厚生労働科学研究費補助金

第3次対がん総合戦略研究事業

血管新生とリンパ管新生の同時制御による制癌法の確立に関する研究

平成19年度～21年度 総合研究報告書

研究代表者 佐藤 靖史

平成22（2010）年 4月

目　　次

厚生労働科学研究費補助金（第３次対がん総合戦略研究事業）
（総合）研究報告書

血管新生とリンパ管新生の同時制御による制癌法の確立に関する研究

研究代表者　佐藤靖史　東北大学加齢医学研究所教授

研究要旨
研究代表者が発見した新規血管新生抑制因子 Vasohibin-1(VASH1)と、そのホモログの Vasohibin-2(VASH2)の血管新生調節における役割について解析し、VASH1 は内皮細胞に発現して血管新生を終息させるのに対し、VASH2 は間質に浸潤する単核球や癌細胞に発現して血管新生を促進することを明らかにした。VASH1 は、広いスペクトルムで血管新生とリンパ管新生を同時に阻害し、腫瘍の発育とリンパ節転移を抑制する活性を有しているが内皮細胞は障害せず、腫瘍血管を成熟化させることで抗癌剤との併用効果が示された。特に、VASH1 は内皮細胞を障害しないばかりか、内皮細胞のストレス耐性を増し、このことは他の血管新生阻害剤と際だった相違点である。大腸菌を用いて、活性を保持した組換え VASH1 蛋白の調整法を確立した。組換え VASH1 蛋白の全身投与による治療は、蛋白のデリバリーの点で未だ改善の余地があるが、腹腔内など局所療法の有用性が示された。一方、がん組織に発現する VASH2 は腫瘍血管新生を促進して腫瘍発育に寄与していることから、がん治療の新たな分子標的になることが示された。

分担研究者
高倉伸幸・大阪大学微生物病研究所　教授
渡部徹郎・東京大学大学院医学系研究科　准教授

A．研究目的

癌の治療標的として血管新生とリンパ管新生が注目されている。理想としては血管新生とリンパ管新生の双方を同時に制御する治療法が望ましいが、そのような治療法は未だ確立していない。主任研究者は、血管新生刺激に反応して血管内皮細胞が産生して血管新生を抑制する新規血管新生抑制因子 Vasohibin-1 とそのホモログの vasohibin-2 を発見した。本研究は、以上のオリジナルな研究成績を背景として、血管新生とリンパ管新生の同時制御による制癌法の確立を目指している。本研究を推進することにより、癌の血管新生とリンパ管新生の双方に対し安全で効果的な制御法を確立し、本邦発の新しい治療法として国民のがん医療に大きく貢献することを目的としている。

B．研究方法

（1）マウスの皮下血管新生モデルを用いて VASH1 と VASH2 の時間・空間的発現様式を明らかにする。作製した VASH1 と VASH2 の遺伝子改変マウスについて、血管新生を惹起させてその過程を野生型マウスと比較する。ヒト VASH1 または VASH2 遺伝子搭載非増殖型アデノウイルスを尾静注して肝臓で発現させ、血管新生に与える効果を観察する。

（２）マウス角膜法を用いて、VEGF-A、VEGF-C、FGF-2、PDGF-BB など種々の増殖因子によって惹起される血管新生、リンパ管新生に対する VASH1 の効果を検討する。

（３）高転移性ヒト非小細胞肺がん細胞を SCID マウスに皮下移植、ヒト VASH1 遺伝子搭載非増殖型アデノウイルスを尾静注して肝臓で発現させ、移植４週間後に屠殺して癌の発育、血管新生、リンパ管新生、リンパ節転移に対する効果を観察する。この治療後の気管粘膜の血管とリンパ管の形態をコントロールの LacZ 遺伝子搭載非増殖型アデノウイルス注射群と比較する。

（４）VASH1 ノックアウトマウスに癌細胞を皮下移植して、腫瘍の発育と血管新生の程度を野生型マウスと比較することで内因性 VASH1 の意義を検討する。　野生型マウスに癌細胞を皮下移植し、ヒト VASH1 遺伝子搭載非増殖型アデノウイルスを尾静注して肝臓で発現させることで、外因性 VASH1 を腫瘍に作用させたときの効果を検討する。

