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T-type calcium channel blockade as a therapeutic strategy against renal injury in rats with subtotal nephrectomy

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T-type calcium channel blockers have been previously shown to protect glomeruli from hypertension by regulating renal arteriolar tone. To examine whether blockade of these channels has a role in protection against tubulointerstitial damage, we used a stereo-selective T-type calcium channel blocker *R*(−)-efonidipine and studied its effect on the progression of this type of renal injury in spontaneously hypertensive rats that had undergone subtotal nephrectomy. Treatment with racemic efonidipine for 7 weeks significantly reduced systolic blood pressure and proteinuria. The *R*(−)-enantiomer, however, had no effect on blood pressure but significantly reduced proteinuria compared to vehicle-treated rats. Both agents blunted the increase in tubulointerstitial fibrosis, renal expression of α-smooth muscle actin and vimentin along with transforming growth factor-β (TGF-β)-induced renal Rho-kinase activity seen in the control group. Subtotal nephrectomy enhanced renal T-type calcium channel α1G subunit expression mimicked in angiotensin II-stimulated mesangial cells or TGF-β-stimulated proximal tubular cells. Our study shows that T-type calcium channel blockade has renal protective actions that depend not only on hemodynamic effects but also pertain to Rho-kinase activity, tubulointerstitial fibrosis, and epithelial–mesenchymal transitions.

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Voltage-dependent Ca^{2+} channels are categorized into two subfamilies by their electrophysiological properties: a high voltage-activated Ca^{2+} channel family, including P-/Q-, L-, N-, and R-type Ca^{2+} channels, and low-voltage-activated Ca^{2+} channels, which correspond to T-type Ca^{2+} channels (TCCs).[1] Although L-type Ca^{2+} channel blockers (L-CCBs), including nifedipine, are widely prescribed for the treatment of hypertension, there is some concern that L-CCBs might aggravate renal damage.[2] As L-type Ca^{2+} channels (LCCs) prevail predominantly in the afferent arteriole but are sparsely expressed in the efferent arteriole,[3,4] L-CCBs would dilate the afferent arteriole preferentially and thus might accelerate glomerular hypertension if the hypotensive effect is insufficient. In contrast, as TCCs are expressed in both afferent and efferent arterioles,[5] it is reasonably conjectured that TCC blockers (T-CCBs) protect glomeruli from systemic hypertension by regulating renal arteriolar tone.[4,6–8] We previously reported that efonidipine, a CCB with a blocking activity on both TCCs and LCCs,[9] ameliorated proteinuria more potently than nifedipine in subtotally nephrectomized spontaneously hypertensive rats (SHR) despite similar blood pressure-lowering effects.[10] Although this result suggests that the T-CCB improves the progression of renal injury, it remains to be determined whether this effect of efonidipine is mediated by the specific blockade of TCCs because efonidipine has substantial effects on LCCs as well. Furthermore, the role of the TCC in mediating the tubulointerstitial changes in the development of renal injury has not been examined.

Several lines of investigations have witnessed that the Rho/Rho-kinase pathway is activated in progressive renal injury.[11–13] Small-molecule G protein, Rho, and its effector Rho-kinase contribute significantly to the vascular contractile response as well as various cellular functions, including cell adhesion/migration and proliferation.[14–16] We have recently demonstrated that the Rho/Rho-kinase pathway participates in the regulation of renal afferent and efferent arteriolar tone and glomerular filtration.[17] Furthermore, the long-term Rho-kinase inhibition by fasudil reduces proteinuria in the rat remnant kidney model.[11] In this regard, it has been

reported that increases in intracellular Ca^{2+} concentration are associated with the activation of Rho-kinase in several types of vascular beds.[18,19] Furthermore, in mesangial cells, efonidipine, but not nifedipine, suppresses nuclear factor-κB expression,[20] which is also a target for the Rho/Rho-kinase pathway.[21] No studies have been conducted, however, examining the role of the TCC-mediated signaling pathway in the activation of Rho-kinase and the subsequent impact on renal injury.

Efonidipine is the raceme of *R*(−)-enantiomer and *S*(+)-enantiomer, and these isomers have been recently isolated. The *R*(−)-enantiomer of efonidipine has been demonstrated to block the T-type Ca^{2+} current almost exclusively in native myocardial cells, *Xenopus* oocytes, and BHK cells, whereas the *S*(+)-enantiomer blocks both T-type and L-type Ca^{2+} current.[22,23] Herein, with the use of the *R*(−)-enantiomer of efonidipine, we investigated the role of the TCC in mediating the development of renal injury in the remnant kidney model. Furthermore, whether the TCC activated the Rho/Rho-kinase pathway in the deterioration process was assessed.

RESULTS

Effect of efonidipine and *R*(−)-efonidipine on blood parameters

At the end of the study, serum creatinine was elevated in subtotally nephrectomized SHR (SHR-Nx, 0.66 ± 0.12 mg per 100 ml, $n = 13$) compared with sham-operated rats (sham, 0.19 ± 0.01 mg per 100 ml, $n = 8$, $P < 0.01$), and was reduced in subtotally nephrectomized SHR given efonidipine (SHR-Nx + Efo, 0.49 ± 0.03 mg per 100 ml, $n = 10$, $P < 0.05$) and subtotally nephrectomized SHR given *R*(−)-efonidipine (SHR-Nx + R, 0.49 ± 0.02 mg per 100 ml, $n = 12$, $P < 0.05$). Serum total protein levels did not differ among four groups (sham, 5.7 ± 0.1 g per 100 ml; SHR-Nx, 5.7 ± 0.2 g per 100 ml; SHR-Nx + Efo, 5.8 ± 0.1 g per 100 ml; SHR-Nx + R, 5.5 ± 0.1 g per 100 ml, $P > 0.5$).

Effect of efonidipine and *R*(−)-efonidipine on blood pressure and proteinuria

After 4 weeks of subtotal nephrectomy, systolic blood pressure (SBP) in the SHR-Nx group was elevated, compared with sham ($P < 0.01$), and remained higher throughout the study period (Figure 1a). Treatment with efonidipine markedly suppressed the elevated SBP at week 4 ($P < 0.01$ vs SHR-Nx), and did not cause further increases in SBP. In contrast, *R*(−)-efonidipine failed to suppress the elevated SBP throughout the study period ($P > 0.5$ vs SHR-Nx).

