Transactivation of RON receptor tyrosine kinase by interaction with PDGF receptor β during steady-state growth of human mesangial cells

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Although it is well known that platelet-derived growth factor (PDGF) causes mesangial cell proliferation (presumably contributing to progression of glomerular disease), targeted inhibition of the PDGF receptor system has shown only limited efficacy against glomerular diseases. To examine whether this discrepancy is due to the involvement of other pathways, we used phosphorylated receptor tyrosine kinase arrays and found that RON (*recepteur d'origine nantais*) was phosphorylated while the PDGF receptor was dephosphorylated (thus inactive) in human mesangial cells (HMCs) at the time of cell cycle entry. Further, RON remained active during steady-state growth. Activation of RON was independent of its canonical ligand, macrophage-stimulating protein, but was mediated by transactivation from the PDGF-engaged PDGF receptor. Following stimulation with PDGF we found that the two receptors physically interacted. Knockdown of RON by siRNA increased the number of apoptotic cells without affecting the rate of DNA synthesis, suggesting that RON has anti-apoptotic functions. Immunohistochemical analysis found phosphorylated RON in glomerular lesions of patients with IgA nephropathy but not those with minimal change nephrotic syndrome, a disease not associated with mesangial proliferation. These results suggest that RON is involved in mesangial cell proliferation under both physiological and pathological conditions, and may be a relevant target for therapeutic intervention.

Kidney International (2009) **75,** 1173–1183; doi:10.1038/ki.2009.44; published online 25 February 2009

KEYWORDS: glomerular disease; mesangial cell; receptor tyrosine kinase; signal transduction; transactivation

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Received 21 August 2008; revised 12 December 2008; accepted 7 January 2009; published online 25 February 2009

Mesangial cell proliferation plays an important role in the progression of glomerular diseases, including IgA nephropathy, a major cause of end-stage renal failure. Elucidation of the mechanisms of mesangial cell proliferation may contribute to the development of effective treatment strategies for glomerular diseases. However, molecular basis of mesangial cell proliferation has not been fully understood, although earlier studies have indicated the involvement of growth factors especially platelet-derived growth factor (PDGF).[1,2]

PDGF is a pleiotropic growth factor that was originally isolated from human platelet-rich plasma. PDGF consists of five dimeric isoforms (AA, AB, BB, CC, and DD) and binds to three structurally related receptors (α/α, α/β, and β/β), which transduce mitogenic signals through their tyrosine kinase domains to downstream effector kinases such as ERK and AKT.[1–3] As PDGF is synthesized by resident renal cells, activated macrophages, and aggregated platelets, it is easy to speculate that this factor accumulates at local sites of renal injury. Furthermore, the overexpression of PDGF-B chain and its cognate receptor PDGFRβ has been reported in many glomerular diseases, and their levels are correlated with the disease severity.[1,3] The involvement of the PDGF system has been shown in both human disease and experimental models, wherein PDGF enhances the progression of glomerular diseases by stimulating the proliferation of mesangial cells and the synthesis of extracellular matrix proteins and transforming growth factor-β.[1,4]

Selective inhibition of PDGF action is therefore considered a major target for specific and effective therapy for glomerular diseases. Imatinib mesylate, a potent inhibitor of PDGF receptor tyrosine kinase (RTK), has ameliorated the pathological findings of experimental mesangial proliferative glomerulonephritis in rats.[5] In addition, specific antagonism of PDGF-B chain[6,7] and PDGF-DD[8] with neutralizing antibodies has reduced mesangial cell proliferation and extracellular matrix protein accumulation in an experimental nephritis model. However, therapeutic effects of PDGF signal inhibition are not satisfactory in animal models, and do not

fulfill the requirement for clinical application. To establish effective molecular-targeted therapy for progressive glomerular diseases, further investigation is required regarding the signal transduction pathways downstream of PDGFR in mesangial cells. In particular, we should consider the involvement of other growth factors and RTKs.

In this study, we attempted to elucidate the mechanisms of human mesangial cells (HMCs) proliferation and survival by focusing on RTK-mediated signal transduction. This study unveiled an additional player in HMC proliferation and survival, RON (*recepteur d'origine nantais*), which is a heterodimeric RTK composed of a 40-kDa α chain and a 150-kDa β chain.[9] The α chain (RONα) is extracellular and serves as a binding platform for macrophage-stimulating protein (MSP), whereas the β chain (RONβ) traverses cell membrane and transduces signals through tyrosine kinase domains. We obtained evidence suggesting that RON is a relevant target of therapeutic intervention for human glomerular diseases.

RESULTS

RONβ is persistently activated during the steady-state growth of human mesangial cells

In an initial step to clarify RTK-mediated signal transduction, which is critical for HMC proliferation, we took advantage of a human phospho-RTK array, which enables us to analyze the phosphorylation states of 42 well-characterized RTKs simultaneously and quantitatively. For this assay, we prepared protein samples from normal primary HMCs in three different cell cycle conditions: quiescence; cells were cultured in the absence of fetal calf serum (FCS) for 24 h, cell cycle entry; cells were stimulated with FCS for 15 min after 24 h of serum starvation, and steady-state growth; cells were cultured in complete medium without starvation. The cell cycle status in each condition was verified by bromodeoxyuridine (BrdU) incorporation and DNA histogram analysis by flow cytometry (data not shown). As shown in Figure 1a, all 42 RTKs showed almost negligible levels of tyrosine phosphorylation in quiescent HMCs. The phosphorylation of PDGFRβ was readily induced at the time of cell cycle entry, consistent with the earlier notion that PDGF acts as a principal growth factor for HMCs. However, our assay revealed that RONβ was also phosphorylated at an equivalent level with PDGFRβ after mitogenic stimulation. More importantly, RONβ phosphorylation persisted during steady-state growth of HMCs, whereas the phosphorylation of PDGFRβ was significantly attenuated (Figure 1a). We confirmed the results of phospho-RTK blots with immunoblotting using antibodies different from those placed on the array (Figure 1b). It is of note that the expression of total RONβ and its heterodimeric partner RONα was stable during mesangial cell proliferation (Figure 1b).

MSP fails to promote cell cycle entry and RONβ phosphorylation in HMCs

Given that RONβ was phosphorylated in response to FCS in HMCs, we evaluated whether MSP, a *bona fide* ligand for RON, stimulated the growth of HMC. To this end, we stimulated HMCs with optimal concentrations of FCS (10%), PDGF-BB (10 ng/ml) and MSP (100 ng/ml) after 24 h of serum starvation, and measured the cell growth using a BrdU incorporation assay after 24 h. Unexpectedly, MSP failed to promote the cell cycle entry of HMC, whereas FCS and PDGF-BB significantly increased BrdU incorporation (Figure 2a). MSP did not affect the growth of HMCs at different concentrations (1–1000 ng/ml) (data not shown). The activity of MSP was verified by activation of MSP-sensitive breast cancer cell line MDA-MB-453 (data not shown).

