Cdc42 Inhibits ERK-mediated Collagenase-1 (MMP-1) Expression in Collagen-Activated Human Keratinocytes

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Abstract

Following injury, keratinocytes switch gene expression programs from one that promotes differentiation to one that supports migration. A common feature of human wounds and ulcerations of any form is the expression of MMP-1 (collagenase-1) by leading-edge basal keratinocytes migrating across the dermal or provisional matrix. Induction of MMP-1 occurs by signaling from the $\alpha_2\beta_1$ integrin in contact with dermal fibrillar type I collagen, and the activity of MMP-1 is required for human keratinocytes to migrate on collagen. Thus, MMP-1 serves a critical role in repair of damaged human skin. Here, we evaluated the mechanisms controlling MMP-1 expression in primary human keratinocytes from neonatal foreskin and adult female skin. Our results demonstrate that shortly following contact with type I collagen, ERK and p38 MAPK were markedly activated, whereas JNK phosphorylation remained at basal levels. ERK inhibition dramatically blocked collagen-stimulated MMP-1 expression in keratinocytes. In contrast, inhibiting p38 or JNK pathways had no effect on MMP-1 production. Moreover, investigating the role of Rho GTPases revealed that Cdc42 attenuates MMP-1 expression by suppressing ERK activity. Thus, our data indicates that injured keratinocytes induce MMP-1 expression through ERK activation, and this process is negatively regulated by Cdc42 activity.

Introduction

The epidermis provides a physical barrier to the outside environment. In intact skin, the epidermis is separated from the underlying dermis by basement membrane. Upon disruption of the epidermis, as occurs in normal wounds and chronic ulcerations, wound edge keratinocytes become exposed to the dermal extracellular matrix (ECM), which is abundant

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in fibrillar type I collagen. Keratinocyte ligate collagen by the $\alpha_2\beta_1$ integrin (Fujisaki and Hattori, 2002). We reported that contact with dermal type I collagen–which keratinocytes do not encounter in intact skin–is a potent activator of keratinocytes, and we have proposed that ligation with dermal collagen provides a clear, spatial cue informing wound edge keratinocytes that they are in a wound environment (Pilcher *et al.*, 1999; Pilcher *et al.*, 1997; Saarialho-Kere *et al.*, 1993; Sudbeck *et al.*, 1997b).

Collagenase-1 (MMP-1) is among the gene products induced in migrating keratinocytes via ligation of the $\alpha_2\beta_1$ integrin with dermal collagen and is a reliable marker of activated keratinocytes in wounded human skin in a variety of conditions (Pilcher *et al.*, 1997; Saarialho-Kere *et al.*, 1993; Saarialho-Kere *et al.*, 1995; Sudbeck *et al.*, 1997b). Furthermore, the ability of MMP-1 to cleave type I collagen is essential for keratinocyte migration on type I collagen, and the process of ligation, proteolysis, release, and renewed ligation provides a means to maintain the directionality of migrating keratinocytes across a wound bed (Chen and Parks, 2009; Dumin *et al.*, 2001; Pilcher *et al.*, 1997). In addition, ligation of keratinocytes to dermal type I collagen induces a variety of gene products (Pilcher et al., 1999). Although MMP-1 is expressed by wound-edge basal keratinocytes in any condition where the epidermis and basement membrane are disrupted, it is expressed at higher levels and by many more keratinocytes in chronic ulcerations compared to normal wounds (Pilcher *et al.*, 1997; Saarialho-Kere *et al.*, 1993; Schultz and Gibson, 2013; Weckroth *et al.*, 1996). Whereas its regulated expression in normal wounds likely provides sufficient collagenolytic activity to promote keratinocyte migration, the over-expression of MMP-1 in chronic ulcerations may lead to unwanted proteolysis that could impede re-epithelialization (Parks and Schultz, 1999). Thus, understanding the pathways controlling MMP-1 expression will shed light on regulatory mechanisms controlling keratinocyte response to injury that may go awry in non-healing wounds.

Signaling downstream of activated integrins activates multiple signal transduction molecules including Rho GTPases and MAP kinases (Miyamoto *et al.*, 1995). Studies of wounded epithelial monolayers demonstrate that ERK activity has a crucial role during re-epithelialization (Matsubayashi *et al.*, 2004). However, the MAPK signaling cascade has pleiotropic effects that influence various cellular responses through different mediators (e.g., transcription factors). The Rho family of small GTPases, RhoA, Rac1 and Cdc42, are key regulators that function by regulating multiple aspects of wound healing (Hall, 1998), including keratinocyte proliferation, differentiation and migration (Jackson *et al.*, 2011; Tscharntke *et al.*, 2007; Wu *et al.*, 2006b). The Rho GTPases are activated in response to integrin signaling and have cell-type specific effects on migration, cell polarity, and gene expression (Brown *et al.*, 2006; Heasman and Ridley, 2008; Marinissen *et al.*, 2001; Matsubayashi *et al.*, 2004; Mythreye and Blobe, 2009; Nakamura *et al.*, 2009; Yang *et al.*, 2006).

