Counteractive effects of HGF on PDGF-induced mesangial cell proliferation in a rat model of glomerulonephritis

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Submitted 9 September 2002; accepted in final form 16 February 2003

Bessho, Kazuhiko, Shinya Mizuno, Kunio Matsumoto, and Toshikazu Nakamura. Counteractive effects of HGF on PDGF-induced mesangial cell proliferation in a rat model of glomerulonephritis. Am J Physiol Renal Physiol 284: F1171–F1180, 2003. First published February 20, 2003; 10.1152/ajprenal.00326.2002.—Activation and proliferation of glomerular mesangial cells play an important role in the development of mesangio-proliferative glomerulonephritis. We investigated the role of hepatocyte growth factor (HGF) in regulating activated mesangial cell proliferation. In glomeruli of normal rats, mesangial cells barely expressed the c-Met/HGF receptor. However, when mesangio-proliferative glomerulonephritis was induced in rats by the administration of an anti-Thy 1.1 antibody, glomerular HGF expression transiently decreased along with mesangiosis, and activation of mesangial cells was associated with upregulation of the c-Met receptor. Activated mesangial cells in culture also expressed the c-Met/HGF receptor. Although addition of HGF to cultured mesangial cells did not increase DNA synthesis, HGF did diminish PDGF-induced DNA synthesis. PDGF induced activation of ERK, which continued for at least 48 h. When PDGF and HGF were simultaneously added, HGF inhibited the prolonged activation of ERK, which suggests that early inactivation of PDGF-induced ERK may be involved in the inhibitory effect of HGF on mesangial cell proliferation. Furthermore, administration of HGF to rats with anti-Thy 1.1 nephritis resulted in a selective suppression of activated mesangial cell proliferation, and this suppressive effect was associated with attenuation of phosphorylated glomerular ERK. These results indicate that HGF counteracts PDGF-induced mesangial cell proliferation and functions as a negative regulator of activated mesangial cell proliferation.

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In the present study, we investigated the role of HGF and the c-Met receptor in the proliferation of activated mesangial cells, using the most widely studied experimental model for mesangio proliferative glomerulonephritis, anti-Thy 1.1 glomerulonephritis (11). Our findings indicate that HGF is a negative regulator of activated mesangial cell proliferation.

**MATERIALS AND METHODS**

**Animals and materials.** Male Wistar rats (7–8 wk old; 190–210 g) were purchased from SLC Japan (Hamamatsu, Japan). All animal experiments were done in accordance with National Institute of Health guidelines, as dictated by the animal care facility at Osaka University Graduate School of Medicine. Recombinant human HGF was purified from cultured media of Chinese hamster ovary cells transfected with National Institute of Health guidelines, as dictated by 190/H9262 obtained from the mice was given intravenously to rats at 25 µl/rat.

To induce anti-Thy 1.1 glomerulonephritis in rats, the ascites obtained from these mice. Hybridoma cells were administered intraperitoneally into male BALB/c mice (SLC Japan), and ascites was obtained under microscopy and stored at 80°C. Six-well culture plates (3 × 10⁴ cells/well), cultured for 24 h, serum-starved for 48 h, and treated with 30 ng/ml HGF for 6 and 12 h. Total RNA from the cells was prepared, using Iso-Gen (Nippon Gene, Toyama, Japan), and 3 µg of total RNA were reverse transcribed into first-strand cDNA with a random hexamer primer using Superscript II RT. TaqMan quantitative PCR for rat PDGF-B chain was done using the same primers and probes as above.

**Histological analysis.** Tissues were fixed in neutral buffered formalin (pH 7.4), embedded in paraffin, and then sections were examined, and the mean number of double-positive light glomerular cross sections examined, and the mean number of nuclei per glomerular cross section was measured. For immunohistochemical staining, tissues were fixed in 70% ethanol for detection of HGF. The primary antibodies were used polyclonal anti-rat HGF (30). Tissue sections were subjected to the avidin-biotin coupling technique, using a commercial kit (Vectastain Elite ABC, Vector Laboratories, Burlingame, CA), according to the manufacturer’s instructions. To identify activated mesangial cells, peroxidase-conjugated monoclonal anti-human α-smooth muscle actin (α-SMA; DAKO, Glostrup, Denmark) was used. Glomerular staining for α-SMA was evaluated by the scoring method principally described by Floege et al. (13). In each sample, 48 glomerular cross sections were examined, and two individual observers made evaluations of all the slides.

