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Sphingosine 1-phosphate-induced Angiogenesis Involves Src Protein Kinases Activated by Phospholipase C-Ca^{2+} Signaling in Human Endothelial Cells

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Several lines of clinical and preclinical evidences have suggested that platelets may importantly contribute to tumor-induced angiogenesis. We have recently reported that sphingosine 1-phosphate (S1P), which is abundantly stored in platelets and released upon platelet activation, induces angiogenesis through G_i protein-coupled receptors. In the present study, we further investigated the signaling pathways of mitogen activated protein kinases (MAPKs) stimulated by S1P in human umbilical vein endothelial cells (HUVECs). S1P rapidly induced extracellular signal-regulated kinases (ERKs) and p38 mitogen- activated protein kinase (p38 MAPK) activation in HUVECs. Notably, S1P-induced ERKs and p38 MAPK activation was almost completely inhibited by the phospholipase C (PLC) inhibitor U73122 but not affected by the phosphatidylinositol 3-kinase (PI-3K) inhibitor wortmannin. As a downstream of PLC, S1P-induced ERK activation was totally blocked by inhibition of intracellular Ca^{2+} mobilization by BAPTA-AM, a calcium chelator, but further stimulated by ionomycin. In contrast, the protein kinase C (PKC) inhibitor GF109203X had no effect. Thus, an increase in intracellular Ca^{2+} is closely involved in S1P-dependent signaling. Moreover, ERK activation by S1P was completely blocked by the calmodulin inhibitor W-7 but not by KN-93, a selective inhibitor of Ca^{2+}/ calmodulin-dependent protein kinases. The Src tyrosine kinase inhibitor PP1 significantly reduced both S1P- and ionomycin-induced ERK activation. In addition, S1P-induced HUVEC migration was blocked by PP1 in a dose-dependent manner. Therefore, these results suggest that Src protein kinases activated by Ca^{2+}/calmodulin signaling may be an important mediator of angiogenesis in response to S1P.

Key Words: Sphingosine 1-phosphate, Angiogenesis, ERK, p38 MAPK, Src

INTRODUCTION

Angiogenesis, the development of new blood vessels from pre-existing endothelium, is a critical process in many physiological and pathological conditions including wound healing, embryonic development, organ regeneration, chronic inflammation and solid tumor growth.[1,2] The process of

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angiogenesis is complex and involves several discrete steps, such as extracellular matrix degradation, proliferation and migration of endothelial cells, and morphological differentiation of endothelial cells to form tubes.[3)]

Under normal conditions, all these steps are tightly regulated in order to avoid the undesired neovascularization, and many control mechanisms have been described to be active.[4)] However, under pathological conditions such as outgrowing solid tumor, these tight regulatory mechanisms have been disrupted in most cases by a number of angiogenic factors constitutively secreted from tumor cells, accessory cells attracted to the site of neoplastic cell growth, and cells in the nearby tissues, when their environment becomes hypoxic or inflammatory.[3~5)]

Recently, Pinedo *et al.* hypothesized that platelets importantly contribute to tumor-induced angiogenesis based on clinical and preclinical findings that tumor angiogenesis is dependent not only on endothelial cells and tumor, but also on platelets- endothelium interaction.[6)] Also, they proposed that the release of proangiogenic and antiangiogenic growth factors by platelets modifies the angiogenic balance of a tumor. Indeed, we have recently demonstrated that S1P, which is abundantly stored in platelets and released upon platelet activation, has a potent angiogenic activity *in vivo*.[7)] Using *in vitro* angiogenesis models, we also showed that S1P strongly stimulated proliferation, migration, and tube formation of endothelial cells. Thus, we proposed that S1P may be an important modulator of platelet-mediated tumor angiogenesis.

