Cancer Science
The official journal of the Japanese Cancer Association

Downregulation of receptor for activated C-kinase 1 (RACK1) suppresses tumor growth by inhibiting tumor cell proliferation and tumor-associated angiogenesis

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(Received March 31, 2011/Revised July 29, 2011/Accepted August 5, 2011/Accepted manuscript online August 16, 2011/Article first published online September 22, 2011)

By behaving as molecular hubs, scaffold proteins can assemble a large number of signaling molecules and organize complicated intracellular signaling networks in time and space. Owing to their crucial role in mediating intracellular signaling related to tumor cell growth and migration, recent studies have highlighted the relevance of scaffold proteins in human cancers and indicated that interfering with their expression and/or their ability to bind effector proteins can inhibit cancer progression. Here, we show that receptor for activated C-kinase 1 (RACK1), a ubiquitously expressed scaffolding protein, plays a crucial regulatory role in tumor growth. Using an RNA silencing approach, we found that downregulation of RACK1 expression in HeLa and A673 tumor cells markedly suppressed the proliferation and invasion of these cells *in vitro* and tumor development *in vivo*. Consequently, we found that significant suppression of constitutive phosphorylation of Akt and MAPK by RACK1 silencing may contribute to the inhibition of tumor growth. Moreover, RACK1 silencing significantly attenuated tumor-associated angiogenesis by, at least in part, inhibiting the expression of two critical angiogenic factors, namely vascular endothelial growth factor-B and fibroblast growth factor 2. The results of the present study show that RACK1 is a potent enhancer of tumor growth and, thus, a potential anti-cancer therapeutic target. (*Cancer Sci* 2011; 102: 2007–2013)

Tumorigenesis is a complicated multistep process promoted by a series of genetic and microenvironmental alterations. The broadly accepted hallmarks of tumorigenesis include uncontrolled autonomous cell proliferative capability acquired through the genetic activation of oncogenes or inactivation of tumor suppressor genes, the impairment of anti-growth or pro-apoptotic signals, the induction of new blood vessel formation, and subsequent acquisition of the ability of invasion and metastasis.[1] Emerging evidence reveals that multiple redundant signaling pathways can be activated within a single cancer cell leading to proliferation, survival, and invasion. Once initiated, these intracellular signaling pathways usually undergo a multistep process, relying mainly on the recruitment of a number of adaptor/scaffold proteins that behave as molecular hubs for signal transduction or organization of complex signaling networks in time or space.[2–6] By assembling a large number of signaling molecules and facilitating protein–protein interactions, scaffold proteins play a crucial role in the tight regulation of cellular pathways, as well as the cross-talk with other signaling pathways. Recent findings have highlighted the relevance of scaffold proteins in many human cancers for their ability to create signaling platforms that drive cellular transformation; thus, interfering with their expression is considered a therapeutic intervention for inhibiting tumorigenesis.[2–6]

As a crucial scaffold protein, receptor for activated C-kinase 1 (RACK1) was originally cloned as an anchoring protein for PKC; it plays a critical role in stabilizing the active form of PKC and in permitting its translocation to different sites within a cell.[7,8] Receptor for activated C-kinase 1 is a 36-kDa protein containing seven internal Trp-Asp 40 (WD40) repeats; it is homologous to the G-protein β-subunit and is expressed ubiquitously in a wide range of tissues, including the brain and spleen.[9] Accumulated evidence reveals that RACK1 can associate with various signaling molecules, including the Src family members, the integrin β-subunit, phosphodiesterase PDE4D5, Flt1, and insulin-like growth factor (IGF)-1 receptors, and is thus involved in the regulation of various cell functions.[10–14] Recently, increased RACK1 expression has been reported in lung, colon, breast, ovarian, and hepatocellular carcinomas and human melanomas.[15–17] Berns *et al.*[18] also provided evidence that RACK1 is upregulated in vascular endothelial cells during angiogenesis and may contribute to tumor growth and spreading. We recently found that RACK1 participates in vascular endothelial growth factor (VEGF)/Flt1-triggered endothelial cell migration.[11] These observations raise the possibility of a crucial role for RACK1 in tumorigenesis and angiogenesis.

In the present study, we investigated the regulatory role of RACK1 in tumor growth. Using an RNAi approach, we found that downregulating RACK1 markedly suppressed the growth and invasion of HeLa and A673 cells *in vitro* and further inhibited solid tumor growth *in vivo*. Significant suppression of Akt and MAPK phosphorylation by RACK1 silencing may partially account for the inhibition of tumor cell growth. In addition, RACK1 silencing significantly attenuated tumor-associated angiogenesis and may thus contribute to the suppression of tumor growth.

Materials and Methods

Antibodies and reagents. Antibodies against Akt, phosphorylated (p-) Akt, MAPK, p-MAPK, phospholipase C (PLC) γ, and p-PLCγ were purchased from Cell Signaling (Beverly, MA, USA). Anti-RACK1 antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Alkaline phosphatase-conjugated anti-mouse and anti-rabbit immunoglobulins were purchased from Promega (Madison, WI, USA). Hamster cell adhesion molecule 1 (CD31) antibody was purchased from Millipore (Beverly, MA, USA) and anti-CD11b was obtained from BD Bioscience (Franklin Lakes, NJ, USA). Lipofectamine RNAiMAX reagent was obtained from Invitrogen (Carlsbad, CA, USA).

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doi: 10.1111/j.1349-7006.2011.02065.x

Cell culture. Human cervical carcinoma HeLa and rhabdomyosarcoma A673 cells were purchased from the American Type Culture Collection (Manassas, VA, USA). The HeLa and A673 cells were maintained in DMEM containing 10% FBS (JRH Biosciences, Victoria, Australia) at 37°C in 5% CO_2.

RNA interference. Stealth siRNAs designed against the human *RACK1* cDNA (GenBank Accession no. GNB2L1) were obtained from Invitrogen. The sequences of the two *RACK1* siRNAs used in the present study were GCCUCUCGAGAUAAGACCAUCAUCA for si1 and GGAACCUGGCUAACUGCAAGCUGAA for si2. Cells were transfected with RACK1 siRNAs or scrambled control siRNA using the RNAiMAX reagent (Invitrogen) according to the manufacturer's instructions. The effect of suppression on RACK1 expression was assessed at 60 h after transfection.