After 15 min of stimulation with FCS and MSP, we prepared whole cell lysates from HMCs, and subjected them to immunoblot analysis for phosphorylation of RONβ. In line with the inability to induce cell proliferation, MSP did not phosphorylate RONβ (Figure 2b). In contrast, RONβ was readily phosphorylated in FCS-stimulated HMCs, indicating that FCS contains a factor other than MSP, which is responsible for RONβ phosphorylation.

PDGF induces RONβ phosphorylation through PDGFR in HMCs

To determine the factor(s) responsible for RONβ phosphorylation, we first examined whether PDGF-BB could phosphorylate RONβ because PDGF-BB is the major growth factor for HMCs. To test this possibility, we compared the kinetics of tyrosine phosphorylation of RONβ with that of PDGFRβ induced by highly purified recombinant PDGF-BB in HMCs. As described earlier,[1,2] the phosphorylation of PDGFRβ was readily observed after 10 min, peaked at 20 min, and declined 50 min after the addition of PDGF at an optimal concentration (Figure 3a). As we speculated, PDGF-BB induced the phosphorylation of RONβ with different kinetics to that of PDGFRβ: It appeared later and was sustained longer than the phosphorylation of PDGFRβ. This is consistent with the pattern obtained in phospho-RTK assays.

Next, we investigated whether PDGF-induced phosphorylation of RONβ was a downstream event of the PDGF receptor signaling pathways. For this purpose, we pretreated serum-starved HMCs with imatinib mesylate, a potent inhibitor of PDGFR tyrosine kinase,[5] stimulated with PDGF-BB for 30 min, and analyzed the phosphorylation of RONβ. As shown in Figure 3b, imatinib mesylate inhibited PDGF-mediated phosphorylation of RONβ concomitantly with a decrease in the phosphorylated species of PDGFRβ in a dose-dependent manner.

Physical interactions between PDGFRβ and RONβ in HMCs

We hypothesized that PDGF-induced phosphorylation of RONβ was mediated through ligand-independent activation of RONβ because it has been reported that interleukin-3 and epidermal growth factor (EGF) transactivate RONβ through interactions with their canonical receptors.[10] To confirm this hypothesis, we first examined the association of RONβ and

