

RhoGTPase Activation Is a Key Step in Renal Epithelial Mesenchymal Transdifferentiation

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ESRD is characterized by an interstitial infiltrate of inflammatory cells in association with tubular atrophy, epithelial mesenchymal transdifferentiation (EMT), and interstitial fibrosis. Human proximal tubular epithelial cells (HK2 cells) undergo EMT in response to activated PBMC conditioned medium (aPBMC-CM), showing acquisition of a fibroblastoid morphology, increased fibronectin-EDA (EDA) expression, loss of junctional E-cadherin localization, and cytokeratin 19 (K19) expression. The signaling pathway(s) that regulates EMT in response to aPBMC-CM is not well understood. This study shows that aPBMC-CM induces a rapid activation of RhoA, Rac1, and Cdc42 activity in HK2 cells from 15 min to 48 h. Moreover, infection with adenovirus expressing constitutively active RhoA, Rac1, and Cdc42 significantly increased the expression of EDA and downregulated expression of E-cadherin and K19. Dominant negative RhoA expression suppressed aPBMC-CM-induced upregulation of EDA but did not restore the expression of E-cadherin and K19. Constitutively active RhoA activated the Rho kinase and its downstream effectors, whereas constitutively active Rac1 and Cdc42 activated the P21-activated protein kinase in epithelial cells. In further experiments, HK2 cells were treated with toxin B, exoenzyme C3, Y-27632, and HA1077. These strategies, inhibiting the Rho/Rho kinase pathway, as well as the Rac1/Cdc42/P21-activated protein kinase pathway, blocked transdifferentiation of HK2 cells in response to aPBMC-CM. To conclude, these results indicate that aPBMC-CM activates RhoA, Rac1, and Cdc42 and their downstream effectors, leading to HK2 cells undergoing transdifferentiation. Therefore, activation of small RhoGTPases is a key step in the mechanism of EMT and likely to be a contributor to tubulointerstitial fibrosis.

J Am Soc Nephrol 16: 1977–1984, 2005. doi: 10.1681/ASN.2004110943

After appropriate stimulation, differentiated epithelial cells are able to adopt a mesenchymal genetic program and form mesenchymal-like cells, such as myofibroblasts, *via* the process of epithelial mesenchymal transdifferentiation (EMT) (1–3). In recent years, an increasing amount of data indicate that EMT may play a pivotal role in the pathogenesis of fibrotic diseases in which myofibroblastic cells become prominent (4–7). Thus, understanding the molecular mechanism of EMT may provide opportunities to formulate new therapeutic interventions.

In a previous study, we investigated the effect of activated PBMC conditioned medium (aPBMC-CM) on human proximal tubular epithelial cell to myofibroblast transdifferentiation. We quantified some putatively active components of aPBMC-CM, including TGF- β 1, EGF, IL-1 β , IL-2, IL-6, OSM, GM-CSF, TNF- α , and INF- γ (8). Neutralizing antibodies to each of these did not provide significant protection against the changes initiated by aPBMC-CM. For example, immunoneutralization of

TGF- β 1 in aPBMC-CM resulted in only 20% inhibition of EMT (8). The results suggested that multiple cytokines, including TGF- β 1, simultaneously activate a number of cellular receptors, initiating signals through key molecular cascades that finally lead to transcriptional regulation of EMT gene expression.

Anticipating that aPBMC-CM would trigger complex changes, we used microarray analysis to uncover possible common pathways that drive EMT. A group of small RhoGTPases, including RhoE, RhoG, RhoA, Rac1, and Cdc42, were found to be associated with aPBMC-CM-mediated EMT. Small RhoGTPases are a subset of the Ras superfamily of GTPases and consist of six subfamilies, including the RhoA-related subfamily, the Rac1-related subfamily, and the Cdc42-related subfamily and others (9). RhoGTPases cycle between an active GTP-bound state and an inactive GDP-bound state (9,10), a process controlled by specialized regulators, Rho guanine nucleotide exchange factors (RhoGEF), which are transiently activated by receptor tyrosine kinases (10). Thus, active RhoGTPases transduce signals from cell-surface receptors to intracellular target molecules and function as essential components of signal transduction pathways that regulate cell growth and differentiation (9). It is known that the RhoGTPases participate in the regulation of both cadherin-mediated cell–cell adhesion (11) and the actin

Received November 16, 2004. Accepted April 12, 2005.

Published online ahead of print. Publication date available at www.jasn.org.