Proliferation assay. The cell proliferation assay was performed as described previously.[11]

Colony formation assay. Cell suspensions (1×10^4 cells diluted in 0.33% low-melting agarose) were overlaid on a bottom 0.5% agar layer (3 mL) in a 60-mm dish. Cells were incubated at 37°C for 2 weeks, and colonies were counted using a light microscope. The numbers observed in eight random visual fields per dish were counted; similar results were obtained in three independent experiments.

TUNEL assay. Cell apoptosis was analyzed using a terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) kit (*in situ* cell death detection kit, fluorescein; Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. Briefly, the cells were fixed in 3.7% formaldehyde in PBS for 1 h, permeabilized with 0.2% Triton X-100 in PBS for 2 min, and then incubated with the TUNEL reaction mixture for 60 min. The specimens were then observed under a fluorescence microscope.

Invasion assay. Cells (1×10^5/well) were seeded in a Cell Culture Insert (8-μm pore size; Falcon, BD Bioscience), precoated with 25 μL of 20% Matrigel (2–3 mg/mL protein), and then placed in a 24-well plate (Falcon). After cells had been cultured at 37°C for 40 h, they were fixed and stained with 0.5% crystal violet. The cells on the top of the Cell Culture Insert were removed by wiping with a cotton swab, and cell invasion was observed with an immunofluorescence microscope by counting the cells that had invaded into the bottom of the Cell Culture Insert.

Western blotting. Western blotting was performed as described previously.[11]

Tumor xenograft assay. All animal experiments were performed in accordance with institutional guidelines, following a protocol approved by the Ethics Committees of the Disease Model Research Center, Tokyo Medical and Dental University. Eight-week-old C57BL/6 mice (CLEA Japan, Kawasaki, Japan) were anesthetized and injected subcutaneously with 1×10^7 tumor cells. Tumor volume was measured every 2 days from Day 4 for approximately 3 weeks. After the mice had been killed, the tumors were removed and directly embedded in an optimal cutting temperature (OCT) compound in a deep freezer at −80°C.

Immunohistochemistry. Cryosections of fresh-frozen mouse tissues were stained with rat anti-CD11b (1:50; BD Bioscience) or hamster anti-CD31 (1:500; Millipore). All antibodies were diluted with tyramide signal amplification blocking reagent solution (Perkin Elmer, Boston, MA, USA). Sections were incubated with appropriate secondary antibodies and the nucleus-staining dye To-Pro-3 (Invitrogen) and analyzed under a confocal microscope (Radiance 2000; Bio-Rad, Hercules, CA, USA).

Reverse transcription and real-time PCR. One milligram of total RNA, extracted from tumor cells using the Isogen reagent (Takara, Shiga, Japan), was reverse transcribed using the Prime Script reverse transcriptase (Takara) according to the manufacturer's instructions. Real-time PCR amplification was performed using the SmartCycler real-time PCR system (Takara); the intercalating dye SYBR Green (Takara) was used for signal detection. Specific primers used in the analysis of *VEGF-A*, *VEGF-B*, *VEGF-C*, *angiopoietin (Ang) 1*, *Ang2*, *hepatocyte growth factor (HGF)*, *FGF2*, *placenta growth factor (PlGF)*, *RACK1*, and *β-actin* are given in Table S1, available as an Accessory Publication to this paper.

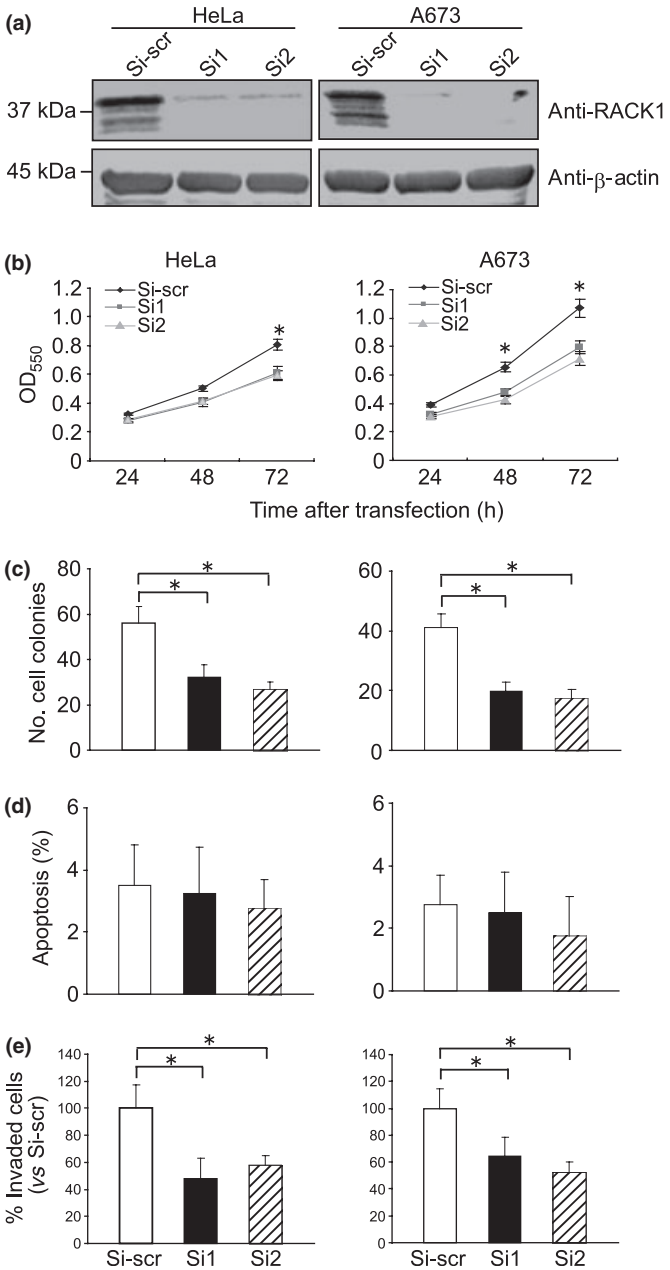

Fig. 1. RACK1 silencing inhibited tumor cell growth and invasion but not apoptosis *in vitro*. HeLa and A673 cells were transfected with scrambled control siRNA (si-scr) or RACK1 siRNAs (si1 and si2), as indicated. (a) Western blotting analysis of RACK1 after RNAi silencing. (b–e) Downregulation of RACK1 by transfection of siRNA significantly suppressed the proliferation (b), colony formation (c), and invasive ability (e) of HeLa and A673 cells, but had no effect on the apoptosis of HeLa and A673 cells (d). Data are the mean ± SEM. *$P < 0.05$ compared with si-scr.

