Akt/mTOR, but not Raf/MEK/ERK signaling, is critical in regulating proliferation and endothelial differentiation of lineage-restricted stem cell lines (Que et al., 2007). However, whether limbal epithelial progenitor cells preferentially adopt the PI3-K/Akt pathway to maintain their proliferation potential and stemness is largely unknown.

Activation of PI3-K also plays an important role in insulin-like growth factor (IGF)-I-induced corneal epithelial cell migration (Lee et al., 2006). At present, the upstream components responsible for activation of PI3-K and MMP-9 production in the context of limbal epithelial cells and intact AM interactions are not clear. PI3-K activation is triggered by the binding of several growth factors such as HGF and IGF-1 to their cell surface receptors (Kakazu et al., 2004; Lee et al., 2006). In mammary epithelial cells, migration and adhesive interactions with the ECM might be mediated by the integrins, a large family of adhesion molecules composed of α and β heterodimers. Several lines of evidence suggest that $\alpha6\beta4$ integrin alone or coupled with EGF receptor signaling is involved in PI3-K activation and epithelial cell migration (Mercurio et al., 2001). This finding is consistent with our recent work indicating that $\beta4$ integrin is preferentially expressed than other β integrins in intact AM-expanded limbal epithelial cells (unpublished data). Subsequent study should be directed to investigate the mechanisms by which integrins are activated and their crosstalk with other growth factors in this model.

Our results provide evidence that (i) transcription factor NF-κB activation is necessary for limbal epithelial outgrowth on intact AM and MMP-9 expression (Fig. 4) and (ii) PI3-K/Akt and JNK pathways are involved in NF-κB activation (Figs. 5 and 6). Our data were supported by recent studies indicating that $\alpha6\beta4$ signaling promotes epidermal growth and wound healing by controlling nuclear translocation of NF-κB and phosphorylation of JNK (Nikolopoulos et al., 2005). Accumulating evidence also suggests that NF-κB can be activated via the EGF receptor (Sun and Carpenter, 1998) and participate in the transcriptional control of cyclin D (Guttridge et al., 1999). Therefore, it is possible that transient activation of NF-κB (Fig. 5A) may be required for progression of limbal epithelial progenitor cells through the G_1 phase of the cell cycle.

Upregulation of MMP-9 depends on the activation of NF-κB and AP-1 which bind to the corresponding regulatory elements within the promoter region of the MMP-9 gene (Sato and Seiki, 1993). Unlike AP-1, which is common to many MMP promoters and is necessary for basal gene expression (Fini et al., 1994), NF-κB is responsible for activation of MMP-9 transcription (Liang et al., 2009). By use of a selective NF-κB inhibitor, Helenalin, we show that NF-κB is critically involved in MMP-9 regulation at both transcriptional and translational levels (Fig. 4B–D). Moreover, we also identified that PI3-K/Akt is upstream of the JNK pathway in activation of NF-κB and MMP-9 expression (Figs. 5B–D and 6). The finding that JNK1/2 phosphorylation was inhibited by LY294002 (Fig. 6D and E) may imply a crosstalk between these two pathways, as reported in another cell system (Aikin et al., 2004). Future studies are required to verify this hypothesis and identify the underlying mechanisms. Taken together, our findings support the hypothesis that divergent kinase activities regulate distinct cellular events associated with cell-cell matrix interactions such as in our model.