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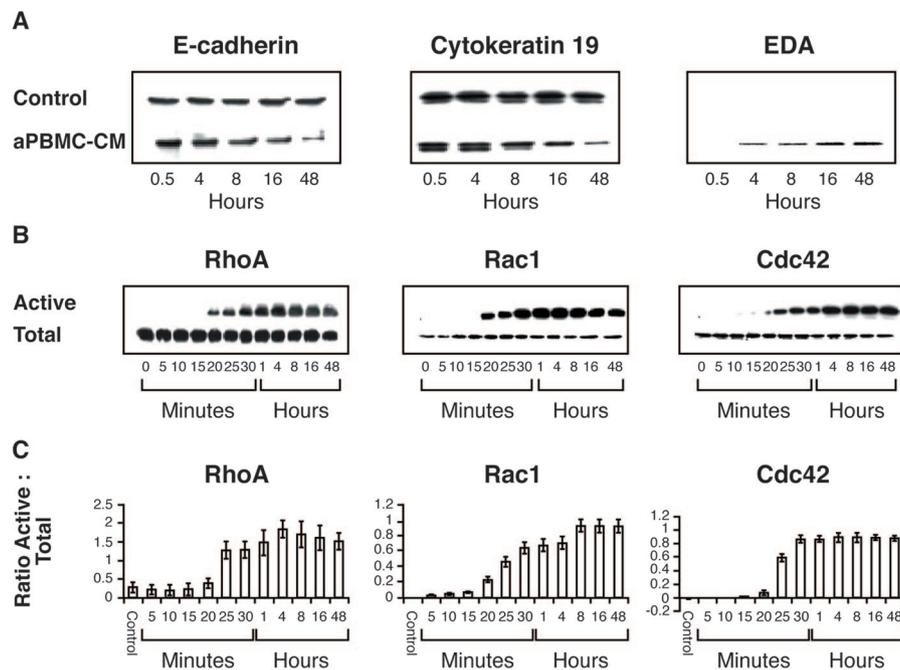


Figure 1. Activated PBMC conditioned medium (aPBMC-CM) induces human kidney tubular epithelial cells (HK2) to undergo transdifferentiation (A) and activates small RhoGTPases (B and C) in a time-dependent manner. (A) Cells were treated with 30% aPBMC-CM over 0.5 to 48 h. The epithelial markers E-cadherin and cytokeratin 19 (K19) and myofibroblast marker EDA were determined. (B) aPBMC-CM induced activation of RhoA, Rac1, and Cdc42 during the treatment period. A pull-down assay was used to capture the active form of the proteins. Twenty micrograms of total protein was loaded in each lane. (C) Densitometric analysis of Western blots (mean \pm 2 SD, $n = 3$) shows the change of band intensities for the ratio between active and total RhoA, Rac1, and Cdc42.

cytoskeleton (9), suggesting that they may play key roles in EMT (12). In this study, we examined the role of RhoA, Rac1, and Cdc42 in EMT of human renal epithelial cells (HK2) induced by aPBMC-CM.

Materials and Methods

Mouse mAb against human E-cadherin, Rac1 (Clone 23A8, which recognizes only 21-kD Rac from human, rat, and mouse), Cdc42 (sc-87, which recognizes only Cdc42 from human, rat, and mouse), phospho-paxillin, and phospho-FAK were purchased from BD Transduction Laboratory (Oxford, UK). Mouse monoclonal anti-human fibronectin-EDA⁺ (EDA) and anti-cytokeratin 19 (K19) were from Abcam Ltd. (Cambridge, UK). Rabbit polyclonal anti-human RhoA (sc-179, which recognizes only p21 RhoA from human, rat, and mouse) and anti-phospho-myosin light chain (Thr18/Ser19) were from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal anti-human phospho-PAK1 (Ser199/204) was from New England Biolabs (Hertfordshire, UK). Rabbit anti-mouse Ig-horseradish peroxidase and peroxidase-conjugated goat anti-rabbit Ig were from DAKO Ltd. (Cambridgeshire, UK). Constitutively active RhoA cDNA (RhoAQ63L), dominant negative RhoA cDNA (RhoAT19N), constitutively active Rac1 cDNA (Rac1Q61L), constitutively active Cdc42 cDNA (Cdc42Q61L), Rhoketin Rho binding domain-agarose (for active RhoA), and PAK1-PBD-agarose (for active Rac1 and Cdc42) were obtained from Upstate Biotechnology (Milton Keynes, UK). *Clostridium botulinum* exoenzyme C3 (C3), *Clostridium difficile* toxin B (toxin B), HA1077, and Y-27632 were from Calbiochem (Lutterworth, UK).

Cell Culture and Treatment

HK2 cells were cultured and aPBMC-CM prepared as reported previously (8). HK2 cell cultures at approximately 70% confluence were treated with 30% aPBMC-CM in REGM (BioWhittaker Inc., Walkersville, MD). After treatment (5 min to 48 h), the medium was collected and the cells were processed for analysis by SDS-PAGE and Western blotting. For inhibition experiments, the cells were pretreated for 1 h with 10 μ M HA1077 or 10 μ M Y-27632 or for 24 h with 100 μ g/ml toxin B or 100 μ g/ml Exoenzyme C3 before the addition of 30% aPBMC-CM. AD293 cells (Stratagene, Amsterdam, Netherlands) were maintained in complete DMEM, supplemented with 10% (vol/vol) heat-inactivated FCS (Life Technologies, Paisley, UK) at 37°C in an atmosphere of 5% CO₂ and were passaged when confluent.