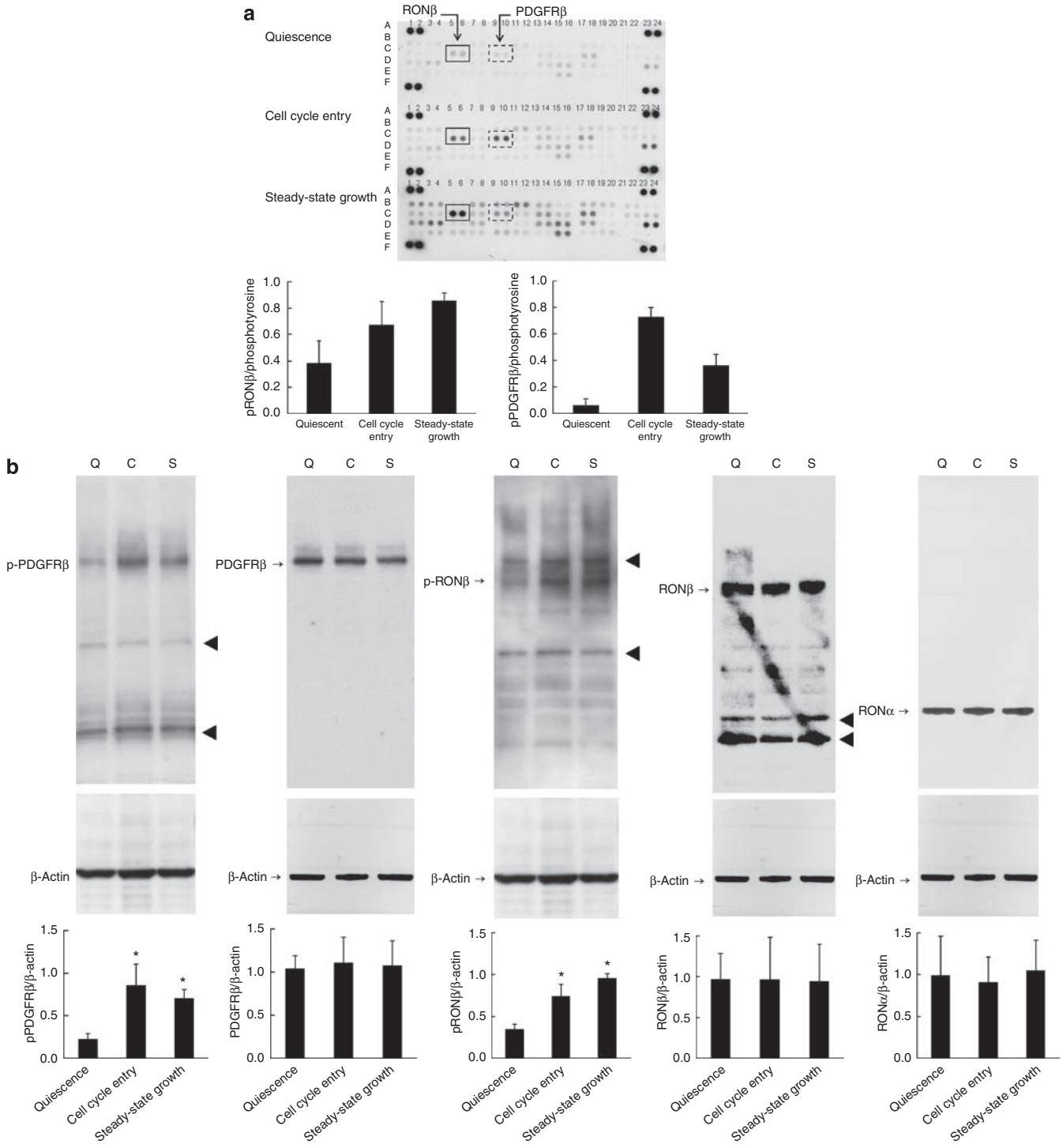

Figure 1 | Persistent phosphorylation of RONβ during steady-state growth of HMCs. (**a**) (Upper panel) HMCs were grown to 70% confluence on 100 mm-diameter tissue culture dishes. After being serum starved for 24 h, cells were stimulated with 10% FCS, and harvested before (quiescence) and after (cell cycle entry) 15 min. Cells cultured without starvation (steady-state growth) were also harvested for the isolation of whole cell lysates. Phospho-RTK arrays were hybridized with 500 μg protein of whole cell lysates, followed by incubation with an anti-phosphotyrosine detection antibody. The locations of RTKs and internal controls are indicated in Table S1. (Lower panel) The signal intensities of phosphorylated RONβ and PDGFRβ were quantified and normalized to the average values of the intensities of anti-phosphotyrosine antibodies placed on A1-2, A23-24, F1-2, and F23-24 (positive controls). The means ± s.d. (bars) of two independent experiments are shown. (**b**) Expressions of phosphorylated PDGFRβ (p-PDGFRβ), total PDGFRβ (PDGFRβ), phosphorylated RONβ (p-RONβ), total RONβ (RONβ), and total RONα (RONα) were examined by immunoblotting in quiescence (Q), at the time of cell cycle entry (C), and during steady-state growth (S) of HMCs. The membranes were reprobed with β-actin as a loading control (some membranes are overlapped). Arrowheads indicate cross-reactive bands inevitably detected by some antibodies as described in manufacturers' datasheets. Nonetheless, the assignment of each band was based on the sizes and signal intensities. The signal intensities of each band were quantified and normalized to those of corresponding β-actin (setting at 1.0). The means ± s.d. (bars) of three independent experiments are shown. Statistical differences were determined by one-way ANOVA with Scheffe multiple comparison test (asterisks denote $P<0.01$).

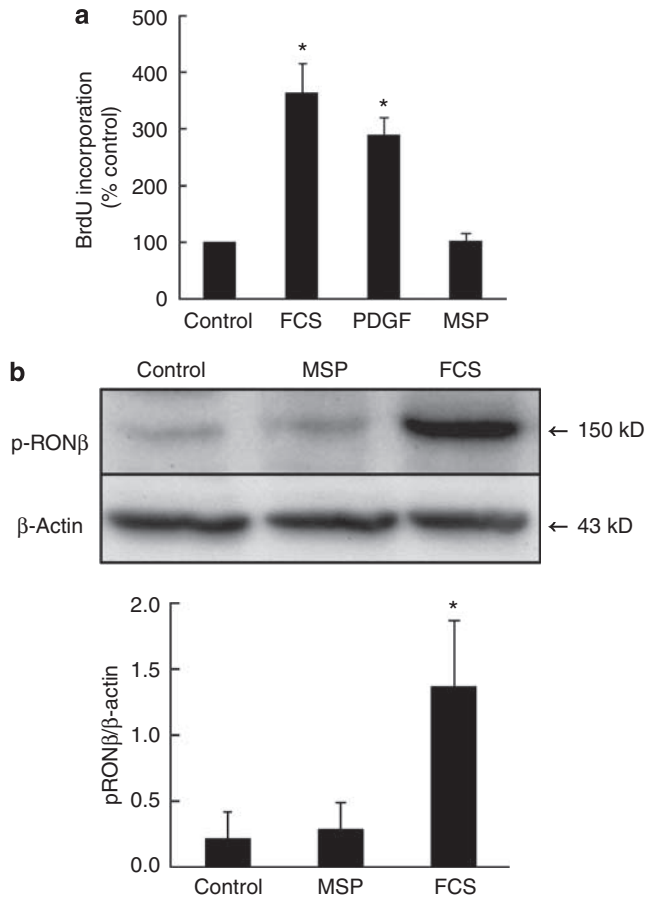

Figure 2 | MSP does not stimulate cell proliferation or induce RONβ phosphorylation in HMCs. (**a**) Serum-starved HMCs were exposed to 10% FCS, 10 ng/ml PDGF-BB, and 100 ng/ml MSP for 24 h, and cell growth was monitored with BrdU incorporation. BrdU incorporation is shown as a percentage of the value obtained with untreated cells (control). Results are expressed as the means ± s.d. (bars) of three independent experiments. (**b**) Serum-starved HMCs were exposed to none (control), 100 ng/ml MSP, and 10% FCS for 15 min, and subjected to immunoblot analysis for the expression of phosphorylated RONβ (p-RONβ). The membrane was reblotted with an antibody to β-actin as a loading control. The signal intensities of each band were quantified and normalized to those of corresponding β-actin (setting at 1.0). The means ± s.d. (bars) of three independent experiments are shown. Statistical differences were determined by one-way ANOVA with Scheffe multiple comparison test (asterisks denote $P<0.01$).

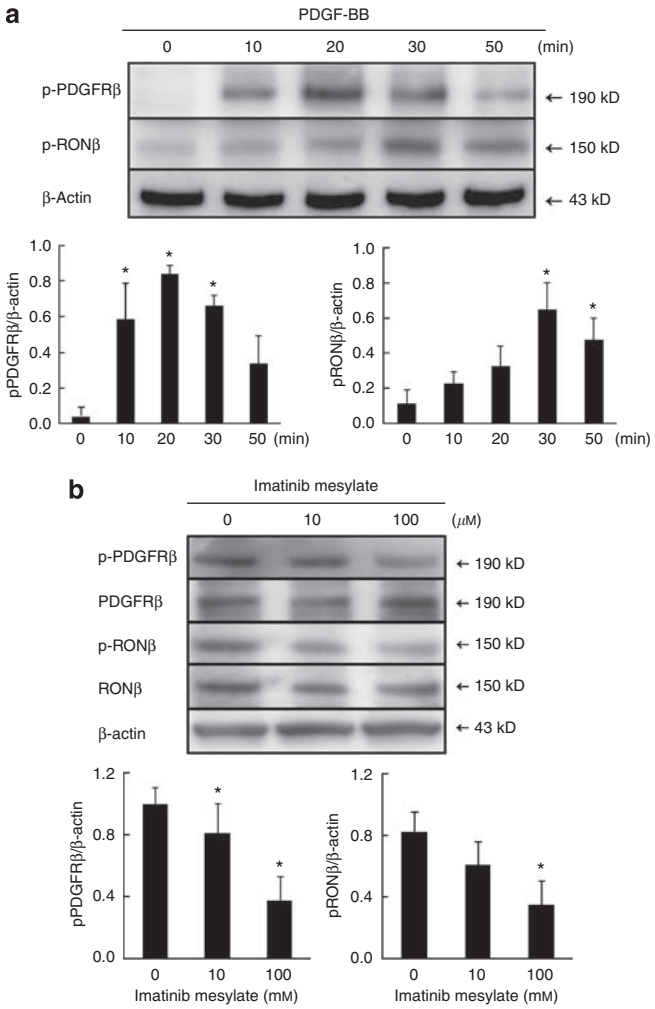

Figure 3 | PDGF induces RONβ phosphorylation through PDGFR in HMCs. (**a**) Serum-starved HMCs were cultured with 10 ng/ml PDGF-BB, and subjected to immunoblot analysis for the expression of phosphorylated PDGFRβ (p-PDGFRβ) and phosphorylated RONβ (p-RONβ at the indicated time points). The membrane was reblotted with an antibody to β-actin as a loading control. (**b**) Serum-starved HMCs were exposed to 10 ng/ml PDGF-BB in the absence or presence of imatinib mesylate at 10 μM or 100 μM. After 15 min, total protein lysates were isolated for the detection of phosphorylated PDGFRβ (p-PDGFRβ), total PDGFRβ (PDGFRβ), phosphorylated RONβ (p-RONβ), total RONβ (RONβ), and β-actin (loading control). The signal intensities of each band were quantified and normalized to those of corresponding β-actin (setting at 1.0). The means ± s.d. (bars) of three independent experiments are shown. Statistical differences were determined by one-way ANOVA with Scheffe multiple comparison test (asterisks denote $P<0.01$).