S1P has been known as a lipid messenger that regulates cell growth, differentiation and cell death, and activates different signal transduction pathways in various cell types.[8~9)] Many of the functions of S1P are previously thought to be due to the second messenger action of S1P produced intracellularly by activation of sphingosine kinase, the enzyme which catalyzes the phosphorylation of sphingosine, in response to various stimuli.[10~13)] Recently, a family of EDG receptors, such as EDG-1, EDG-3 and EDG-5/H218/AGR16, were identified as plasma membrane receptors for S1P, and the extracellular action mechanism of S1P has been delineated. These receptors are shown to be associated with various G proteins, and modulate multiple signaling pathways including PLC activation, Ca^{2+} mobilization, Ras/MAPK activation, and adenylate cyclase inhibition in various cell types.[9)]

However, the signaling properties of S1P in endothelial cells are not elucidated. In the present study, we investigated the MAPK signaling pathway induced by S1P in human endothelial cells. We have found that Src protein tyrosine kinases (PTKs) activated by a PLC-Ca^{2+} signal are closely involved in S1P-induced ERK activation. Thus, we suggest that these signaling pathways may be responsible for mediating the angiogenic activities of S1P in endothelial cells.

MATERIALS AND METHODS

1) Materials

S1P, U73122, U73343, LY294002, GF109203X, ionomycin, BAPTA-AM, W-7, KN-93, and PP1 were purchased from BIOMOL Research Laboratory. Phorbol 12-myristate 13-acetate (PMA), wortmannin, N-acetyl-L-cysteine (NAC), and pyrrolidine dithiocarbamate were from Sigma. PTX was from Research Biochemicals International. Antibodies for phospho-specific ERK and phospho-specific p38 MAPK were obtained from New England Biolabs. Antibody for p38 MAPK was from Santa Cruz Biotechnology and anti-phosphotyrosine antibody was from Transduction Laboratories.

2) Cell culture

HUVECs were kindly provided by Dr. Y.-H. Kang (Hallym University, Korea). The cells were grown onto a gelatin-coated 75-cm^2 flask in M199 with 20% fetal bovine serum (FBS), 100 units/ml penicillin, 100 μg/ml streptomycin, 3

ng/ml basic fibroblast growth factor (bFGF), and 5 units/ml heparin at 37°C under 5% CO_2, 95% air. Cells at 2~7 passages were used in these experiments. HUVECs were rendered quiescent by incubation with M199 containing 1% FBS for 6 h prior to the experiments.

3) Western blotting

Cell lysates from HUVECs were loaded into a 10% SDS-PAGE gel, and transferred to polyvinyl-difluoride membrane. The blocked membranes were then incubated with the indicated antibody, and the immunoreactive bands were visualized using chemiluminescent reagent as recommended by Amersham Pharmacia Biotechnology. The signals of the bands were quantitated using a densitometer.

4) Chemotaxis assay

The chemotactic motility of HUVECs was assayed using a Transwell chamber with 6.5 mm diameter polycarbonate filters (8 μm pore size). Briefly, the lower surface of the filter was coated with 10 μg of gelatin. S1P prepared in 600 μl of M199 with 1% FBS was placed in the lower wells. HUVECs were trypsinized and suspended at a final concentration of 1×10^6 cells/ml in M199 containing 1% FBS. Inhibitors were given to the cells for 30 min at room temperature before seeding. 100 μl of the cell suspension was loaded into each of the upper wells. The chamber was incubated at 37°C for 4 h. Cells were fixed and stained with hematoxylin and eosin. Nonmigrating cells on the upper surface of the filter were removed by wiping with a cotton swab, and chemotaxis was quantified by counting the cells that migrated to the lower side of the filter with optical microscopy at ×200 magnification. Ten fields were counted for each assay. The data are presented as percentage±S.E. of control. Each sample was assayed in triplicate, and the assays were repeated three times.

RESULTS

1) S1P-induced ERK and p38 MAPK activation involve PLC but not PI-3K or PKC in HUVECs

Recent studies have indicated that ERK is required for morphological differentiation of endothelial cells in response to PMA,[14] and p38 MAPK is involved in vascular endothelial growth factor (VEGF)-induced actin reorganization and cell migration in human endothelial cells.[15] We have recently identified that S1P rapidly induces activation of ERK and p38 MAPK via G_i protein-coupled receptors, and the ERK activity is critical for morphological differentiation of endothelial cells to form tube-like structures. However, the intracellular signaling molecules engaged in S1P-induced ERK activation are not understood.