The present study was undertaken to determine the signaling pathways regulating collagen-mediated MMP-1 expression in migrating keratinocytes. Our findings demonstrate that Cdc42 modulates MMP-1 expression through ERK signaling, which underscore the cell type specific mechanism involved in regulation of MMP-1 production in keratinocytes.

Results

Keratinocyte contact with type I collagen induced activation of ERK and p38 MAPK

To determine the signaling mechanisms mediating collagen induction of MMP-1 in human keratinocytes, we assessed the activation status of the ERK, p38, and JNK MAPK pathways (Fig. 1). ERK phosphorylation increased as early as 15 min after contact with native type I collagen (hereafter referred to as "collagen"). Phosphorylation of p38 was also enhanced following collagen contact. However, unlike ERK, which was persistently activated, p38 phosphorylation was transient and diminished by 60 min after stimulation. In contrast, JNK had a constitutively low level of activation that diminished following plating cells on collagen.

Collagen induction of MMP-1 expression in keratinocytes was ERK-dependent

To determine if ERK and p38 regulated collagen-mediated expression of MMP-1, we selectively inhibited each MAPK pathway (Fig. 2). Keratinocytes cultured on collagen prominently induced MMP-1 expression. However, keratinocytes cultured with PD98059, an ERK pathway inhibitor, had much reduced levels of MMP-1 protein production (Fig. 2A). In contrast, p38 had no effect on MMP-1 expression.

Because MMP-1 is largely regulated by transcriptional expression after keratinocyte stimulation (Pilcher *et al.*, 1999), we evaluated if ERK signaling attenuated MMP-1 promoter activity using a MMP1 promoter luciferase reporter assay. MMP-1 promoter activity was almost completely abrogated when keratinocytes plated on collagen were treated with an ERK inhibitor (Fig. 2B). Consistent with our previous results, p38 and JNK inhibition had no effect on collagen-stimulated MMP-1 promoter activity. In addition, ERK inhibition had a dose-dependent suppression of MMP-1 transcription in collagen-stimulated keratinocytes (Fig. 2C).

ERK regulated MMP-1-dependent keratinocyte migration

Migration of keratinocytes across a type I collagen substratum requires MMP-1 proteolytic activity (Pilcher *et al.*, 1997). Because collagen-induced MMP-1 expression required ERK signaling (Fig. 2), we predicted blockade of the ERK pathway would attenuate keratinocyte migration. Cell migration was quantified with a colloidal gold motility assay (Fig. 3A). Keratinocytes migrated efficiently over collagen. Whereas ERK inhibition attenuated keratinocyte migration across collagen, inhibition of p38 and JNK did not. Thus, ERK signaling promotes keratinocyte motility on fibrillar collagen.

Using a more physiologic model of keratinocyte migration, we evaluated ERK-dependent MMP-1 expression and cell migration in *ex vivo* wounded human skin explants (Fig. 3B,C), a model we established in other studies (Dumin *et al.*, 2001). A robust keratinocyte migration response was observed in skin specimens treated with an inactive analog of the ERK inhibitor. In contrast, in the presence of the ERK inhibitor, keratinocyte migration was blocked. Using *in situ* hybridization, we found that expression of MMP-1 was also blocked in skin explants incubated in the presence of the ERK inhibitor (Fig. S1)

Rho GTPases modulated MMP-1 expression

Because Rho GTPase are activated by integrins and promote keratinocyte migration (Ho and Dagnino, 2012), we determined the effect of Rho GTPase signaling on MMP-1 expression. Keratinocytes were transfected with siRNA targeting RhoA, Rac1, or Cdc42, which specifically and effectively silenced their respective expression (Fig. S2). Knockdown of Cdc42 significantly augmented MMP-1 expression by 6.6 fold (Fig. 4A & B; $p < 0.05$). In contrast, knockdown of RhoA modestly downregulated MMP-1 expression, and knockdown of Rac1 had no effect. Using selective small molecule inhibitors of Rho GTPases and phorbol myristate acetate (PMA) to stimulate MMP-1, we found that blocking Cdc42 augmented MMP-1 expression in a dose-dependent manner (Fig. 4C; $p < 0.05$). This effect was also seen in unstimulated cells treated with the Cdc42 inhibitor (data not shown). Furthermore, inhibition of ROCK (Rho-associated protein kinase), a downstream mediator of RhoA, blocked expression of MMP-1 (Fig. 4D; $p < 0.05$), while inhibition of Rac1 had no effect (Fig. 4E).

