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Keratinocyte Differentiation Is Regulated by the Rho and ROCK Signaling Pathway

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Summary

The epidermis comprises multiple layers of specialized epithelial cells called keratinocytes. As cells are lost from the outermost epidermal layers, they are replaced through terminal differentiation, in which keratinocytes of the basal layer cease proliferating, migrate upwards, and eventually reach the outermost cornified layers. Normal homeostasis of the epidermis requires that the balance between proliferation and differentiation be tightly regulated [1]. The GTP binding protein RhoA plays a fundamental role in the regulation of the actin cytoskeleton and in the adhesion events that are critically important to normal tissue homeostasis [2]. Two central mediators of the signals from RhoA are the ROCK serine/threonine kinases ROCK-I and ROCK-II [3]. We have analyzed ROCK's role in the regulation of epidermal keratinocyte function by using a pharmacological inhibitor and expressing conditionally active or inactive forms of ROCK-II in primary human keratinocytes. We report that blocking ROCK function results in inhibition of keratinocyte terminal differentiation and an increase in cell proliferation. In contrast, activation of ROCK-II in keratinocytes results in cell cycle arrest and an increase in the expression of a number of genes associated with terminal differentiation. Thus, these results indicate that ROCK plays a critical role in regulating the balance between proliferation and differentiation in human keratinocytes.

Results and Discussion

Inhibition of ROCK Prevents Keratinocyte Terminal Differentiation

C3 ADP-ribosyltransferase (C3), derived from *Clostridium botulinum*, irreversibly inactivates all three isoforms of Rho (RhoA, -B, and -C) [4]. It has previously been reported that EDIN, a *Staphylococcus aureus* homolog of C3, inhibits keratinocyte differentiation [5]. We asked whether inhibiting Rho function by using C3 affected keratinocyte terminal differentiation by placing keratinocytes in single-cell suspension for 24 hr in the presence or absence of recombinant C3. Treatment of differentiating cells with 5 $\mu\text{g ml}^{-1}$ C3 resulted in a statistically significant ($p < 0.001$) decrease in the number of cells expressing Involucrin, a marker for keratinocyte differentiation (Figure 1A) [6]. We observed a similar decrease when we used Transglutaminase-1 as an alternative marker for keratinocyte differentiation (Figure 1B) [6]. Having observed regulation of keratinocyte differentiation by C3, we analyzed whether patterns of RhoA expression and activity changed during keratinocyte differentiation. No change in expression of RhoA was observed when keratinocytes were induced to undergo terminal differentiation by culture in single-cell suspension (Figure 1C). Similarly, using an in vitro assay in which the Rho binding domain of Rhotekin was used to pulldown Rho in its active GTP bound form, we observed no significant difference in RhoA activity during the differentiation process (Figure 1D). This is in contrast to a recent study that reported increased Rho activity during calcium-induced keratinocyte differentiation [7]. The reasons for this discrepancy are not clear but may reflect differing experimental conditions.

One of the principal downstream effectors of Rho is the serine/threonine kinase, ROCK. Both isoforms of ROCK are expressed in keratinocytes, but the predominant isoform is ROCK-II (data not shown). To indirectly analyze ROCK activity during suspension-induced keratinocyte differentiation, we used a phospho-specific antibody against Myosin Phosphatase Target Subunit 1 (MYPT1), a known substrate of ROCK [8]. Although expression of ROCK-II did not significantly change during keratinocyte differentiation, a significant increase in phosphorylation of MYPT1 was observed (Figure 1E). To analyze further the role of ROCK, we used Y-27632, a pharmacological inhibitor of ROCK [9]. Cells were placed in suspension in the presence of increasing concentrations of Y-27632 for 12 hr, and differentiation was assessed by analysis of Involucrin expression. Statistically significant ($p < 0.05$) inhibition of differentiation was observed in the presence of 1 μM Y-27632, with almost complete inhibition in the presence of 5 μM or 10 μM Y-27632 (Figure 1F). No evidence of increased cell death was observed (data not shown). These data are consistent with ROCK acting downstream of RhoA to regulate keratinocyte differentiation.

