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Downregulation of exocyst Sec10 accelerates kidney tubule cell recovery through enhanced cell migration

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ABSTRACT

Migration of surviving kidney tubule cells after sub-lethal injury, for example ischemia/reperfusion (I/R), plays a critical role in recovery. Exocytosis is known to be involved in cell migration, and a key component in exocytosis is the highly-conserved eight-protein exocyst complex. We investigated the expression of a central exocyst complex member, Sec10, in kidneys following I/R injury, as well as the role of Sec10 in wound healing following scratch injury of cultured Madin-Darby canine kidney (MDCK) cells. Sec10 overexpression and knockdown (KD) in MDCK cells were used to investigate the speed of wound healing and the mechanisms underlying recovery. In mice, Sec10 decreased after I/R injury, and increased during the recovery period. In cell culture, Sec10 OE inhibited ruffle formation and wound healing, while Sec10 KD accelerated it. Sec10 OE cells had higher amounts of diacylglycerol kinase (DGK) gamma at the leading edge than did control cells. A DGK inhibitor reversed the inhibition of wound healing and ruffle formation in Sec10 OE cells. Conclusively, downregulation of Sec10 following I/R injury appears to accelerate recovery of kidney tubule cells through activated ruffle formation and enhanced cell migration.

1. Introduction

With sub-lethal kidney injury, such as 30 min of bilateral kidney ischemia and reperfusion (I/R), some kidney tubule cells undergo necrosis and apoptosis leading to cell death, while others survive. Migration and proliferation of the surviving kidney tubular epithelial cells are critical events for restoration of kidney tubules following injury [1].

In vitro wound healing assays after scratch have been widely used to study recovery from injury in a variety of cells such as fibroblasts [2], skin cells [3], and kidney tubule cells [4,5]. The involvement of Aquaporin-1 in proximal tubule cell migration after scratch injury, and in renal ischemia-induced kidney injury have been reported [4]. Polarized cell migration is a tightly regulated process that occurs in wound healing [6]. Exocytosis is involved in the establishment of epithelial polarity and polarized cell migration [7].

The exocyst, an evolutionarily conserved octameric protein complex, plays a crucial role in the exocytosis [7]. The exocyst complex is made up of eight proteins: Sec3, Sec5, Sec6, Sec8, Sec10, Sec15, Exo70, and Exo84. Sec10 is known to be a central component of the exocyst complex and knockdown of Sec10 results in loss of other exocyst complex members [8]. Overall, Sec10 is known to play a protective role in renal injury. Knockdown of Sec10 led to a polycystic kidney disease-phenotype *in vivo* [9] as well as defects in ciliogenesis and cystogenesis *in vitro* [10]. In addition, Sec10 protected epithelial barrier integrity and enhanced recovery from

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oxidative stress in cultured kidney epithelial cells [11]. Yet, our knowledge regarding the role of Sec10 in renal tubular epithelial cell migration after injury is very limited.

Membrane ruffling enhances cell's motility and migration [12]. One of the Rho-like GTPases Rac1 as major determinant of cytoskeletal organization has been identified as key regulator of cell migration, which requires membrane ruffling and lamellipodia formation [13]. Rac1 can be activated by focal adhesion kinase [14], type I phosphatidylinositol-4-phosphate 5-kinases [15]. On the other hand, Diacylglycerol kinase (DGK)γ serves as an upstream suppressor of Rac1 and lamellipodia formation [16]. EGFR, which was activated by Sec10-mediated endocytosis [17], activated Rac1 through various guanine nucleotide exchange factors (GEF) [18], and also inactivated Rac1 through DGKγ-Rac-specific GAP beat2-Chimaerin axis [19]. Here, we examined the role of Sec10 during the recovery following scratch injury of kidney tubule cells, and the underlying mechanism with a focus on the lamellipodia formation and cell migration.

2. Materials and methods

2.1. Animal experiment

All animal experiments were conducted in accordance with the guidelines provided by the Animal Care and Use Committee of Kyungpook National University. Experimental protocol (KNU 2015-32) was approved by institutional Animal Care and Use Committee at Kyungpook National University. Eight-week-old C57BL/6 male mice (Koatech, Gyounggido, Korea) were used in experiments. For the induction of ischemia, kidneys were exposed through a flank incision, and the renal pedicles were clamped completely for 30 min using microaneurysm clamps, while mice were anesthetized with pentobarbital sodium (50 mg/kg BW, ip). The same procedure, except for the clamping of the renal pedicle, was done as a sham treatment. For use in biochemical studies, kidneys were frozen in liquid nitrogen immediately after extraction at indicated time points after I/R insult.