Cdc42 attenuated ERK-mediated MMP-1 expression

Next, we tested for crosstalk between Rho GTPase and ERK activity in keratinocytes. ERK inhibition had no effect on RhoA, Rac1, and Cdc42 activation (Fig. 5A). In contrast, Cdc42 inhibition augmented ERK activation (Fig. 5B). These results indicate Cdc42 functions upstream of ERK, and its activity suppresses phosphorylation of ERK. To determine if ERK is the main effector downstream of Cdc42 in regulation of MMP-1, we inhibited ERK and Cdc42 simultaneously and found that ERK inhibition abrogated the augmented MMP-1 expression induced by Cdc42 inhibition in keratinocytes stimulated by collagen ligation (Fig. 5C & D) or PMA (Fig. 5E).

Cell-cell contact promoted Cdc42 activation

Our results imply that Cdc42 expression must be higher in an intact epithelium compared to the injured state when MMP-1 is induced. Indeed, our previous studies found keratinocytes at higher confluence had less MMP-1 expression (Pilcher *et al.*, 1997). Therefore, we measured Cdc42 activation in keratinocytes plated on collagen as sub-confluent cultures and compared them to confluent monolayers. Our results showed that Cdc42 activity directly correlates with the re-establishment of cell-cell contacts (Fig. 6A & B).

Discussion

The controlled expression of MMP-1 by basal keratinocytes at the wound edge is critical for cell migration during re-epithelialization (Pilcher et al., 1997). However, over-expression of the collagenase is evident in a wide variety of non-healing chronic ulcerations (Pilcher *et al.*, 1997; Saarialho-Kere *et al.*, 1993; Schultz and Gibson, 2013; Weckroth *et al.*, 1996), and it is possible that a stoichiometric excess of this metalloproteinase may lead to unwanted proteolysis that impairs re-epithelialization. Thus, understanding how the expression of MMP-1 is controlled in normal wounds may shed light on pathways that become dysfunctional in chronic ulcerations. Here, using models that mirror normal repair process, we begun to define the intracellular pathways that transduce signals from extracellular matrix into the keratinocyte to start the migratory machinery in response to skin injury. Our

key findings are that i) interaction of keratinocytes with collagen, as occurs during injury, activated ERK to induce MMP-1 expression and cell migration; ii) Cdc42 negatively regulated MMP-1 expression by governing ERK activation; and iii) increased cell-cell contact augmented Cdc42 activation and effectively shuts off MMP-1 expression. Furthermore, we confirmed that the same mechanisms control MMP-1 expression in neonatal male and adult female keratinocytes (Fig. S1, S3).

Members of the MAPK family are activated upon skin epidermal injury *in vitro* and *in vivo* (Chen *et al.*, 2013; Kobayashi *et al.*, 2003). Additionally, ERK signaling is vital for the skin development, and abrogation of this pathway causes profound defects in epidermal development and keratinocyte migration resulting in tissue hypoplasia and barrier dysfunction (Li *et al.*, 2004; Scholl *et al.*, 2007). MAPK signaling has also been shown to regulate expression of other MMPs in epithelial cells (Cheng *et al.*, 2012; Nagai *et al.*, 2009; Tseng *et al.*, 2013; Uttamsingh *et al.*, 2008). Like most MMPs, production of MMP-1 is regulated primarily at transcription and is induced by both p38 and ERK in response to transforming growth factor β1 (TGF-β1) or tumor necrosis factor-α (TNF-α) (Johansson *et al.*, 2000; Ravanti *et al.*, 1999a; Ravanti *et al.*, 1999b; Ravanti *et al.*, 2001; Westermarck *et al.*, 2001; Xu *et al.*, 2001). Our findings indicate that cell-matrix interactions during the wound healing process (e.g., collagen ligation by keratinocyte) can also signal via MAP kinases to induce MMP-1 expression.

We found that MMP-1 expression in keratinocytes was specifically inhibited by Cdc42. Rho GTPase-dependent mechanisms modulating MMP-1 expression seem to be organ and cell-specific. For example, whereas rabbit synovial fibroblasts require Rac1 GTPase for expression of MMP-1, expression of MMP-1 by human dermal fibroblasts is modulated via Rac1 and Cdc42 (Deroanne *et al.*, 2005; Kheradmand *et al.*, 1998). Moreover, we show that Cdc42 had an inhibitory effect on ERK activation, which is consistent with findings from other groups studying other cell models (Deroanne *et al.*, 2005; Zuo *et al.*, 2011).

The newly injured epithelium has asymmetric cell-cell contracts, which drive directional migration toward the free edge (Desai *et al.*, 2009). Our data indicate that keratinocytes use cell-cell cues (or lack thereof) to attenuate Cdc42 activity, which induces MMP-1 expression. Indeed, MMP-1 expression is largely localized to the keratinocytes at the wound front that have reduced cell-cell contacts. Conversely, as the epidermal barrier is reconstituted after injury, cell-cell contacts are re-established causing keratinocytes to active Cdc42 and effectively turn off MMP-1 expression.