SDS-PAGE and Western Blotting

Cell lysates were prepared in either ice-cold M-PER (Pierce, Cheshire, UK) or RIPA buffer supplemented with proteinase inhibitors (8), protein was measured using the BCA kit (Pierce), and SDS-PAGE and Western blotting were performed as described previously (8).

Pull-Down Assay for Active RhoGTPases

Rhoketin Rho binding domain-agarose beads and PAK1-P21-binding domain-agarose beads were used to pull down GTP-Rho and GTP-Rac/Cdc42, respectively, following the manufacturer's instructions. Active RhoA, Rac1, and Cdc42 were detected by Western blotting with antibodies specific for RhoA, Rac1, and Cdc42.

Generation of Recombinant Adenoviruses

Replication-deficient recombinant adenoviruses carrying expression plasmids encoding RhoAQ63L, Rac1Q61L, Cdc42Q61L, and RhoAT19N were generated using the AdEasy XL Adenoviral vector system (Stratagene). Briefly, the corresponding cDNA were cloned into the adenoviral transfer vector, pShuttle-IRES-hrGFP-1 (Stratagene), allowing co-expression of GFP with the FLAG-tagged RhoGTPase mutants. After recombination of linearized shuttle vector and adenovirus backbone vector in *Escherichia coli* BJ5183, the recombined adenoviral constructs were transfected into XL10 Gold (Stratagene) to allow higher plasmid yield. The resultant recombinant virusmids then were transfected into AD293 (Stratagene). Crude lysates that contained recombinant adenoviruses were prepared as viral stocks and used to infect AD-293 cells for amplification. Adenoviruses were purified using the Adeno-X virus purification kit (BD Transduction). Primary viral stock was serially diluted with complete DMEM supplemented with 10% FCS, and the diluted virus was applied to AD-293 cells that were plated in six-well plates. After incubation, the medium was removed and the cells were overlaid with 1.25% agarose/growth medium. Plates were incubated at 37°C until plaques formed. Plaques were counted under a light microscope.

Adenoviral Infection

HK2 cells (2×10^5 /6-cm dish) were infected with the recombinant adenovirus at titers ranging from a multiplicity of infection (MOI) of 20 to 50 and then incubated in complete REBM supplemented with 0.5% FCS for 48 h. Cells were viewed under an ultraviolet microscope to assess GFP expression. Cells that were infected with dominant negative RhoA were stimulated further with 30% aPBMC-CM for 24 h. The lysates were subjected to pull-down assay for active RhoGTPases and Western blot analysis for EMT markers.

Statistical Analyses

Data are expressed as mean \pm 2 SD, unless otherwise stated. Comparisons between control and treated samples were performed using *t* test for paired data. Values of *P* < 0.05 were considered significant.

Results

aPBMC-CM Induces EMT via the Activation of RhoA, Rac1, and Cdc42

Figure 1A shows the changes in the epithelial cell markers E-cadherin and K19 and in the myofibroblast marker EDA after the addition of 30% aPBMC-CM for various periods (0.5 to 48 h). As reported previously (8), epithelial markers were lost with time, whereas the myofibroblast marker was increasingly expressed. The effect of aPBMC-CM on the activation of RhoA, Rac1, and Cdc42 in HK2 cells was determined by Western blot analysis after affinity precipitation (Figure 1B). This showed that the three RhoGTPases were activated from 25 min up to 48 h after stimulation (Figure 1C) and that activation occurred earlier than the changes of EMT markers.

Inhibitors were used to examine whether activation of RhoGTPases is associated with aPBMC-CM-mediated EMT. HK2 cells were pretreated with either C3 or toxin B for 24 h before stimulation with aPBMC-CM. C3 is regarded as a RhoA subfamily inhibitor (13), whereas toxin B inhibits almost all members of the RhoGTPases, including RhoA-, Rac1-, and Cdc42-related subfamilies (14). There were no morphologic changes in the cells that were treated with inhibitors, and MTS assay showed that they had no effect on HK2 cell viability (data

not shown). Activation of RhoA after aPBMC-CM stimulation was substantially suppressed in the presence of either inhibitor (Figure 2, A and C). Toxin B but not C3 inhibited the aPBMC-CM-mediated activation of Rac1 and Cdc42. Toxin B blocked the loss of E-cadherin and K19 and gain of EDA (Figure 2, B and D). C3 prevented gain of EDA but had no effect on loss of E-cadherin and K19. HA1077 and Y27632, inhibitors of Rho kinase (ROCK) (15), a downstream target of RhoA activation, had no effect on aPBMC-CM-induced activation of RhoA, Rac1, and Cdc42; however, they prevented gain of EDA but had no effect on E-cadherin and K19. These data suggest that the transdifferentiation that occurs in response to aPBMC-CM is dependent on signaling through activation of RhoGTPases.