Many previous studies have determined the links between G protein-coupled receptor and ERK activation, and indicated that stimulation of G_i protein-coupled receptors activates ERKs by the $\beta\gamma$ dimers but not by the α_i subunit.[16,17] Moreover, PI-3K or PLC was shown to be involved in $\beta\gamma$ signaling to the ERK cascade.[18,19] Thus, we have investigated whether PI-3K or PLC is involved in ERK activation induced by S1P. When subconfluent HUVECs were exposed to 5 μM S1P for 5 min, the activation of ERK and p38 MAPK was prominently detected by Western blotting using rabbit polyclonal phospho-specific anti-ERK or p38 MAPK, which only detect phosphorylated and activated form of ERK and p38 MAPK, respectively (Fig. 1). Pretreatment of HUVECs with 100 nM wortmannin, an inhibitor of PI-3K, did not block S1P-induced activation of ERKs (Fig. 1A). The same results were obtained from the treatment of higher concentrations of wortmannin (200 and 300 nM) or LY294002 (20 and 50 μM), another PI-3K inhibitor (data not shown). In contrast, pretreatment of HUVECs with U73122 (5 μM for 30 min) markedly suppressed S1P-induced ERK activation, while a structurally similar derivative of U73122 (U73343) failed to exert a similar effect (Fig. 1A).

PLC is known to generate inositol triphosphate

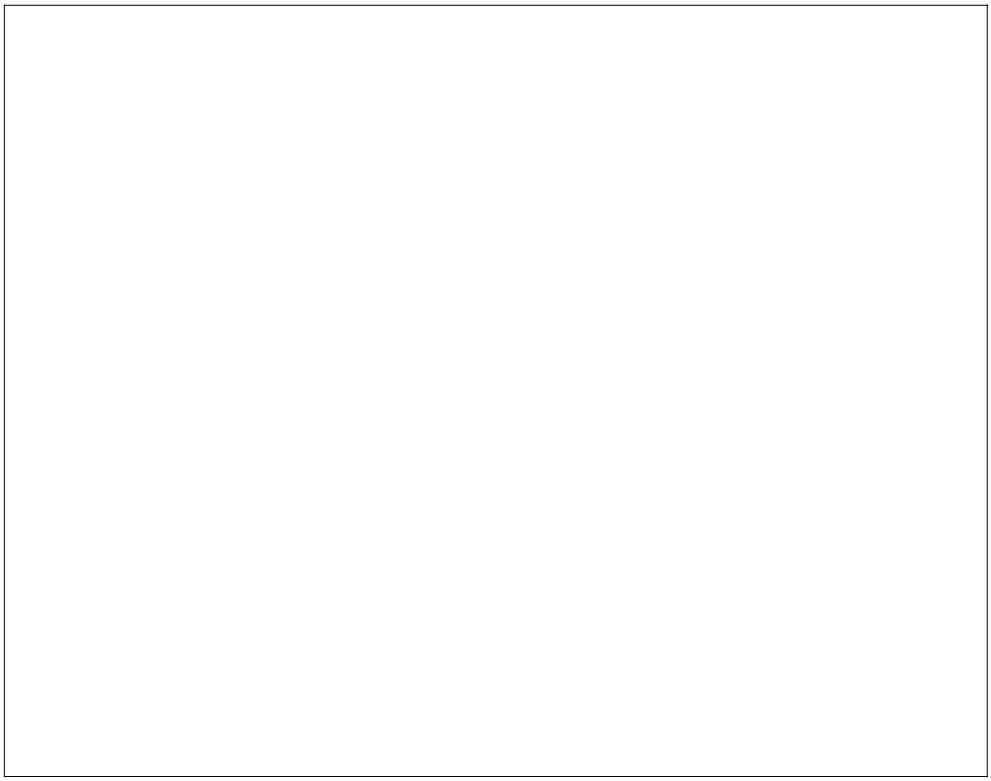

Fig. 1. Effects of wortmannin, U73343, U73122, or GF109203X on S1P-induced ERK and p38 MAPK activation in HUVECs. Quiescent subconfluent HUVECs were pretreated with 100 nM wortmannin (Wort), 5 μM U73343, 5 μM U73122, or 2 μM GF109203X (GF) for 30 min, and stimulated with 5 μM S1P or 100 nM PMA for 5 min. Cell lysates were separated on 10% SDS-PAGE, and followed by Western blotting. (A) The effect of drugs on ERK activation by S1P. (B-D) The densities of the bands corresponding to phosphorylated and non-phosphorylated p38 MAPKs were quantitated using a densitometer. Results are expressed as fold-stimulation above control. Data represent mean±SE of three separate experiments. *Inset*, a representative experiment is shown.