Cdc42 has several important effects to help maintain a proper epithelial surface (Desai *et al.*, 2009). Activated Cdc42 is recruited to adherens and tight junctions to help maintain cell-cell adhesion and establish the polarity in epithelial surfaces (Cunliffe *et al.*, 2012; Etienne-Manneville and Hall, 2002). Keratinocytes also require Cdc42 activity for secretion of laminin 332 and maintenance of the basement membrane (Wu *et al.*, 2006a). Therefore, as the injured skin completes its migration over the wound and re-establishes cell-cell contacts, Cdc42 activation serves several mutually beneficial purposes, which are to terminate migration while establishing a polarized epithelium that can create a new basement membrane.

Several reports from us and others demonstrated that the patterns and control of MMP-1 expression observed in *ex vivo* human skin wound models mirrors findings in actual human skin biopsies (Dumin *et al.*, 2001; Inoue *et al.*, 1995; Pilcher *et al.*, 1999; Sudbeck *et al.*, 1997a). Moreover, others have reported an agreement in data from cultured skin explants and *in vivo* situation (Yasuoka *et al.*, 2008). Despite these assurances, there are important caveats to consider. *In vivo*, the wounded setting is more complex than our *ex vivo* or keratinocyte models, in which piece of normal skin or cells are cultured under defined conditions. *In vivo*, the keratinocyte response to wounding can be shaped by numerous concurrent influences, such as leukocytes and other cell types, cytokines and other mediators, and microorganisms, all of which could affect how MMP-1 is controlled. However, the defined models allowed us to focused on how specific cell-matrix interactions – which have been established to be key regulators of not just MMP-1 expression but to the keratinocyte response to injury (Manohar *et al.*, 2004; Margadant *et al.*, 2010)– signal to control MMP-1 expression. (As MMP-1 is not in the mouse genome (Balbin *et al.*, 2001), we cannot easily validate our findings in mice.)

With skin injury, keratinocytes activate a migration phenotype largely through contextual clues such as ligation with type I collagen. As they complete their migration over the wound, mechanisms must be in place to shift toward cell differentiation. Collectively, our results show that keratinocytes respond to injury by suppressing Cdc42 activation, which in turn induces MMP-1 expression through ERK signaling. As cell-cell contact is re-established, the reverse occurs with augmented Cdc42 activity, which can switch the cell from migration to processes required for establishing the epidermal barrier. Wound healing is not simply about the active stages of repair, and improper control of these processes may lead to conditions such as hypertrophic scars, chronic ulcerations, and malignances. Hence, a better understanding of the healing process, and as such the role for ERK and Cdc42 that we showed in this study with focus on cell-matrix interaction, may raise the prospect for new treatment of some skin diseases.

Materials & Methods

Materials

Rat tail type I collagen was purchased from BD Bioscience (Bedford, MA). ERK inhibitors (PD98059, U0126) and the control inactive analog (U0124), p38 MAPK inhibitor (SB203580), and Rac1 inhibitor (NSC23766) were purchased from Calbiochem (La Jolla, CA). Curcumin, an inhibitor of JNK/SAPK, and the selective inhibitor for ROCK, Y-27632, were purchased from Sigma Chemical (St. Louis, MO). ML 141, an inhibitor of Cdc42, was obtained from Tocris Bioscience (Bristol, UK). Anti-Rac1 antibody (23A8) was purchased from Chemicon International (Temecula, CA). Phospho-p44/42 MAPK XP[TM] rabbit monoclonal antibody, p44/42 MAPK rabbit monoclonal antibody, Phosphoplus® p38 MAPK antibody, Phosphoplus® SAPK/JNK antibody, and the rabbit monoclonal antibodies for RhoA (67B9) and Cdc42 (11A11) were obtained from Cell Signaling Technology (Beverly, MA). Anti-MMP1 rabbit polyclonal antibody was purchased from Millipore (Temecula, CA).

Human Keratinocytes

Primary human keratinocytes were obtained from discarded neonatal foreskins of newborn males undergoing circumcision. (IRB approval for this has been granted by the University of Washington. Because we used exclusively discarded material, signed informed consent was not required.) For each experiment, keratinocytes were pooled from 3 or 4 donors. Keratinocytes were cultured on a feeder layer of irradiated 3T3 fibroblasts in Dulbecco's modified Eagle's medium containing 10% calf serum (Iuchi *et al.*, 2006; Rheinwald and Green, 1975). After the first passage, cells were cultured in serum-free, defined medium (Epilife; Invitrogen, Carlsbad, CA). Biological replicates of all experiments were done with cells at passage three to four. To expanding cell populations, keratinocytes were grown in low Ca^{2+} (0.06 mM). However, for experiments, the Ca^{2+} concentration was increased to 1.8 mM to promote differentiation. Keratinocyte were plated on tissue culture dishes coated with 100 μg/ml (equal to 5 μg/cm^2) type I collagen, which is necessary for matrix-induced activation.