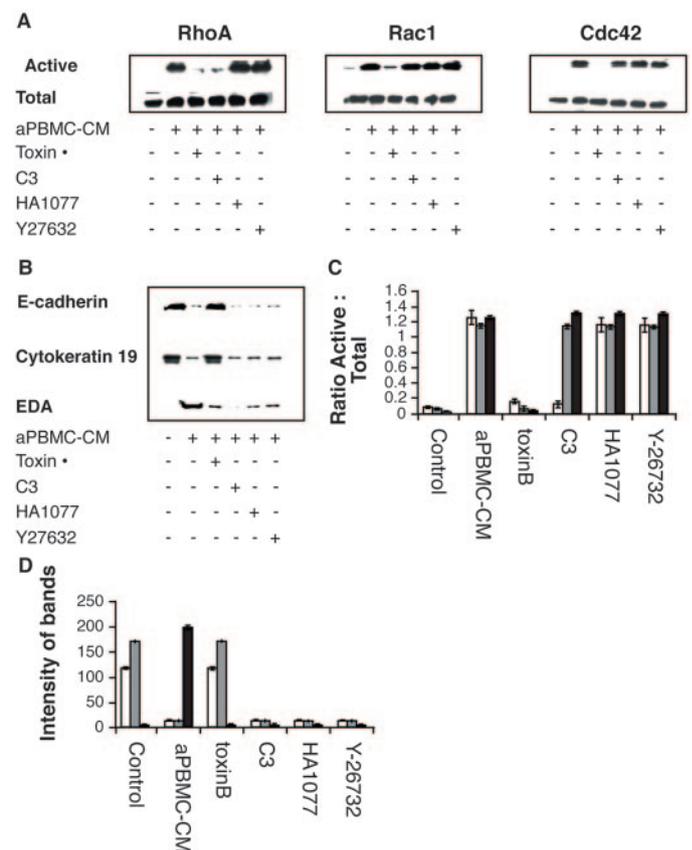


Figure 2. Blockade of RhoGTPase activation results in the inhibition of human renal epithelial-myofibroblast transdifferentiation. HK2 cells were treated with 100 μ g/ml toxin B (for 24 h), 100 μ g/ml exoenzyme C3 (for 24 h), 10 μ M HA1077 (for 1 h), or 10 μ M Y-27632 (for 1 h) before addition of 30% aPBMC-CM for an additional 48 h in the presence of the inhibitors. (A) Total and active RhoA, Rac1, and Cdc42 were determined by Western blotting. A pull-down assay was used to capture the active form of the proteins. (B) HK2 cells were analyzed for E-cadherin, K19, and EDA by Western blotting after the same treatment. Equal amounts of total protein were loaded per lane. (C) Densitometric analysis of Western blots (mean \pm 2 SD, *n* = 3) showing the change of band intensities for the ratio between active and total RhoA \square , Rac1 \blacksquare , and Cdc42 \blacksquare . (D) Densitometric analysis of Western blot (mean \pm 2 SD, *n* = 3) showing the change of band intensities for E-cadherin \square , K19 \blacksquare , and EDA \blacksquare expression.

Transient Expression of Mutant RhoGTPases in HK2 Cells

The use of IRES in the pShuttle-IRES-hrGFP-1 vector allowed the coexpression of GFP and FLAG-tagged RhoGTPases. The presence of GFP-positive cells correlated with the MOI value applied (data not shown). These results agreed well with the increased expression of FLAG in HK2 cells for each adenoviral construct that was found to be proportional to the increased MOI value (Figure 3), with a LacZ infection control showing no expression of FLAG at the highest MOI value (data not shown).

The levels of active RhoA, Rac1, and Cdc42 also increased with increasing level of the MOI value used for infection of the constitutively active constructs, but infection had no effect on