（５）培養内皮細胞の内因性 VASH1 の発現を siRNA でノックダウンするか、あるいは VASH1 遺伝子導入して高発現させ、血清飢餓

あるいはH_2O_2処理して、VASH1 の内皮細胞の viability に与える影響を検討する。
（６）大腸菌用コドンに置き換えることで大腸菌でのレコンビナント VASH1 蛋白の産生を行い、蛋白の調整法について、精製方法やリフォールディングなどの条件を検討する。その抗血管新生活性を角膜アッセイで確認する。さらに、レコンビナント VASH1 蛋白をポリエチレングリコール(PEG)化修飾して体内動態を検討する。
（７） 精製したレコンビナントVASH1の抗腫瘍活性を、マウスモデルを用いて検証する。
（８） Vasohibinファミリーのもう１つの分子であるVASH2のがん組織における発現と局在を検証する。また、がん細胞におけるVASH2の発現を変動させ、腫瘍発育や腫瘍血管新生における意義を動物モデルを用いて検証する。

（倫理面への配慮）
全ての動物実験は所属施設での審査を受けた後に行う。

Ｃ．研究結果
平成１９年度
（１） マウス皮下の血管新生モデルを用いて VASH1, VASH2 の時間的・空間的発現を詳細に検討したところ、野生型マウスでは、VASH1 は、血管新生先端の発芽部位でなく、後方の血管新生終止部位の血管内皮に発現するのに対し、VASH2 は主に発芽部位に浸潤する骨髄由来の単核球に発現していた。これに対し、VASH1 遺伝子改変マウスでは血管新生が終息しにくかったのに対して、VASH2 遺伝子改変マウスでは発芽部位での血管新生が障害されていた。アデノウイルスベクターを用いて VASH1 や VASH2 を補充すると、それらの異常は正常化した。
（２）VASH1 は種々の増殖因子によって惹起される血管新生、リンパ管新生を広い作用スペクトルムで抑制した。
（３）高転移性ヒト非小細胞肺がん細胞の移植実験では、LacZ 遺伝子搭載非増殖型アデノウイルス注射群と比較して、ヒト VASH1 遺伝子搭載非増殖型アデノウイルス注射群では、腫瘍の発育、腫瘍内血管密度、腫瘍周囲リンパ管密度、所属リンパ節の転移はいずれも有意に抑制された。また、気管粘膜の血管とリンパ管の形態はヒト VASH1 遺伝子搭載非増殖型アデノウイルス注射群で変化を認めなかった。
（４）培養内皮細胞に VASH1 を発現させると、血管内皮細胞の増殖は抑制され、血清飢餓や H_2O_2 など各種の細胞死を惹起する処理に曝されても血管内皮細胞の細胞死は顕著に抑制された。
平成２０年度
（１）VASH1 ノックアウトマウスでは、移植腫瘍は腫瘍血管新生に富んでおり、内因性 VASH1 は腫瘍血管新生制御に機能していることが確認された。
腫瘍を移植した野生型マウスにヒト VASH1 遺伝子搭載非増殖型アデノウイルスを尾静注すると、外因性 VASH1 は腫瘍血管を成熟化させた。また、その結果として血流が改善し、抗癌剤との併用効果が観察された。
（２）一般に血管新生抑制因子は内皮細胞のアポトーシスを促進するが、VASH1 は内皮細胞のストレス耐性を増し、内皮細胞の生存を助長することが確認された。その機序として VASH1 は SOD2 の発現を介して ROS の生成量を制御すると共に、長寿遺伝子 SIRT1 の発現を増すことが判明した。
（３）治療応用に資するためレコンビナント VASH1 蛋白の大量調整法の検討を進め、大腸菌を用いて活性を有するレコンビナント蛋白の調整を可能とし、本蛋白が、動物実験においても活性を発揮することを確認した。さらに、レコンビナント VASH1 蛋白の PEG 化修飾による体内での安定性の改良を行った。
平成２１年度
（１）内皮細胞を障害せずに、広いスペクトルムで血管新生とリンパ管新生を抑制する VASH1 について、大腸菌由来組換え蛋白のリフォールディングによる分散画分（活性画分）調製法の改良を進め、活性を有する組換え蛋白の調整法を確立した。
（２）動物モデルにおいて、このレコンビナント VASH1 蛋白を腫瘍局所に注射すると抗腫瘍効果は得られるが、血中での安定化を目的に PEG 化修飾した蛋白の全身投与では、PEG 化によって活性化が低下するため十分な抗腫瘍効果は得られなかった。一方、腹膜播種すると腹水を形成して急速に個体を死に至らしめるヒト卵巣癌由来 SKOV-3 細胞に VASH1 遺伝子を安定導入すると、mock コントロールではヌードマウスの腹腔内移植で 4 週間以内に全て死亡するのに対し、VASH1 遺伝子導入群では腹水も生じず、全て 10 週間以上生存するという顕著な効果を観察した。
（３）Vasohibin ファミリーメンバーの VASH2 は、血管新生局所に浸潤する骨髄由来単核球に発現し、VASH1 と拮抗して血管新生を促進することが判明しているが、癌細胞の一部にも発現することが判明した。そこで