Immuno-assays

Total proteins were solubilized in 1X NuPage LDS sample and reducing buffer and resolved by SDS-PAGE. Proteins were electrophoretically transferred to Immobilon™-P PVDF membrane (Sigma), and incubated with specific antibodies overnight at 4°C. Bound antibodies were visualized using horseradish peroxidase-linked secondary antibodies, followed by detection using SuperSignal West Pico Chemiluminescent substrate (Thermo Scientific, Rockford, IL) according to the manufacturer's instructions. The amount of MMP-1 in keratinocyte conditioned medium was measured by a human MMP-1 Duoset ELISA kit (R&D Systems, Minneapolis, MN) per manufacturer's instruction. Results were normalized to total cellular protein as quantified by BCA protein assay (Pierce, Rockford, IL). To detect active Cdc42, protein lysates (100 μg) was incubated with 20 μg GST-PBD fusion protein containing GST fused to the Cdc42 binding region of PAK1B on glutathione Sepharose beads (Cytoskeleton, Denver, CO). After 2 h incubation at 4°C, beads were pelleted and re-suspended in LDS sample buffer and denatured at 70°C for 10 min. Samples were analyzed by western blot procedure using the Cdc42 specific antibody.

Luciferase Reporter Assay

An expression construct containing the firefly luciferase reporter gene with a 2.2-kilobase pair fragment of the human *MMP1* promoter, pGL3-*MMP1*, was used to determine *MMP1* promoter activity. The *MMP1* promoter fragment was subcloned into the pGL3 basic luciferase reporter vector (Promega) via restriction digestion from a pCLCAT construct as described (Doyle *et al.*, 1997; Pilcher *et al.*, 1999; Sudbeck *et al.*, 1997b) and has been demonstrated to mirror transcription from the endogenous gene. Primary human keratinocytes were cultured on collagen and transfected at 75% confluence with 1.0 μg/well of pGL3-empty or pGL3-MMP-1, and *Renilla* pRL-SV40 control vector using 4 μl/well Lipofectamine (Invitrogen) according to manufacturer's instructions. After 16 h the medium was replaced and the cells were incubated with control or experimental solutions for an additional 24 h and harvested. Quantification of firefly and *Renilla* luciferase activities was accomplished using the Dual-Luciferase® Reporter Assay System (Promega) with a

TD20/20 luminometer (Turner Designs, Sunnyvale, CA) according to manufacturer's instructions.

mRNA Analyses

Total RNA was isolated using RNeasy mini kit (Qiagen, Valencia, CA) and was reverse transcribed using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Grand Island, NY) under the manufacturer's recommended conditions. Expression of RhoA, Rac1, Cdc42, and MMP-1 was quantified using qRT-PCR and normalized to GAPDH. TaqMan expression assays were purchased from Applied Biosystems.

Cell Migration Assay

To assess keratinocyte migration across type I collagen, chamber slides were pre-coated with a mixture of 100 μg/ml type I collagen and colloidal gold particles as described (Pilcher *et al.*, 1999). Keratinocytes were pre-incubated in the presence of control medium or 10 μM of U0126, SB203580, or curcumin before plating onto chamber slides. Non-adherent cells were removed 20 min after plating the cells, and the medium (+/− inhibitors) was replaced. Cells were incubated for 20 h, fixed in 4% formalin, washed in PBS, and dehydrated through graded ethanol. Paths of cell migration (phagokinetic tracks) were identified as areas devoid of gold particles. A migration index was determined using image analysis software by measuring the area of the phagokinetic tracks associated with cells in randomly chosen fields of view under dark-field illumination.

siRNA

SMARTpool siRNA reagents were purchased from Dharmacon (Lafayette, CO) to specifically knock down RhoA, Rac1, or Cdc42. As a control, cells were transfected with non-silencing siRNA. For transfection, cells were plated at 30% confluency in low calcium medium (0.06 mM Ca^{+2}), and after 6 h, cells were transfected with 30 nM siRNA for target genes using Lipofectamine 2000 (1μg/ml) (Invitrogen). After 1 day, the medium was replaced with fresh medium containing 1.8 mM Ca^{+2}. 48 h after transfection cells were harvested and analysis was done on cell lysate, total RNA or conditioned medium.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgment

Abbreviations

qRT-PCR	Quantitative reverse transcription polymerase chain reaction
ECM	extracellular matrix
MMP	matrix metalloproteinase