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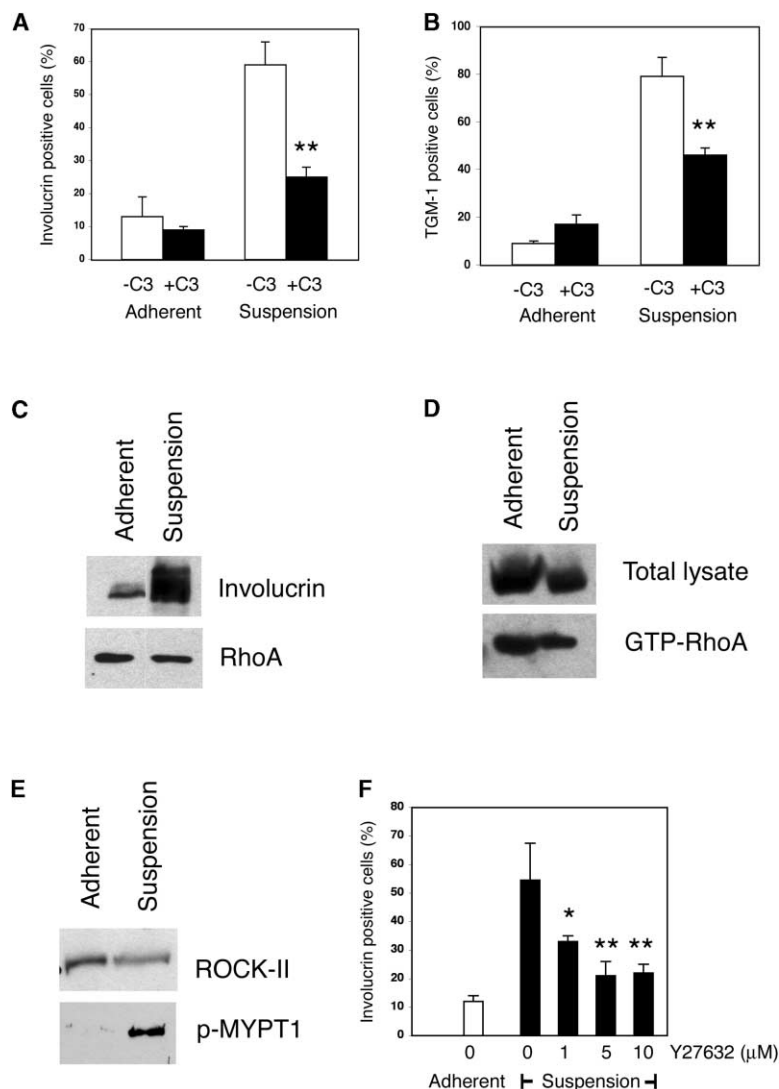


Figure 1. ROCK Acts Downstream of Rho to Regulate Keratinocyte Differentiation

(A and B) To induce terminal differentiation, we placed cells in single-cell suspension in the presence or absence of 5 $\mu\text{g/ml}$ C3 for 24 hr. After harvesting, cells were immunostained with antibodies against Invulcrin (A) or Transglutaminase-1 (TGM-1) (B), and the percentage of positive cells was calculated. (C) Cell lysates were prepared from adherent, preconfluent keratinocytes, and cells were induced to differentiate by being placed in single-cell suspension for 24 hr. Expression of invulcrin and RhoA was detected by Western blotting. (D) The Rho binding domain of Rhotekin was used for precipitating GTP bound active RhoA from cell lysates. The GTP bound RhoA was then detected with RhoA-specific antibodies after SDS-PAGE and Western blotting. As controls, total cell lysates were also prepared and Western blotted with an antibody to RhoA. (E) Cell lysates were prepared from adherent, preconfluent keratinocytes, and cells were induced to differentiate by placement in single-cell suspension for 24 hr. Expression of ROCK-II and phospho-MYPT1 was detected by Western blotting with specific antibodies. (F) The effect of ROCK inhibition on keratinocyte differentiation was assessed via placement of cells in suspension in the presence of increasing concentrations (1–10 μM) of the pharmacological inhibitor Y-27632 for 12 hr. After harvesting, cells were immunostained and invulcrin-positive cells were counted. Results from (A), (B), and (F) are the mean and standard error of three separate experiments, and statistical comparisons were made via the Student's T-test (* $p < 0.05$; ** $p < 0.001$).