2.2. Plasma creatinine concentration

Blood was taken from the retrobulbar vein plexus. Plasma creatinine concentrations were measured using a Vitros 250 (Johnson & Johnson, Rochester, NY, USA).

2.3. Western blot analysis

Protein samples were prepared and Western blot analyses were performed as previously described [20]. Protein samples were electrophoresed on 10–12% polyacrylamide gel with 0.1% SDS and transferred to PVDF membranes, and then subjected to an immunoblotting with antibodies against Sec10 (Proteintech, Rosemont, IL, USA), DGKγ (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), and GAPDH (Novus Biologicals, Littleton, CO, USA) as a loading control. Horseradish peroxidase-conjugated secondary antibodies (anti-rabbit: Santa Cruz, Santa Cruz, CA, USA; anti-mouse: Bethyl, Montgomery, TX, USA) were applied. Immunoblots were visualized using chemiluminescence reagent (PerkinElmer Life Sciences, Waltham, MA, USA). Densities of immunoblots were quantified using image analysis software ImageJ (NIH, Bethesda, ML, USA).

2.4. ATP depletion

ATP depletion experiment was performed as previously described [21]. Murine proximal tubular epithelial cells (mProx24 cells) were cultured in DMEM with 10% FBS (Mediatech Inc., Manassas, VA, USA) and 100 unit/mL of streptomycin/penicillin (WelGENE Inc., Daegu, Korea). To induce ATP depletion, cells were incubated in a Krebs–Henseleit buffer (115 mM NaCl, 3.6 mM KCl, 1.3 mM KH_2PO_4, 25 mM $NaHCO_3$, 1 mM $CaCl_2$, 1 mM $MgCl_2$; pH 7.4) with or without sodium cyanide (5 mM) and 2-deoxyglucose (5 mM) for 60 min.

2.5. Generation of a Sec10-overexpressing and Sec10-knock down stable cell line

All MDCK cell lines used were derived from low passage type II MDCK cells that were obtained from Dr. K. Mostov (University of California, San Francisco, CA) and that were originally cloned by Dr. D. Louvard (European Molecular Biology Laboratory, Heidelberg, Germany). Human Sec10 cDNA with a myc epitope tag added to the COOH terminus was cloned into the pcDNA3 mammalian expression vector, where expression is driven by a CMV promoter. This plasmid was stably transfected into type II MDCK cells, and monoclonal lines were selected that stably overexpress Sec10, as described in Lipschutz et al. [22]. Stable monoclonal MDCK cells with shRNA-mediated Sec10 knockdown were previously generated and characterized [10].

2.6. Cell culture

Control or Sec10 gene-regulated Madin-Darby canine kidney (MDCK) cells were cultured on coverslips to confluence in MEM (Gibco, GrandIsland, NY, USA) with 5% fetal bovine serum (FBS) (Gibco, GrandIsland, NY, USA) and 100 unit/mL streptomycin/penicillin (S/P) (Welgene Inc., Daegu, Korea) at 37 °C in a humidified atmosphere containing 5% CO_2. 70 μg/mL G418 disulfate salt (Sigma Aldrich, St. Louis, Mo, USA) was used for the selection of Sec10 overexpressing cell for the culture.

2.7. In vitro wound healing assay

Following scratch injury, cells migrate or grow to close the wound. *In vitro* wound healing was assayed as described previously [5]. Briefly, confluent cells grown on coverslips were scratched with a sterile 10-μl or 100-μl micropipette tip. The wounded cells were washed to remove the detached cells. Using a microscope, points where the width of scratch was exactly the same in control and Sec10-regulated cells were selected and marked on the outside of the cell culture dish so the same spots could be found later. 6–13 h later, the images of marked spots were captured with a digital camera under bright field microscopy (Leica, Leica instruments, Wetzlar, Germany). Wound healing was evaluated from images captured at 0 and 6–13 h after scratching. Wound width was measured using ImageJ software (NIH, USA). Data were collected from 3 independent experiments. Cells were fixed with 4% paraformaldehyde (PFA) for 15 min and processed for immunofluorescence or Phalloidin staining. Wound healing rate was also evaluated by time-lapse photography using IncuCyte ZOOM system (Essen BioScience, Hertfordshire, UK) for 5 h. Sec10 over-expression delayed the wound healing and Sec10 knock down accelerated wound healing compared to own control cells (Supplementary Fig. 1).