Conclusions

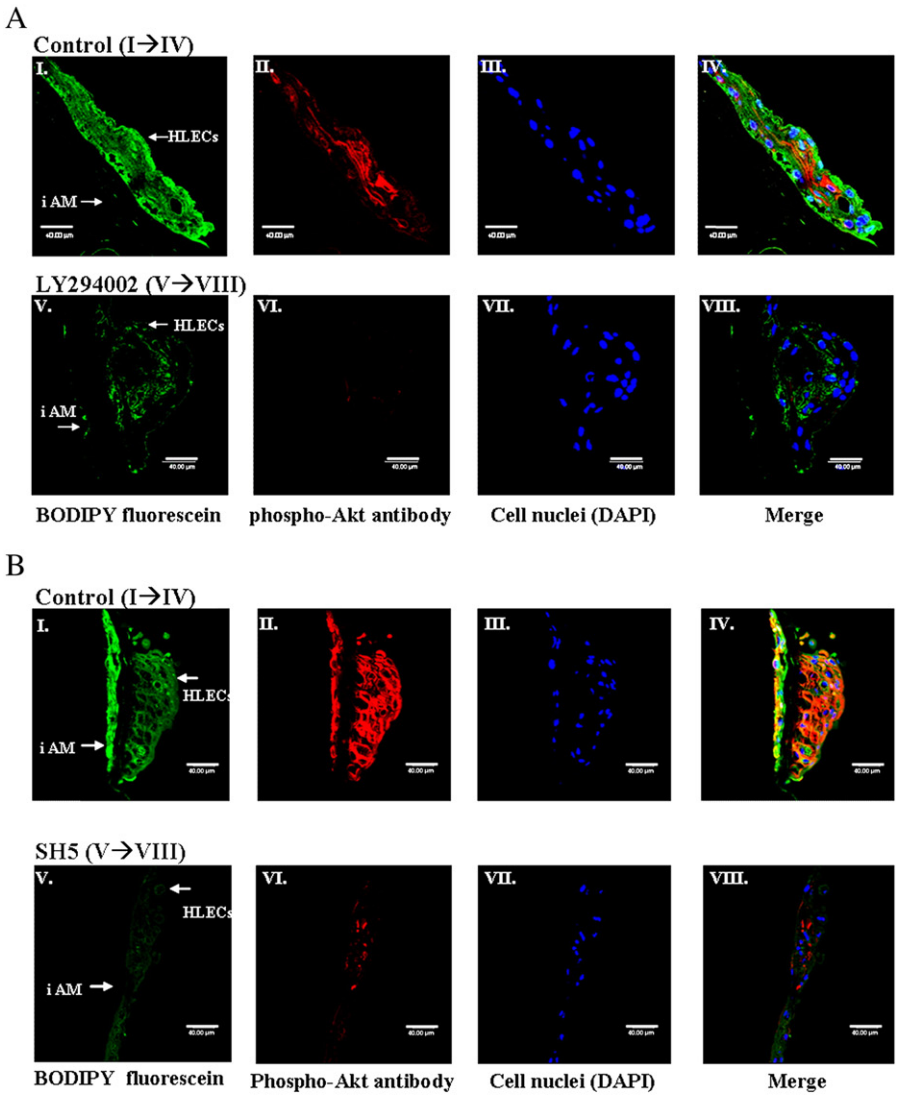
We demonstrate that both PI3-K/Akt and MAPK pathways provide the downstream signaling for outgrowth of limbal epithelial cells expanded on intact AM. Moreover, we also found that the expression of MMP-9 in this culture system is regulated via the PI3-K/Akt/JNK/NF-κB cascade. The relevant signaling network is depicted in Fig. 7. Further understanding of the molecular mechanisms underlying interactions between MMP-9 and other proteases as well as their crosstalk with specific growth factors (such as EGF) or adhesion molecules (such as integrins) would facilitate to formulate a new therapeutic strategy for corneal tissue engineering in the future.