Inhibition of ROCK Results in Increased Keratinocyte Proliferation

Having observed that pharmacological inhibition of ROCK significantly inhibited keratinocyte differentiation, we analyzed cell cycle progression by examining the incorporation of the nucleoside analog bromodeoxyuridine (BrdU) into nascent DNA. Subconfluent keratinocytes were incubated in BrdU-labeling medium in the presence or absence of 5 μM Y-27632 for 1, 8, 12, and 16 hr and then immunostained with an antibody against BrdU. After 12 hr of Y-27632 treatment, a statistically significant increase ($p < 0.001$) in the number of BrdU-positive cells was observed in treated versus untreated control cells (Figure 2A). The increased proportion of S phase cells suggested that inhibition of ROCK might increase the proliferative capacity of these cells. To analyze this, we determined the colony-forming efficiencies of keratinocytes cultured in the presence or absence of 5 μM Y-27632. We observed a statistically significant increase ($p < 0.05$) in the number of keratinocyte colo-

nies present after 14 days of culture in the presence of Y-27632 (Figure 2B). Thus, we observed that inactivation of ROCK resulted both in inhibition of keratinocyte terminal differentiation and in a increase in proliferative capacity. Although it is not possible to establish the exact timing of events, this clearly suggests that ROCK plays a key role in determining keratinocyte fate. In some cell types there is evidence that Rho and Rac act antagonistically and that Rac1 activity is required for cell cycle progression [10–12]. Thus, the possibility existed that ROCK inhibition might result in altered Rac1 activity and that this might contribute to the observed increase in proliferation. However, pulldown assays that utilized the ability of the Rac binding domain of PAK1 to associate with active GTP bound but not inactive GDP bound Rac1 revealed no difference in Rac1 activity in cells treated with Y-27632 (Figure 2C). Thus, it is unlikely that the inhibition of differentiation and increase in proliferation we observed were a consequence of altered Rac1 activity.

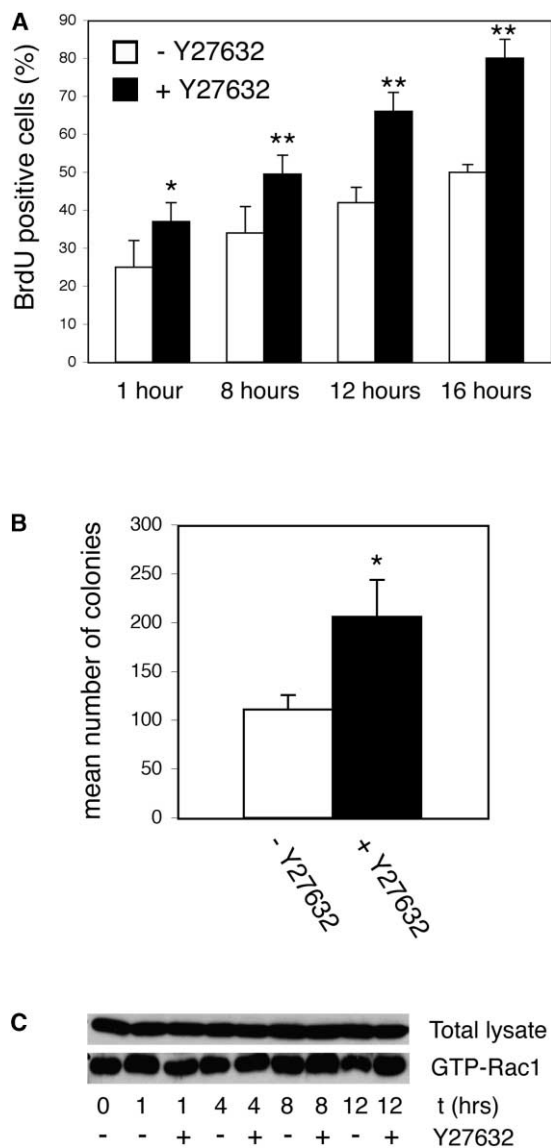


Figure 2. Inhibition of ROCK Results in Increased Cell Cycle Progression without Altering Rac Activity