For double immunohistochemical staining of c-Met and Thy 1.1 or c-Met and α-SMA, tissues were fixed in 70% ethanol and incubated with polyclonal anti-mouse c-met (SP260, Santa Cruz Biotechnology, Santa Cruz, CA) plus anti-Thy 1.1 ascites (see above) or monoclonal anti-human α-SMA. After being washed with PBS, tissue sections were sequentially incubated with Alexa Fluor 546 anti-mouse IgG conjugate and Alexa Fluor 488 anti-rabbit IgG conjugate (Molecular Probes, Eugene, OR). Tissue sections were examined under a laser-scanning microscope.

For immunohistochemical staining for BrdU, tissues were fixed in 70% ethanol and incubated with murine anti-BrdU monoclonal IgG (Takara Bio, Kyoto, Japan). The sections were then incubated with peroxidase-conjugated anti-murine IgG, and BrdU-positive cells were identified based on the enzymatic reaction using Vectastain Elite ABC. In the doublestaining for BrdU and α-SMA, tissue sections stained for BrdU were successively incubated with a commercially available blocking reagent (MOM Immunodetection kit, Vector Laboratories) and monoclonal anti-human α-SMA. The sections were next incubated with alkaline phosphatase-conjugated anti-murine IgG, and α-SMA-positive cells were detected using a commercial kit (Vectastain ABC, Vector Laboratories). In each sample, 48 glomerular cross sections were examined, and the mean number of double-positive light glomerular cross section were determined. All antibodies and control IgG were used at 1 µg/ml, and no significant signal was obtained on substitution of the primary antibody with equivalent concentrations of normal rabbit or mouse IgG and with the c-Met primary antibody preabsorbed with
an excess of c-Met blocking peptide (SP260P, Santa Cruz Biotechnology).

Cell culture and measurement of cell growth. Mesangial cells were prepared from 8-wk-old male Wistar rats by a differential sieving method and characterized as described (25). Cells were cultured in DMEM supplemented with 20% FCS, 0.5 μg/ml streptomycin, 100 U/ml penicillin G, and 0.25 μg/ml amphotericin B. For all experiments, we used cells of passages 4–10. For measurement of DNA synthesis, cells were seeded in 96-well culture plates (5 × 10^4 cells/well) and cultured for 24 h. Under these conditions, a cell confluence of ~95% was reached. Cells were serum-starved for 48 h in medium supplemented with 0.5% FCS, and then the medium was replaced with fresh medium and cells were further cultured for 48 h in the absence or presence of PDGF and/or HGF. The cells were pulse-labeled with [3H]thymidine (1.0 μCi/ml) for 5 h, and the amount of [3H]thymidine incorporated into nuclei was measured, as described elsewhere (32). Six wells were used for each condition.

Western blotting. To detect c-Met receptor expression in the cultured cells, the cells were seeded in 90-mm culture dishes (1 × 10^5 cells/dish), cultured for 4 days, serum-starved for 24 h, then lysed in sample buffer for SDS-PAGE. The lysate was subjected to SDS-PAGE on a 6% polyacrylamide gel, and proteins were electroblotted on polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA). After blocking, the membrane was sequentially incubated with the anti-mouse c-Met antibody and horseradish peroxidase-conjugated anti-rabbit IgG. Signals were visualized using an enhanced chemiluminescence reagent (Amersham Pharmacia Biotech).

For analysis of ERK phosphorylation in cultured mesangial cells, cells were seeded in six-well culture plates (3 × 10^4 cells/well) and cultured for 24 h. Cells were serum-starved for 48 h then treated with 20 ng/ml PDGF-BB and/or 30 ng/ml HGF for the indicated periods. Cells were washed once with ice-cold PBS, snap-frozen, thawed, and lysed with lysis buffer including endothelial and mesangial cells) caused by anti-Thy 1.1 glomerulonephritis. In normal glomeruli, HGF expression became predominant in mesangial areas rather than capillary areas, from 4 days after anti-Thy 1.1 treatment (data not shown). The overshooting mesangial cell growth was observed on days 4 and 8. To determine the potential involvement of HGF in pathophysiological changes in anti-Thy 1.1 nephritis, we first analyzed changes in glomerular expression of HGF during progression of glomerulonephritis (Fig. 1). Glomerular HGF mRNA levels decreased on day 2 (~29% compared with the value at day 0), the time when glomerular cell loss was most prominent (Fig. 1A). The HGF mRNA level then increased toward day 8 and returned to ~80% of the pretreatment level.