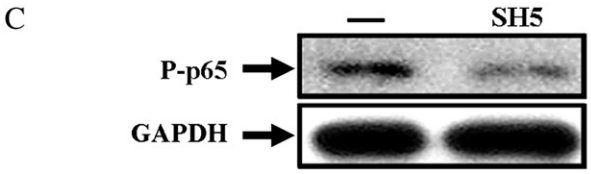
Materials and methods

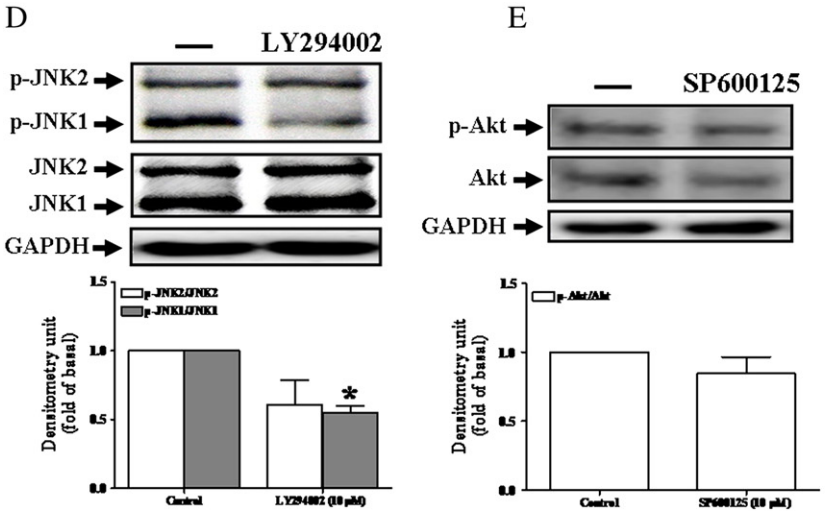
Materials

The tissue culture plastic dishes (40×10 mm) were purchased from Orange Scientific (Waterloo, Belgium). DMEM/

Figure 5 Involvement of PI3-K and JNK in NF-κB nuclear translocation and P65 phosphorylation in ex vivo-expanded human limbal epithelial cells on intact AM. (A) At day 21 in culture, NF-κB nuclear translocation (white arrows) as shown by immunofluorescence staining was differentially expressed in intact AM-expanded human limbal epithelial cells, the phenomenon of which was more prominent while the outgrowth was approaching the leading edge. (B) PI3-K inhibitor, LY294002 at 30 μM, completely abolished NF-κB nuclear translocation (white arrows) in human limbal epithelial cells. (C) NF-κB (p65) nuclear translocation was also prevented by LY294002 as evidenced by western blotting analysis. (D) Similarly, p65 phosphorylation was inhibited by a selective JNK inhibitor, SP600125 at 10 μM. GAPDH was used as internal control. The dotted line indicates the boundary of limbal explant. Inlets represent cell nuclei counterstained by DAPI. Scale bar, 40 μm.


A
Control (I→IV)
I.
HLECs
iAM
II.
III.
IV.
LY294002 (V→VIII)
V.
HLECs
iAM
VI.
VII.
VIII.
BODIPY fluorescein
phospho-Akt antibody
Cell nuclei (DAPI)
Merge
B
Control (I→IV)
I.
HLECs
iAM
II.
III.
IV.
SH5 (V→VIII)
V.
HLECs
iAM
VI.
VII.
VIII.
BODIPY fluorescein
Phospho-Akt antibody
Cell nuclei (DAPI)
Merge


C
—
SH5
P-p65
GAPDH


D
—
LY294002
p-JNK2
p-JNK1
JNK2
JNK1
GAPDH
p-JNK2/JNK2
p-JNK1/JNK1
Densitometry unit (fold of basal)
1.5
1.0
0.5
0.0
*
Control
LY294002 (10 μM)
E
—
SP600125
p-Akt
Akt
GAPDH
p-Akt/Akt
Densitometry unit (fold of basal)
1.5
1.0
0.5
0.0
Control
SP600125 (10 μM)