Daily urinary protein excretion in SHR-Nx was elevated at week 4 ($P < 0.01$ vs sham), reaching 96 ± 13 mg day^{-1} at week 8 ($P < 0.01$ vs sham; Figure 1b). Treatment with efonidipine blunted the increase in proteinuria at week 4 ($P < 0.05$ vs SHR-Nx) and week 8 ($P < 0.01$ vs SHR-Nx). Similarly, in SHR-Nx + R, the increase in proteinuria was suppressed at week 4 ($P < 0.05$ vs SHR-Nx), at week 6 ($P < 0.05$), and at week 8 ($P < 0.01$).

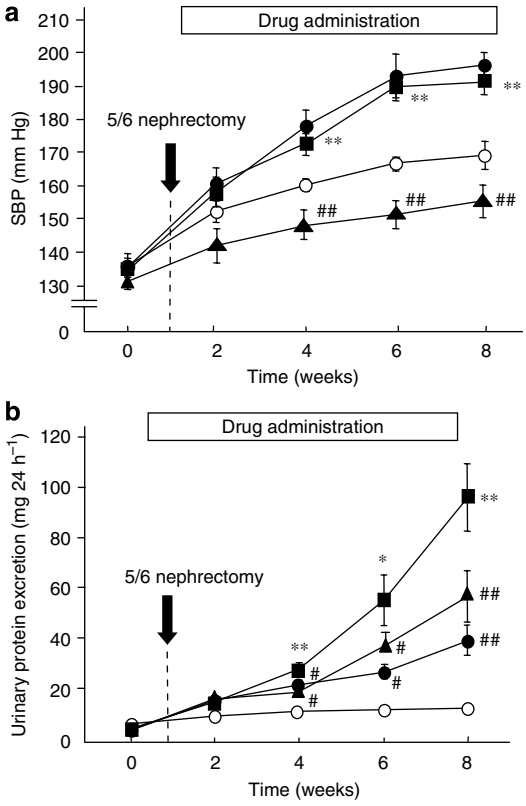

Figure 1 | Effects of efonidipine and *R*(−)-efonidipine on the changes in SBP (a) and urinary protein excretion (b) in subtotally nephrectomized rats. Open circles: sham; squares: SHR-Nx; triangles: SHR-Nx + efonidipine; closed circles: SHR-Nx + *R*(−)-efonidipine. *$P < 0.05$ vs sham, **$P < 0.01$ vs sham, #$P < 0.05$ vs SHR-Nx, ##$P < 0.01$ vs SHR-Nx.

Morphological changes by efonidipine and *R*(−)-efonidipine

In SHR-Nx, several glomeruli revealed pathological manifestation, including mesangial expansion (Figure 2a). These alterations were ameliorated by both efonidipine and *R*(−)-efonidipine. Glomerular size was increased in SHR-Nx, compared with sham ($P < 0.01$; Figure 2b). This change was restored partially by treatment with efonidipine and *R*(−)-efonidipine ($P < 0.01$ vs SHR-Nx).

Masson-trichrome staining revealed increased levels of renal interstitial fibrosis and tubular casts in kidneys from SHR-Nx (Figure 2c). Treatment with efonidipine and *R*(−)-efonidipine markedly suppressed the changes observed in SHR-Nx ($P < 0.01$). When the tubulointerstitial changes were semiquantified, it was evident that both agents potently inhibited the tubulointerstitial fibrosis (Figure 2d).

TCC expression

We examined whether TCC expression was altered in the remnant kidney. Thus, immunohistochemical analysis revealed that the expression of α1G, an α1 subunit of the TCC, was enhanced widely in the kidney, especially in renal arterioles and glomerular areas, from SHR-Nx (Figure 3a). In analogy, the expression of α1G was upregulated in the renal cortical tissues of SHR-Nx, with a 3.2-fold increase compared with sham ($P < 0.05$; Figure 3b).