Statistical analysis. Data are presented as the mean ± SEM. Data were analyzed using two-tailed *t*-tests and $P < 0.05$ was considered significant.

doi: 10.1111/j.1349-7006.2011.02065.x

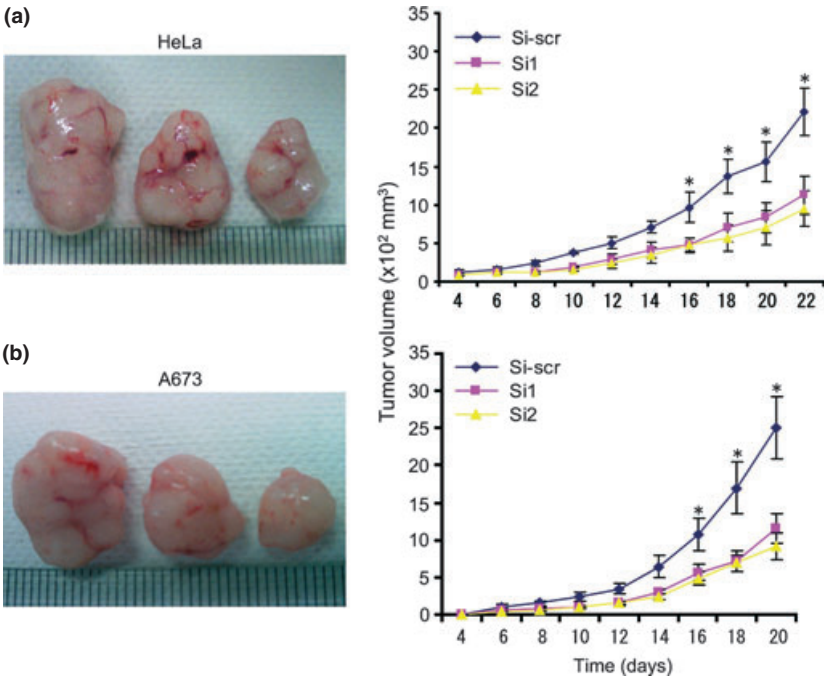

Fig. 2. RACK1 silencing significantly suppressed tumor growth *in vivo*. Representative images of xenograft tumor models and tumor growth curves of HeLa (a) and A673 (b) cells transfected with scrambled control siRNA (si-scr) or *RACK1* siRNAs (si1 and si2). Data are the mean ± SEM. $*P < 0.05$ compared with si-scr.

Results

RACK1 silencing suppressed tumor cell growth and invasion *in vitro*. To investigate the function of RACK1 in tumor growth, we used a gene-silencing approach. Two siRNAs designed from the human RACK1 sequence showed a very strong suppressing effect, with appropriately 90% reduction in RACK1 expression (Fig. 1a). In addition, *RACK1* mRNA expression was significantly suppressed to approximately 10–20% by Day 2 after siRNA transfection (Fig. S1), and this strong inhibitory effect continued up until Day 4. Expression gradually recovered and reached near-normal levels by Day 8. Using these siRNAs, we examined the effects of RACK1 silencing on tumor cell growth *in vitro* and observed a significant decrease in the growth rate of HeLa and A673 tumor cells after RACK1 silencing compared with control cells (scrambled siRNA) in both the proliferation (Fig. 1b) and colony formation assays (Figs 1c,S2). Conversely, RACK1 silencing had no effect on the apoptosis rate of these cells, as determined using a TUNEL assay (Fig. 1d).

Because RACK1 controls the interaction of signaling pathways involved in the coordination of cell adhesion and movement,[10,11,14] we next examined the effect of RACK1 silencing on the invasion of these tumor cells. As shown in Figures 1(e) and S3, downregulation of RACK1 greatly impaired the invasive ability of HeLa and A637 cells. This inhibitory effect was not due to the suppression of cell growth (Fig. S4). Together, these observations indicate that RACK1 plays an important regulatory role in tumor cell growth and movement.

Tumor growth was suppressed by RACK1 silencing *in vivo*. To obtain definitive evidence that RACK1 is required for tumor growth *in vivo*, we conducted a xenograft assay by injecting the control (si-scrambled) and RACK1-silenced tumor cells subcutaneously into mice and comparing the growth rate of the solid tumors. Interestingly, tumor growth after RACK1 silencing was significantly slower for both HeLa (Fig. 2a) and A673 (Fig. 2b) tumor cells compared with control cells. These results indicate that suppression of RACK1 expression in HeLa and A673 cells markedly suppresses their tumorigenicity in mice.

RACK1 silencing inhibited the phosphorylation of Akt and MAPK but not of PLCγ *in vitro*. Because downregulation of RACK1 inhibited the growth of human tumor xenografts, we then attempted to clarify the molecular mechanisms involved. Although multiple binding partners, including PKCs, Src, and IGF-1, highlight the complexity of RACK1-mediated signaling,[5,10,19] in the present study we focused on the effects of RACK1 silencing on the activation of MAPK, Akt, and PLCγ, which are implicated in the main intracellular signaling required for cell proliferation and survival.[20,21] As shown in Figure 3, compared with control siRNAs, RACK1 silencing resulted in a significant 30–40% suppression of Akt and MAPK activation, but not of PLCγ phosphorylation, in both HeLa and A673 cells. These results indicate that RACK1 silencing inhibits tumor cell growth, at least in part, by suppressing MAPK- and Akt-mediated signaling, but not the PLCγ-related signaling pathway.