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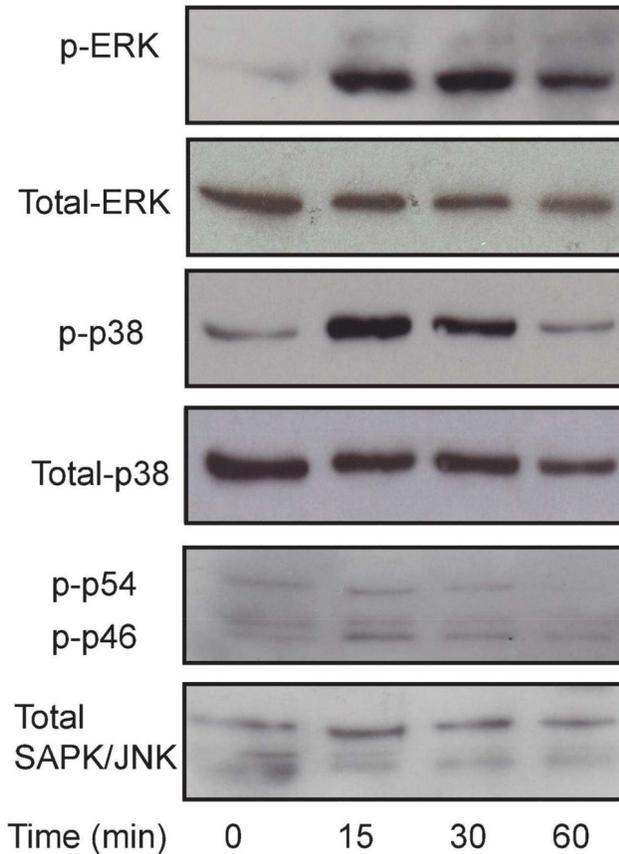
Figure 1. Keratinocyte ligation to fibrillar type I collagen induced phosphorylation of ERK and p38 MAPK but not JNK

Primary keratinocytes were plated on fibrillar type I collagen, and cell lysates were harvested at the designated time points. The 0-min cells were keratinocytes sampled before plating on collagen. Phosphorylated (p-) and total ERK, p38, and JNK were visualized by immunoblot using specific antibodies. Results are representative of three independent experiments with keratinocytes from 3 different batches of cells.
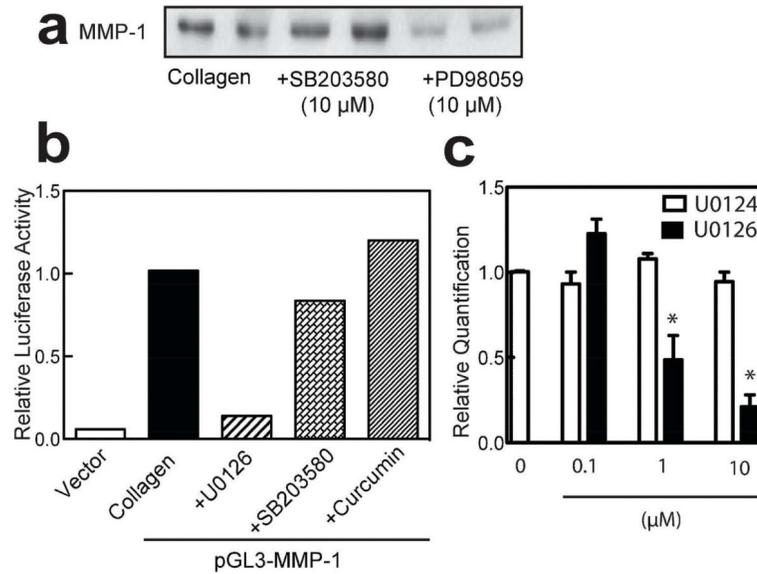
Figure 2. Collagen induction of MMP-1 by keratinocytes was mediated by ERK signaling
(a) Keratinocytes were pre-treated for 2 h with control medium, PD98059 (10 μM; ERK inhibitor), SB203580 (10 μM; p38 inhibitor). Then, cells were plated on type I collagen for 48 h. Equal volumes of conditioned medium were resolved by SDS-PAGE, and MMP-1 was detected by immunoblotting. The panel is representative of four independent experiments. (b) Keratinocytes cultured on collagen were transfected at 60% confluence with a 2.26kb human *MMP1* promoter construct containing the luciferase reporter gene and a construct containing the Renilla luciferase gene for normalization of transfection efficiency. After 16 h, keratinocytes were treated with vehicle control, U0126 (10 μM; ERK inhibitor), SB203580 (10 μM), or curcumin (10 μM; JNK inhibitor). This panel is representative of reproducible experiments. (c) Keratinocytes were pre-treated for 2 h with increasing concentrations of U0126, or its inactive analog U0124 (1-10 μM), and then plated on collagen-coated plate and incubated for 24 h in the presence of inhibitors. Expression of MMP1 was quantified using qRT-PCR method. (*$p < 0.05$, two way Anova followed by Bonferroni test).
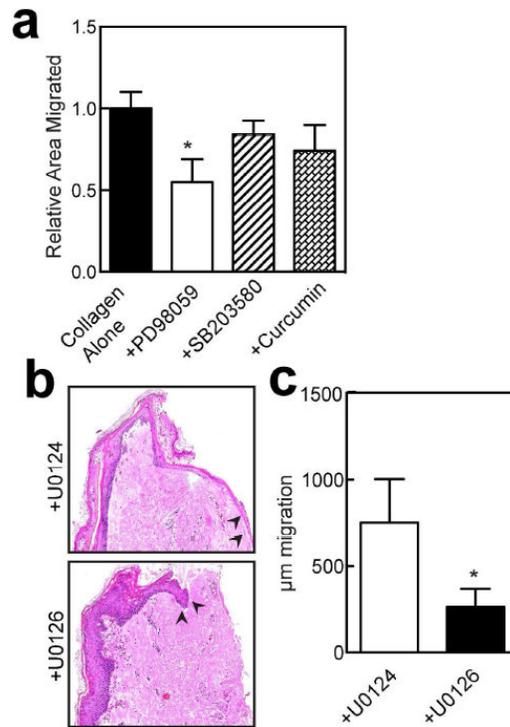
Figure 3. Keratinocyte migration across type I collagen required ERK signaling, and its blocking inhibited keratinocyte MMP-1 and migration in *ex vivo* wounded epidermis