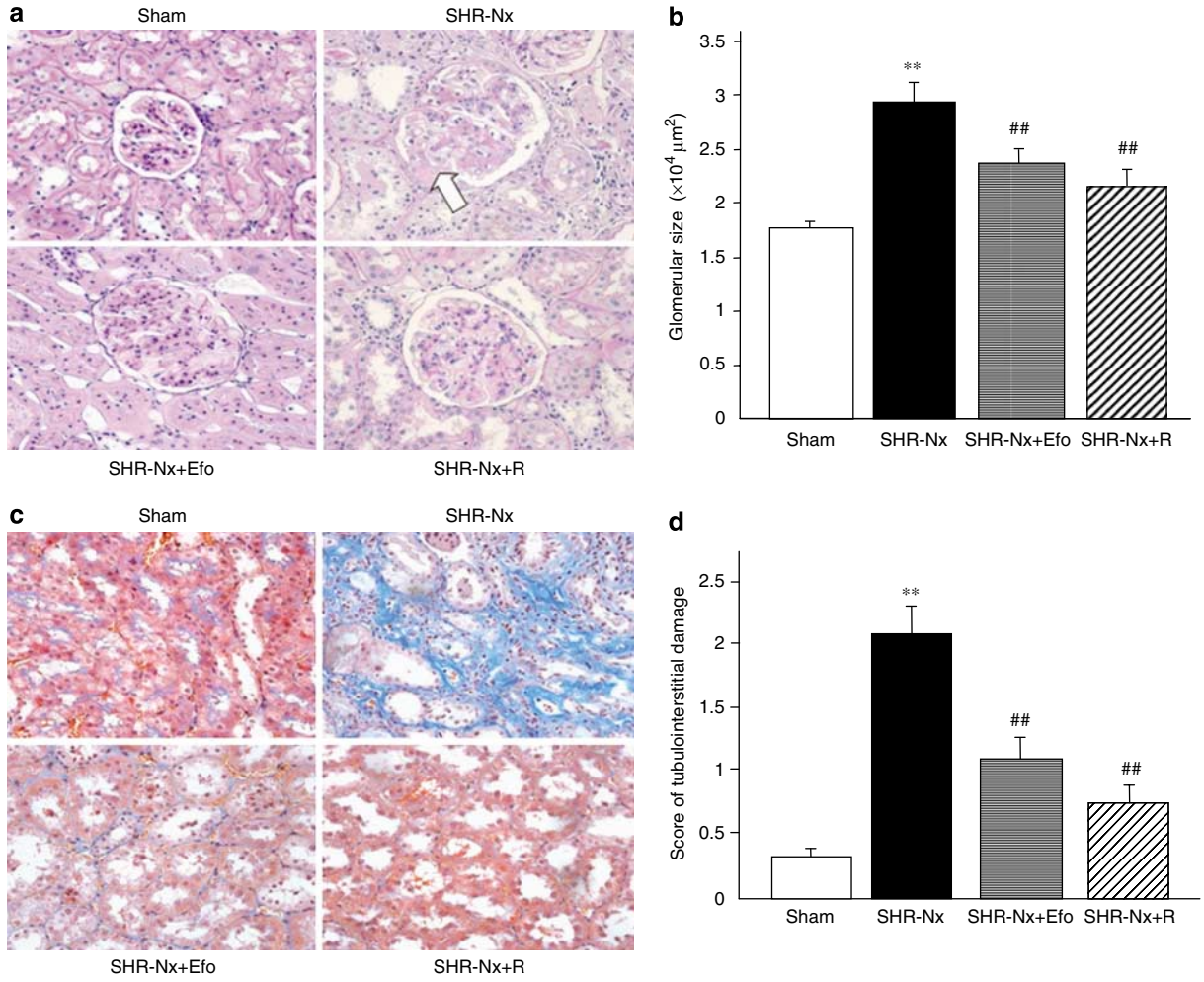

Figure 2 | Effects of efonidipine and *R*(−)-efonidipine on morphological changes in subtotally nephrectomized rats. (**a**) An open arrow indicates glomerular sclerotic changes in SHR-Nx. (**b**) Glomerular size in SHR-Nx. (**c**, **d**) Interstitial fibrosis and tubular casts in kidneys from SHR-Nx. $^{**}P<0.01$ vs sham, $^{\#\#}P<0.01$ vs SHR-Nx. (**a**, **b**: periodic acid-Schiff (PAS) stain; **c**, **d**: Masson-trichrome stain).

To elucidate the role of humoral factors in mediating the upregulation of α1G expression in renal injury, angiotensin II (Ang II) and transforming growth factor-β (TGF-β) were administered in cultured mesangial cells and human kidney proximal tubular epithelial cells (PTECs), respectively. Thus, Ang II (100 nmol l^{-1}) upregulated α1G expression in mesangial cells, with a 1.5-fold increase compared with control ($n=4$, $P<0.05$; Figure 3c). Similarly, treatment with TGF-β (5 ng ml^{-1}) caused a 1.8-fold increase in α1G expression in PTECs (Figure 3d).

Effect of efonidipine and *R*(−)-efonidipine on TGF-β and Rho-kinase

Immunohistochemical analysis showed an enhanced expression of TGF-β in the remnant kidney from SHR-Nx ($P<0.01$ vs sham; Figure 4a and b). Furthermore, the upregulated TGF-β expression was suppressed by treatment with R(−)-efonidipine (SHR-Nx + R, $P<0.01$ vs SHR-Nx), but not by efonidipine (SHR-Nx + Efo).

Previously, we demonstrated that the renal cortical tissue from SHR-Nx manifested enhanced Rho-kinase activity, which was responsible in part for the progression of renal injury.[11] We therefore investigated whether treatment with efonidipine and *R*(−)-efonidipine affected the Rho/Rho-kinase pathway in this experimental rat. Thus, the phosphorylation level of MYPT1, which indicated Rho-kinase activity, was enhanced in the renal cortical tissue from SHR-Nx ($P<0.01$ vs sham) (Figure 5a). Treatment with efonidipine and *R*(−)-efonidipine inhibited the increased Rho-kinase activity, with similar suppression in SHR-Nx + Efo and SHR-Nx + R ($P<0.01$ vs SHR-Nx).

We further evaluated the effect of TGF-β on Rho-kinase activity and the role of TCCs in TGF-β-mediated Rho-kinase activation in PTECs. Treatment with TGF-β elicited substantial activation of Rho-kinase (as assessed by MYPT-1 phosphorylation) in PTECs ($P<0.05$; Figure 5b). Furthermore, this upregulation was completely prevented by both efonidipine and *R*(−)-efonidipine ($P<0.01$ vs TGF-β).

Expression of α-smooth muscle actin and vimentin in the renal cortex

In the renal cortical interstitial area, the expression of α-smooth muscle actin (α-SMA) was increased in SHR-Nx