2.8. In vitro migration assay

Migration was assayed as described previously using a modified Boyden chamber (Corning Costar, Cambridge, MA, USA) that contains a polycarbonate transwell membrane filter (6.5 mm diameter, 8 μm pore size) [4] with a little modification. Number of 10^4 Cells

were plated on the upper chamber in MEM (Gibco, GrandIsland, NY, USA) that contained 1% FBS (Gibco, GrandIsland, NY, USA). The lower chamber contained MEM with 10% FBS. Cells were incubated for 8 h at 37 °C in a humidified atmosphere containing 5% CO_2. Cells were stained with hematoxylin and eosin (H&E), and migrated cells that remained on the bottom surface were counted after non-migrated cells were scraped from the upper surface of the membrane with a cotton swab. Pictures were taken using a microscope (Leica, Leica instruments, Wetzlar, Germany).

2.9. Immunofluorescence staining

Fixed cells were permeabilized with 0.1% Triton-X 100 for 5 min. Cells were incubated with 3% BSA for 30 min for blocking, then incubated with antibodies against DGKγ (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) diluted at 1:100 overnight at 4 °C. After incubation, the cells were washed three times in PBS for 5 min each, incubated with FITC-conjugated secondary antibody (Vector Laboratories, Inc., Burlingame, CA, USA) for 60 min at room temperature (RT), and washed three times with PBS for 5 min each. To detect the cell nuclei, 4′-6-diamidino-2-phenylindole (DAPI; Sigma Aldrich, St. Louis, MO, USA) was applied. Finally, the cells were observed and pictures were taken randomly at the leading edge of the cells using a fluorescence microscope (Leica, Leica instruments, Wetzlar, Germany). DGKγ-positive areas were evaluated using i-Solution software (IMT, Vancouver, Canada) and normalized with the number of cells at the leading edge.

2.10. Phalloidin staining

Phalloidin staining for F-actin was performed using phalloidin-TRITC (Sigma Aldrich, St. Louis, MO, USA) following the manufacturer's instructions. Briefly, fixed cells were permeabilized with 0.1% Triton-X 100 for 5 min. Cells were incubated with 50 μg/mL phalloidin-TRITC in PBS for 40 min at RT. Cells were washed and pictures were taken using a fluorescence microscope (Leica, Leica instruments, Wetzlar, Germany). Ruffle area was outlined along the F-actin staining inside and additional structure to the outer lining of the cells, and evaluated using i-Solution software (IMT, Vancouver, Canada) and normalized with the number of cells at the leading edge.

2.11. Statistics

The results were expressed as the means ± SE. Statistical differences between the groups were evaluated with an analysis of variance using a two-tailed Student's *t*-test. Differences between the groups were considered statistically significant with a *P* value of <0.05.

3. Results

3.1. Exocyst component Sec10 expression is dynamically altered during kidney I/R injury and repair

Plasma creatinine significantly increased one day following I/R injury and then decreased over time (Fig. 1a) with functional recovery. In order to investigate the role of Sec10 in kidney I/R injury and repair we first determined the expression levels of excocyst Sec10. Sec10 decreased 1 day after I/R injury, and increased as the kidney recovered functionally (Fig. 1b and c) suggesting that decrease in Sec10 may trigger restoration of kidney. In order to test if the decrease in Sec10 after I/R is a response to hypoxia we checked the expression of Sec10 in ATP depletion condition, which is *in vitro* model of hypoxia. Sec10 greatly decreased after ATP depletion (Fig. 1d and e).

3.2. Sec10 negatively regulated wound healing through inhibition of cell migration

In order to investigate the role of Sec10 in wound healing, we determined the wound healing rate per hour following the scratching of Sec10-regulated cells. We first confirmed higher expression of Sec10 in OE cells, and reduced expression of Sec10 in the KD cells by Western blot analysis (Fig. 2a). Wound healing in Sec10 OE cells was delayed (6.46±0.27 μm/h) compared to control MDCK II cells (11.04±0.41 μm/h) ($P < 0.001$ vs. control) (Fig. 2b and c), while wound healing in Sec10 KD cells was enhanced (15.00±0.36 μm/h) compared to control T23 cells (9.51±0.39 μm/h)