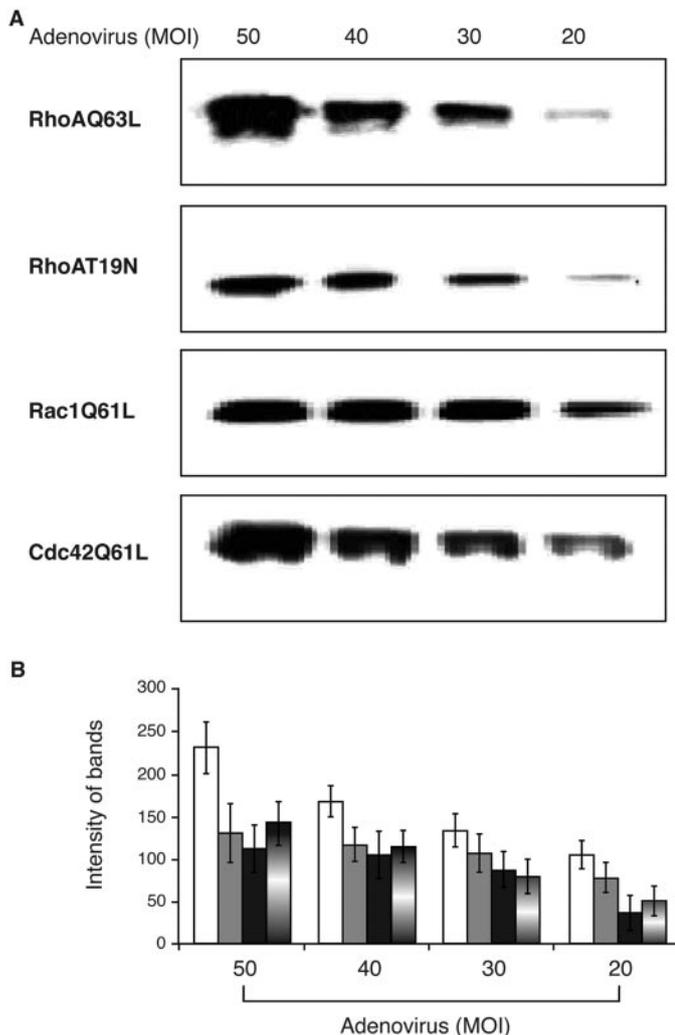


Figure 3. FLAG expression after infecting HK2 cells with constitutively active and dominant negative RhoA (RhoAQ63L □ and RhoAT19N ▨), constitutively active Rac1 (Rac1Q61L ■), and constitutively active Cdc42 (Cdc42Q61L ▩). (A) HK2 cells were infected with various amounts of adenovirus (50, 40, 30, and 20 multiplicity of infection [MOI]). The cells were harvested 48 h later and analyzed for FLAG expression using Western blotting. Twenty micrograms of protein was loaded per lane. (B) Densitometric analysis of Western blots (mean ± SEM for five experiments) showing the change of band intensities for FLAG expression.

the total level of RhoA, Rac1, and Cdc42 (data not shown). A MOI of 50 achieved approximately the same level of active RhoA, Rac1, and Cdc42 as stimulation of the cells with 30% aPBMC-CM. When cells were infected with virus that contained RhoAT19N and stimulated for an additional 24 h with aPBMC-CM, a MOI of 50 viral-delivered RhoAT19N almost completely blocked the formation of activated RhoA.

Constitutively Active RhoGTPases Induce EMT in HK2 Cells

We examined the effect of each mutant on cell morphology (Figure 4) and on the expression the EMT markers (Figure 5). Noninfected cells and cells that were infected with a LacZ-expressing adenovirus had an epithelial morphology, whereas HK2 cells that were treated with aPBMC-CM for 48 h had a fibroblastic morphology. Expression of constitutively active RhoA, Rac1, and Cdc42 all promoted a fibroblastic morphology, especially at MOI of 50. Western blot analysis confirmed that the expression of constitutively active RhoA, Rac1, and Cdc42 in HK2 cells results in these cells undergoing EMT at higher MOI values (Figure 5A). With MOI of 20 and over, cellular expression of EDA was substantially elevated, whereas the level of E-cadherin and K19 was reduced at MOI of 40 and above. The shift of molecular markers from epithelial to myofibroblast, accompanied by the cells adopt-