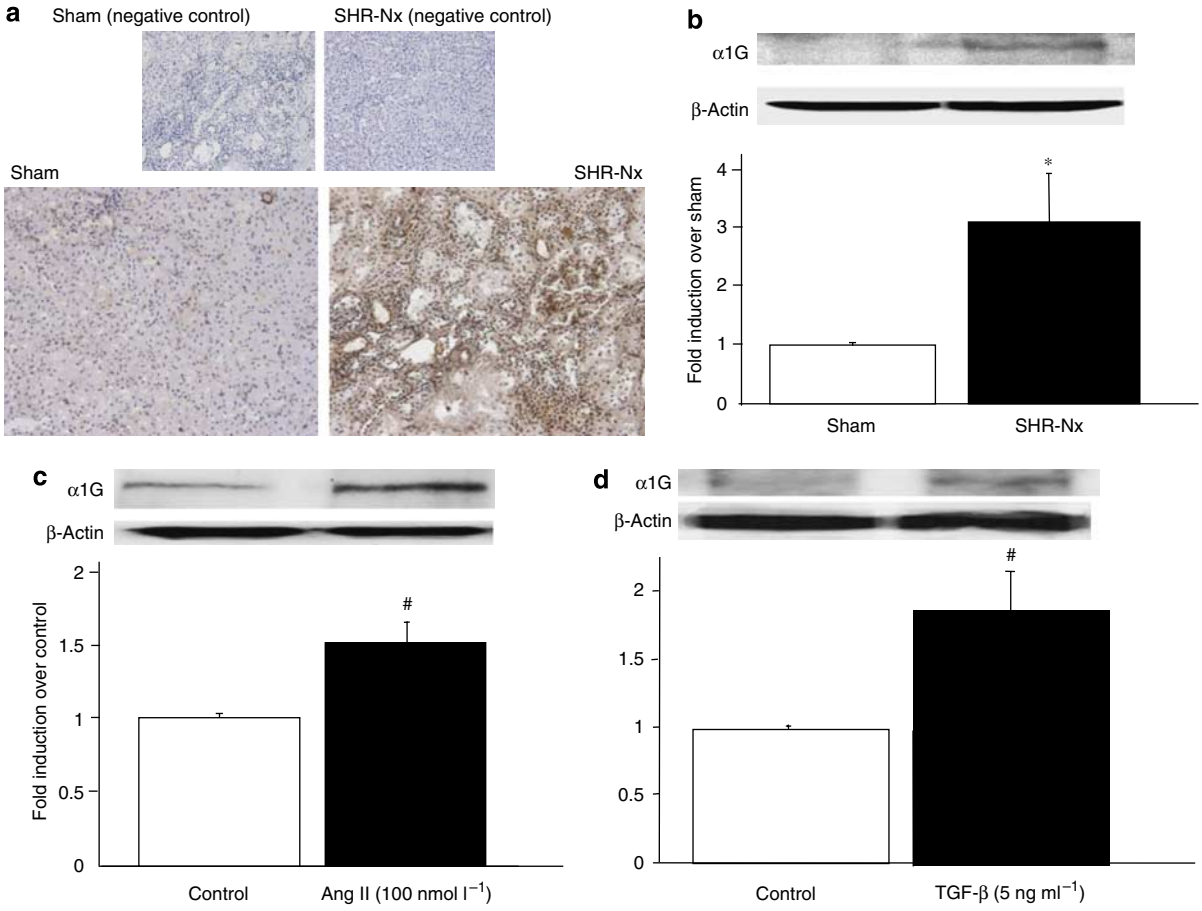

Figure 3 | α1G expression in the renal cortex of subtotally nephrectomized rats, rat mesangial cells, and PTECs.
(**a**) Immunohistochemistry for α1G expression in kidneys from SHR-Nx. (**b**) Immunoblot for α1G protein in kidneys from SHR-Nx. (**c**) Upregulation of α1G expression by Ang II in cultured mesangial cells. (**d**) Upregulation of α1G expression by TGF-β in PTECs. $^{*}P<0.05$ vs sham, $^{\#}P<0.05$ vs control.

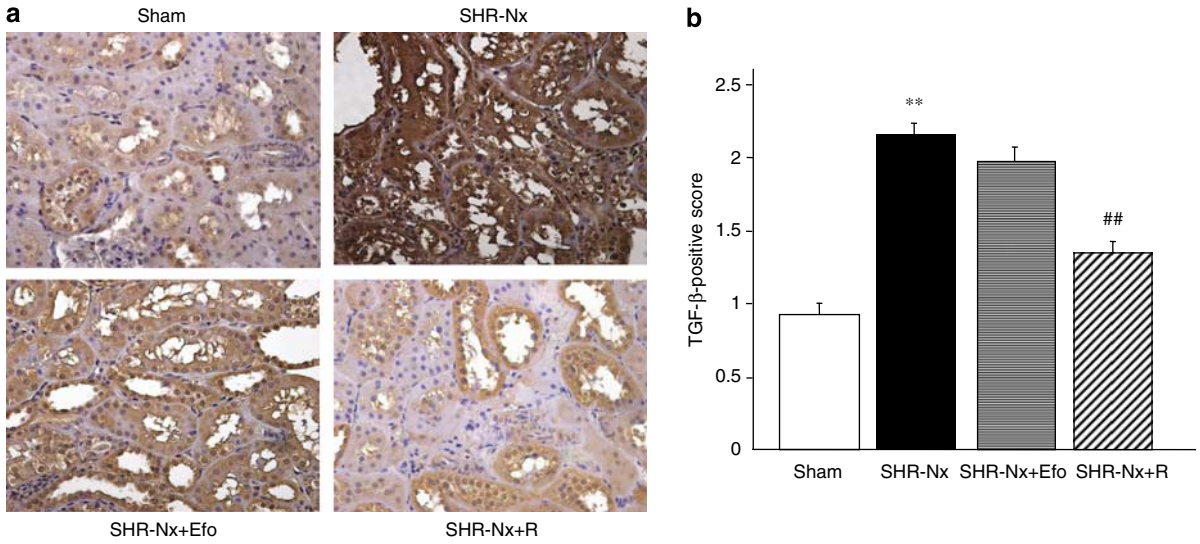

Figure 4 | Effects of efonidipine and *R*(−)-efonidipine on renal TGF-β expression in subtotally nephrectomized rats.
(**a**) Immunohistochemical analysis for TGF-β expression in the renal cortical tubulointerstitial area of SHR-Nx. (**b**) Effects of CCBs on the expression of TGF-β-positive cells. $^{**}P<0.01$ vs sham, $^{\#\#}P<0.01$ vs SHR-Nx.