VASH2 発現陰性の腫瘍細胞に VASH2 を導入してマウスに移植すると、腫瘍血管新生と腫瘍発育が増強することが判明した。

D．考察

VASH 1 は、発芽部位より後方の血管新生終止部位の血管内皮に発現して、血管新生を止める働きがあるのに対して、VASH2 は主に発芽部位に局在し、VASH1 と拮抗して血管新生を促進すると考えられた。

VASH1 は、血管新生のみならずリンパ管新生をも広い作用スペクトルムで抑制するが、正常内皮を障害せず、癌のリンパ節転移制御に対しても有用と考えられた。

VASH1 は、広いスペクトルムで血管新生とリンパ管新生を阻害するが、内皮細胞を障害せず、逆にストレス耐性を増ことは他の血管新生阻害剤と際だった相違点である。腫瘍血管を成熟化させるため、抗癌剤との併用が効果的と考えられる。

レコンビナント VASH1 蛋白の調整法は確立できたが、血管をルートとして全身投与する治療法への応用は、現時点では未だ困難である。しかし、腹腔内投与など、局所療法への応用は、可能性の高い手法と考えられた。VASH2 はがん細胞に発現し、腫瘍血管新生を促進して腫瘍発育に寄与している。従って、VASH1 を外因性に投与すると同時に VASH2 の作用を阻害することが、がん治療における望ましい治療法となると期待される。

E．結論

VASH1 は、血管新生のみならずリンパ管新生に対しても広いスペクトルムで抑制作用を有しており、血管新生とリンパ管新生の同時制御による制癌法への応用が期待される。

内皮細胞が産生する血管新生・リンパ管新生抑制因子 VASH1 の特異な性質を明らかにし、さらに治療応用の一環としてのレコンビナント蛋白の大量調整と体内安定化に目処がついた。

レコンビナント VASH1 蛋白の大量調整法を確立すると共に、がんにおける VASH2 の意義が明らかにし、VASH2 が、がん治療の新たな分子標的となえう可能性を示した。がん治療においては、外因性のレコンビナント VASH1 蛋白を投与に VASH2 の作用阻害を併用することの有用性が示唆された。

F．研究発表

1．論文発表

Sato, Y., and Sonoda, H. The vasohibin family: a negative regulatory system of angiogenesis genetically programmed in endothelial cells. Arterioscler. Thromb. Vasc. Biol. 27: 37-41, 2007.

Suzuki, T., Abe, M., Miyashita, H., Kobayashi, T., and Sato, Y.: Puromycin insensitive leucyl-specific aminopeptidase (PILSAP) affects RhoA activation in endothelial cells. J. Cell. Physiol. 211: 708-715, 2007.

Sato, Y. VEGFR1 for lymphangiogenesis: an alternative signaling pathway? Arterioscler. Thromb. Vasc. Biol. 28: 604-605, 2008.

Yoshinaga, K., Moriya, T., Nagase, S., Takano, T., Niikura, H., Ito, K., Nobuo Yaegashi, N., and Sato, Y. Vasohibin, a novel endothelium-derived angiogenesis inhibitor: its expression on endometrial carcinoma in relation to tumor vascularity. Cancer Sci. 99:914-914, 2008.

Wakusawa, R., Abe, T., Sato, H., Yoshida, M., Kunitaka, H., Sato, Y., and Nishida K. Expression of vasohibin, an antiangiogenic factor, in human choroidal neovascular membranes. Am. J. Ophthalmol. 146:235-243, 2008.

Tamaki K, Moriya T, Sato Y, Ishida T, Maruo Y, Yoshinaga K, Ohuchi N, Sasano H. Vasohibin-1 in human breast carcinoma: a potential negative feedback regulator of angiogenesis. Cancer Sci. 100:88-94, 2009.

Lefter LP, Dima S, Sunamura M, Furukawa T, Sato Y, Abe M, Chivu M, Popescu I, and Horii A. Transcriptional silencing of ETS-1 efficiently suppresses angiogenesis of pancreatic cancer. Cancer Gene Ther. 16:137-148, 2009.