PDGFRβ using immunoprecipitation–immunoblot assays. As shown in Figure 4a, the physical interaction between RONβ and PDGFRβ was readily detected in quiescent HMCs, and was apparently enhanced during steady-state growth. We further showed the intracellular association of two molecules using immunocytochemistry under confocal laser microscopy. Figure 4b showed that RONβ and PDGFRβ were co-localized on the cell surface, and the former was also detected in the nucleus of HMCs. Furthermore, we evaluated the localization of the phosphorylated form of RONβ, and found that RONβ was predominantly present in the nucleus of HMCs on phosphorylation (Figure 4c, left panel). This unexpected finding was confirmed by immunoblotting of enriched nuclear fractions from serum-stimulated HMCs using an antibody specifically recognizing the phosphorylated form of RONβ (Figure 4c, right panel). These results suggest that RONβ translocates into the nucleus when activated by phosphorylation. The biological significance and underlying mechanism of this phenomenon remain to be determined, but a similar observation has been reported in KDR, a type 2 receptor of vascular endothelial cell growth factor.[11]

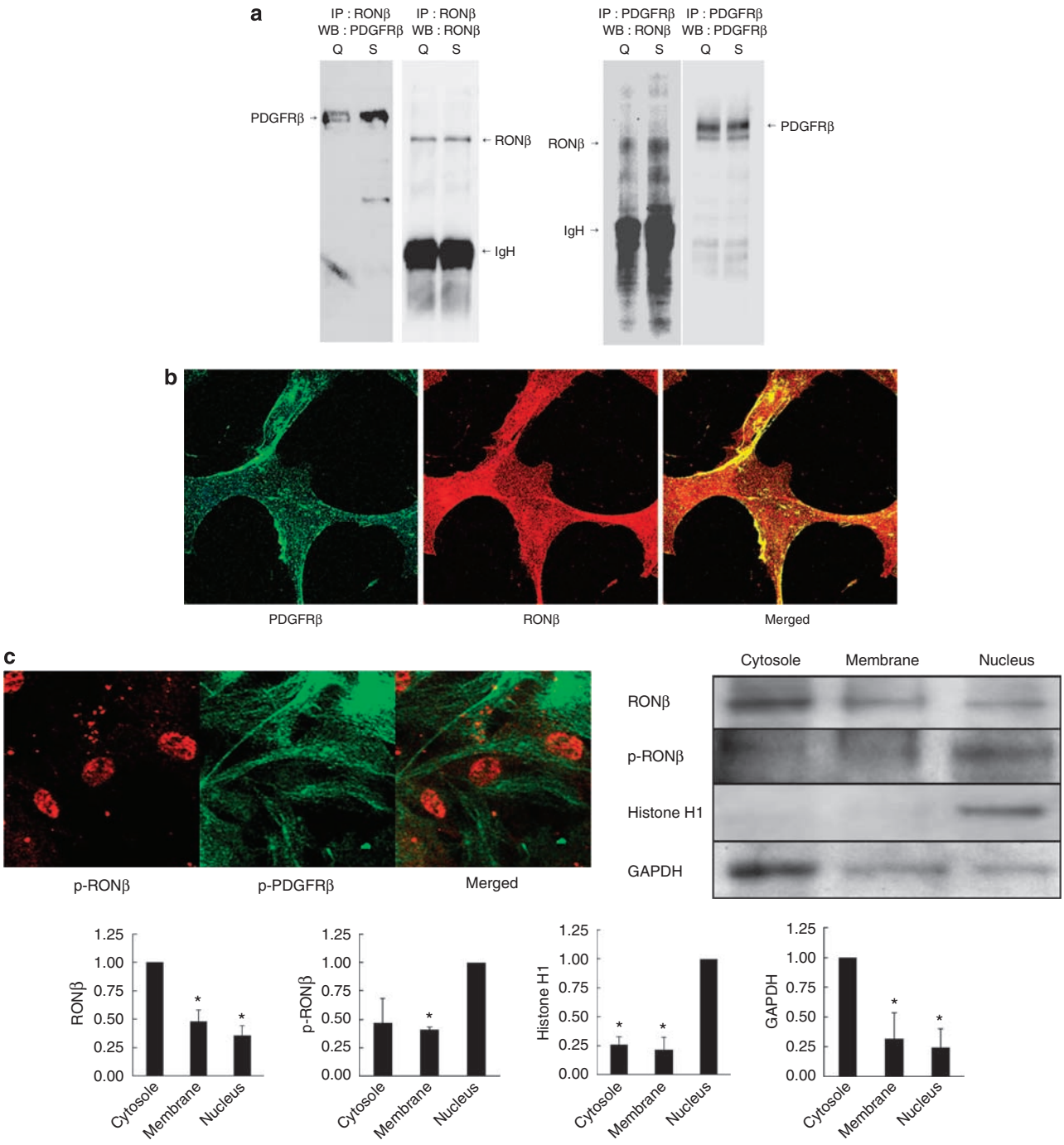

Figure 4 | Physical interactions between PDGFRβ and RONβ in HMCs. (**a**) Intracellular association of PDGFRβ and RONβ was detected by immunoprecipitation–immunoblot assays. (Left panel) RONβ-containing protein complex was immunoprecipitated from HMCs during quiescence (Q) and steady-state growth (S), and separated on SDS-PAGE, followed by immunoblotting with anti-PDGFRβ and anti-RONβ antibodies. IgH indicates a heavy chain of immunoglobulin. (Right panel) The same experiment was performed in reverse order. (**b**) HMCs were cultured on glass slides, and fixed in 4% paraformaldehyde in PBS. *In situ* detection of PDGFRβ (green) and RONβ (red) was carried out with confocal laser microscopy. (**c**) (Left panel) *In situ* detection of phosphorylated RONβ (red) and phosphorylated PDGFRβ (green) was carried out with confocal laser microscopy. (Right panel) The fractions enriched for cytosol, membrane, and nucleus were prepared from HMCs and subjected to immunoblot analysis for RONβ, phosphorylated RONβ, histone H1 (nuclear marker), and GAPDH (cytosolic marker). We calculated the relative intensities of each band with the strongest signal setting at 1.0, and show the means ± s.d. (bars) of three independent experiments. Statistical differences were determined by one-way ANOVA with Scheffe multiple comparison test (asterisks denote $P < 0.01$).