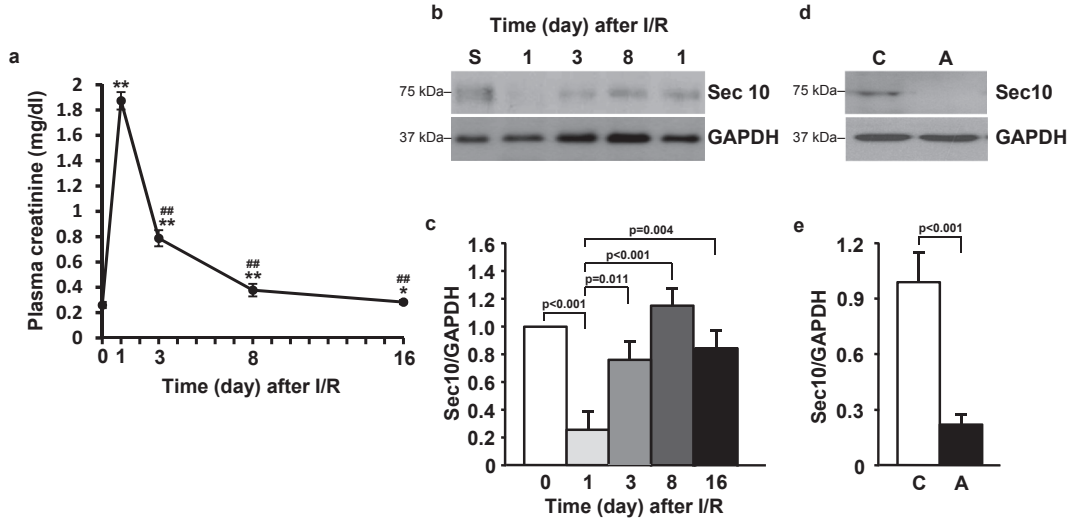

Fig. 1. Exocyst Sec10 expression correlated with kidney injury and repair following ischemia/reperfusion (I/R). (a) Plasma creatinine (PCr) was measured at the indicated time points after 30 min of bilateral renal I/R or sham operation. (b) Representative Western blots for Sec10 in the kidney at the indicated time points after I/R. (c) The graph summarizes Sec10 expression in the kidney at the indicated time points after I/R. Data are presented as means ± SE ($n = 4–6$). S = sham, I/R = ischemia and reperfusion. (d) Representative Western blots for Sec10 in cultured mouse proximal tubule cells with (A) or without (C) ATP depletion. (e) The graph summarizes Sec10 expression in cultured mouse proximal tubule cells. Data are presented as means ± SE ($n = 3$). C = control, A = ATP depletion.