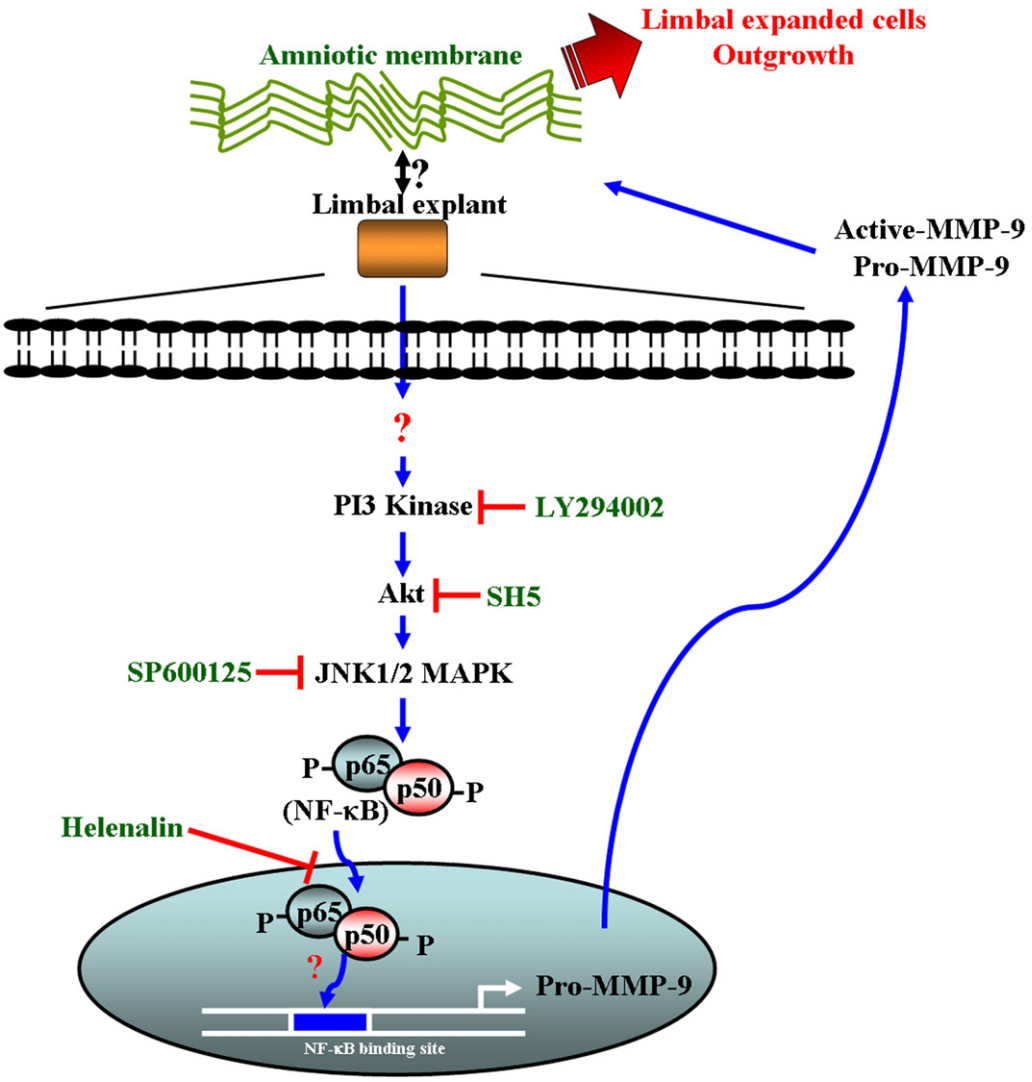


Figure 7 The schematic diagram summarized the molecular mechanisms by which MMP-9 was activated in ex vivo expansion of human limbal epithelial cells on intact AM. Both PI3-K/Akt and MAPK pathways provide the downstream signaling for outgrowth of limbal epithelial cells expanded on intact AM. Moreover, the interaction between intact AM and human limbal epithelial cells-induced MMP-9 expression was mediated through a PI3-K/Akt/JNK-dependent NF-κB translocation and activation, in turns to activate MMP-9 transcriptional activity. Up-regulation of MMP-9 by these signaling molecules may contribute to outgrowth of human limbal epithelial cells on intact AM.