PDGF-induced transactivation of RONβ is specific for mesangial cells

The above results strongly suggested that RONβ could be activated in a ligand-independent manner through cross-talk with PDGFRβ in HMCs. The next question was whether this PDGF–PDGFRβ–RONβ signaling pathway is specific to HMCs or is also active in other cell types. To address this question, we used normal human epidermal keratinocytes expressing both PDGFRβ and RONβ. Normal human epidermal keratinocytes were serum-starved for 24 h, exposed to purified human recombinant PDGF-BB at an optimal concentration, and harvested for immunoblotting at the indicated time points (Figure 5). As is clearly shown, PDGF-BB failed to induce phosphorylation of RONβ in normal human epidermal keratinocytes. In addition, we carried out the same experiments with peripheral blood-derived adherent monocytes, which express both PDGFRβ and RONβ. Phosphorylation of RONβ was not observed in adherent monocytes cultured with PDGF (data not shown), suggesting that PDGF-mediated transactivation of RONβ is specific for mesangial cells within the renal system.

Persistent phosphorylation of RONβ during steady-state growth is due to relatively early dissociation from SHP2 and PP1 phosphatases

To clarify the mechanism by which RONβ phosphorylation persisted during steady-state growth of HMCs, we investigated the kinetic difference in the affinity with phosphatases between PDGFRβ and RONβ. For this purpose, we first screened for the expression of various phosphatases in HMCs by immunoblotting. HMCs expressed SHP-2 and PP1, but not SHP-1, SHIP, and SIRP1 (data not shown). We therefore examined the association of SHP-2 and PP1 with either PDGFRβ or RONβ using immunoprecipitation–immunoblot assays at the time of cell cycle entry and during steady-state growth. The association of PDGFRβ with SHP-2 and PP1 was detectable at the time of cell cycle entry, and persisted during steady-state growth of HMCs (Figure 6, left panel). This may underlie the transient nature of PDGF-induced phosphorylation of PDGFRβ. In contrast, the association of RONβ with SHP-2 and PP1 almost disappeared during steady-state growth (Figure 6, right panel). The relatively lower affinity to phosphatases contributes, at least in part, to the sustained phosphorylation of RONβ during steady-state growth of HMCs.

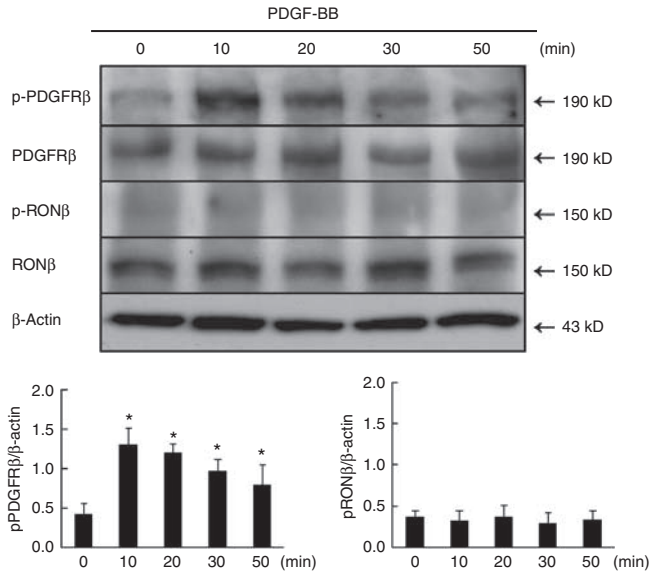

Figure 5 | Specificity of PDGF-induced transactivation of RON. Normal human epidermal keratinocytes were cultured in serum-free medium for 24 h and were exposed to 10 ng/ml human recombinant PDGF-BB. Whole cell lysates were isolated at the indicated time points, and subjected to immunoblot analysis for phosphorylated PDGFRβ (p-PDGFRβ), total PDGFRβ (PDGFRβ), phosphorylated RONβ (p-RONβ), and total RONβ (RONβ). The signal intensities of each band were quantified and normalized to those of corresponding β-actin (setting at 1.0). The means ± s.d. (bars) of three independent experiments are shown. Statistical differences were determined by one-way ANOVA with Scheffe multiple comparison test (asterisks denote $P < 0.01$).

RONβ mediates anti-apoptotic function in human mesangial cells

Sustained phosphorylation of RONβ suggests its additional role(s) during the steady growth phase of HMCs. To clarify this point, we examined the biological consequence of the knockdown of RONβ by the aid of siRNA. HMCs were transfected with either siRNA targeting the RON gene or scrambled control, and assessed for cell growth and death using BrdU incorporation and propidium iodide staining, respectively, after 48 h. As shown in Figure 7a, siRNA-mediated targeting of RON resulted in a significant increase in the proportion of apoptotic cells, but not cells undergoing DNA synthesis. These results suggest that RON contributes to the sustained growth of HMCs primarily by suppressing apoptosis, but not directing cell proliferation.

Next, we investigated the mechanisms by which RON suppresses apoptosis in HMCs. Immunoblot analysis

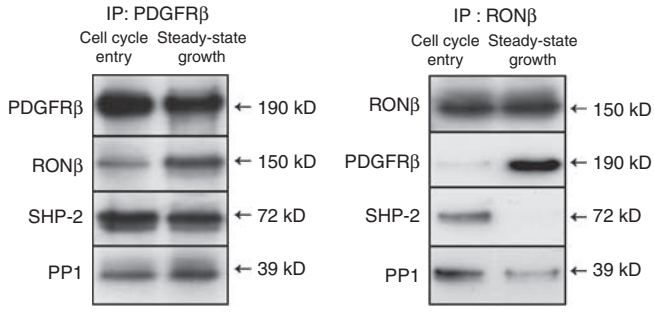

Figure 6 | Association of PDGFRβ and RONβ with SHP-2 and PP1 phosphatases. (Left panel) PDGFRβ-containing protein complex was immunoprecipitated from HMCs at the time of cell cycle entry and during steady-state growth, followed by immunoblotting with antibodies against PDGFRβ, RONβ, SHP-2, and PP1. (Right panel) RONβ-containing protein complex was immunoprecipitated from HMCs at the time of cell cycle entry and during steady-state growth, followed by immunoblotting with antibodies against RONβ, PDGFRβ, SHP-2, and PP1. Data shown are representative results of multiple independent experiments.