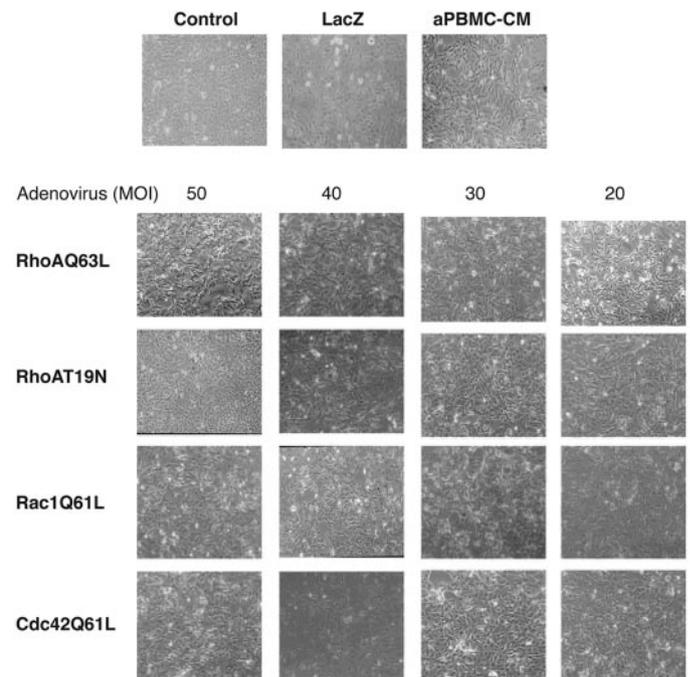


Figure 4. Fibroblastic changes in HK2 cells cultured for 48 h with varying amounts of adenovirus (50 to 20 MOI). Adenoviral expression of constitutively active RhoA (RhoAQ63L), Rac1 (Rac1Q61L), or Cdc42 (Cdc42Q61L) or treatment with 30% aPBMC-CM for 48 h induced a fibroblastic morphology. Control cultures and cells that were infected with a LacZ-expressing adenovirus had epithelial morphology. Dominant negative RhoA (RhoAT19N) prevented the appearance of a fibroblastic morphology in 30% aPBMC-CM-treated cells. Magnification, ×100.

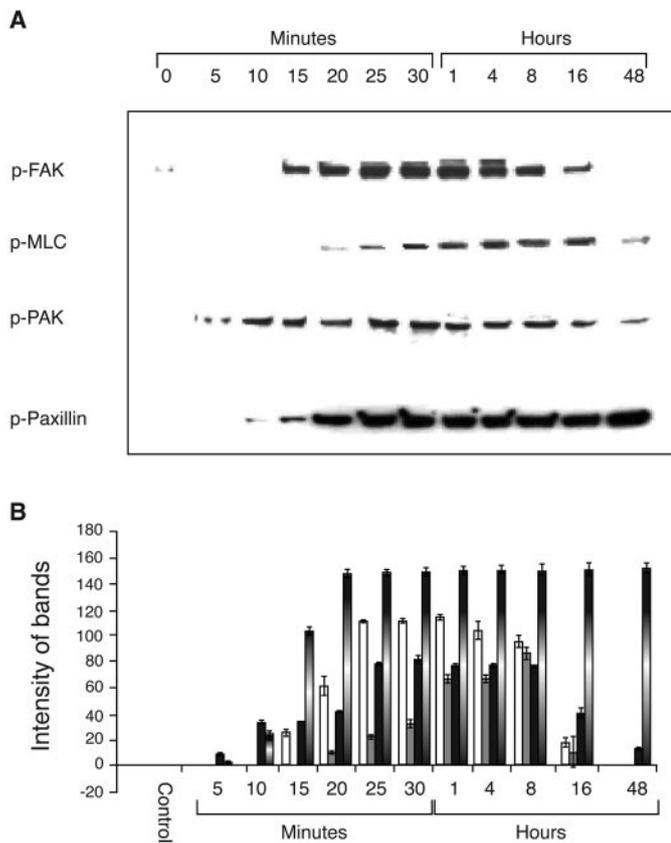


Figure 6. HK2 cells were treated over 48 h with 30% aPBMC-CM. (A) The cell lysates were analyzed for focal adhesion kinase (FAK), myosin light chain (MLC), P21-activated protein kinase (PAK), and paxillin by Western blotting using phospho-specific antibodies. Twenty micrograms of protein was loaded per lane. (B) Densitometric analysis of Western blots (mean \pm 2 SD, $n = 3$) show the change of band intensities for phosphorylated FAK \square , MLC \square , PAK \blacksquare , and paxillin \blacksquare .

undergo EMT (8,12,16,17). However, in more physiologically relevant conditions in which aPBMC-CM was applied to epithelial cells, blockade of single cytokines by neutralizing antibodies showed little inhibition of EMT (8). Thus, it seems likely that multiple cytokines may act synergistically to trigger EMT *in vivo*. The many cytokines present in aPBMC-CM are likely to activate various signaling pathways that then disturb the dynamic equilibrium state between a cell's transcription and transduction network and the local environment (2). The global changes of gene expression determined by microarray provide valuable information of putative molecular mechanisms that drive EMT (8). One set of factors that are involved with the EMT signaling network belong to the small RhoGTPases family. In this study, we found that RhoA, Rac1, and Cdc42 were activated in kidney epithelial cells shortly after the aPBMC-CM stimulation and directly contribute to the suppression of E-cadherin expression, as this could be reversed by toxin B, an inhibitor of small RhoGTPases. Our results are consistent with previous reports. For example, a RhoA-dependent mechanism was implicated in TGF- β -induced EMT (12), whereas activation of Cdc42 and Rac directly related to hepatocyte growth factor-induced cell scattering/spread by dis-