To determine the localization of HGF, immunohistochemical examination was made on the glomeruli of anti-Thy 1.1 glomerulonephritis. In normal glomeruli, HGF was identified mainly in the capillary luminal areas composed of endothelial cells, whereas weak signals were sparse in mesangial areas (Fig. 1B, arrowhead and inset, day 0), the time when glomerular cell loss was most prominent (Fig. 1B, inset, day 0). On day 2 after anti-Thy 1.1 treatment, glomerular cells markedly decreased, and HGF expression was noted also on remaining mesangial-like cells, which were multipolar and located between clusters of capillary loops (Fig. 1B, arrow, day 2). HGF expression became predominant in mesangial areas rather than capillary areas, from 4 days after anti-Thy 1.1 treatment. On day 8, HGF expression became extensive, accompanied by glomerular hypercellularity, and this was consistent with the recovery of HGF mRNA levels. At this time, HGF-positive signals were mainly noted in mesangial areas (Fig. 1B, arrow and inset, day 8). These results suggest that the temporal decrease in HGF mRNA levels in this model may be due to glomerular injuries of intrinsic cells (possibly including endothelial and mesangial cells) caused by mesangioytic events. On the other hand, plasma HGF...
levels did not change significantly during the progression of glomerulonephritis.

**Induction of the c-Met receptor in activated mesangial cells.** Next, we analyzed expression of the c-Met/HGF receptor in glomeruli (Fig. 2A). In the normal glomerulus, c-Met receptor expression was evident in cells, presumably epithelial and endothelial cells based on their localization and morphology (Fig. 2A, center top). When c-Met staining was merged with Thy 1.1 staining, a marker of mesangial cells, c-Met-positive cells and Thy 1.1-positive cells were mostly noncolocalized but not exclusively, thereby indicating that mesangial cells in normal glomeruli are mostly c-Met negative (Fig. 2A, top). On the other hand, when glomerular c-Met expression was analyzed in rats with anti-Thy 1.1 nephritis, based on double immunohistochemistry of c-Met and α-SMA (Fig. 2A, bottom center and left), α-SMA-positive cells were mostly c-Met positive, thus indicating that the α-SMA-positive activated mesangial cells expressed the c-Met receptor (Fig. 2A, bottom right). These results indicate that the c-Met receptor is inducible in mesangial cells during activation toward myofibroblast-like cells, and hence HGF may play a role in regulating activated mesangial cell behavior. On the other hand, when we measured the change in c-Met mRNA expression in isolated glomeruli using quantitative PCR, glomerular c-Met mRNA expression did not change significantly.

To obtain further evidence for induction of the c-Met receptors in mesangial cells, mesangial cells isolated from glomeruli of the normal rat kidney were used. During cultivation, mesangial cells autonomously underwent a phenotypic change into α-SMA-positive activated mesangial cells (data not shown), as reported elsewhere (8). When expression of the c-Met receptor was analyzed by Western blotting, activated mesangial cells in culture clearly expressed the c-Met receptor (Fig. 2B), which means that activated mesangial cells may be targets of HGF.

**Reduction of PDGF-induced DNA synthesis by HGF in cultured mesangial cells.** Because induction of the c-Met receptor in activated mesangial cells and regulation of HGF expression suggested the potential involvement of HGF in the pathophysiology of mesangio proliferative glomerulonephritis, we next asked whether HGF would regulate the proliferation of activated mesangial cells in culture. Because PDGF is the most potent known mitogen for mesangial cells (12) and PDGF induced in glomeruli with anti-Thy 1.1 nephritis is involved in the proliferation of mesangial cells (10, 16), mesangial cells were cultured in the absence or presence of HGF, PDGF, or their combination and subjected to measurement of DNA synthesis (Fig. 3A). Consistent with reported data (14), addition of PDGF stimulated DNA synthesis of mesangial cells and the stimulatory effect of PDGF was maximal at concentrations over 20 ng/ml (Fig. 3A and data not shown). HGF alone up to 100 ng/ml had no significant effect on DNA synthesis of mesangial cells. However, the simultaneous addition of HGF and PDGF dose dependently inhibited PDGF-induced DNA synthesis, and 10–30 ng/ml HGF inhibited DNA synthesis to the basal level seen without PDGF.