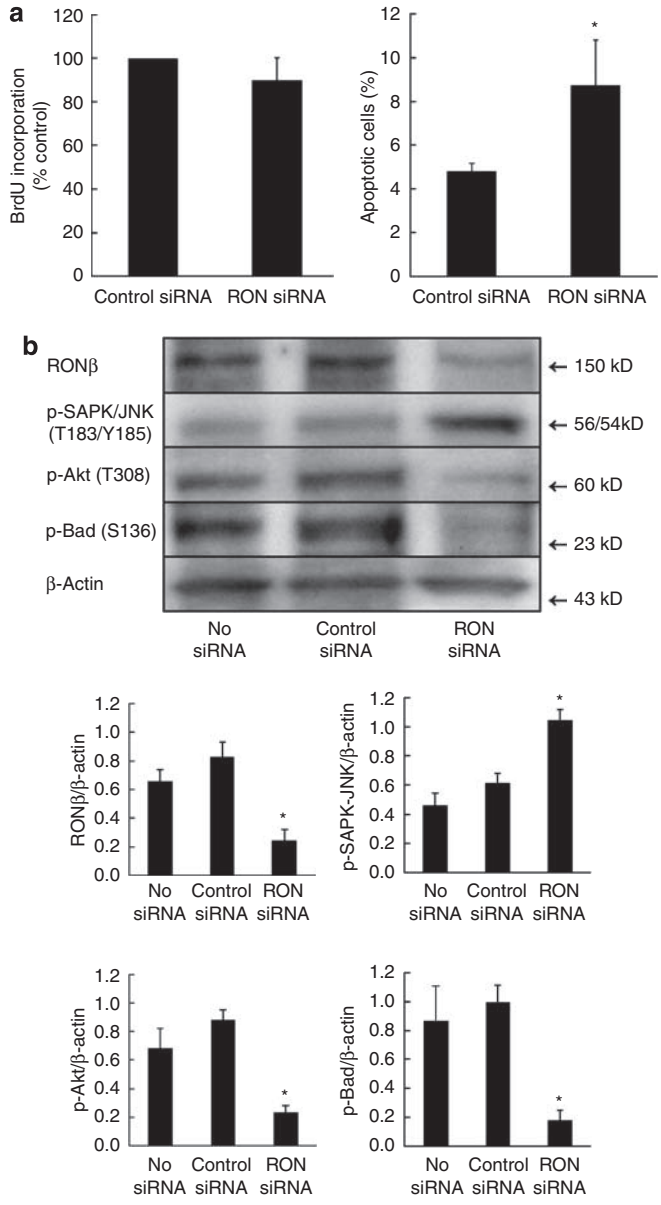

Figure 7 | RON regulates apoptosis, but not cell growth in HMCs. (**a**) HMCs were transfected with 50 nM siRNA targeting the RON gene (RON siRNA) or non-targeting siRNA (control siRNA). After 48 h, the cells were subjected to BrdU incorporation (left panel) and Annexin-V assay (right panel). BrdU incorporation is shown as a percentage of the value obtained with non-targeting siRNA. The results are expressed as the means ± s.d. (bar) of three independent experiments. Statistical analysis was performed with Student's *t*-test (asterisks denote $P<0.01$). (**b**) HMCs were transfected with 50 nM siRNA targeting the RON gene (RON siRNA) or non-targeting siRNA (Control siRNA). After 48 h, total protein lysates were prepared from the transfectants and untreated HMCs (no siRNA) and analyzed by immunoblotting using antibodies against RONβ, phosphorylated-SAPK/JNK, phosphorylated-Akt, and phosphorylated-Bad. The same membrane was reblotted with an antibody to β-actin for loading control. The signal intensities of each band were quantified and normalized to those of corresponding β-actin (setting at 1.0). The means ± s.d. (bars) of three independent experiments are shown. Statistical differences were determined by one-way ANOVA with Scheffe multiple comparison test (asterisks denote $P<0.01$).

revealed that an increase in the amount of phosphorylated SAPK/JNK coincided with the downregulation of RONβ in HMCs treated with siRNA against RON (Figure 7b). In addition, there was a decrease in the abundance of Akt phosphorylated at threonine-308 and Bad phosphorylated at serine-136 (Figure 7b). It has been shown that SAPK/JNK promotes apoptosis on phosphorylation[12] and the phosphorylation of Akt and Bad at threonine-308 and serine-136, respectively, favors cell survival.[13–15] Therefore, it is plausible that RON suppresses apoptosis of HMCs by modulating the activities of these molecules.

RONβ is phosphorylated in glomerular lesions of human mesangial proliferative glomerulonephritis

Finally, we attempted to clarify the biological significance of RONβ phosphorylation *in vivo*. For this purpose, we examined the phosphorylation status of RONβ in glomerular lesions of mesangial IgA nephropathy, a prototype mesangial proliferative disease, using immunohistochemistry. As controls, we stained PDGFRβ in the same samples and performed the same experiments using biopsy specimens from patients with minimal change nephrotic syndrome (MCNS), which is not associated with mesangial cell proliferation. As shown in Figure 8c, a marked increase in PDGFRβ expression was observed in samples from patients with IgA nephropathy, but not in those from patients with MCNS, consistent with the role of the PDGF/PDGFR system in the proliferation of HMCs.[1–3] It is of note that the expression of phosphorylated RONβ was also upregulated in glomerular lesions of IgA nephropathy compared with those of MCNS (Figure 8d). Quantification of the positivity of phosphorylated RONβ revealed that the difference between IgA nephropathy (56.5 ± 2.1%) and MCNS (37.6 ± 6.8%) was statistically significant ($P<0.01$) (Figure 8e). These results suggest that RONβ activation is implicated in the development of mesangial proliferative glomerulonephritis, and is an additional target of therapeutic intervention.

DISCUSSION

Although the importance of PDGF in the proliferation of HMCs has been established, targeted inhibition of the PDGF/PDGFR system has shown only limited efficacy against mesangial proliferative diseases. This implies that additional signal-transducing molecules are involved in the proliferation of HMCs. In this study, we found that RON is activated during the steady-state growth of HMCs, in which PDGFR is inactivated by dephosphorylation, and suppresses apoptosis through the SAPK/JNK and Akt pathways. The activation of RON is ligand-independent and is mediated through transactivation from PDGFR after engagement with PDGF. We also showed that RONβ is phosphorylated in glomerular lesions of patients with IgA nephropathy. These results suggest that RON is involved in mesangial cell proliferation under both physiological and pathological conditions, and is a relevant target of therapeutic intervention for human glomerular diseases.