F-12 medium, fetal bovine serum (FBS) and Trizol were purchased from Invitrogen (Carlsbad, CA). MMP-9 monoclonal antibody was from NeoMarkers (Fremont, CA). Anti-phospho-SAPK/JNK, anti-phospho-Akt (Ser^{473}), and anti-phospho-NF-κB (p65) antibodies were from Cell Signaling (Danver, MA). Anti-JNK1, anti-JNK2, anti-Akt and

Figure 6 PI3-K/Akt/JNK-regulated NF-κB nuclear translocation was involved in MMP-9 activation in human limbal epithelial cells expanded on intact AM. (A) Immunofluorescence staining demonstrated that PI3-K inhibitor, LY294002 at 30 μM, profoundly attenuated both *in situ* casein zymographic activity (I and V) and phospho-Akt expression (II and VI) as compared to the control. (B) Akt selective inhibitor, SH5 at 10 μM, also attenuated *in situ* zymographic activity (I and V) and phospho-Akt expression (II and VI), confirming that PI3-K/Akt signaling was involved in MMP-9 activation. (C) A selective Akt inhibitor, SH5 at 10 μM, attenuated NF-κB (p65) phosphorylation. (D, E) JNK was downstream of PI3-K/Akt pathway in regulating MMP-9 activation via NF-κB nuclear translocation. Proteins were collected from co-culture system pretreated without (control) or with (D) LY294002 (30 μM) or (E) SP600125 (10 μM)-treated AM^{Intact}-expanded HLECs were subjected to western blot analysis (upper panel) using an anti-phospho-JNK1/2 or anti-JNK1/2 (D) and anti-phospho-Akt and anti-Akt (E) or anti-GAPDH antibody (as an internal control). The activation of JNK1 (D, gray bar), JNK2 (D, open bar), and Akt (E) was determined as the phosphorylation level normalized to their respective total protein (lower panel), respectively. Data were summarized and expressed as the mean ± SEM of six independent experiments (n = 6). *$P < 0.05$ compared with the control. The cell nuclei were counterstained by DAPI. Scale bar, 40 μm.

anti-NF-κB (p65) antibodies were from Santa Cruz (Santa Cruz, CA). Anti-GAPDH antibody was from Affinity BioReagents™ (Golden, CO). LY294002, SP600125, U0126, SB202190, and Helenalin were from Biomol (Plymouth Meeting, PA). SH5 was from Alexis Biochemicals (San Diego, CA). EnzChek protease assay kit was from Molecular Probes (Eugene, OR). Enzymes and other chemicals were from Sigma (St. Louis, MO). All studies were performed with the approval of the institutional ethics committee at Chang Gung Memorial Hospital.

Human limbal explant culture on intact and denuded AM

Human tissue was handled according to the tenets of the Declaration of Helsinki. Corneoscleral buttons procured after conventional penetrating keratoplasty from human donor eyes, aged 15–65 years, were obtained from the Chang Gung Memorial Hospital Eye Bank. The tissue was rinsed thrice with DMEM/F-12 containing 50 μg/ml gentamicin and 1.25 μg/ml amphotericin B. After careful removal of excessive sclera, iris, corneal endothelium, conjunctiva and Tenon's capsule, the remaining tissue was placed in a tissue culture plastic and cut into cubes of approximately $1.5 \times 2 \times 3\ mm^3$ by a scalpel. Human AM was obtained by elective cesarean section from Chang Gung Memorial Hospital (Keelung, Taiwan) with properly informed consent and was processed as previously described (Lee and Tseng, 1997). Briefly, the AM was aseptically washed thrice in 200 ml of phosphate-buffered saline (PBS) containing 50 μg/ml penicillin, 50 μg/ml streptomycin, 2.5 μg/ml amphotericin B, and 25 ng/ml gentamicin. The AM with epithelial cells remained (intact AM) was preserved sterile in DMEM/F-12 with 50% glycerin at −80 °C for at least six months. In preparation of denuded AM, the membranes were treated with 0.1% EDTA for 30 min and then gently scrubbed with an epithelial scrubber to remove the amniotic epithelium without breaking the underlying basement membrane (Sun et al., 2005). Before using, the AM was thawed and placed on a tissue culture plastic with the basement membrane side up and incubated at 37 °C in a humidified incubator under 95% air and 5% CO_2 overnight. On the centre of AM, a limbal explant was placed and cultured in a medium made of an equal volume of HEPES-buffered DMEM containing bicarbonate and F-12, and supplemented with 5% FBS, 0.5% dimethyl sulphoxide, 2 ng/ml mouse EGF, 5 μg/ml insulin, 5 μg/ml transferrin, 5 ng/ml selenium, 0.5 μg/ml hydrocortisone, 30 ng/ml cholera toxin, 50 μg/ml gentamicin, and 1.25 μg/ml amphotericin B. Cultures were incubated at 37 °C under 5% CO_2 and 95% air and the medium was changed and saved for gelatin or casein zymographic analyses every 2 to 3 days. When addition of pharmacological inhibitors was necessary to detect their effects on outgrowth and MMP-9 expression of limbal epithelial cells, the inhibitors of PI3-K (LY294002, 30 μM), Akt (SH5, 10 μM), JNK (SP600125, 10 μM), or NF-κB (Helenalin, 10 μM) was added in the culture media every 2–3 days from day 14 for one more week, and then subjected to microscopic photography. The extent of each outgrowth was monitored with a phase contrast microscope and calculated by computer software (Motic Image Plus 2.0, Germany).