Sato H, Abe T, Wakusawa R, Asai N, Kunikata H, Ohta H, Sonoda H, Sato Y, and Nishida K. Vitreous levels of vasohibin-1 and vascular endothelial growth factor in patients with proliferative diabetic retinopathy. Diabetologia. 52:359-361, 2009.

Nishida Y, Shibata K, Yamasaki M, Sato Y, and Abe M. A possible role of vimentin on the cell surface for the activation of latent transforming growth factor-β. FEBS Lett. 583: 308–312, 2009.

Suzuki H, Ohkuchi A, Matsubara S, Takei Y, Murakami M, Shibuya M, Suzuki M, Sato Y. The effect of recombinant PlGF-2 on hypertension induced by full-length mouse sFlt-1 adenoviral vector in pregnant mice. Hypertension 54:1129-1135, 2009.

Taniguchi K, Sasaki K, Watari K, Yasukawa H, Imaizumi T, Ayada T, Okamoto F, Ishizaki T, Kato R, Kohno R, Kimura H, Sato Y, Ono M, Yonemitsu Y, Yoshimura A. Suppression of Sproutys has a therapeutic effect for a mouse model of ischemia by enhancing angiogenesis. PLoS ONE 4:e5467, 2009.

Hosaka T, Kimura H, Heishi T, Suzuki Y, Miyashita H, Ohta H, Sonoda H, Moriya T, Suzuki S, Kondo T, Sato Y. Vasohibin-1 expressed in endothelium of tumor vessels regulates angiogenesis. Am. J. Pathol. 175:430-439, 2009.

Kimura H, Miyashita H, Suzuki Y, Kobayashi M, Watanabe K, Sonoda H, Ohta H, Fujiwara T, Shimosegawa T and Sato Y. Distinctive localization and opposed roles of vasohibin-1 and vasohibin-2 in the regulation of angiogenesis. Blood 113:4810-4818, 2009.

Naito H, Kidoya H, Sato Y, and Takakura N. Induction and expression of anti-angiogenic vasohibins in the hematopoietic stem/progenitor cell population. J. Biochem. 145: 653-659, 2009.

Miura S, Mitsui K, Heishi T, Shukunami C, Sekiguchi K, Kondo J, Sato Y, Hiraki Y. Impairment of VEGF-A-stimulated lamellipodial extensions and motility of vascular endothelial cells by Chondromodulin-I, a cartilage-derived angiogenesis inhibitor. Exp Cell Res. 316: 775-788 2010.

Komi Y, Sogabe Y, Ishibashi N, Sato Y, Moriwaki H, Shimokado K, Kojima S. Acyclic retinoid inhibits angiogenesis via suppressing MAPK pathway. Lab. Invest. 90: 52-60, 2010.

Heishi T, Hosaka T, Suzuki Y, Miyashita H, Oike Y, Takahashi T, Nakamura T, Arioka S, Mitsuda Y, Takakura T, Hojo K, Matsumoto M, Yamauchi C, Ohta H, Sonoda H, Sato Y. Endogenous angiogenesis inhibitor vasohibin1 exhibits a broad-spectrum anti-lymphangiogenic activity and suppresses lymph node metastasis. Am. J. Pathol. 176:1950-1958, 2010..

Tamaki K, Sasano H, Maruo Y, Takahashi Y, Miyashita M, Moriya T, Sato Y, Hirakawa H, Tamaki N, Watanabe M, Ishida T, Ohuchi N. Vasohibin-1 as a potential predictor of aggressive behavior of ductal carcinoma in situ of the breast. Cancer Sci. 101:1051-1058, 2010.

Yoshida T, Sato Y, Morita I, Abe M. Pigpen, a nuclear coiled body component protein is involved in angiogenesis. Cancer Sci. 2010 (Epub ahead of print).

２．学会発表

Sato, Y. The role of vasohibin family in the regulation of angiogenesis. Seventh ESH Euroconference on Angiogenesis, Albufeira, Portugal, 2007.

Sato, Y. The role of vasohibin family in the regulation of angiogenesis. Ang iogenesis & Microcriculation, Newport, USA, 2007.

Sato, Y. The vasohibin family: novel regulators of angiogenesis and lymphangiogenesis. OLS/NUS-Tohoku/COE Joint Symposium, Singapore/Bintan, 2007.

Sato, Y. Role of vasohibin family in the regulation of angiogenesis. The Joint Meeting of Vascular Biology Organiz ations in Asia Pacific Region, Tainan, Taiwan, 2007.