(a) Keratinocytes were pre-treated for 2 h with ERK, p38 or JNK inhibitor (10 μM). After incubation, cells were plated on a colloidal gold-type I collagen substratum and allowed to migrate for 16 h. Relative area migrated was quantified by averaging the total phagokinetic track area in four separate fields of view per treatment group. *$p < 0.05$ by one way ANOVA followed by the Bonferroni post-test, n= 4. (b) Punch biopsies (5 mm) of normal human skin from neonatal foreskin were cultured for 4 days in serum free DMEM (formulated with 1.8 mM Ca^{2+}) in the presence of ERK inhibitor U0126 (10 μM) or its inactive analog (U0124) in air-liquid interface in 24 well transwell plate. The tissues were fixed, and stained with H&E. Arrowheads indicate the tip of migrating epithelium. (c) Epithelium migration was quantified in samples from four different donors (*$p < 0.05$, paired t-test).
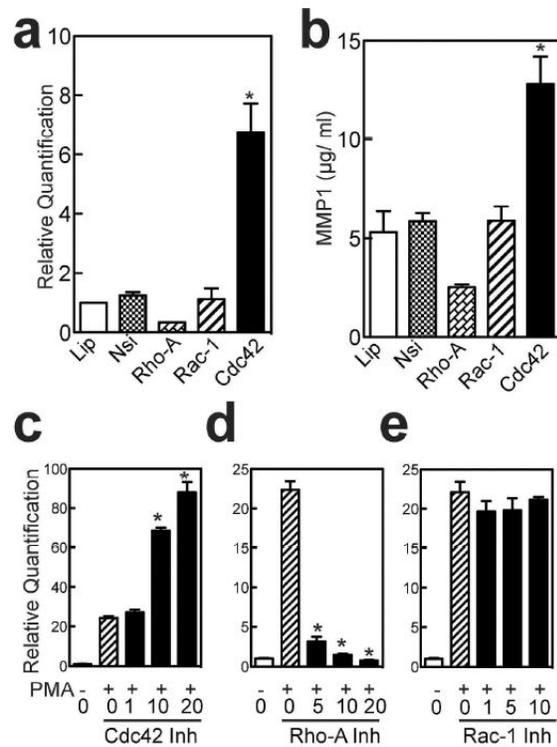
Figure 4. Rho GTPase signaling modulated MMP-1 expression
Keratinocytes plated on type I collagen were transfected with siRNA specific for RhoA,
Rac-1, Cdc42 or non-silencing siRNA. After 48 h, cells were harvested, and (a) MMP-1
expression was determined by QRT-PCR method; (b) MMP-1 secretion into the conditioned
medium was assessed by ELISA. (c-e) Keratinocytes plated on type I collagen were pre-
treated for 2 h with the small molecule inhibitors for (c) Cdc42, (d) ROCK, or (e) Rac-1.
Cells were stimulated for 6h with PMA (10 ng/ml), and then RNA was isolated and
processed for MMP-1 QRT-PCR. Data bars are mean ± SEM of normalized samples to
mock control (Lipofectamine 2000 [a,b] or untreated cells [c-e]). Nsi: Non silencing siRNA,
Lip: Lipofectamine 2000. *$p < 0.05$ by one way ANOVA followed by the Bonferroni post-
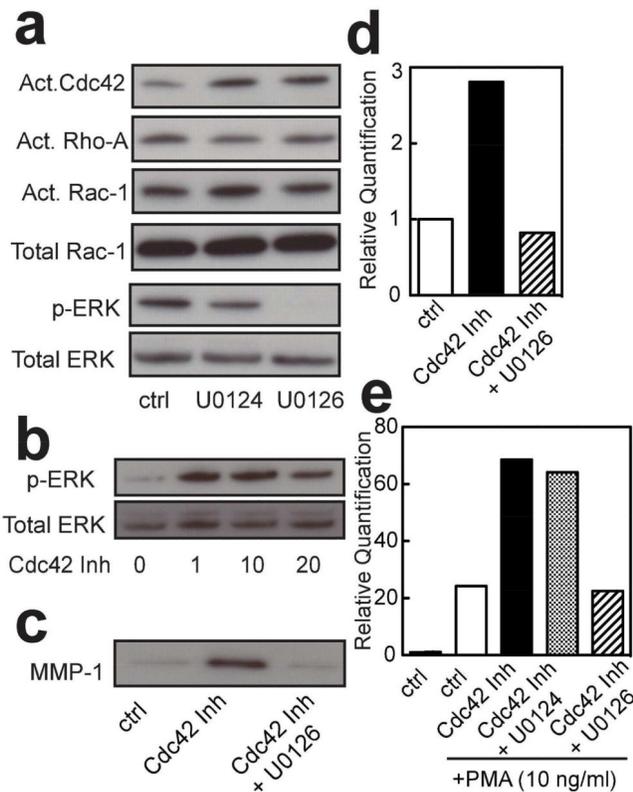test, n=3.