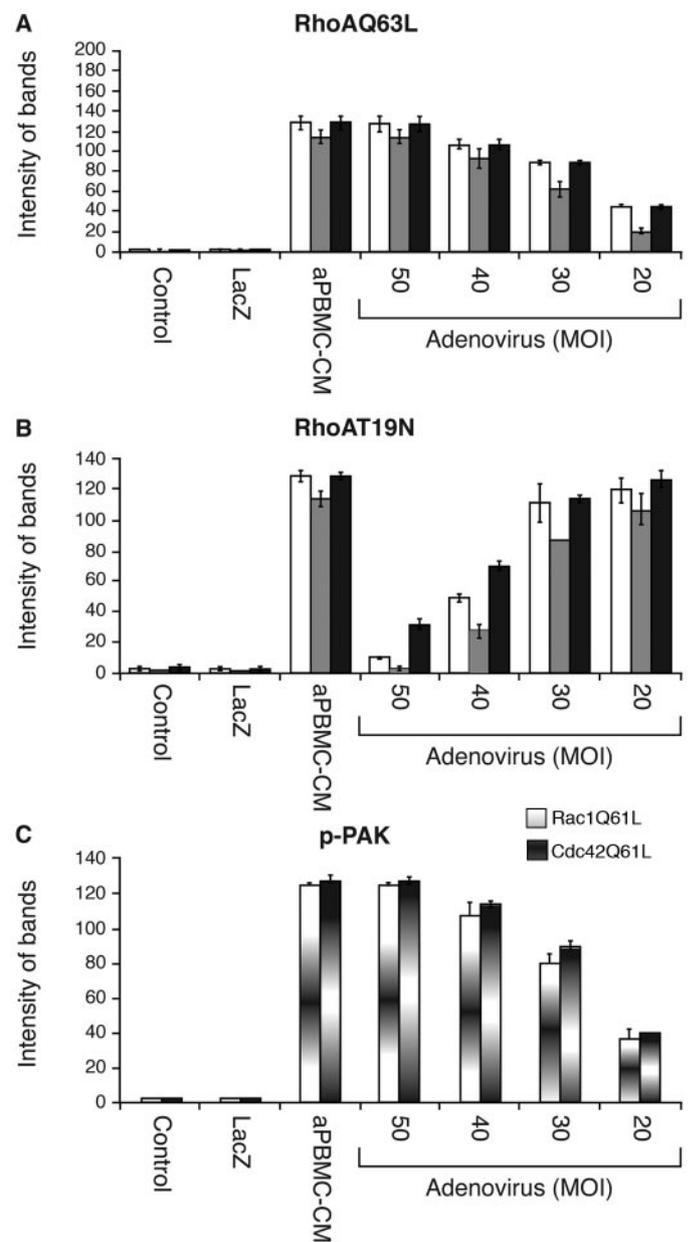


Figure 7. Phosphorylation of FAK, MLC, PAK, and paxillin in HK2 cells expressing RhoAQ63L, RhoAT19N, Rac1Q61L, and Cdc42Q61L. HK2 cells were treated for 24 h with 50 to 20 MOI of RhoAQ63L, Rac1Q61L, and Cdc42Q61L. HK2 cells were also infected with various concentrations of RhoAT19N for 24 h and stimulated for an additional 24 h with 30% aPBMC-CM. Cell lysates were analyzed by Western blotting using phospho-specific antibodies and densitometry (mean \pm 2 SD, $n = 3$). Band intensities for phosphorylated FAK \square , MLC \square , and paxillin \blacksquare after infection with RhoAQ63L (A) and RhoAT19N (B) and for phosphorylated PAK after infection with Rac1Q61L and Cdc42Q61L (C).

rupting cell-cell junctions and rearrangement of the actin cytoskeleton (18,19). Using dominant negative forms of small RhoGTPases the formation of filopodia and lamellipodia and cell scattering were disrupted (18,20). Thus, our data, together with that of others, support the notion that small RhoGTPases play key