Gelatin/casein zymography

The culture medium was collected and centrifuged at 14,000 rpm for 5 min at 4 °C to remove cell debris. The supernatant was mixed with 5x nonreducing sample buffer (4:1, vol/vol) and electrophoresed on a 10% SDS-polyacrylamide gel containing 0.1% gelatin or casein as a substrate for MMP-2 and −9. After electrophoresis, gels were washed in 3% Triton X-100 for 1 h to remove SDS and then incubated at 25 °C for 16 h in developing buffer (50 mM Tris, 40 mM HCl, 200 mM NaCl, 5 mM $CaCl_2$, and 0.2% Briji) on a rotary shaker. After incubation, gels were stained in 30% methanol, 10% acetic acid, and 0.5% (wt/vol) Coomassie brilliant blue for 1 h followed by destaining. Mixed human MMP-2 and −9 were used as positive controls. Gelatinolytic or caseinolytic activities manifested as horizontal white bands on a blue background.

In situ casein zymography

Frozen sections taken from the limbal explant cultures were mounted on silane-coated slides and incubated with EnzChek® protease assay kit for the activity of MMP-9 at 37 °C in a light-protected, humidified chamber for 20 h. After incubation, the samples were rinsed twice with PBS to remove nonspecific binding. To block nonspecific staining, the slides were incubated in blocking solution including 1% normal goat serum and 4% bovine serum albumin (BSA) for 30 min. For double staining, sections were then incubated with an anti-MMP-9 or anti-phospho-Akt antibody at a dilution of 1:100 for 1 h at room temperature. After incubation, the sections were washed with PBS and then incubated with AMCA-conjugated secondary antibody for MMP-9 and Rhodamine-conjugated secondary antibody for phospho-Akt, respectively, at a dilution of 1:100 for 1 h at room temperature. The slides were then washed and scanned using a confocal microscope (Leica Confocal Laser Scanning Microscopy, Deerfield, IL, USA). Proteases activate the quenched BODIPY dye-labeled casein substrates (fluorescein and Texas red), producing areas of green and red fluorescence against a dark background.

Immunohistochemical and immunocytochemical staining

Six-micrometer-thickness, frozen-sectioned specimens taken from the limbal explant culture or intact AM-expanded limbal epithelial cells grown on coverslides were fixed and mounted on silane-coated slides. Endogenous peroxidase activity was blocked by placing sections in 2% hydrogen peroxide for 30 min. Samples were rinsed in deionized water and then in Tris-buffered saline containing 0.1% BSA. To block nonspecific staining, the slides were incubated in 20% normal goat serum for 10 min. Samples were incubated overnight at 4 °C with an anti-MMP-9 or anti-NF-κB (p65) antibody at a dilution of 1:100. After thoroughly washing with PBS, the specimens were incubated with fluorescein-conjugated secondary antibody at a dilution of 1:400 for 1 h. The slides were then washed and scanned using a confocal microscope. Negative controls had the primary antibody omitted and DAPI was used for nuclear counterstaining.

RT-PCR and quantitative real time-PCR analysis

Total RNA was isolated from intact AM-expanded human limbal epithelial cells (treated with or without indicated pharmacological inhibitors) with Trizol (Invitrogen) according to the manufacturer's instructions.