Figure 5. Cdc42 inhibited MMP-1 expression via ERK phosphorylation
(a) Cells pretreated with an ERK inhibitor (U0126; 0.5 μM) or its inactive analog (U0124; 0.5 μM) for 1 h at 4 °C and were plated on collagen and incubated at 37 °C for another hour before processing for immunoblots of active Cdc42, RhoA or Rac-1. (b) Keratinocytes were incubated with a Cdc42 inhibitor (ML 141; 1-20 μM) for 1 h at 4 °C prior to collagen stimulation for 30 min at 37 °C and then processed for ERK and phospho-ERK immunoblots. (d) Keratinocytes plated on collagen or (e) stimulated with PMA (10 ng/ml) were incubated with the inhibitors for ERK (U0126, 0.5 μM) and Cdc42 (ML 141, 10 μM). Expression of MMP-1 was assessed (c) in equal amount of conditioned medium with immunoblotting (after 48 hr) or (d, e) in total mRNA with QRT-PCR (after 8 h). Expression results were normalized to GAPDH. Panels are representative of reproducible experiments.
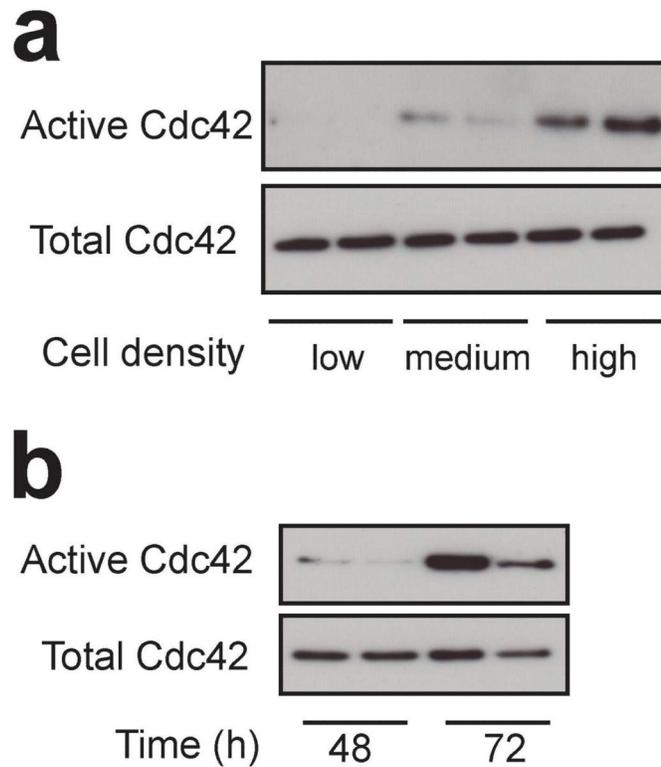
Figure 6. Cell-cell contact induces Cdc42 activation

(a) Keratinocytes were plated on collagen at low, medium, and high seeding densities (approximately 30%, 50%, and 70% confluent, respectively). After 24 h, cell lysates were harvested and equal amounts used for the Cdc42 activation assay. (b) Keratinocytes were plated at 30% confluence (low density) and allowed to proliferate to higher confluence for 48 h and 72 h before harvesting for the Cdc42 activation assay. These are representative figures of reproducible results.