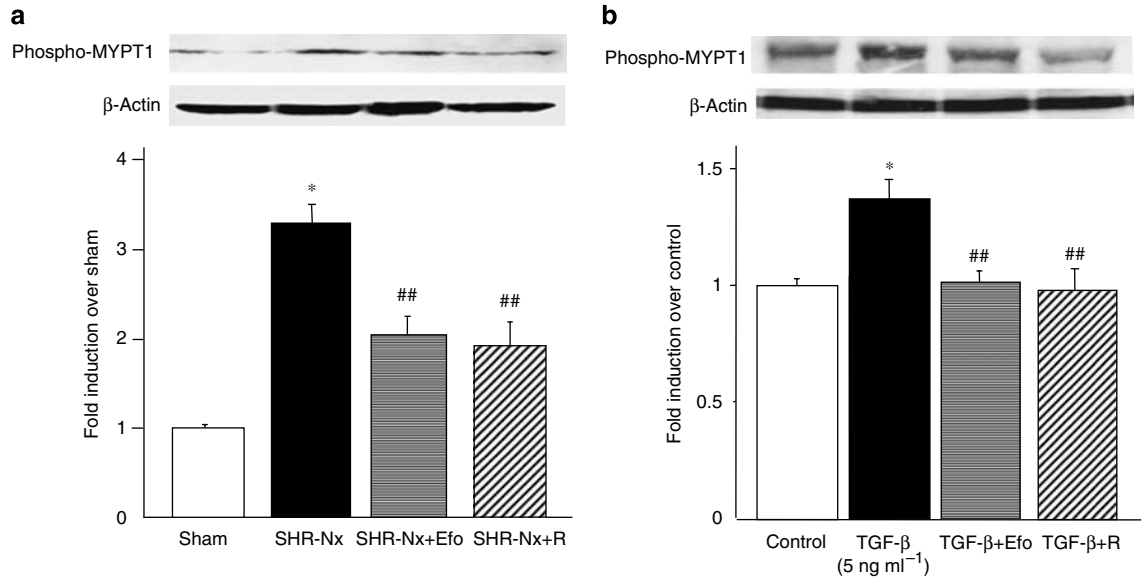

Figure 5 | Effects of efonidipine and *R*(−)-efonidipine on renal Rho-kinase activity and TGF-β-induced Rho-kinase upregulation. (**a**) The phosphorylation level of MYPT1, as a marker for Rho-kinase activity, in the renal cortex of SHR-Nx. $^{*}P<0.05$ vs sham, $^{\#\#}P<0.01$ vs SHR-Nx. (**b**) Effects of Ca^{2+} channel blockers on TGF-β-induced phosphorylation of MYPT1 in PTECs. $^{*}P<0.05$ vs control, $^{\#\#}P<0.01$ vs TGF-β.

($P<0.01$ vs sham; Figure 6a and b). Treatment with efonidipine and *R*(−)-efonidipine completely suppressed the enhanced expression of α-SMA ($P<0.05$ vs SHR-Nx). The expression of vimentin was also enhanced in the tubulointerstitial area in SHR-Nx ($P<0.01$ vs sham; Figure 6c and d). The enhanced expression of vimentin was markedly downregulated in SHR-Nx + Efo and SHR-Nx + R ($P<0.01$ vs SHR-Nx). No significant difference in vimentin expression levels was observed between SHR-Nx + Efo and SHR-Nx + R.

DISCUSSION

A large amount of evidence has been accumulated that lowering systemic blood pressure constitutes a pivotal determinant of renal protection in the treatment of hypertension. Renal protection is also afforded by the correction of glomerular hypertension with specific agents that elicit vasodilation of the postglomerular (i.e., efferent) arteriole. Our previous study demonstrated that efonidipine improved renal injury much more than nifedipine in subtotally nephrectomized SHR, although both CCBs equally lowered SBP.[10] These data suggest that the renal protective effect of efonidipine results from its salutary action on glomerular hemodynamics independent of SBP, based on the finding that efonidipine elicits dilation of the efferent as well as the afferent arteriole. Nevertheless, it has not been determined whether the renal protective action of efonidipine depends on the inhibition of TCC activity, because efonidipine has the ability to block both TCCs and LCCs.

This study has demonstrated that efonidipine improves proteinuria and glomerular hypertrophy and reduces SBP in subtotally nephrectomized SHR. As subtotal nephrectomy predisposes the afferent arteriole to vasodilation[24] and the effect of CCBs on the afferent arteriolar tone may not be apparent in chronic renal disease, it can be speculated that the glomerular capillary pressure is determined by the systemic blood pressure and the efferent arteriolar tone. Thus, the beneficial action of efonidipine on glomerular hemodynamics may depend on a marked reduction in blood pressure and the efferent arteriolar dilation.[2,4,7,10] In contrast, *R*(−)-efonidipine, a specific T-CCB, fails to lower blood pressure but ameliorates proteinuria and glomerular hypertrophy to the same extent as efonidipine (Figures 1 and 2). Because *R*(−)-efonidipine possesses more potent inhibitory activity on TCCs than efonidipine,[23] efferent arteriolar dilation could play a major role in preventing glomerular hypertrophy. In concert, our findings clearly indicate that specific blockade of TCCs offers renal protective action independent of systemic blood pressure.

Alternatively, the T-CCB may directly prevent the pathological process of renal injury. This study demonstrates that the renal tissue from SHR-Nx manifests enhanced expression of α1G subunits of the TCC (Figure 3a and b). Furthermore, in cultured mesangial cells, the expression of α1G is upregulated by Ang II (Figure 3c). Ang II constitutes a pivotal vasoactive substance involved in the progression of renal injury,[25] and the renal Ang II content is increased in renal injury induced by five-sixths nephrectomized rats.[26] Moreover, TGF-β, which is enhanced and is responsible for the development of renal injury,[27] upregulates α1G expression (Figure 3d). Our current observation therefore implies that the blockade of TCCs would provide exaggerated mitigatory action on the progression of glomerular injury. Furthermore, it has been reported that mibefradil, a mixed TCC and LCC blocker, improves proteinuria and glomerular damage in DOCA-salt rats whereas amlodipine fails to blunt