RACK1 silencing inhibited tumor-associated angiogenesis, partly due to the suppression of 2 angiogenic factors, VEGF-B and FGF2. Tumor-associated angiogenesis is a crucial hallmark of tumorigenesis, which contributes significantly to tumor progression and metastasis.[22] Berns *et al.*[18] identified RACK1 as being significantly upregulated during angiogenesis and in carcinogenesis. To clarify whether RACK1 plays an important role in tumor-associated angiogenesis, we assessed the effects of downregulation of RACK1 in tumor cells on blood vessel formation using immunohistochemical staining with CD31, a specific marker for endothelial cells. In our *in vivo* experiments, silencing RACK1 significantly reduced blood vessel density in both HeLa and A673 tumor tissues (Fig. 4). Considering that numerous angiogenic factors, including VEGF-A, VEGF-B, VEGF-C, Ang1, Ang2, HGF, FGF2, and PlGF, are released from tumor cells for angiogenesis,[22,23] we analyzed the expression of these angiogenic factors using real-time PCR. As shown in Figure 5, the mRNA expression of *VEGF-B* and *FGF2* after RACK1 silencing was reduced by 40–50% compared with that of scrambled siRNA-transfected HeLa and A673 cells. These results suggest that RACK1 silencing suppresses tumor-associated angiogenesis via the downregulation of angiogenic factors and, thus, contributes to the reduction of tumor growth.

To identify a correlation between the suppression of angiogenic factors and the phosphorylation of Akt and MAPK after RACK1 knockdown, we examined the time course of these effects. As shown in Figure S5, suppression of p-Akt and

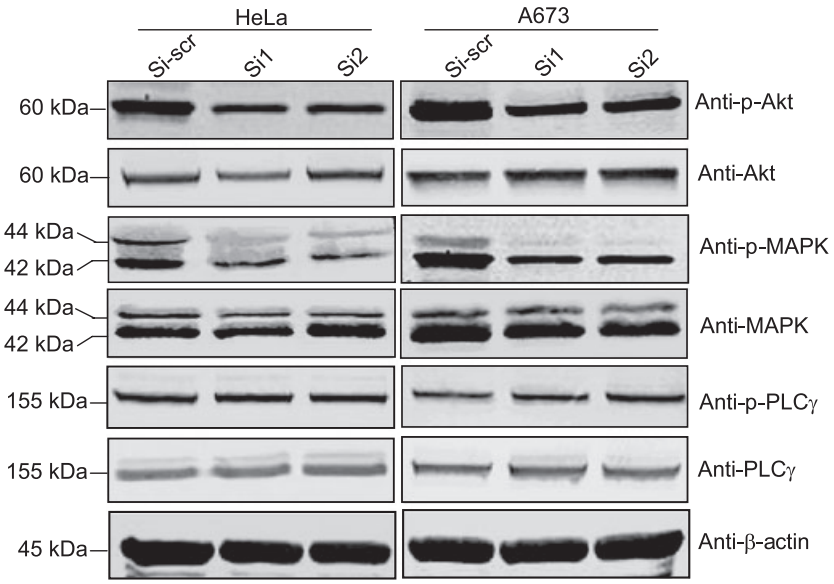

Fig. 3. Effect of RACK1 silencing on the phosphorylation of Akt, MAPK, and phospholipase C (PLC) γ *in vitro*. HeLa and A673 cells were transfected with scrambled control siRNA (si-scr) or *RACK1* siRNAs (si1 and si2) and the cell lysates were subjected to western blotting, as described in the Materials and Methods. Downregulation of RACK1 resulted in significant suppression of Akt and MAPK, but not PLCγ, phosphorylation. p, phosphorylated.

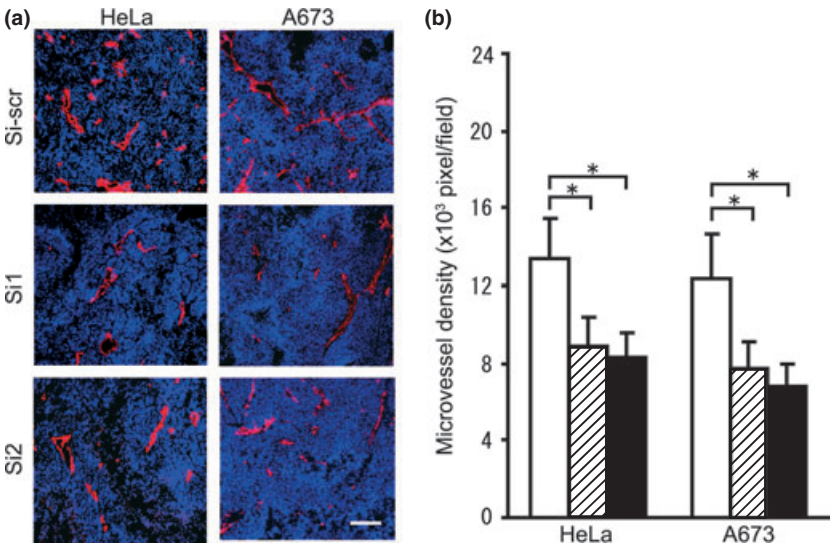

Fig. 4. RACK1 silencing significantly inhibited tumor-associated angiogenesis. (a) Representative micrographs of immunohistochemical staining from xenograft tumor models of HeLa and A673 cells transfected with scrambled control siRNA (si-scr) or *RACK1* siRNAs (si1 and si2). Endothelial cells and the nuclei were stained with CD31 (red) and To-Pro-3 (blue), respectively. Scale bar, 150 μm. (b) Microvessel density of HeLa or A673 xenograft tumor models, quantified using an Angiogenesis Image Analyzer (Kurabo, Osaka, Japan), following transfection of HeLa and A673 cells with si-scr (□), si1 (▨), and si2 (■). Data are the mean ± SEM. $*P < 0.05$.

p-MAPK was seen 60 h after RACK1 knockdown in HeLa and A673 cells, but not after 30 h. Conversely, suppression of *VEGF-B* and *FGF2* mRNA expression was detected 30 h after RACK1 knockdown (Fig. S6). These results suggest that suppression of p-Akt and p-MAPK occurs at a later stage, and is not involved in the upstream signaling of the suppression of VEGF-B and FGF2 transcription.

To clarify the effect of RACK1 silencing *in vivo*, we examined changes in the expression of RACK1, VEGF-B, and FGF2 and the activation status of signaling molecules such as Akt, MAPK, and PLCγ in xenograft tumor models. Because the duration of the silencing effect on RACK1 expression is approximately 8 days (Fig. S1), we excised the xenograft tumor tissues transfected with control siRNA (Si-scr) or *RACK1* siRNA (Si1) from mice on Days 2 (Fig. S7), 10, and 20. As expected, RACK1 expression on Day 2 after xenograft transplantation was strongly suppressed by *RACK1* siRNA (Si1) compared with that of the control scrambled RNA (Fig. 6a). However, this inhibitory effect had disappeared by Day 10. Accordingly, on Day 2 RACK1 knockdown had significantly suppressed the phosphorylation of Akt and MAPK (Fig. 6a) and the expression of *VEGF-B* and *FGF2* (Fig. 6b). Those inhibitory effects disappeared in association with a gradual recovery of RACK1 expression. We did not detect any change in the phosphorylation PLCγ at these time points. These results suggest that all the changes in the xenograft angiogenic factors and signal molecules depend on low RACK1 expression.