Because a previous study showed that mesangial cells expressed PDGF in this model (15), regulation of PDGF expression by HGF might be involved in a mechanism by which HGF suppressed PDGF-induced cell proliferation. We thus analyzed the effect of HGF on PDGF expression in cultured mesangial cells, using real-time quantitative RT-PCR (Fig. 3B). At both 6 and 12 h after HGF treatment, there was no significant
expression of c-Met receptor in cultured mesangial cells, as detected by Western immunoblotting.

c-Met at glomerulus on day 4, top left middle), and the c-Met receptor (middle, top and bottom left), and the c-Met receptor tyrosine kinases, and inhibition of ERK in cultured mesangial cells potently diminishes PDGF-induced proliferation (5). In growth-arrested mesangial cells, ERK was marginally phosphorylated or mostly unphosphorylated, whereas it was strongly phosphorylated 10 min after stimulus with either PDGF or HGF. It is noteworthy, however, that activation/phosphorylation of ERK continued for at least 48 h after the PDGF stimulus, whereas activation/phosphorylation of ERK by HGF was transient. ERK was strongly phosphorylated by the HGF stimulus at 10 min, which rapidly decreased after 10 min, and only marginal phosphorylation remained 2 h after the HGF stimulus. When PDGF and HGF were simultaneously added, HGF significantly inhibited the PDGF-dependent prolonged phosphorylation of ERK, although levels of phosphorylated ERK were higher than seen in the case of HGF alone. Levels of phosphorylated ERK in cells treated with PDGF plus HGF were significantly lower than those in PDGF-treated cells later than 2 h after treatment, and weakly phosphorylated ERK was seen 24–48 h after treatment with PDGF plus HGF.

Suppression of mesangial cell proliferation, glomerular ERK phosphorylation, and α-SMA expansion in anti-Thy 1.1 glomerulonephritis by HGF. Based on the finding that HGF inhibited the proliferation of activated mesangial cells stimulated by PDGF in vitro, we considered it important to determine whether administration of HGF would exert negative regulatory functions in the expansion of mesangial cells during progression of anti-Thy 1.1 nephritis. For this purpose, recombinant human HGF was repeatedly administered later than 2 days after injection of anti-Thy 1.1 antibody (Fig. 5A). Because disruption of glomerular construction occurs during the first 2 days after anti-Thy 1.1 treatment (11), treatment with HGF later than 2 days after anti-Thy 1.1 treatment excludes the possibility that HGF would affect the onset of nephritis itself in this model. Compared with control saline-treated rats, the mean number of total cells per glomerulus significantly decreased on days 4 and 8 in HGF-treated rats (Fig. 5B). To observe whether a decrease in the number of cells in glomeruli after HGF treatment was due to a decrease in the number of proliferating cells, cells undergoing DNA synthesis were determined based on BrdU incorporation and subsequent immunohistochemistry (Fig. 6A).
number of BrdU-positive cells per glomerulus in HGF-treated rats decreased to 74.7% on day 4 and 58.0% on day 8, compared with findings in saline-treated rats. On the other hand, apoptotic cell death in activated mesangial cells was proposed to be a cell clearance mechanism in anti-Thy 1.1 nephritis, thereby contributing to the resolution of glomerular hypercellularity (2). However, during our present observation, the number of terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labeling-positive apoptotic cells was few, and there was not a significant change with HGF treatment (data not shown).