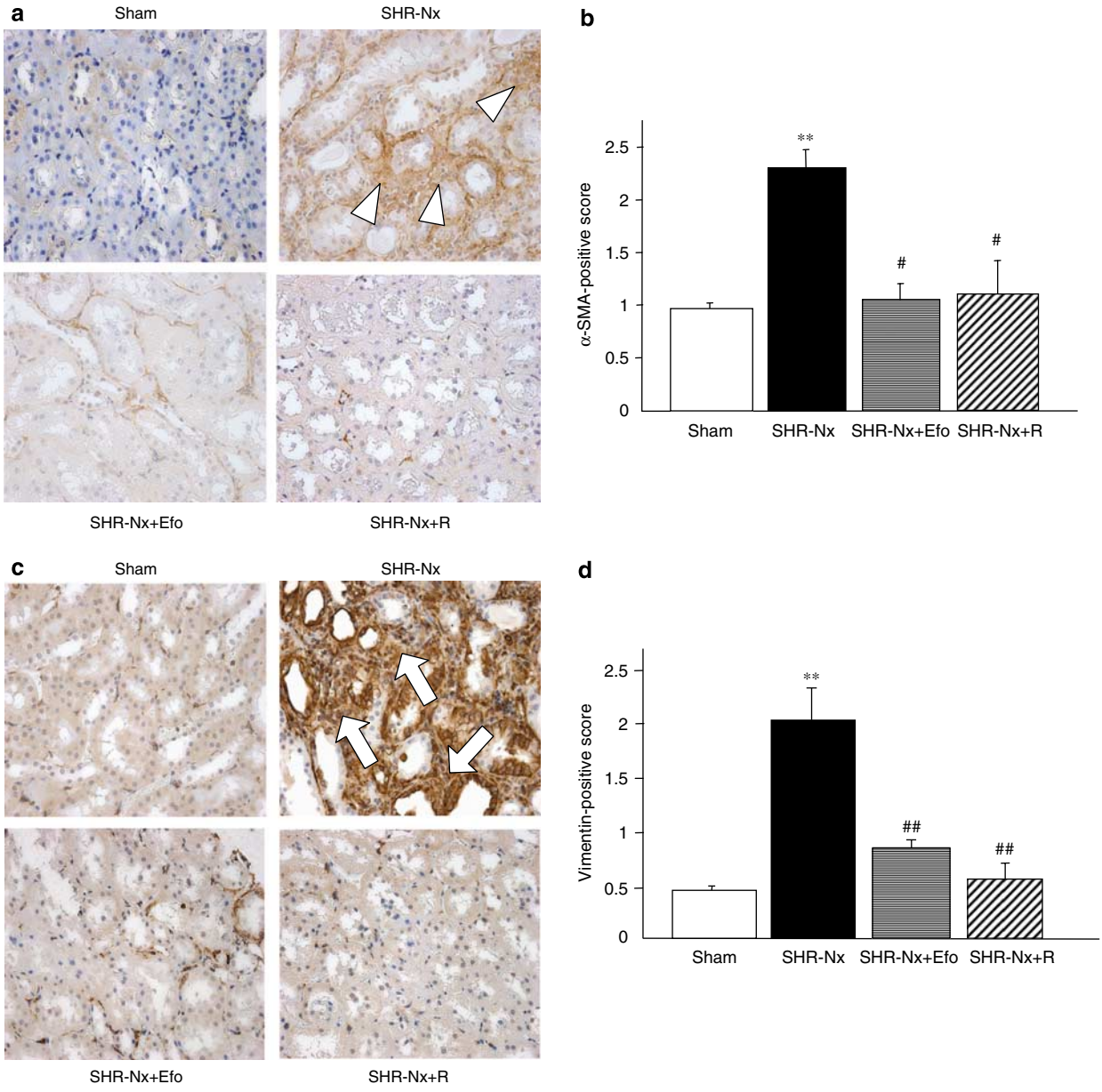

Figure 6 | Effects of efonidipine and *R*(−)-efonidipine on renal expressions of α-SMA and vimentin in kidneys from subtotally nephrectomized rats. (**a**) Arrowheads indicate α-SMA-positive cells in the renal cortical interstitial area of SHR-Nx. (**b**) Effects of Ca^{2+} channel blockers on the expression of α-SMA in the renal cortical area of SHR-Nx. (**c**) Arrows indicate vimentin expression in the tubulointerstitial area of SHR-Nx. (**d**) Suppressed expression of vimentin in SHR-Nx + Efo and SHR-Nx + R. $^{**}P<0.01$ vs sham, $^{\#}P<0.05$ vs SHR-Nx, $^{\#\#}P<0.01$ vs SHR-Nx. (× 400 magnification).

the progression of renal injury.[28] Of note, both amlodipine and mibefradil exert similar antihypertensive actions and decrease glomerular capillary pressure to the same level. It appears therefore that the T-CCB confers greater benefit distinct from that provided by the L-CCB. In concert, available evidence indicates that the TCC *per se* regulates glomerular pathological process, which does not absolutely require the effect on systemic blood pressure or glomerular capillary pressure for the renal action of the TCC.

The expression of the TCC has been reported in several pathological conditions. It has been demonstrated that TCCs are expressed in the fetal myocardium[29] and in the condition of morbid cardiac muscle, such as cardiac hypertrophy[30] and heart failure.[31] In our model of subtotally nephrectomized SHR, the expression level of α1G subunits of the TCC is markedly upregulated (Figure 3a and b). These results support the idea that the T-CCB has the potential to block the pathological processes, and suggest the role of the TCC in 'atavism' of damaged organs. In the morbid state, the suppression of the TCC might be exceedingly important for arresting the process that builds up vicious circle. Indeed, Ang II is reported to increase T-type Ca^{2+} current and TCC

expression in cardiac cells.[32] We therefore propose that exaggerated TCC expression plays a substantial role in Ang II-associated organ damage. Further studies are required to elucidate the role of the TCC in Ang II-associated organ remodeling and cellular phenotypic changes.

Although it is well established that TGF-β plays a crucial role in the progression of renal fibrosis, the mechanism whereby TCCs affect renal tubulointerstitial fibrotic process remains undetermined. In this study, we observed enhanced expression levels of TGF-β, particularly in the interstitial area of kidneys from SHR-Nx, and the increased TGF-β expression was prevented by a specific TCC-B, *R*(−)-efonidipine, and tended to be suppressed by efonidipine (Figure 4a and b). These observations suggest that TCCs participate in the induction of TGF-β and the subsequent renal fibrosis. In this regard, amlodipine, a CCB with L-type and N-type calcium channel blocking activity, is reported to stimulate TGF-β production in human mononuclear cells.[33] Thus, the failure of efonidipine to suppress renal TGF-β expression might be related to variable degrees of blocking activity on the TCC and LCC.