RACK1 silencing did not affect the infiltration of macrophage lineage cells into tumor tissue. Recent reports have indicated that the infiltration of various inflammatory cells, particularly macrophages, in tumor tissue is associated with prognosis and tumor angiogenesis in a variety of clinical conditions.[24,25] To elucidate whether RACK1 is required for the infiltration of macrophage lineage cells into tumor tissue, we measured the number of macrophage lineage cells that had infiltrated the si-scramble control or RACK1-silenced tumor tissues. As shown in Figure 7, downregulation of RACK1 did not significantly affect the infiltration of macrophage lineage cells into these tumor tissues. This suggests that RACK1 in tumor cells is not involved in the infiltration of macrophage-lineage cells into tumor tissue.

Discussion

In the present study, we studied the intrinsic function of RACK1 in tumor growth. Our findings show that downregulation of RACK1 results in the inhibition of proliferation and colony for-

doi: 10.1111/j.1349-7006.2011.02065.x

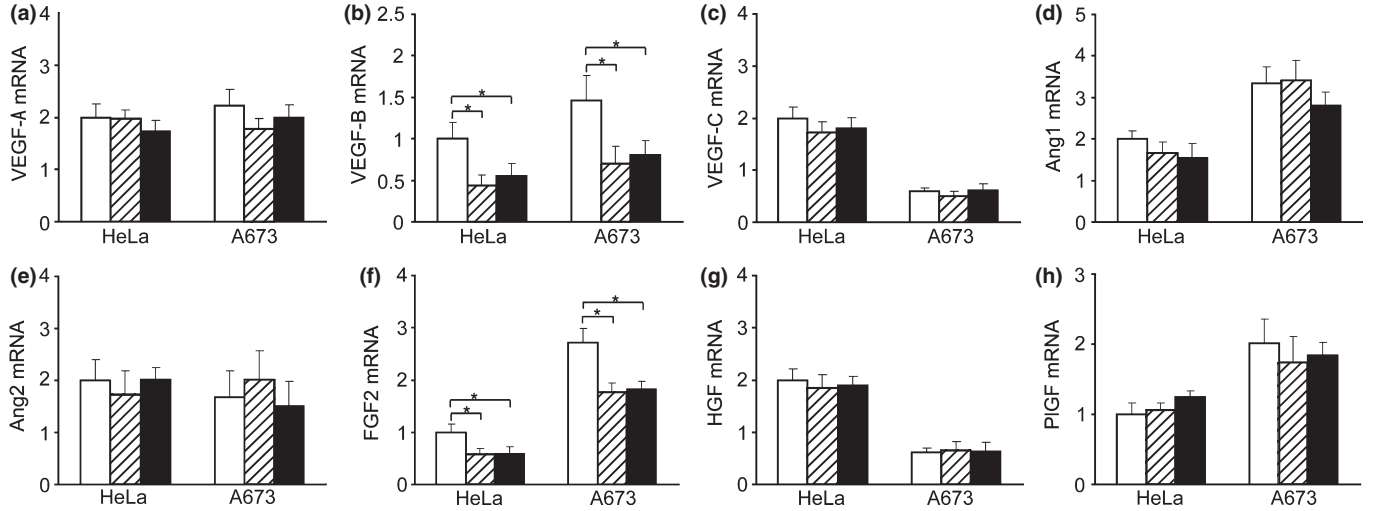

Fig. 5. Expression of proangiogenic factors released by RACK1-silenced tumor cells, determined using quantitative real-time PCR. Total RNA was extracted from HeLa or A673 cells transfected with scrambled control siRNA (si-scr; □) or *RACK1* siRNAs (▨, si1; ■, si2) and was used for reverse transcription of cDNA for the determination of mRNA expression of *vascular endothelial growth factor (VEGF)-A*, *VEGF-B*, *VEGF-C*, *angiopoietin (Ang) 1*, *Ang2*, *fibroblast growth factor (FGF) 2*, *hepatocyte growth factor (HGF)*, and *placenta growth factor (PlGF)*. RACK1 silencing significantly suppressed the expression of *VEGF-B* and *FGF2* by 50% and 40%, respectively, compared with si-scr-transfected control cells. Data are the mean ± SEM. **P* < 0.05.