(A) Cells were incubated with BrdU and 5 μ M Y-27632 for 1, 8, 12, and 16 hr. Cells incorporating BrdU were detected by indirect immunofluorescence. Data presented are the mean and standard error of three separate experiments, and statistical significance was determined via a Student's T-test (* $p < 0.05$; ** $p < 0.001$) (B). Proliferative capacity in the presence or absence of Y-27632 was assessed from colony-forming efficiency. Keratinocytes were seeded at low density and cultured in the presence or absence of 5 μ M Y-27632 for 14 days. After this time, cells were fixed, stained with 1% rhodanile blue, and the number of colonies was counted. Data are the mean and standard error from three separate experiments (* $p < 0.05$). (C) For the analysis of Rac activity, cells were treated with 5 μ M Y-27632 for 0, 1, 4, 8, and 12 hr and lysed, and the Rac binding domain of PAK was used for precipitation of GTP bound active Rac from lysates. GTP-Rac was subsequently detected via SDS-PAGE and Western blotting.

Activation of ROCK-II Results in Cell Cycle Arrest and Increased Expression of Involucrin

To analyze further the role of ROCK in regulating keratinocyte function, retroviral transduction was used to establish primary human keratinocytes stably expressing a chimeric protein consisting of the catalytic domain of ROCK-II fused at the carboxy-terminal end to a mutated ligand binding domain of the estrogen receptor (ER) and to enhanced green fluorescence protein at the amino terminus (D.R.C. and M.F.O., unpublished data). The ROCK:ER fusion protein is constitutively expressed but is inactive because of the binding of a heat shock protein (HSP90) to the ER ligand binding domain [13]. Addition of the estrogen homolog, 4-hydroxytamoxifen (4-HT), results in displacement of HSP90 and activation of the kinase. In addition, cell lines stably expressing a kinase-dead form of ROCK-II (KD ROCK:ER) were established. To analyze ROCK-II activity, we performed in vitro kinase assays in which we assessed the ability of immunoprecipitated ROCK-II to phosphorylate Histone H1. ROCK-II activity was induced 7-fold by 4-HT in lysates prepared from ROCK:ER cells (Figure 3A), whereas no significant ROCK activity was observed in the absence or presence of 4-HT in the KD ROCK:ER control lysates (Figure 3A). We saw no evidence that 4-HT affected expression of ROCK-II (Figure 3B).

Having observed that Y-27632 treatment resulted in increased keratinocyte cell cycle progression (Figure 2), we analyzed BrdU incorporation in keratinocytes expressing ROCK:ER in the presence or absence of 4-HT. Significant ($p < 0.001$) inhibition of BrdU incorporation was observed in ROCK:ER-expressing cells after treatment with 4-HT compared to no 4-HT (Figure 3C). No inhibition of cell cycle progression was observed in cells expressing the kinase-dead form of ROCK-II when they were treated with 4-HT (data not shown). Previous work has indicated that RhoA is required for cell cycle progression in Swiss 3T3 fibroblasts [10]. The observation that, in primary keratinocytes, activation of ROCK-II results in cell cycle arrest is novel and suggests that the role of Rho signaling in cell cycle progression may be cell-type specific.

We next analyzed the effect of activating ROCK-II on keratinocyte differentiation. We induced keratinocytes expressing ROCK:ER to undergo terminal differentiation by placing cells in suspension, in the presence or absence of 100 nM 4-HT, for 24 hr. No inhibition of differentiation was observed after activation of ROCK-II. (Figure 1, Supplemental Data). Having observed that activation of ROCK-II did not affect the ability of cells to undergo differentiation when in suspension, we analyzed expression of Involucrin in adherent keratinocytes expressing KD ROCK:ER and ROCK:ER in the presence or absence of 4-HT. In the presence of 4-HT, we observed a statistically significant increase ($p < 0.05$) in the numbers of adherent ROCK:ER cells expressing Involucrin but no increase in numbers of Involucrin-positive KD ROCK:ER cells (Figure 3D). Thus, these data indicate that ROCK is involved not only in the regulation of suspension-induced differentiation but also in the initiation of differentiation in adherent keratinocytes.