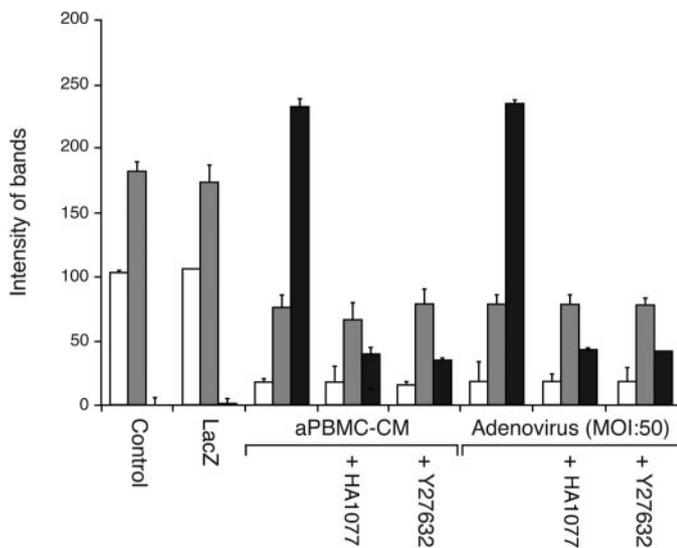


Figure 8. HK2 cells were treated for 1 h with 10 μ M HA1077 or 10 μ M Y-27632 before infecting with 50 MOI of RhoA_{Q63L} or stimulating them with 30% aPBMC-CM. The cell lysates were analyzed for E-cadherin, K19, and EDA by Western blotting and densitometric analysis (mean \pm 2 SD, n = 3). Twenty micrograms of protein was loaded per lane.

roles in transducing extracellular signals to switch on the program of EMT.

RhoA promotes the formation of stress fibers and increased contractility, but a RhoA-induced increase in contractility is not sufficient to disrupt adherens junctions, because inhibition of RhoA only partially restores the epithelial phenotype of Ras transformed cells (21–24). However, Rac1 and Cdc42 are believed to be involved in the establishment and maintenance of epithelial intercellular adhesions (25–27). In our experiment, even low levels of overexpression of constitutively active RhoA cause human epithelial cells to switch on the expression of the mesenchymal cell marker EDA, whereas higher levels of overexpression also decrease that of E-cadherin and K19. In contrast to the putative role of Rac1 and Cdc42 (25–27), we found that overexpression of constitutively active Rac1 and Cdc42 in HK2 cells stimulated them to turn on the EMT program in a similar pattern to that observed in epithelial cells that were exposed to aPBMC-CM. Our data are more consistent with the observation that activated Rac promotes cell–cell adhesion breakdown in T47D mammary carcinoma cells (28). One possibility is that these differences in response to Rac and Cdc42 are due to the differences in the cell types investigated. Another possibility is that when virally encoded constitutively active small RhoGTPases are expressed, their level of activity may be superphysiologic, inducing effects and cross-talk between signaling pathways that would not normally occur. However, in our experiments, we infected cells with a range of concentrations of adenoviruses to establish a dose-response effect and avoid this problem as far as possible. Finally, another factor that may relate to the paradoxical observations of Rac's involvement in transdifferentiation is that the nature of the extracellular matrix to which the cells adhere may account for different observations. For example, Tiam-expressing RasV12-transformed MDCK cells showed a fi-

broblast-like migratory phenotype when plated on collagen, but an epithelial phenotype when plated on laminin or fibronectin (29).

The activation of small RhoGTPases is associated with that of specific downstream proteins. Thus, serine/threonine protein kinases of the ROCK family have been identified as downstream targets of RhoA, transducing Rho activation into stress fiber formation and focal adhesion assembly (30). Understanding the molecular cascades of the small RhoGTPase pathways may help us to identify new therapeutic targets. For example, the ROCK inhibitor Y-27632 was shown to reverse tubulointerstitial fibrosis after unilateral ureteric obstruction (15), a model that develops tubulointerstitial fibrosis *via* EMT (31). Our data showed that aPBMC-CM induced phosphorylation of MLC, FAK, and paxillin and that this effect was inhibited by expression of dominant negative RhoA. Moreover, constitutively active RhoA stimulated phosphorylation of MLC, FAK, and paxillin in HK2 cells. Although our data suggest that RhoA initiates EMT *via* the ROCK/MLC/FAK/paxillin pathway, it leaves open the possible involvement of other members of the small RhoGTPases, such as RhoB and RhoC (32). Because HK2 cells that were treated with aPBMC-CM leads to activation of RhoA pathways, as well as to activation of the Rac1/Cdc42 signaling pathway, and induces transdifferentiation events similar to those initiated by TGF- β (12), it is likely that small RhoGTPase pathways play key roles in the process of EMT. Clearly, further investigation of the role of RhoA, Rac1, and Cdc42 in transdifferentiation and in renal fibrosis is warranted.

Our results strongly suggested that small RhoGTPase signaling plays important roles in aPBMC-CM-mediated EMT. However, the contributions of some other pathways, such as the Smad pathway, should not be excluded (4,33). The latter's importance is illustrated in a mouse model of chronic renal injury, in which recombinant human BMP-7 showed therapeutic efficacy by inhibiting Smad-dependent TGF- β 1-induced EMT (34). It is likely that parallel signaling pathways, such as Smad/ILK and RhoA/ROCK, work together *in vivo* (7).

Acknowledgments

This work was supported by Teijin Ltd. (Japan) and UK Medical Research Council Technology.

We are grateful to Professors Chris Marshall and El Nahas for helpful discussion. We also thank Parvin Ahmed for preparing aPBMC-CM.

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