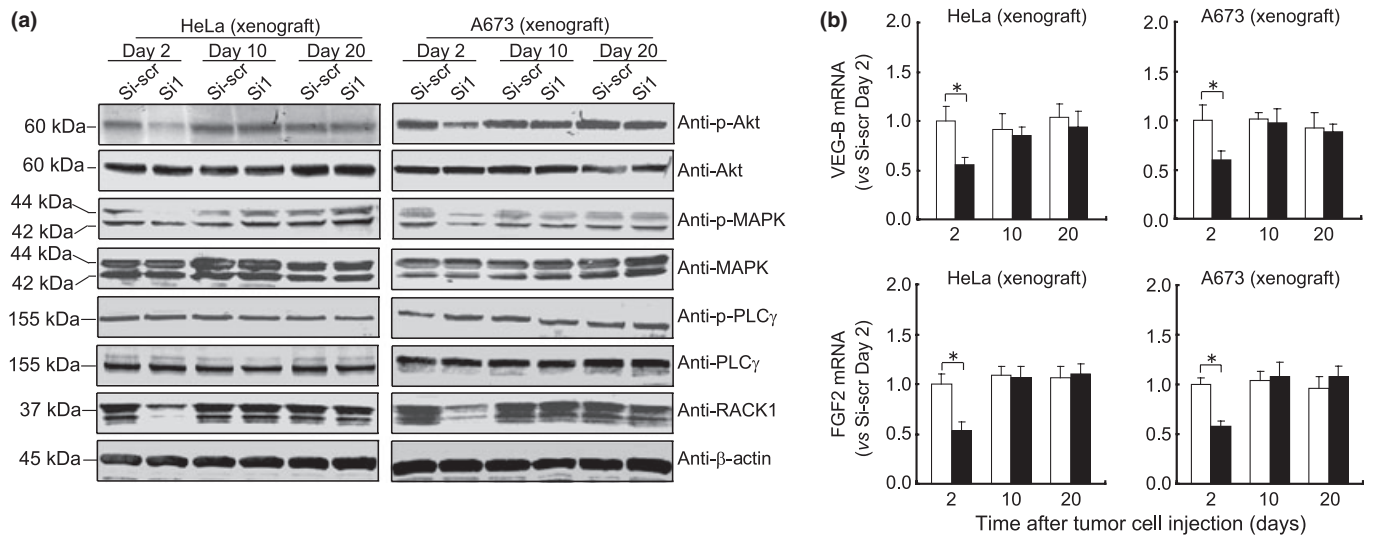

Fig. 6. RACK1 silencing significantly suppressed the phosphorylation of Akt and MAPK, and the expression of vascular endothelial growth factor (VEGF)-B and fibroblast growth factor (FGF) 2 *in vivo*. (a) Control (si-scr) and RACK1-silenced (si1) tumor cells were injected subcutaneously into mice and xenograft tumor tissues were excised on Days 2, 10, and 20. Tumor tissue lysate was subjected to western blotting with antibodies against phosphorylated (p-)Akt, Akt, p-MAPK, MAPK, p-phospholipase C (PLC) γ, PLCγ, RACK1, or β-actin. (b) Real-time PCR analysis of *VEGF-B* and *FGF2* expression in xenograft tumor tissues 2, 10, 20 days after subcutaneous injection of control (□) and RACK1-silenced (■) tumor cells into mice. Data are the mean ± SEM. **P* < 0.05.

mation of HeLa and A673 cells *in vitro*, and suppression of solid tumor growth *in vivo*. These results provide evidence that the RACK1 is required for tumor growth and that it may be an oncogenic signal transducer.

Is RACK1 a potent enhancer of tumorigenesis? Emerging evidence, as described below, supports the view that RACK1 plays a crucial role in tumorigenesis. First, upregulation of RACK1 expression has been observed in a range of human cancers, including lung, colon, breast, ovarian, and hepatocellular carcinomas, as well as human melanoma.[15–18] These findings imply that an increase in RACK1 expression is required for tumor growth and also suggest a new use for RACK1 as a biomarker for several lines of cancers. Second, it has been shown that RACK1 is associated with a variety of signaling molecules, including PKC, Src, and IGF-1R.[7,13,15] It is of note that the relevance of the signaling mediated by these proteins in tumor cell growth is well established. In addition, it is broadly accepted that aberrant activation of several PKC isozymes leads to tumorigenesis; therefore, as an important stabilizer of PKC activity, RACK1 may play a pivotal role in regulating PKC signaling-related tumor progression.[7,8,26] Furthermore, Keily *et al.*[27] showed that the proliferation of MCF-7 cells was enhanced by overexpression of RACK1, whereas IGF-1-mediated protection from etoposide-dependent death was greatly reduced. Separately, another two groups also reported that knockdown of RACK1 suppressed IGF-1- and androgen receptor-dependent tumor cell growth.[19,28] These reports suggest that RACK1 plays a crucial role in intracellular signaling related to tumor cell

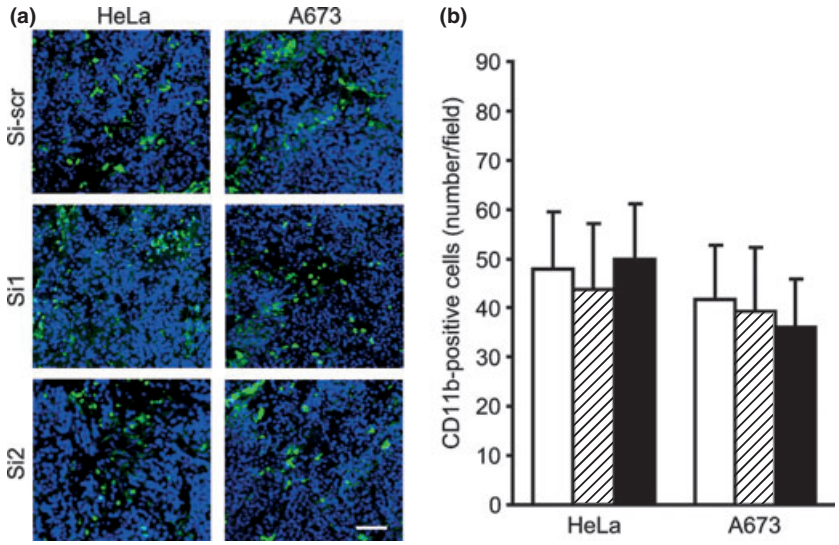

Fig. 7. Downregulation of RACK1 did not affect the infiltration of macrophage-lineage cells into tumor tissue. (a) Representative micrographs of monocytes/macrophages infiltrating into the xenograft tumor tissue of HeLa and A673 cells transfected with scrambled control siRNA (si-scr) or *RACK1* siRNAs (si1 and si2). Scale bar, 75 μm. (b) Number of macrophages infiltrating into tumor tissue following injection of cells transfected with si-scr (□) or *RACK1* siRNAs (▨, si1; ■, si2). There was no significant effect of RACK1 silencing on the infiltration of macrophage-lineage cells into tumor tissue.

growth and, accordingly, in regulating tumor development. Moreover, our results showed that RACK1 knockdown inhibited the invasion of HeLa and A673 cells. Studies have revealed that RACK1 is involved in extracellular matrix (ECM)/integrin signaling by acting as a scaffold to recruit other proteins to focal adhesion complexes.[10,14,19] Thus, by controlling interactions with the ECM and by coordinating cell adhesion, RACK1 may play an important role in governing cell survival as well as cell chemotaxis. However, in contrast with a previous report that overexpression of RACK1 induced apoptosis of HT-29 colon carcinoma cells, partly by inhibiting Src activity,[29] our results showed that RACK1 knockdown inhibited cell growth but did not affect cell apoptosis. To date, several reports have suggested that RACK1 has opposing effects on the proliferation of different types of tumor cells. Although, the mechanisms involved remain to be determined, the discrepancies may arise from differences in the cell types used or in the experimental conditions and served to highlight the complexity of RACK1 involvement in signaling regulation.