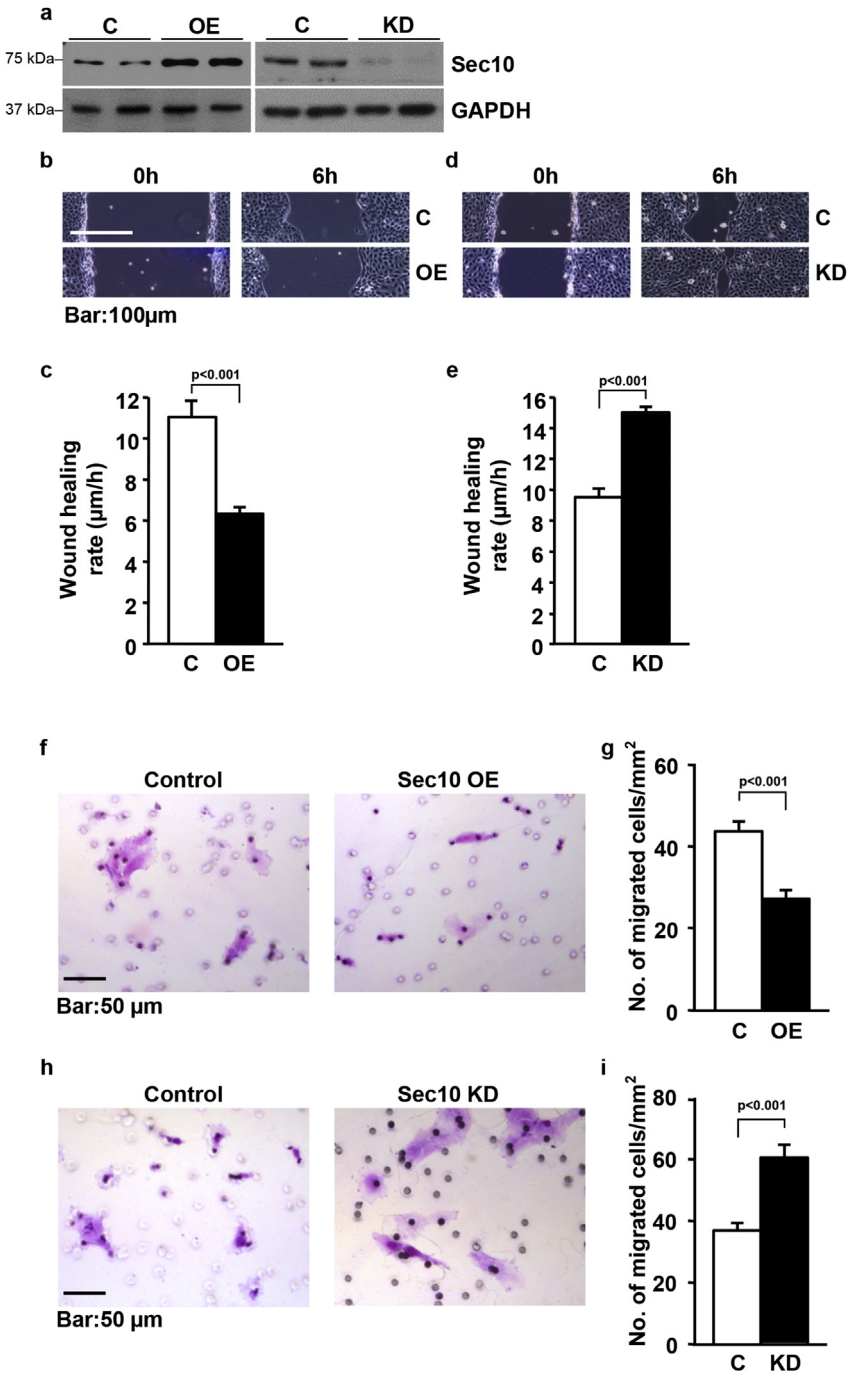

Fig. 2. Kidney tubule cell recovery after scratch injury inversely correlated with expression levels of Sec10. Sec10 overexpressing (OE) and knockdown (KD), along with control, cells were scratched and cultured for 6 h. Wound healing was determined by measuring the newly grown area. (a) Representative Western blots of Sec10 levels in Sec10 OE and knockdown KD cells. (b, d) Representative pictures of Sec10 OE, KD, and control cells at the indicated times following the scratch. (c, e) The graphs summarize the wound healing rates in Sec10 OE (c) and Sec10 KD (e) cells. (f, h) Representative pictures of H&E-stained migrated cells of Sec10 OE, KD, and control cells. Cells are pinkish stained. Small circles are pores of the membrane filters. (g, i) Graphs summarize the number of migrated cells of Sec10 OE and control (g) and Sec10 KD and control (i) cells. Data are presented as means ± SE. Data were obtained from 3 independent experiments. C = control.

($P < 0.001$ vs. control) (Fig. 2d and e). In order to unveil the mechanism underlying Sec10-dependent wound healing we investigated cell migration according to Sec10 expression. Cell migration of Sec10 OE cells was attenuated (27.92±1.96 cells/mm^2) compared to control MDCK II cells (44.52±2.69 cells/mm^2) ($P < 0.001$ vs. control) (Fig. 2f and g), while wound healing in Sec10 KD cells was enhanced (62.62±3.89 cells/mm^2) compared to control T23 cells (37.24±1.64 cells/mm^2) ($P < 0.001$ vs. control) (Fig. 2h and i).

3.3. Sec10 negatively regulated ruffle formation at the leading edge of MDCK cells during wound healing

To investigate the underlying mechanism of Sec10-regualted wound healing, we examined the effect of Sec10 expression on migration through ruffle formation. F-actin staining showed that ruffle formation was inhibited in Sec10 OE cells (6.25±1.39 μm^2) compared to control MDCK II cells (44.52±2.69 μm^2) ($P < 0.03$ vs.