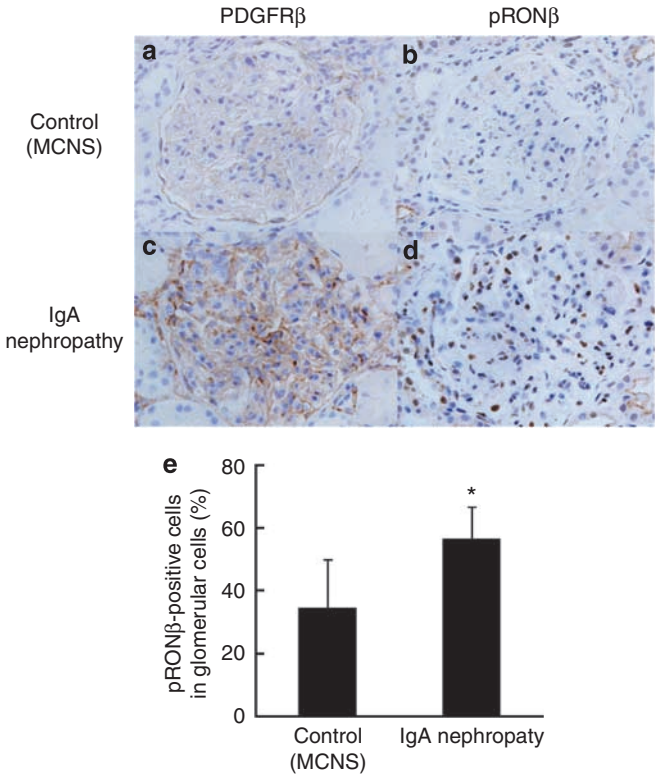

Figure 8 | Expression of phosphorylated RON in the glomerular lesions of mesangial proliferative diseases. Renal biopsy specimens from patients with minimal change nephrotic syndrome (MCNS) (**a** and **b**) and IgA nephropathy (**c** and **d**) were stained for the detection of PDGFRβ (**a** and **c**) and phosphorylated RONβ (**b** and **d**). Representative photographs are shown (original magnification, ×400). (**e**) The proportions of phosphorylated RON-positive cells in glomerular cells were quantified in 6 and 24 samples from MCNS and IgA nephropathy, respectively (asterisk denotes $P<0.01$ by Student's *t*-test). Data shown are the means ± s.d. (bars) of the percentages of pRONβ-positive cells in each glomerulus (10 glomeruli were examined in each case).

RON belongs to Met family RTKs. It was originally isolated from a human keratinocyte cDNA library,[16] and the murine homolog, also called STK (stem cell-derived tyrosine kinase), was cloned from hematopoietic stem cells.[17] RON is mainly expressed in resident peritoneal macrophages,[18] adherent monocytes,[19] osteoclasts,[20] mesangial cells,[21] and epithelial tissues in the skin, colon, breast, lung and kidney.[22–25] In the kidney, RON is expressed in tubular epithelium cells, mesangial cells, and glomerular-infiltrating monocytes in experimental glomerulonephritis model.[26] Recent studies have shown that RON expression is frequently altered in several primary human cancers, including those of breast, colon, and ovary.[27–29] In addition, truncation of RON has been found in primary tumors of the gastrointestinal tract.[30] Although these findings point to a role of RON in the regulation of physiological and pathological cell growth, the precise functions of RON in HMCs remain elusive.

MSP, a member of the plasminogen-related growth factor family, is known as a sole ligand for RON.[31] The MSP/RON system elicits diverse biological effects in a number of cell types. These include the inhibition of inducible nitric oxide synthase by macrophages,[32] contribution to wound healing and liver regeneration,[33] proliferation and migration of keratinocytes,[34] and a potential role in bone resorption.[20] The studies *in vivo* suggest that RON regulates inflammatory responses in the skin,[34] liver,[35] and lung.[36,37]

In this study, MSP failed to phosphorylate RONβ, but instead PDGF-BB induced the phosphorylation of RONβ in HMCs. The reason for the insensitivity of HMCs to MSP is at present unknown, but evidence from gene-targeted mice provides a clue. Complete disruption of the RON gene leads to the death of mouse embryos in the early stage, suggesting that RON is absolutely required for embryonic development.[38] Unlike with RON deficiency, MSP knockout mice are not lethal and do not show any visible phenotypic changes, indicating that MSP is dispensable for cell growth and basal cellular functions.[33] The striking difference in the consequences of RON and MSP deficiencies raises the possibility that additional ligand(s) for RON and/or alternate mechanism(s) of RON activation exist. Our findings suggest that PDGF may compensate for the loss of MSP by transactivating RON in MSP knockout mice.

PDGFR is not an exclusive partner of RON. Recent studies have shown that RON is spatially and functionally associated with other transmembrane molecules including adhesion receptors (integrins and cadherins) and cytokine and growth factor receptors (IL-3 receptor, erythropoietin receptor, EGF receptor, and Met).[10,39–41] The interaction between RON and IL-3 receptor is known to be unidirectional: MSP induces the phosphorylation of both RON and IL-3 receptor, whereas IL-3 does not phosphorylate RON.[39] In contrast, cross talk of RON with either Met or EGF receptor is bidirectional.[40,41] As shown in this study, the interaction between RON and PDGFR is also unidirectional. PDGF-mediated transactivation of RON seems to be unique to HMCs in the renal system; it was not observed in activated monocytes, and tubular epithelial cells express RON, but not PDGFR (data not shown).

As a mechanism of the persistent phosphorylation of RON during sustained growth of HMCs, we propose a difference in the affinity to phosphatases, SHP-2 and PP1, between RON and PDGFR. Activities of RTKs are regulated by protein tyrosine phosphatases in many ways.[42] SHP-2 has been described as a regulator of multiple RTK signaling pathways.[43,44] In addition, RTKs also contain several serine and threonine residues. There is increasing evidence that serine/threonine phosphorylation also plays a relevant role in modulating RTK functions.[45] It has been shown that PP1 is involved in serine dephosphorylation of RON.[46]

Finally, we have shown for the first time the increased phosphorylation of RON in glomerular lesions of patients with IgA nephropathy, a prototype mesangial proliferative nephritis. This finding may be a clue to understanding the mechanisms of chronic mesangial proliferation more precisely, and provides a rational basis for the development of better treatment strategies for human glomerular diseases.

MATERIALS AND METHODS

Cells and cell culture

Normal primary human mesangial cells were purchased from Cambrex BioScience (Walkersville, MD, USA). Cells were maintained in RPMI1640 medium (Sigma-Aldrich Co., St Louis, MO, USA) supplemented with 10% FCS and 10 μg/ml insulin, 5.5 μg/ml transferrin, and 6.7 ng/ml sodium selenite (Life Technology, Carlsberg, CA, USA). Normal human epidermal keratinocytes were purchased from Kurabo (Osaka, Japan). Cells were grown in Keratinocyte-SFM medium (Invitrogen, NY, USA) supplemented with 30 μg/ml bovine pituitary extract and 0.2 ng/ml recombinant EGF. Cells used in the present experiments were from four to eight passages.

Cell proliferation assay

HMCs were growth-arrested for 24 h in serum-free RPMI1640 medium, and incubated with either 10 ng/ml PDGF-BB (R&D Systems, Minneapolis, MN, USA), 100 ng/ml MSP (R&D Systems), or 10% FCS for 24 h. DNA synthesis was monitored for the final 6 h using a BrdU incorporation assay kit (Roche Diagnostics, Mannheim, Germany).