Possible regulatory mechanisms of RACK1 at the molecular level in tumor growth. One critical question is how RACK1 affects the signaling of cell growth at the molecular level. Because RACK1 is simultaneously involved in widespread signaling pathways owing to its association with PKC, Src family members, and others, multiple pathways related to these proteins appear to be activated in the process of tumor growth. Thus, RACK1 is implicated in a complicated signaling network rather than a simple pathway. In the present study we focused on the effect of RACK1 on the activation of central players in growth signaling, such as Akt, MAPK, and PLCγ, which are often constitutively activated in a variety of cancers.[20,21] Importantly, we found that RACK1 silencing significantly inhibited the phosphorylation of Akt and MAPK by 30–40%, but not that of PLCγ, both *in vitro* and *in vivo*. Although the suppressive effects were not very strong, the suppression of Akt- or MAPK-related pathways contributes, at least in part, to the inhibition of tumor growth.

Effects of RACK1 silencing on tumor-associated angiogenesis may be another key for the inhibition of tumor growth. Changes in the tumor microenvironment induced by the suppression of angiogenesis markedly affect tumor growth and metastasis.[22,30] Berns *et al.*[18] have reported that RACK1 is upregulated during angiogenesis and in human carcinoma cells. We also found that inhibition of RACK1 expression has a negative effect on tumor angiogenesis, but it did not affect macrophage infiltration into the tumor tissue (Figs 4,7). Tumor angiogenesis is known to be required for tumor growth to >3 mm in diameter so that nutrients and oxygen can be supplied and waste products removed. Therefore, inhibition of blood vessel formation by downregulation of RACK1 delays tumor growth.

Another important question is how RACK1 regulates tumor angiogenesis. Analysis of the levels of a group of proangiogenic factors released by tumor cells showed that the expression of the *FGF2* and *VEGF-B* genes was suppressed by RACK1 silencing. It has been shown that RACK1 is involved in the transcriptional regulation of genes such as those coding for brain-derived neurotropic factor (BDNF) and TGF-β-stimulated collagen as a modulator of chromatin remodeling and a Smad3-binding protein.[31,32] Smad3 has not been shown to regulate *FGF2* or *VEGF-B* transcription; thus, RACK1-induced chromatin remodeling may be a possible mechanism by which mRNA levels of *FGF2* and *VEGF-B* are modulated. Further investigations are necessary to clarify the relationship between RACK1 and these angiogenic and/or growth factors. Importantly, increases in both FGF2 and VEGF-B expression can enhance tumor angiogenesis and promote tumor growth.[22,33–35] Thus, reduced expression of two proteins by RACK1 silencing may lead, in part, to the reduction of angiogenesis and, finally, reduced tumor growth. However, as a complicated multistep process, tumor-associated angiogenesis is implicated in the extensive interplay among cells, soluble factors, and ECM components. Moreover, the ''angiogenic switch'' via the change of the local balance of pro- and anti-angiogenic factors, such as thrombospondin-1, is critical for the initiation of tumor angiogenesis.[22] Thus, we cannot rule out the possibility that RACK1 also plays an important role in the regulation of other complicated processes in angiogenesis. It is necessary to investigate the mechanism by which angiogenesis is regulated by RACK1 in more detail.

In conclusion, the present study has demonstrated that RACK1 plays a critical role in tumor growth by affecting cell proliferation and tumor angiogenesis. A number of scaffold proteins have recently been highlighted for their roles in tumor growth, and new anti-cancer drugs targeting these genes have been developed. Based on the multiple functions of RACK1 in tumor growth, it may be considered a potential anticancer therapeutic target.

Acknowledgements

The authors thank the members of their laboratory at the Department of Molecular Oncology, Tokyo Dental and Medical University, Tokyo, Japan, for help and advice throughout this study. This work was

doi: 10.1111/j.1349-7006.2011.02065.x

supported by a Grant-in-Aid for Special Project Research on Cancer-Bioscience 17014020 from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

Disclosure Statement

The authors have no conflict of interest.