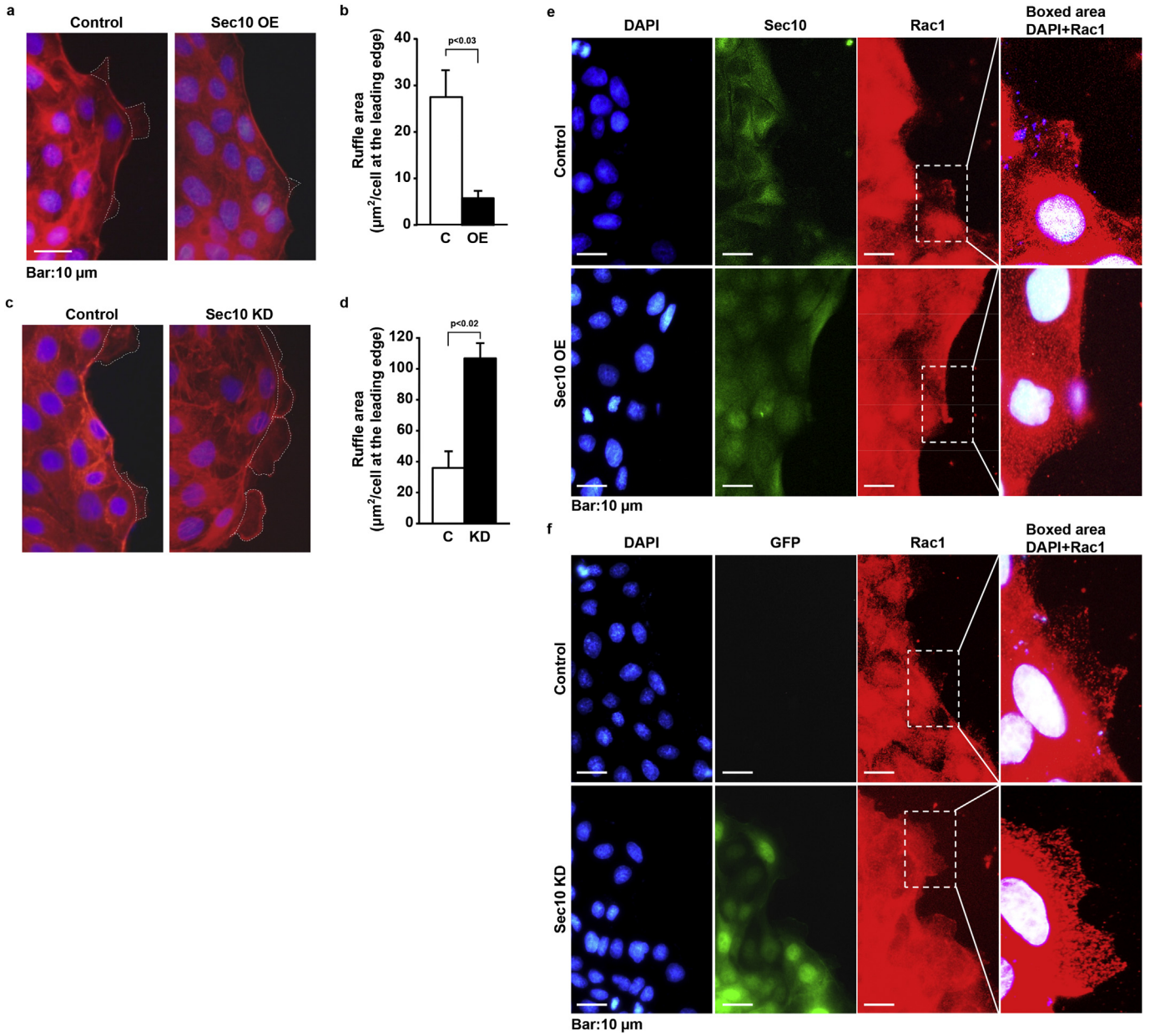

Fig. 3. Ruffle formation in kidney tubule cells during wound healing was dependent on the level of Sec10 expression. (a, c) Representative immunofluorescence (IF) pictures of actin staining using Phalloidin (red) in Sec10 OE (a) and KD cells (c). White lines range ruffles at the leading edge during wound healing following the scratch injury. (b, d) Graphs summarize the area of ruffles of Sec10 OE and control (b), and Sec10 KD and control (d) cells. Data are presented as means ± SE. Data were obtained with 18–21 cells from 3 independent experiments. Sec10 OE=Sec10 overexpressing cells; Sec10 KD=Sec10 knockdown cells.

control) (Fig. 3a and b), while ruffle formation in Sec10 KD cells was enhanced (112.38±11.10 μm^2) compared to control T23 cells (38.44±10.40 μm^2) ($P < 0.02$ vs. control) (Fig. 3c and d). These data indicate that Sec10 negatively regulates wound healing of kidney tubule cells through regulation of ruffle formation.

3.4. Diacylglycerol kinase (DGK) inhibitor reversed Sec10-mediated delay of wound healing

To elucidate the underlying mechanism of Sec10-regulated migration and ruffle formation, we determined the expression of DGKγ, which is known to be a negative regulator of ruffle formation. DGKγ expression was higher at the leading edge of Sec10 OE cells, where the ruffle appeared less, than in control cells (Fig. 4a-c). A DGK inhibitor did not affect the wound healing rate of the control cells, whereas it increased the wound healing rate in Sec10 OE cells (Fig. 4d and e). Western blot analysis with cell culture in resting condition showed the higher expression of Sec10 in Sec10 OE cells than in control MDCK II cells (Fig. 4f and g). However, unlike the staining at the leading edge, the levels of DGKγ were not different between Sec10 OE and control cells (Fig. 4f, h) suggesting that it is a local phenomenon.