PCR was performed with the following primers (100 ng/μl concentration):

MMP-9
Sense: 5′-GGCGCTCATGTACCCTATGT-3′
Antisense, 5′-TCAAAGACCGAGTCCAGCTT-3′
TIMP-1
Sense: 5′-ATCCTGTTGTTGCTGTGGCTGATAG-3′
Antisense: 5′-TGCTGGGTGGTAACTCTTTATTTCA-3′
β-actin
Sense: 5′-GACGGGGTCACCCACACTGTGCCCATCTA-3′
Antisense: 5′-CTAGAAGCATTTGCGGTGGACGATG-GAGGG-3′

The expression of β-actin was used as an internal control for the assay of a constitutively expressed gene. The annealing temperature was 58 °C for MMP-9 and TIMP-1, and 55 °C for β-actin. Amplified fragment sizes for MMP-9, TIMP-1 and β-actin were 468 bp, 692 bp and 636 bp, respectively. The resulting PCR product was analyzed by 2% agarose gel electrophoresis.

Furthermore, using the cDNA templates, quantitative real time-PCR (QRT-PCR) was performed according to the manufacturer's protocols (Applied Biosystems, Foster City, CA). Assays were performed using a real-time PCR system using a mix (StepOnePlus with TaqMan Universal PCR Master Mix; Applied Biosystems; http://www.appliedbiosystems.com). The TIMP-1 (Hs00171558_m1) and GAPDH (Hs99999905_m1) primers and probes were designed using commercial software (ABI PRISM Sequence Detection System; Applied Biosystems). The probes were labeled with a FAM dye. The data are calculated with ΔΔCt. All quantitative PCR assays were performed in triplicates. Results were expressed as ratios of target gene mRNA copies to GAPDH copies.

Preparation of cell extracts and western immunoblot analysis

Expanded limbal epithelial sheets on intact AM at day 21 were washed and scraped off with a spatula. When selective inhibitors were used, they were added at day 14 and incubated for one week. Cell lysates were prepared as previously described (He et al., 2006) and the protein concentration was determined by using the BCA reagents according to the instructions of the manufacturer. Samples from these cell lysates (25 μg protein) were denatured and subjected to SDS-PAGE using a 12% (w/v) running gel. Protein was transferred to nitrocellulose membrane and the membrane was incubated successively at room temperature with 5% BSA in TTBS for 1 h, and then incubated overnight at 4 °C with an anti-MMP-9, anti-phospho-p65, anti-NF-κB (p65), anti-phospho-JNK, anti-phospho-Akt, or anti-GAPDH polyclonal antibody at a dilution of 1:1000 in TTBS. Membranes were washed four times with TTBS for 5 min each, incubated with a 1:2000 dilution of an anti-rabbit or anti-mouse horseradish peroxidase antibody for 1 h, followed by washing with TTBS. The immunoreactive bands detected by ECL reagents were developed by Hyperfilm-ECL.

Translocation of NF-κB

AM^{intact}-expanded human limbal epithelial cells cultured at the end of the second week were treated with or without 30 μM LY294002. After one week, the cells were washed, scraped, and centrifuged to prepare membrane, cytosolic, and nuclear fractions, as previously described (Lin et al., 2009). The translocation of NF-κB p65 was determined by western blot using an NF-κB (p65) antibody. Membranes were stripped and re-probed using an anti-GAPDH Ab as a control.

Statistical analysis of data

Quantitative data were analyzed by a computer program (Prism 4; GraphPad, San Diego, CA, USA), expressed as the mean ± SEM, and analyzed with a two-tailed Student's *t*-test with $P<0.05$ set as the level of significance between individual groups.

Acknowledgements

We thank Dr. Chi-Chin Sun for providing the limbal tissues after conventional penetrating keratoplasty from human donor eyes. This work was supported by National Science Council (NSC96-2320-B-182-003 and NSC96-2320-B-182-047-MY3) and Chang Gung Medical Research Foundation (CMRPD32043 and CMRPD170332).

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