References

1 Hanahan D, Weinberg RA. The hallmarks of cancer. *Cell* 2000; **100**: 57–70.
2 Defilippi P, Di Stefano P, Cabodi S. p130Cas: a versatile scaffold in signaling networks. *Trends Cell Biol* 2006; **16**: 257–63.
3 Nishihara H, Tanaka S, Tsuda M *et al.* Molecular and immunohistochemical analysis of signaling adaptor protein Crk in human cancers. *Cancer Lett* 2002; **180**: 55–61.
4 White CD, Brown MD, Sacks DB. IQGAPs in cancer: a family of scaffold proteins underlying tumorigenesis. *FEBS Lett* 2009; **583**: 1817–24.
5 Sato T, Gotoh N. The FRS2 family of docking/scaffolding adaptor proteins as therapeutic targets of cancer treatment. *Expert Opin Ther Targets* 2009; **13**: 689–700.
6 Bouras T, Lisanti MP, Pestell RG. Caveolin-1 in breast cancer. *Cancer Biol Ther* 2004; **3**: 931–41.
7 Ron D, Chen CH, Caldwell J, Jamieson L, Orr E, Mochly-Rosen D. Cloning of an intracellular receptor for protein kinase C: a homolog of the beta subunit of G proteins. *Proc Natl Acad Sci USA* 1994; **91**: 839–43.
8 Ron D, Mochly-Rosen D. Agonists and antagonists of protein kinase C function, derived from its binding proteins. *J Biol Chem* 1994; **269**: 21395–8.
9 McCahill A, Warwicker J, Bolger GB, Houslay MD, Yarwood SJ. The RACK1 scaffold protein: a dynamic cog in cell response mechanisms. *Mol Pharmacol* 2002; **62**: 1261–73.
10 Liliental J, Chang DD. Rack1, a receptor for activated protein kinase C, interacts with integrin beta subunit. *J Biol Chem* 1998; **273**: 2379–83.
11 Wang F, Yamauchi M, Muramatsu M, Osawa T, Tsuchida R, Shibuya M. RACK1 regulates VEGF/Flt1-mediated cell migration via activation of a PI3K/Akt pathway. *J Biol Chem* 2011; **286**: 9097–106.
12 Yarwood SJ, Steele MR, Scotland G, Houslay MD, Bolger GB. The RACK1 signaling scaffold protein selectively interacts with the cAMP-specific phosphodiesterase PDE4D5 isoform. *J Biol Chem* 1999; **274**: 14909–17.
13 Chang BY, Chiang M, Cartwright CA. The interaction of Src and RACK1 is enhanced by activation of protein kinase C and tyrosine phosphorylation of RACK1. *J Biol Chem* 2001; **276**: 20346–56.
14 Besson A, Wilson TL, Yong VW. The anchoring protein RACK1 links protein kinase Cepsilon to integrin beta chains. Requirements for adhesion and motility. *J Biol Chem* 2002; **277**: 22073–84.
15 Zhang W, Zong CS, Hermanto U, Lopez-Bergami P, Ronai Z, Wang LH. RACK1 recruits STAT3 specifically to insulin and insulin-like growth factor 1 receptors for activation, which is important for regulating anchorage-independent growth. *Mol Cell Biol* 2006; **26**: 413–24.
16 Bourd-Boittin K, Le Pabic H, Bonnier D, L'Helgoualc'h A, Theret N. RACK1, a new ADAM12 interacting protein. Contribution to liver fibrogenesis. *J Biol Chem* 2008; **283**: 26000–9.
17 Egidy G, Jule S, Bosse P *et al.* Transcription analysis in the MeLiM swine model identifies RACK1 as a potential marker of malignancy for human melanocytic proliferation. *Mol Cancer* 2008; **7**: 34.
18 Berns H, Humar R, Hengerer B, Kiefer FN, Battegay EJ. RACK1 is up-regulated in angiogenesis and human carcinomas. *FASEB J* 2000; **14**: 2549–58.
19 Hermanto U, Zong CS, Li W, Wang LH. RACK1, an insulin-like growth factor I (IGF-I) receptor-interacting protein, modulates IGF-I-dependent integrin signaling and promotes cell spreading and contact with extracellular matrix. *Mol Cell Biol* 2002; **22**: 2345–65.
20 Falasca M. PI3K/Akt signalling pathway specific inhibitors: a novel strategy to sensitize cancer cells to anti-cancer drugs. *Curr Pharm Des* 2010; **16**: 1410–6.
21 Katz M, Amit I, Yarden Y. Regulation of MAPKs by growth factors and receptor tyrosine kinases. *Biochim Biophys Acta* 2007; **1773**: 1161–76.
22 Folkman J. Angiogenesis: an organizing principle for drug discovery? *Nat Rev Drug Discov* 2007; **6**: 273–86.
23 Cao Y, Liu Q. Therapeutic targets of multiple angiogenic factors for the treatment of cancer and metastasis. *Adv Cancer Res* 2007; **97**: 203–24.
24 Dirkx AE, Oude Egbrink MG, Wagstaff J, Griffioen AW. Monocyte/macrophage infiltration in tumors: modulators of angiogenesis. *J Leukoc Biol* 2006; **80**: 1183–96.
25 Leek RD, Landers RJ, Harris AL, Lewis CE. Necrosis correlates with high vascular density and focal macrophage infiltration in invasive carcinoma of the breast. *Br J Cancer* 1999; **79**: 991–5.
26 Schechtman D, Mochly-Rosen D. Adaptor proteins in protein kinase C-mediated signal transduction. *Oncogene* 2001; **20**: 6339–47.
27 Kiely PA, Sant A, O'Connor R. RACK1 is an insulin-like growth factor 1 (IGF-1) receptor-interacting protein that can regulate IGF-1-mediated Akt activation and protection from cell death. *J Biol Chem* 2002; **277**: 22581–9.
28 Kraus S, Gioeli D, Vomastek T, Gordon V, Weber MJ. Receptor for activated C kinase 1 (RACK1) and Src regulate the tyrosine phosphorylation and function of the androgen receptor. *Cancer Res* 2006; **66**: 11047–54.
29 Mamidipudi V, Dhillon NK, Parman T, Miller LD, Lee KC, Cartwright CA. RACK1 inhibits colonic cell growth by regulating Src activity at cell cycle checkpoints. *Oncogene* 2007; **26**: 2914–24.
30 Ferrara N, Kerbel RS. Angiogenesis as a therapeutic target. *Nature* 2005; **438**: 967–74.
31 He DY, Neasta J, Ron D. Epigenetic regulation of BDNF expression via the scaffolding protein RACK1. *J Biol Chem* 2010; **285**: 19043–50.
32 Okano K, Schnaper HW, Bomsztyk K, Hayashida T. RACK1 binds to Smad3 to modulate transforming growth factor-beta1-stimulated alpha2(I) collagen transcription in renal tubular epithelial cells. *J Biol Chem* 2006; **281**: 26196–204.
33 Fahmy RG, Dass CR, Sun LQ, Chesterman CN, Khachigian LM. Transcription factor Egr-1 supports FGF-dependent angiogenesis during neovascularization and tumor growth. *Nat Med* 2003; **9**: 1026–32.
34 Mould AW, Greco SA, Cahill MM *et al.* Transgenic overexpression of vascular endothelial growth factor-B isoforms by endothelial cells potentiates postnatal vessel growth *in vivo* and *in vitro*. *Circ Res* 2005; **97**: e60–70.
35 Albrecht I, Kopfstein L, Strittmatter K *et al.* Suppressive effects of vascular endothelial growth factor-B on tumor growth in a mouse model of pancreatic neuroendocrine tumorigenesis. *PLoS ONE* 2010; **5**: e14109.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. The duration of the silencing effect on RACK1 expression by specific RNAi.

Fig. S2. Downregulation of RACK1 markedly inhibited colony formation of HeLa and A673 cells.

Fig. S3. RACK1 knockdown significantly suppressed the invasive ability of HeLa and A673 cells.

Fig. S4. Inhibition of the invasion of HeLa and A673 cells by RACK1 silencing was not due to the suppression of cell growth.

Fig. S5. Effect of RACK1 silencing on Akt and MAPK phosphorylation from 0 to 60 h *in vitro*.

Fig. S6. Effect of RACK1 silencing on VEGF-B and FGF2 expression from 0 to 60 h *in vitro*.

Fig. S7. Representative image of xenograft tumors on Day 2 after transplantation.

Table S1. Sequences of specific primers used for angiogenic factors and RACK1.

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