Transfection of siRNA

Transfection of siRNA was carried out using a Dharmafect transfection reagent (GE Healthcare UK Ltd, Buckinghamshire, England) according to the manufacturer's instructions. HMCs were grown in six-well dishes, and transfected with either siRNA against RON (macrophage stimulating 1 receptor (MST1R) ON-TARGET plus SMARTpool) or control siRNA (siCONTROL Non-targeting siRNA pool) (Dharmacon, Inc., Lafayette, CO, USA) at 50 nM for 24 h. The sequences of RON and control siRNAs are 5′-UAAAUCUAUAGGUCUGCAGUU, 5′-UCACACUCAAACUCCUCUAUU, 5′-UAUGACAUUCACCAUCUACUU, and 5′-CUAUCGGAGAAGGAUGCUCUU; and 5′-UGGUUUACAUGUCGACUAA, 5′-UGGUUUACAUGUUUUCUGA, 5′-UGGUUUACAUGUUUUCCUA, and 5′-UGGUUUACAUGUUGUGUGA, respectively.

Flow cytometry

Cell cycle profile was determined by staining DNA with propidium iodide, and the size of the sub-G1 fraction was calculated as a percentage with the ModFitLT 2.0 program (Verity Software, Topsham, ME, USA). For the detection of apoptosis, cells were stained with PE-conjugated annexin-V (Biovision, Mountain View, CA, USA).

Cell fractionation

Cells were fractionated into cytosol, membranes, nuclei, and cytoskeleton using a Subcellular ProteoExtract kit (EMD Chemicals, San Diego, CA, USA) according to the manufacturer's directions.

RTK array analysis

We used a human Phospho-RTK Array kit (R&D Systems) to simultaneously detect relative tyrosine phosphorylation levels of 42 different RTKs. Whole cell lysates were prepared in chilled cell lysis buffer (50 mM Tris-HCL, pH 8.0, 120 mM $NaCl_2$, 0.5% Nonidet P-40, 100 mM sodium fluoride, and 200 μM sodium orthovanadate). Array membranes were blocked and hybridized with 500 μg of protein samples overnight at 4°C, followed by incubation with an anti-phosphotyrosine antibody conjugated with horseradish peroxidase for 2 h at room temperature. After being washed, membranes were developed with ECL Western blotting detection reagent (GE Healthcare, UK).

Immunoblotting

Immunoblotting was carried out according to the standard method. The following primary antibodies were used: anti-human MSPR/Ron beta (R&D Systems), anti-RON alfa (BD Transduction Laboratories, San Jose, CA, USA), anti-phosphorylated RON (Tyr1238/Tyr1239) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-PDGFRβ (Cell Signaling Technology, Danvers, MA, USA), anti-phosphorylated PDGFRβ (Tyr751) (Cell Signaling Technology), anti-phosphorylated SAPK/JNK (Thr183/Tyr185) (Cell Signaling Technology), anti-phosphorylated Akt (Thr308) (Cell Signaling Technology), anti-phosphorylated Bad (Ser136) (Cell Signaling Technology), anti-histone H1 (Santa Cruz Biotechnology), anti-GAPDH (Santa Cruz Biotechnology), and anti-β-actin (C4; ICN Biomedicals, Aurora, OH, USA).

Immunoprecipitation–immunoblotting assays

After being precleared with protein A-sepharose, whole cell lysates (500 μg protein) were incubated with 2 μg of either anti-RON polyclonal antibody (ABGENT, San Diego, CA, USA), anti-PDGFRβ polyclonal antibody (Cell Signaling Technology), or rabbit IgG in 200 μl of cell lysis buffer. After brief centrifugation, the supernatants were rocked overnight at 4°C in the presence of protein A-sepharose beads. Immune complexes were collected on the beads, washed three times in cell lysis buffer, and applied to 10% SDS-PAGE, followed by immunoblotting with either anti-RON, anti-PDGFRβ, anti-SHP2 (BD Biosciences, Franklin Lakes, NJ, USA), or anti-PP1 (Santa Cruz Biotechnology) monoclonal antibody.

Confocal laser microscopy

Cells were cultured on Shandon cytoslides for 24 h (Thermo Scientifics, Pittsburgh, PA, USA) and fixed in 4% paraformaldehyde in phosphate-buffered saline. RONβ was stained with anti-MST1R/RON polyclonal antibody (MBL, Japan) and a goat antibody to rabbit immunoglobulin conjugated with Alexa 488 (Molecular Probes, Eugene, OR, USA). PDGFRβ was stained with anti-PDGFRβ monoclonal antibody (Cell Signaling Technology) and a donkey antibody to mouse immunoglobulin conjugated with Cy3 (Amersham Biosciences, Buckinghamshire, England). Phosphorylated RONβ was stained with anti-phosphorylated RONβ (Tyr1238/1239) and a goat antibody to rabbit immunoglobulin conjugated with Alexa 488. Phosphorylated PDGFRβ was stained with anti-phosphorylated PDGFRβ and a donkey antibody to mouse immunoglobulin conjugated with Cy3. Confocal microscopic analysis was performed as described earlier.[47]

Immunohistochemistry

We examined renal biopsy specimens from 30 patients with renal diseases, including 24 patients with IgA nephropathy (age: 16–65, sex: male/female = 11/13) and six patients with MCNS (age: 19–70, sex: male/female = 3/3), admitted to Jichi Medical University Hospital. The ethics committee of Jichi Medical University approved this study, and all patients gave written informed consent before entry into the study. The expressions of phosphorylated RONβ and PDGFRβ were evaluated by immunohistochemistry. Formalin-fixed paraffin-embedded sections (3 μm) were deparaffinized in xylene, rehydrated in graded ethanol, and subjected to antigen retrieval procedure in 10 mmol/l of boiling citrate buffer solution (pH 6.0). Non-specific protein staining was blocked by preincubation for

10 min with normal swine serum. Tissue sections were incubated overnight at 4°C with either anti-phosphorylated RONβ antibody (Santa Cruz Biotechnology) or anti-PDGFRβ antibody (Cell Signaling Technology) at final dilutions of 1:200 and 1:50, respectively. After being washed with PBS, tissue sections were treated with the Envision Plus polyclonal system (Dako, Santa Barbara, CA, USA). Samples were developed with liquid diaminobenzidine and counterstained with Mayer's hematoxylin.

Statistical analysis

The results of immunoblot experiments including phospho-RTK assays were quantified using the Scion Image software (Scion Corporation, Frederick, MD, USA). Statistical differences were determined by either Student's *t*-test or one-way analysis of variance (ANOVA) with Scheffe multiple comparison test on the Stat View program (SAS Institute, Cary, NC, USA) with $P<0.01$ being considered significant.

DISCLOSURE

All the authors declared no competing interests.

ACKNOWLEDGMENTS

We are grateful to Ms Tomoko Kamiakito for her excellent technical assistance in immunohistochemistry.

This work was supported in part by the High-Tech Research Center Project for Private Universities: Matching Fund Subsidy from MEXT 2002-2006, and grants from the Vehicle Racing Commemorative Foundation and Sankyo Foundation of Life Science (to YF).

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/ki

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