3.5. A DGK inhibitor reversed Sec10-mediated inhibition of ruffle formation at the leading edge

To elucidate the mechanism by which DGKγ restores wound healing in Sec10 OE cells, we determined the effects of a DGK

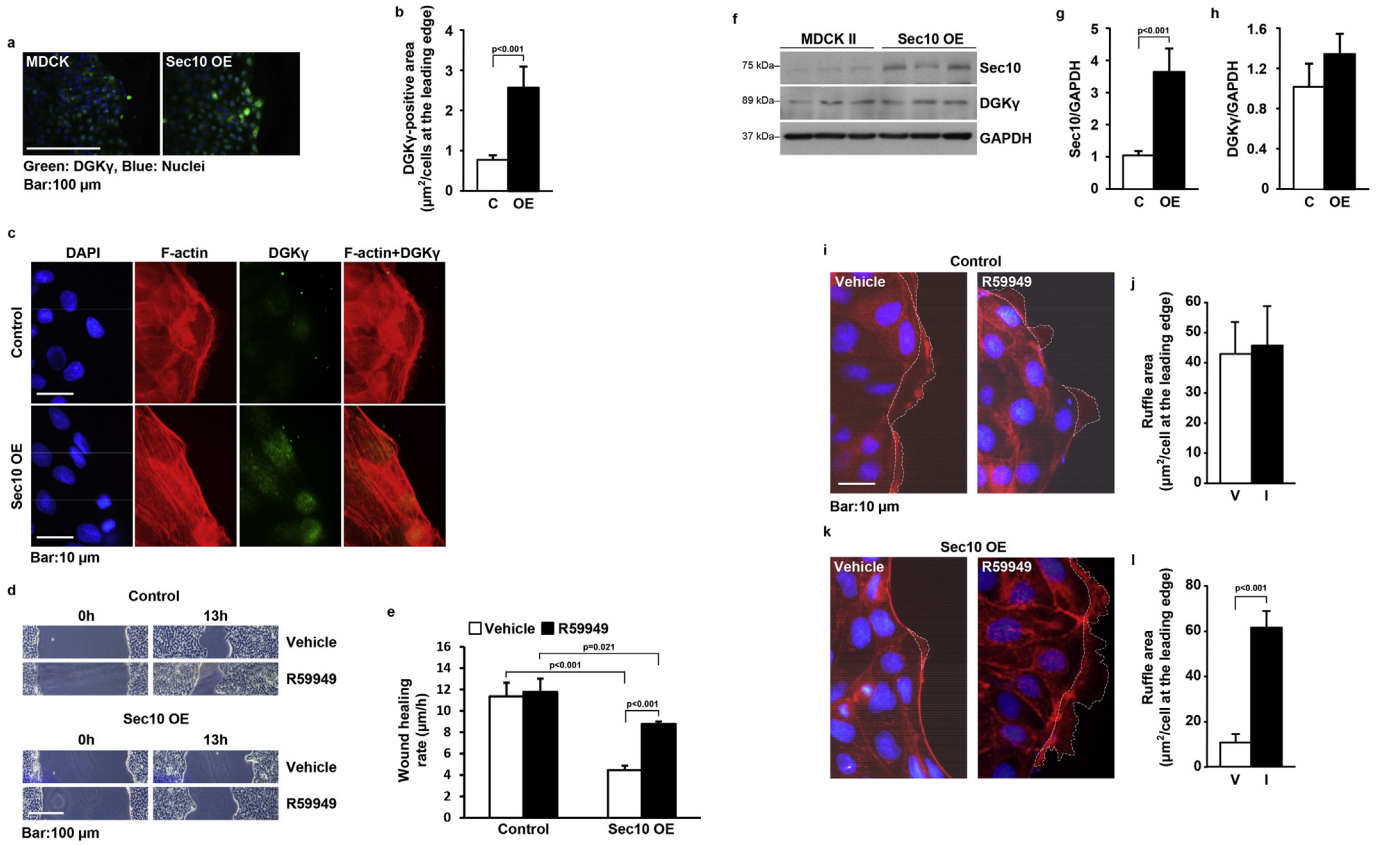

Fig. 4. Pharmacological inhibition of DGKγ reversed Sec10-mediated inhibition of ruffle formation and wound healing of MDCK cells. Control and Sec10 OE cells were scratched and treated with either vehicle or 1 μM of R59949. Cells were further incubated for 13 h. Wound healing was determined by measuring newly grown area. (a) Representative IF of DGKγ at the leading edge during wound healing. (b) The graph summarizes expression of DGKγ at the leading edge. Data are presented as means ± SE. Data were obtained with 68–72 cells from 3 independent experiments. (c) Representative IF of F-actin and DGKγ at the leading edge during wound healing in control and Sec10 OE cells. (d) Representative bright field images of control and Sec10 OE cells before and after scratch with/without treatment of DGK inhibitor. (e) The graph summarizes wound healing rates in control and Sec10 OE cells with/without treatment of DGK inhibitor after scratch injury. (f) Representative Western blots for Sec10 and DGKγ in control and Sec10 OE cells. (g, h) The graphs summarize Sec10 (g) and DGKγ (h) expression in control and Sec10 OE cells. (i, k) IF of actin staining using Phalloidin (red) in control (i) and Sec10 OE cells (k) with/without treatment of DGK inhibitor R59949 after scratch injury. (j, l) Graphs summarize the ruffle area at the leading edge in control (j) and Sec10 OE (l) cells. White lines indicate ruffles. Data are presented as means ± SE. Data were obtained with 75–90 cells from 3 independent experiments. Sec10 OE=Sec10 overexpressing cells.