This study demonstrates that Rho-kinase is activated in SHR-Nx and is suppressed by efonidipine and *R*(−)-efonidipine (Figure 5a). In this regard, we have recently shown that Ang II-induced activation of Rho-kinase is inhibited by efonidipine and mibefradil but not by nifedipine in vascular smooth muscle cells (unpublished observation). Furthermore, *R*(−)-efonidipine blunts the Ang II-induced enhancement in GTP-Rho. As Ang II is responsible in large part for TGF-β stimulation and the aggravation and/or initiation of renal injury, TCCs may mediate or modulate the activation of Rho/Rho-kinase in renal injury. Of note, a growing body of evidence has been accumulated that the Rho/Rho-kinase pathway participates in the control of glomerular hemodynamics[17] and the pathological process of renal disease.[11,12] We have recently demonstrated that fasudil, a Rho-kinase inhibitor, improves renal injury in subtotally nephrectomized SHR.[11] Moreover, the Rho/Rho-kinase pathway is involved in the development of tubulointerstitial fibrosis.[34] Finally, this study shows that TGF-β stimulates Rho-kinase activity in PTECs and this effect is inhibited by T-CCBs (Figure 5b). Previous studies demonstrated that Rho-kinase plays a substantial role not only in inducing TGF-β[12,13,35] but also in mediating the renal tubular action of TGF-β.[36] It is thus conjectured that T-CCBs ameliorate renal tubulointerstitial fibrosis, and the suppression of the Rho/Rho-kinase pathway by T-CCBs is responsible in part for the improvement of this pathological process.

Of note, this study shows that *R*(−)-efonidipine ameliorates renal injury with no appreciable effect on blood pressure. As Rho-kinase is one of the important factors causing hypertension, the suppression of the Rho/Rho-kinase pathway by T-CCBs might efficiently correct hypertension in this experiment. In our previous study using the rat remnant kidney model, however, fasudil reduced proteinuria, but failed to induce a significant decrease in blood pressure.[11] Another study also demonstrates that fasudil significantly improves renal function, proteinuria, and histological findings, without changing blood pressure in Dahl salt-sensitive rats.[12] Thus, the inhibition of TCCs and Rho-kinase may offer renal protection, which may not exclusively require a reduction in systemic blood pressure.

The role of epithelial–mesenchymal transition (EMT) in renal fibrosis merits comments. This study demonstrates that subtotal nephrectomy induces renal tubulointerstitial fibrosis (Figure 2) and upregulates the expression of α-SMA and vimentin (Figure 6). Furthermore, these changes are inhibited by treatment with efonidipine and *R*(−)-efonidipine. Once epithelial cells acquire mesenchymal phenotypic characteristics, the expression levels of α-SMA and vimentin are increased,[37,38] which implies enhanced mesenchymal transdifferentiation of tubular epithelial cells[39] and the subsequent tubulointerstitial fibrosis. To the extent that activation of Rho-GTPase serves as an important mechanism for EMT,[40] the potent inhibitory action of T-CCBs on Rho-kinase would halt the process of EMT and alleviate renal tubulointerstitial fibrosis. Of interest, treatment with efonidipine or *R*(−)-efonidipine mitigates but does not completely abolish the activated Rho-kinase observed in SHR-Nx, whereas it nearly totally suppresses the expression of α-SMA and vimentin. It is surmised therefore that other mechanisms contribute to the inhibition of EMT and fibrosis, including the suppression of nuclear factor-κB[20] and cell proliferation.[41]

In summary, the blockade of the TCC exerts potent renal protective action, reflected by the suppression of glomerular hypertrophy and tubulointerstitial fibrosis in the model of chronic kidney disease. The beneficial action does not absolutely require a reduction in systemic blood pressure, but is mediated by multiple mechanisms, including inhibition of EMT, in which activation of the Rho/Rho-kinase pathway is involved (Figure 7). Finally, as TCCs are upregulated in the chronic renal injury model, T-CCBs may constitute a novel therapeutic tool for the treatment of chronic kidney disease.

MATERIALS AND METHODS

Animal preparations

Six-week-old male SHR ($n = 43$) (Japan SLC Inc., Shizuoka, Japan) were placed in individual metabolic cages and were initially fed a standard rat chow ($15\,g\,day^{-1}$; Nippon Clea, Tokyo, Japan) containing 0.3% NaCl, 0.74% potassium, and 20.6% protein, and were given water *ad libitum*. Subtotal nephrectomy was performed by removal of two-thirds of the left kidney (week 0), followed by total right nephrectomy (week 1) under ether anesthesia. Rats were then randomly assigned to four groups: group 1, sham-operated rats (sham, $n = 8$); group 2, subtotally nephrectomized rats (SHR-Nx, $n = 13$); group 3, SHR-Nx given efonidipine (Efo, 100 mg per kg per day, $n = 10$; Nissan Chemical Industries, Tokyo, Japan), and group 4, SHR-Nx given *R*(−)-efonidipine (R, 100 mg per kg per day, $n = 12$; Nissan Chemical Industries). SBP, measured by the tail-cuff method, and daily urinary protein excretion were evaluated every other week. At week 8, rats in each group were decapitated in the morning under the fasting condition. Blood was collected, and serum total protein