and diacylglycerol, which activate intracellular Ca^{2+} mobilization and PKC, respectively. Thus, we examined whether PKC is involved in S1P-induced ERK activation in HUVECs. At 100 nM, PMA strongly activated ERK, but not p38 MAPK (Fig. 1A and D) and PMA-induced ERK activation was completely inhibited by pretreatment with specific PKC inhibitor GF109203X (2 μM for 30 min). However, GF109203X had no effect on S1P-induced ERK activation (Fig. 1A). These data indicate that S1P-stimulated ERK activation is independent of PKC in HUVECs.

Similar to the ERK pathway, both wortmannin and GF109203X did not block S1P-induced p38 MAPK activation (Fig. 1B and D). In contrast, U73122 markedly inhibited S1P-stimulated p38 MAPK activation (Fig. 1C). These results suggest that upstream signaling mechanism leading to ERK and p38 MAPK activation by S1P may parallel in a signaling paradigm which involves G_i protein- mediated PLC activation in HUVECs.

2) S1P-induced ERK activation is mediated by Ca^{2+}/calmodulin-dependent pathway

To determine whether ERK and p38 MAPK activation by S1P were Ca^{2+}-dependent, we

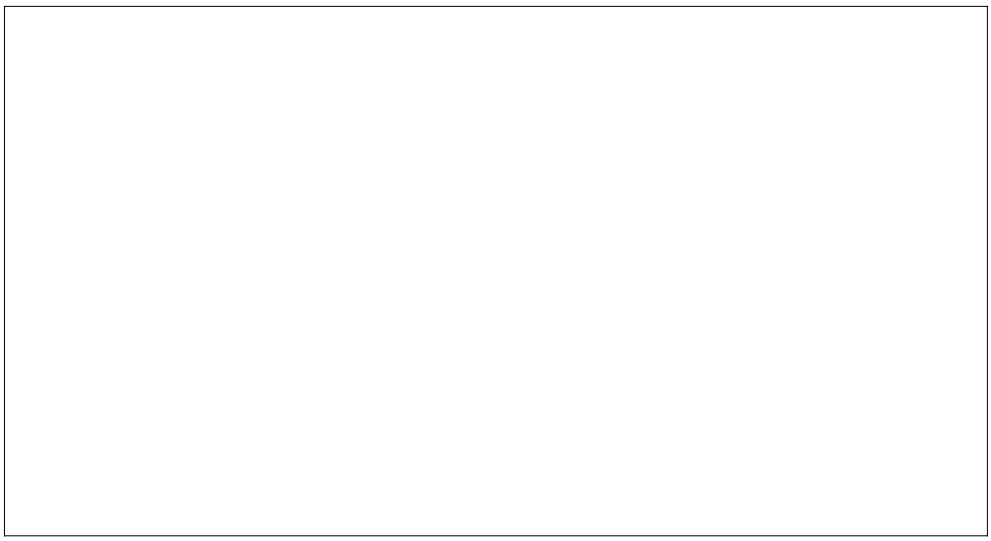

Fig. 2. Ca^{2+} induces ERK and p38 MAPK activation, and S1P-induced ERK activation is dependent on calmodulin. (A) Quiescent HUVECs were exposed to ionomycin (1 μM) for the indicated time periods. (B) HUVECs were pretreated with 100 ng/ml of PTX for 6 h, and stimulated by 5 μM S1P alone or co-treatment with 5 μM S1P plus 1 μM ionomycin (Iono) for 5 min. (C) HUVECs were pretreated with 30 μM BAPTA-AM (BAPTA) for 30 min, 20 mM N-acetyl-L-cysteine (NAC) for 5 h, 25 μM W-7 for 30 min, or 10 μM KN-93 for 30 min. Then, cells were stimulated with 5 μM S1P for 5 min. Cell lysates were separated on 10% SDS-PAGE, followed by Western blotting using anti-phospho-specific ERK antibody (P-ERK1 and P-ERK2) and anti-phospho-specific p38 MAPK antibody (P-p38). Results are representatives of three independent experiments.

treated HUVECs with Ca^{2+} ionophore, ionomycin (1 μM). Ionomycin was sufficient to evoke ERK and p38 MAPK activation with similar time courses of those by S1P (Fig. 2A) and the inhibition of S1P-induced ERK and p38 MAPK activities by PTX were significantly restored by ionomycin (Fig. 2B). Further, pretreatment with BAPTA-AM (30 μM for 30 min), an intracellular Ca^{2+} chelator, resulted in a significant loss of ERK activation induced by S1P (Fig. 2C). These findings indicate that an increase in intracellular Ca^{2+} is responsible for ERK activation induced by S1P in HUVECs.