We then asked whether a decrease in the proliferating glomerular cells with HGF treatment is related to changes in proliferation of activated mesangial cells. When the numbers of proliferating activated mesangial cells and nonmesangial cells were determined by double staining for /H9251-SMA and BrdU, the number of /H9251-SMA-positive mesangial cells undergoing DNA synthesis decreased on day 4 to 52.0% of the control value seen with HGF treatment (Fig. 6B). In contrast, HGF treatment had no statistically significant effect on the proliferation of nonmesangial cells. Similarly, accompanied by a decrease in the number of proliferating activated mesangial cells, expansion of /H9251-SMA-positive cells in glomeruli, as evaluated by immunohistochemical staining and its scoring, was suppressed by HGF treatment on both days 4 and 8 (Fig. 6C). To further confirm the suppressive effect of HGF on activated mesangial cell proliferation, we analyzed the activation status of ERK in glomerular lysates (Fig. 6D).

Glomerular ERK was phosphorylated/activated in rats with anti-Thy 1.1 glomerulonephritis on day 4, whereas glomerular ERK activation was significantly attenuated by HGF treatment. The attenuation of glomerular ERK activation by HGF may possibly be involved in the...
suppressive effect of HGF on activated mesangial cell proliferation. Taken together, these results indicate that HGF treatment preferentially suppressed proliferation of activated mesangial cells but not of non-mesangial cells during development of anti-Thy 1.1 nephritis.

When we analyzed expression of the PDGF-B chain mRNA and PDGF-BB protein levels in glomeruli on day 4 after disease induction with real-time quantitative RT-PCR and ELISA, respectively, HGF treatment did not significantly change either PDGF-B mRNA expression or PDGF-BB levels in glomeruli (Table 1). Thus HGF suppressed PDGF-induced mesangial cell proliferation without affecting PDGF expression in glomeruli.

**DISCUSSION**

In the kidney, activation of glomerular mesangial cells in response to glomerular damage and proliferation of these cells are thought to be risk factors for the progression of glomerular nephritis to irreversible glomerular scarring and play an important role in the pathogenesis of a variety of glomerular diseases (12). Therefore, understanding regulatory mechanisms governing proliferation of mesangial cells is important in designing effective treatments for glomerular nephritis.
and gaining a better understanding of pathophysiological aspects. In this study, we demonstrated for the first time that the c-Met receptor was induced in mesangial cells on stimulus toward activated mesangial cells and that HGF suppressed PDGF-induced proliferation of activated mesangial cells both in vivo and in vitro.

During successive cultivation, mesangial cells spontaneously undergo a phenotypic change into activated, myofibroblast-like cells (8). Previous reports showed that mesangial cells express the c-Met receptor in vitro, but the effects of HGF on cultured mesangial cells and in vivo expression of the c-Met receptor in mesangial cells were controversial (19, 21, 37). In the present study, we found that mesangial cells barely expressed the c-Met receptor in the normal glomerulus, but after anti-Thy 1.1 treatment the c-Met receptor was induced in α-SMA-positive activated mesangial cells. Thus in glomerular mesangial cells, expression of the c-Met receptor is inducible, when mesangial cells are activated to become α-SMA-positive myofibroblast-like cells. Although mechanisms for induction of the c-Met receptor during activation of mesangial cells remain to be addressed, Liu et al. (24) reported that in cultured mesangial cells the c-Met receptor was upregulated by several growth factors, including PDGF. Therefore, the increased expression of growth factors such as PDGF in this model (15) may be partially responsible for c-Met receptor induction in this model.

Infusion of PDGF into the kidney or transfection of the PDGF gene in the kidney selectively induced mesangial cell proliferation (10, 16). Conversely, neutralization of PDGF resulted in the suppression of mesangial cell proliferation (18, 33). Similarly, it was reported that glomerular PDGF and PDGF receptor expression are increased in patients with various forms of glomerulonephritis (12). Thus considering the close involvement of PDGF in mesangiproliferative glomerulonephritis, we investigated a potential role for HGF, focusing on mesangial cell proliferation in combination with a PDGF stimulus. Other studies have shown that HGF alone had no apparent effect on rat, mouse, or human mesangial cell proliferation (21, 37), or that it is weakly mitogenic (19). In the present study, HGF alone had no apparent effect on cultured mesangial cells, but HGF did suppress the proliferation of activated mesangial cells promoted by PDGF. Because HGF did not alter PDGF expression in cultured mesangial cells, we studied the activation of ERK, an intracellular event closely associated with mesangial cell proliferation. Recent reports demonstrated that ERK is a mediator of the proliferative response in mesangioproliferative glomerulonephritis (4) and that inhibition of ERK in the proliferative phase of anti-Thy 1.1 nephritis prevented mesangial cell proliferation (5). Importantly, we found that ERK was differently regulated after stimulation of cells with PDGF, HGF, or both combined. The earlier inactivation of ERK by the simultaneous addition of PDGF and HGF seems to explain the suppressive effect of HGF on PDGF-dependent growth in activated mesangial cells. Our study clearly showed the counteractive interaction of two different growth factors and their receptors, either of which has the tyrosine kinase receptor.