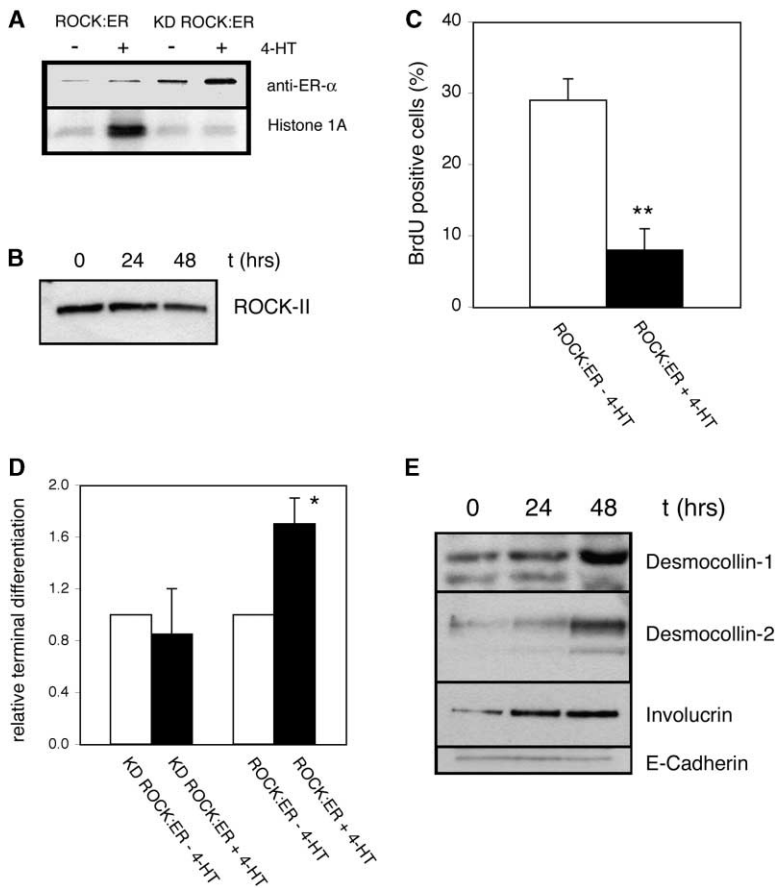


Figure 3. Activation of ROCK-II Inhibits Cell Cycle Progression and Increases Expression of Involucrin in Normal Adherent Keratinocytes

(A) The activity of exogenous ROCK-II was assessed via an in vitro kinase assay in which the ability of immunoprecipitated ROCK-II to phosphorylate an artificial substrate, Histone H1, was measured. Cells expressing kinase-dead ROCK-II (KD ROCK) or active ROCK-II (ROCK) were incubated in the presence or absence of 100 nM 4-HT for 12 hr and lysed, and the kinase assay was performed. (B) Cells expressing active ROCK-II were cultured in the presence of 100 nM 4-HT for up to 48 h, and protein lysates were prepared. Expression of ROCK-II was determined by Western blotting. (C) Cell cycle progression after activation of ROCK was assessed via BrdU incorporation during DNA synthesis. Cells expressing ROCK:ER were incubated in the presence or absence of 100 nM 4-HT for 12 hr and then BrdU labeled for 1 hr. The number of BrdU-positive cells was determined by indirect immunofluorescence, and data presented are the mean and standard error of three separate experiments. Statistical analysis was performed via the Student's T-test (**p < 0.001). (D) Adherent keratinocytes expressing wild-type ROCK-II (ROCK) or the kinase-dead form of ROCK-II (KD ROCK) were incubated in the absence or presence of 100 nM 4-HT for 12 hr and then immunostained for the differentiation marker involucrin. Numbers of involucrin-positive cells were counted, and results were expressed relative to cells cultured in the absence of 4-HT. Data are the mean and standard error of three separate experiments. Statistical analysis was performed via the Student's T-test (*p < 0.05). (E) Protein lysates were prepared from adherent keratinocytes that expressed the conditionally active form of ROCK (ROCK:ER) and had been cultured in the presence of 100 nM 4-HT for 0, 24, and 48 hr. Expression of Involucrin, Desmocollin-1, Desmocollin-2, and E-Cadherin was determined by Western blotting.