A previous report has indicated that S1P stimulates H_2O_2 generation through activation of PLC-Ca^{2+} system in FRTL-5 thyroid cells.[20] In cardiomyocytes, H_2O_2 was shown to increase ERK activity.[21] Thus, the potential involvement of H_2O_2 in S1P-induced ERK pathway was investigated. Pretreatment of antioxidants, NAC (20 μM for 5 h) and pyrrolidine dithiocarbamate (100 μM for 1 h) had no effect on ERK activation (Fig. 2C and data not shown). Alternatively, it has been also suggested that elevation of cytosolic Ca^{2+} activates ERK through its interaction with calmodulin.[22] In order to examine the role of calmodulin in ERK activation in response to S1P, HUVECs were preincubated with the calmodulin inhibitor W-7 (25 μM for 30 min). W-7 almost completely blocked S1P-induced ERK activity (Fig. 2C). However, KN-93, a selective inhibitor of Ca^{2+}/calmodulin- dependent protein kinases, had no effect on ERK activation by S1P (Fig. 2C). These results suggest that S1P stimulates ERK activity through a Ca^{2+}/ calmodulin-dependent mechanism, but independent of Ca^{2+}/calmodulin-dependent protein kinases.

3) Role of protein tyrosine kinases in S1P-induced ERK and p38 MAPK activation

In HUVECs, S1P induced a rapid tyrosine of

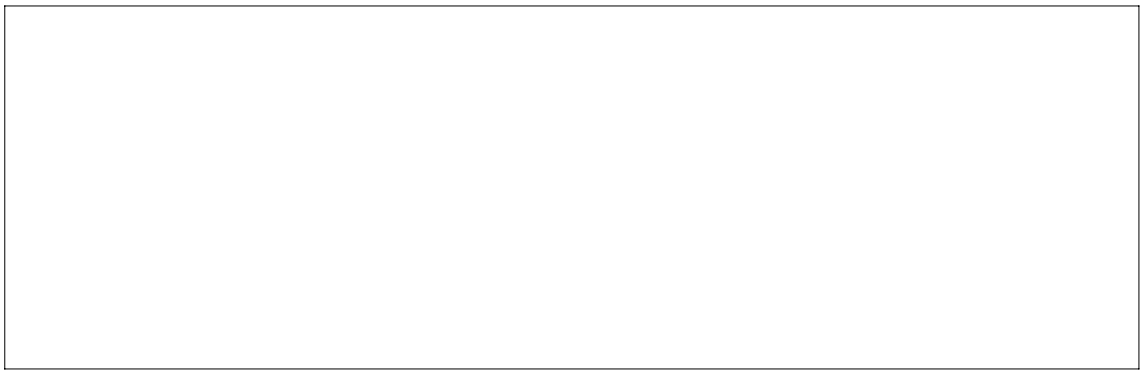

Fig. 3. Role of Src PTKs in S1P-induced ERK and p38 MAPK. (A) Quiescent HUVECs were stimulated with 5 μM S1P for the indicated time periods. Cell lysates were separated on 10% SDS-PAGE, and analyzed for tyrosine phosphorylation of proteins by Western blotting using anti-phosphotyrosine antibody. The positions of phospho-tyrosine signals apparently increased by S1P are indicated by *arrowheads*. The positions of molecular weight markers are indicated on the *right*. Cells were pretreated for 30 min with PP1 (10 μM), then treated for 5 min with 5 μM S1P (B) or 1 μM ionomycin (Iono) (C). Cell lysates were separated on 10% SDS-PAGE, followed by Western blotting using anti-phospho-specific ERK antibody (P-ERK1 and P-ERK2) and anti-phospho-specific p38 MAPK antibody (P-p38). Results are representatives of three independent experiments.