Consistent with in vitro findings on the counteractive effects of HGF on PDGF-induced proliferation of activated mesangial cells, administration of HGF to rats with anti-Thy 1.1 nephritis suppressed glomerular ERK phosphorylation and the proliferation of activated mesangial cells, and these events were associated with a reduction in glomerular expansion of α-SMA expression. Because HGF did not significantly change the glomerular expression of PDGF, the intracellular counteraction of HGF against PDGF-dependent mesangial cell proliferation seems to be a predominant mechanism by which HGF suppressed expansion of activated mesangial cells in mesangiproliferative glomerulonephritis. Because endogenous glomerular HGF expression was decreased, rather than increased, after anti-Thy 1.1 treatment, decreased glomerular HGF expression might to some extent and in part allow for the proliferation of activated mesangial cells.

Ostendorf et al. (33) showed that the transient inhibition of PDGF-B chain by a specific aptamer during the mesangioproliferative phase in the irreversible glomerulosclerosis model diminished proliferation of activated mesangial cells and that this resulted in almost complete prevention of the development of renal failure and glomerular as well as tubulointerstitial scarring. Their results provide a strong argument against concerns that inhibition of overshooting mesangial cell growth after injury might lead to the inhibition of healing and thus exacerbate glomerular damage. A reduction in early glomerular proliferation may be important for any sequent reduction of glomerular scarring and renal failure. Whether antiproliferative effects of HGF on activated mesangial cells are associated with the prevention of glomerulosclerosis remains to be addressed, using a model for glomerulosclerosis.

Previous approaches using distinct animal models of tissue fibrosis provided evidence that HGF has antifibrogenic actions on tissue fibrosis, including liver cirrhosis (26, 34), renal tubulointerstitial fibrosis (29), and lung fibrosis (36). Based on the notions that the c-Met receptor is preferentially expressed in epithelial cells and endothelial cells under physiological condi-

Table 1. Changes in PDGF mRNA expression and PDGF-BB protein level after HGF treatment in rats with anti-Thy 1.1 glomerulonephritis

<table>
<thead>
<tr>
<th>PDGF Expression</th>
<th>Saline</th>
<th>HGF</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDGF-B chain mRNA, PDGF-B/GAPDH × 10^4</td>
<td>1.46 ± 0.09</td>
<td>1.72 ± 0.14</td>
<td>0.18</td>
</tr>
<tr>
<td>PDGF-BB protein, %total protein × 10^2</td>
<td>2.98 ± 0.58</td>
<td>2.46 ± 0.42</td>
<td>0.50</td>
</tr>
</tbody>
</table>

Values are means ± SD; n = 6 rats/group. Hepatocyte growth factor (HGF) was administered to rats with anti-Thy 1.1 glomerulonephritis, as described in the text. On day 4 after anti-Thy 1.1 treatment, glomerular PDGF-B chain mRNA expression and PDGF-BB protein levels, respectively, were measured using quantitative RT-PCR and ELISA.
tions and that transforming growth factor-β (TGF-β) plays a role in tissue fibrosis, these studies addressed mechanisms involved in antifibrotic actions of HGF, focusing on cell division and antiapoptosis in epithelial cells, expression of TGF-β, and proteases involved in the breakdown of ECM (23, 26, 29, 34, 36). However, little attention was directed to the potential role of HGF to directly attenuate stromal cell expansion. Our original findings here are that HGF exerts antiproliferative actions, directly targeting stromal myofibroblast-like cells. Our observations provide a better understanding of the pathogenic mechanisms of as well as therapeutic approaches to fibrotic disorders, from the aspect of tissue remodeling regulated by growth factor networks.

We are grateful to M. Ohara for comments and language assistance. This study was supported by a grant-in-aid from the Ministry of Education, Science, Technology, Sports, and Culture of Japan.

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