Activation of ROCK-II in Human Keratinocytes Induces Expression of a Number of Differentiation Markers

To determine further the role of ROCK-II in regulating keratinocyte function, we used Affymetrix gene arrays

to analyze gene expression after activation of ROCK-II. Initially, as a control, we analyzed the gene expression profile of keratinocytes expressing the kinase-dead form of ROCK-II (KD ROCK:ER) in the presence or absence of 4-HT. Analysis of data from this experiment revealed

Table 1. Differentiation-Related Genes Upregulated More than 2-Fold upon Activation of ROCK-II

Description	Gene	CE	EDC	Reference
Involucrin	IVL	+	+	[6]
Keratin 1	KRT1	+	-	[6]
Keratin 10	KRT10	+	-	[6]
Desmocollin 1	DSC1	-	-	[15]
Desmocollin 2	DSC2	-	-	[15]
Desmoglein 1	DSG1	-	-	[16]
Desmoglein 3	DSG3	-	-	[16]
Desmoplakin I/II	DPI/DPII	+	-	[6]
Serine protease inhibitor kazal type 5	SPINK5	-	-	[19]
Elafin	PI3	+	-	[6]
Fatty acid binding protein 5	FABP5	-	-	[20]
Small proline-rich protein 3	SPRR3	+	+	[6]
Psoriasin 1	S100A7	+	+	[6]
Calgranulin A	S100A8	+	+	[6]
Sciellin	SCEL	+	-	[14]
Periplakin	PPL	+	-	[6]

CE indicates genes encoding proteins involved in formation of the epidermal cornified envelope. EDC indicates genes clustered in the Epidermal Differentiation Complex located on chromosome 1q21.

only 14 genes with an increase in expression greater than 1.5-fold and three genes with a decrease in expression greater than 1.5-fold. Only one gene demonstrated a greater-than-2-fold change in expression (see Appendix 1, Supplemental Data). We next compared the gene expression profile of the KD ROCK:ER cells cultured in the presence of 4-HT with those expressing ROCK:ER cultured in the presence of 4-HT. Analysis of these data revealed 210 genes with a greater-than-2-fold increase in expression and 105 genes with a greater-than-2-fold decrease in expression (Appendix 2, Supplemental Data). Analysis of these data revealed upregulation of a number of genes encoding proteins associated with keratinocyte differentiation (Table 1). These included Involucrin and a number of other genes involved in cornified envelope formation, including those encoding Desmoplakin I/II, SPRR3, Elafin, Sciellin, and Periplakin [6, 14]. In addition, we also observed increases in expression of the genes encoding suprabasal keratins and desmosomal proteins, all of which have been demonstrated to be expressed solely in suprabasal, differentiating cells or to be upregulated during differentiation [6, 15, 16]. Interestingly, a number of these genes are clustered together on chromosome 1 (1q21) in a region known as the "Epidermal Differentiation Complex" [17]. To confirm that transcriptional changes were reflected in altered protein expression, we prepared protein lysates from ROCK:ER keratinocytes cultured for 24 and 48 hr in the presence of 4-HT. We observed increased expression of Involucrin, Desmocollin-1, and Desmocollin-2 48 hr after addition of 4-HT, confirming that activation of ROCK results in increased expression of proteins associated with keratinocyte differentiation (Figure 3E). As a control, we analyzed expression of E-Cadherin by Western blotting with no change in protein expression observed (Figure 3E).

Thus, expression of an activated form of ROCK-II in human keratinocytes inhibited cell cycle progression and led to increased expression of a number of genes associated with keratinocyte differentiation. A recent report implicates ROCK in the cytoskeletal changes that take place during epidermal stratification [18]. Our data suggest a more fundamental role for ROCK in regulating keratinocyte fate.

Supplemental Data

Supplemental Experimental Procedures as well as a supplemental figure and two appendices are available with this article online at <http://www.current-biology.com/cgi/content/full/13/24/2185/DC1/>.

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