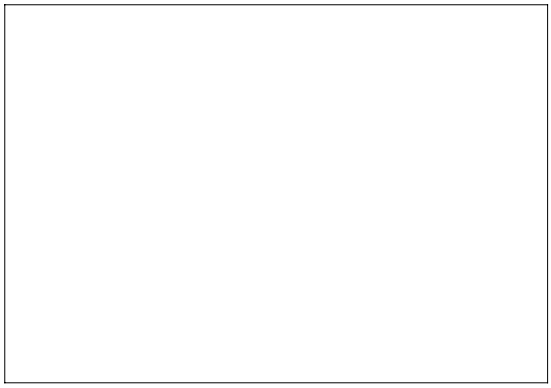

Fig. 4. Role of Src PTKs in S1P-induced HUVEC migration. Chemotactic motility of HUVECs was assayed in the absence (*white bar*) and presence of 5 μM S1P (*black bar*). Cells were preincubated for 30 min without or with PP1 (1, 2 μM) prior to treatment with 5 μM S1P. Results are expressed as percentage±S.E. of control. Each sample was assayed in duplicate, and the assays were repeated three times.

multiple proteins as indicated in anti-phospho-tyrosine blot (Fig. 3A). To determine whether tyrosine kinase activity is required for Ca^{2+}-dependent ERK activation in response to S1P, HUVECs were pretreated with PP1 (10 μM for 30 min), a selective Src PTK inhibitor, and then stimulated with either S1P or ionomycin. PP1 completely abolished both S1P- and ionomycin-induced ERK and p38 MAPK activation, with no effects on basal kinase activity (Fig. 3B and 3C). Taken together, these findings suggest that Src family PTKs activated downstream of the Ca^{2+}/calmodulin pathway may be closely involved in S1P-induced ERK and p38 MAPK activation in HUVECs.

4) The role of Src family PTKs in S1P- induced HUVEC migration

Since we found the activation of Src PTKs in response to S1P, we investigated the potential role of Src protein kinases in S1P-induced HUVEC migration. In a chemotaxis assay using Transwell chamber, S1P stimulated HUVEC migration by 2 ~ 3 folds over the control (Fig. 4). The Src PTK inhibitor PP1 significantly reduced S1P-induced HUVEC migration in a dose dependent manner (Fig. 4). The similar results were obtained from the treatment of genistein, another Src PTK inhibitor (data not shown). Thus, these results indicate that Src PTKs are closely involved in S1P-induced HUVEC migration.

DISCUSSION

S1P has been recognized as an important regulator of multiple cellular physiologies in various biological systems. Recently, there is a growing interest in the potential roles of S1P in angiogenesis-dependent disease states such as thrombosis, atherosclerosis, tumor growth, and inflammation as S1P is one of major bioactive molecules in platelets which is central to thrombosis upon their activation and adhesion. In a previous study, we provided several lines of *in vivo* and *in vitro* evidences that S1P induced angiogenesis. Importantly, here we presented the S1P-induced signaling mechanism in human endothelial cells.

The present data showed that S1P-induced MAPK activation was dominantly mediated by a PLC-dependent mechanism in HUVECs in addition to the role of G_i protein-coupled receptor in the S1P- induced signaling pathways as we described previously.[7] Our data also provide evidences that intracellular calcium mobilization is critically involved in S1P-induced ERK signaling pathways, and Src PTKs may be the downstream signal of Ca^{2+}/calmodulin in HUVECs and the Src activity is important for S1P-induced endothelial cell migration. Therefore, these findings suggest that intracellular Ca^{2+} increased upon stimulation of endothelial cells with S1P may be an important messenger triggering the downstream signaling in angiogenesis. This postulation is in part supported by previous report that the blockade of intracellular Ca^{2+} mobilization inhibits adhesion, collagenic activity, migration, and proliferation of human endothelial cells and capillary outgrowth *in vivo*.[23]

In conclusion, we here propose that S1P-induced angiogenesis may be mediated via the G_i protein- linked PLC-Ca^{2+} pathway, at least involving activation of Src PTKs. These findings may be useful to predict the potential angiogenic activity of other bioactive molecules based on their signaling properties, and also will contribute to understand the intracellular signaling mechanism engaged in angiogenesis.

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