inhibitor on ruffle formation. The DGK inhibitor did not affect ruffle formation in control cells (Fig. 4i, j), whereas it greatly increased ruffle formation at the leading edge of Sec10 OE cells (Fig. 4k and l).

4. Discussion

The principal finding of this study is that migration and wound healing of kidney epithelial cells following scratch injury is inhibited by upregulation of a key exocyst complex member, Sec10. Sec10 negatively regulated ruffle formation and kidney tubule cell migration via upregulation of DGKγ.

Restoration of the kidney tubules following sub-lethal kidney injury, such as ischemic kidney injury, depends on the migration and proliferation of surviving cells [1]. Therefore, elucidating the factors and underlying mechanisms of kidney tubule cell migration during the repair following injury may provide novel targets for treatment. Several studies showed that certain proteins play a critical role in epithelial repair through the modulation of proliferation and/or migration after a scratch injury. Aquaporin-1 (AQP1), which is a principal water-transporting protein in kidney tubule cell membranes, was unexpectedly shown to be involved in migration and lamellipodia formation at the leading edge of migrating cells. Migration of AQP-1 deficient cells was inhibited, and restoration of AQP-1 corrected the migration defect. AQP1 null mice showed significantly greater tubular injury than wild type mice in response to kidney ischemia, suggesting that AQP-1-mediated migration of cells is essential for recovery [4]. In the present study, Sec10 decreased after I/R injury and then increased with recovery. Downregulation of Sec10 accelerated ruffle formation and wound healing. Overexpression of Sec10 led to opposite findings. This was surprising to us as we previously showed that Sec10 overexpression accelerated recovery of renal tubular cells from injury through ERK [11] or EGFR/MAPK activation [17]. However, on the second thought, these data demonstrate that, in terms of proliferation, Sec10 have a protective effect as we previously reported. However, when it comes to migration, Sec10 negatively regulates it through reduced ruffle formation. These data suggest that I/R injury induced a temporary decrease in Sec10 resulting in migration of surviving renal tubule cells, and Sec10 restores with time enhancing proliferation of the tubule cells to speed recovery.

It is a surprise that Sec10 over-expression decreased ruffle formation, because ruffle is induced by Rac1 activation. Rac1 can be activated by EGFR activation [23], which is potentiated by Sec10 [17]. However, Rac1 is regulated many signals including focal adhesion kinase [14], type1 phosphatidylinositol-4-phosphate-5-kinases [15] positively, and DGKγ [16] and even EGFR [19] negatively through DGKγ-Rac-specific GAP beta2-Chimaerin axis. DGKγ

suppresses cell migration by inhibiting lamellipodia/ruffle formation through inhibition of Rac1. Expression of a dominant negative mutant and inhibition of endogenous DGKγ activity using DGK inhibitor R59949 induced membrane ruffle formation [16]. In the present study DGKγ expression determined by western blot analysis with cell culture in resting status was not different between control and Sec10 OE cells. However, over-expression of Sec10 increased DGKγ at the leading edge, likely inactivating Rac1 resulting in inhibited ruffle formation and delayed wound healing, and the treatment with R59949 in Sec10 OE cells reversed the delayed wound healing with restored ruffle formation. At this time, it is not clear how Sec10 upregulates DGKγ at the leading edge after scratch injury but it is clear that delay of wound healing and inhibition of ruffle formation is due to DGKγ.

In summary, here we show that temporal regulation of exocyst Sec10 is important for kidney tubule recovery following injury. We describe a model in which a dramatic decrease in Sec10 during I/R injury induces migration of surviving cells, then followed by an increase in Sec10, which allows for cell proliferation and tubule recovery. We also found that DGKγ, an upstream suppressor of ruffle formation, is an effector molecule for Sec10 and acts to decrease migration. These data suggest that Sec10 and its downstream signals are candidate targets for treatment following kidney I/R injury.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.bbrc.2018.01.013.

Transparency document

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