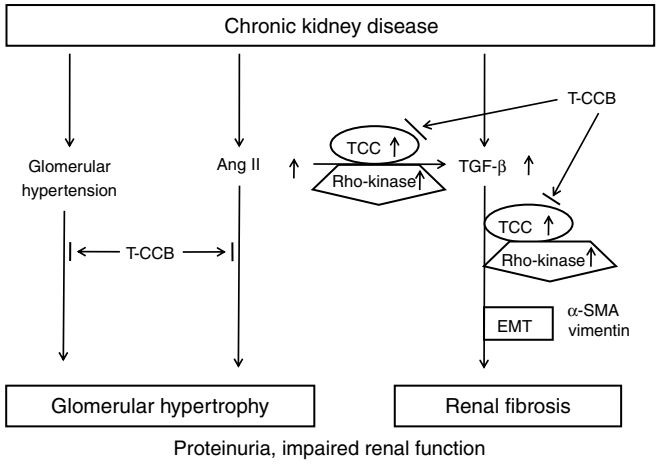

Figure 7 | Schematic diagram illustrating the putative role of TCCs in the progression of chronic kidney disease. Besides hemodynamic factors affecting glomerular pressure, chronic kidney disease stimulates the production of intrarenal Ang II and TGF-β, where TCCs and Rho-kinase are involved. The enhanced TGF-β activity stimulates the EMT, as reflected by the upregulation of α-SMA and vimentin, and subsequently induces renal fibrosis. In this setting, the TCC blocker (T-CCB) ameliorates glomerular hypertension by dilating efferent arteriolar dilation[2,4,8] and prevents the mesangial cell hypertrophy. Furthermore, the T-CCB inhibits Rho-kinase activity and suppresses the expression of TGF-β and the EMT process. All these effects act in concert to contribute to amelioration of chronic kidney disease by the T-CCB.

and creatinine were measured by an autoanalyzer. The kidneys were harvested for further analysis. All experiments were performed in accordance with the animal experimentation guidelines of Keio University School of Medicine.

Morphological examination

Each paraffin section of rat kidneys was stained with periodic acid-Schiff and Masson-trichrome stain. Glomerular changes were assessed as the size of mesangial and vascular areas at the level of vascular pole using a computer-aided manipulator (microscope, Leica DM4000B; camera, Leica DFC300FX; software, Leica IM500). Tubulointerstitial fibrotic areas stained in light blue with Masson-trichrome stain were picked on the digital images using a computer-aided manipulator, and were graded as follows: 0, none; +1, <25%; +2, 25–50%; +3, >50%. The scores were determined in each section selected at random and more than 15 fields were examined under ×400 magnification. Morphological evaluation was conducted by two independent observers in a blinded fashion.

Immunohistochemistry

Immunohistochemical analysis for renal fibrosis was performed using antibodies against α-SMA (Dako Cytomation, Glostrup, Denmark) and vimentin (Dako). α-SMA-positive cells, vimentin-positive cells, and TGF-β-positive cells were counted and graded as follows: 0, none; +1, <25%; +2, 25–50%; +3, >50% of α-SMA- or vimentin-positive area. We further examined whether the expression of the TCC was altered in kidneys from SHR-Nx. Immunohistochemical evaluation was conducted using an antibody against α1G subunits of the TCC (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Cell culture

Mesangial cells were prepared from 3-week-old male Sprague–Dawley rats, using the mesh sieving technique. Briefly, the renal cortical tissue was removed, washed with Hank's balanced salt solution (Nissui pharmaceutical Co, Ltd, Tokyo, Japan), and passed through 212 and 150 μm mesh sieves. Glomeruli were finally recovered after sieving through a 53 μm mesh filter. The cells were cultured in D-MEM/F-12 medium (Invitrogen, Carlsbad, CA, USA) containing 10% fetal calf serum (Biological Industries, Kibbutz Beit Haemek, Israel), 10 μl ml^{-1} ITS + culture supplement (Becton Dickinson, Frankin Lakes, NJ, USA), 100 U ml^{-1} penicillin (Invitrogen), 100 mg ml^{-1} streptomycin (Invitrogen), and 200 mmol l^{-1} L-glutamine (Invitrogen). Early passaged (passages 4–8) mesangial cells were grown to 60–70% confluence and made quiescent by serum starvation for 24 h. Ang II (100 nmol l^{-1}; Sigma, St Louis, MO, USA) was added and the cells were harvested after 24 h of Ang II treatment.

PTECs (Cambrex Bio Science Walkersville Inc., Walkersville, MD, USA) were cultured in renal epithelial cell basal medium (Cambrex Bio Science Walkersville Inc.) supplemented with 0.5 μg ml^{-1} hydrocortisone, 10 ng ml^{-1} human recombinant epidermal growth factor, 5 μg ml^{-1} insulin, 0.5 μg ml^{-1} epinephrine, 6.5 ng ml^{-1} triiodothyronine, 10 μg ml^{-1} transferrin, 0.5 ml GA-1000, 0.5% fetal bovine serum, 100 U ml^{-1} penicillin (Invitrogen), 100 mg ml^{-1} streptomycin (Invitrogen), and 200 mmol l^{-1} L-glutamine (Invitrogen). Each cell was grown at 37°C in 5% CO_2 and the medium was changed every other day. After starvation, recombinant human TGF-β1 (R&D Systems, Minneapolis, MN, USA) was added before the treatment of efonidipine or *R*(−)-efonidipine, and the cells were harvested after 24 h of TGF-β treatment.

Immunoblotting

Renal cortical tissues, mesangial cells, and PTECs were lysed and fornicated in solubilization buffer. Immunoblotting was performed as described previously,[42] using specific antibodies against phospho-MYPT1 (Upstate Biochemistry, Lake Placid, NY, USA) and α1G (Alomone, Jerusalem, Israel). Immunoreactive bands were detected using an ECL detection kit (Amersham Biosciences, Uppsala, Sweden). Immunoblots were analyzed by computer-aided planimetry using the Scion Image software (Scion Corp., Fredrick, MD, USA). In detail, images were digitized and captured with a CCD camera connected to a personal computer, and scanned into Photoshop (Adobe Systems, San Jose, CA, USA). A binary overlay was created automatically, and the threshold of the binary overlays was adjusted by comparing image to optimal match of each spot. The value of the threshold setting was kept constant.

Statistical analysis

Data were expressed as the mean ± s.e.m. Data were analyzed by one- or two-way analysis of variance as appropriate, followed by Newman–Keuls *post hoc* test. Histological results were analyzed by Kruskal–Wallis nonparametric test. $P<0.05$ was considered statistically significant.

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