Heishi, T., Tamai, T., Suzuki, H., Hosaka, T., Kimura, H., Kobayashi, M., Suzuki, Y., Miyashita, H., Takahashi, T.,and Sato, Y. Vasohibin inhibits lymphangiogenesis in mouse cornea and xenograft cancer model. OLS/NUS-Tohoku/COE Joint Symposium, Singapore/Bintan, 2007.

佐藤靖史．血管新生制御における vasohibin family の役割．第 39 回日本動脈硬化学会総会・学術集会，大阪，2007.

佐藤靖史. 血管新生制御における vasohibin family の役割. 第 28 回日本炎症・再生医学会, 東京, 2007.

佐藤靖史. The role of vasohibin in the regulation of angiogenesis. 第 66 回日本癌学会学術総会, 横浜, 2007.

宮下浩輝, 佐藤靖史. 転写因子 VEZF の標的遺伝子であるメタロチオネインは血管新生制御に関与する. メタロチオネインおよびメタルバイオサイエンス研究会 2007, 徳島, 2007.

佐藤靖史. リンパ管新生の内因性抑制因子に関する研究. 第 15 回日本血管生物医学会学術大会, 福岡, 2007.

佐藤靖史. The role of vasohibin in the regulation of angiogenesis in tumors. The U.S.-Japan Cooperative Cancer Research Program, 京都, 2008.

瓶子隆弘, 保坂智子, 木村洋, 宮下浩輝,高橋隆, 佐藤靖史. Vasohibin inhibits lymphangiogenesis in mouse cornea and xenograft cancer model. 第 66 回日本癌学会学術総会, 横浜, 2007.

Komi, Y., Sogabe. Y., Ishibashi. N., Nishiwaki, R., Moriwaki, H., Sato, Y., Shimokado, K., and Kojima, S. Study on the mechanism of suppression of blood vessel formation by acyclic retinoid. 第 66 回日本癌学会学術総会, 横浜, 2007.

木村洋, 宮下浩輝, 佐藤靖史. 第 15 回日本血管生物医学会学術大会, 福岡, 2007.

瓶子隆弘, 宮下浩輝, 高橋隆, 尾池雄一,佐藤靖史. Vasohibin suppresses lymph node metastasis via the inhibition of lymphangiogenesis in mice. The U.S.-Japan Cooperative Cancer Research Program, 京都, 2008.

Sato, Y. The Role of Vasohibin in the Regulation of Angiogenesis. 15th International Vascular Biology Meeting, Sydney, Australia, 2008.

Sato, Y. The Role of Vasohibin in the Regulation of Tumor Angiogenesis. 9th International Conference Angiogenesis: Basic Science and Clinical Applications, Patras, Greece, 2008.

Heishi T, Miyashita H, Takahashi T, Oike Y and Sato Y. Vasohibin Suppresses Lymph Node Metastasis via the Inhibition of Lymphangiogenesis in Mice. 15th International Vascular Biology Meeting, Sydney, Australia, 2008.

佐藤靖史. 内因性血管新生抑制因子と癌. 第 67 回日本癌学会学術総会, 名古屋, 2008.

佐藤靖史. がんにおける血管新生制御の分子基盤. 平成 20 年度がん特定領域研究 5 領域合同シンポジウム, 東京, 2009.

佐藤靖史. Common molecular basis for the regulation of angiogenesis and vascular aging. 第 4 回研究所ネットワーク国際シンポジウム, 東京, 2009.

佐藤靖史. 血管新生阻害剤の分子標的. 第 7 回日本臨床腫瘍学会学術集会, 名古屋, 2009.

宮下浩輝, 佐藤靖史. 血管内皮細胞における Vasohibin-1 の標的遺伝子の解析. 第 81 回日本組織培養学会大会, つくば, 2008.

鈴木康弘, 小林美穂, 宮下浩輝, 太田英樹, 園田光, 佐藤靖史. Vasohibin 結合性低分子量蛋白 SVBP の単離とその分泌における役割. 第 67 回日本癌学会学術総会, 名古屋, 2008.

鈴木康弘, 小林美穂, 宮下浩輝, 太田英樹, 園田光, 佐藤靖史. Isolation of a small Vasohibin-binding protein (SVBP) and its impormant role in the secretion of Vasohibin. 第 16 回日本血管生物医学会学術総会, 名古屋, 2008.

宮下浩輝, 佐藤靖史. Vasohibin-1 is involved in the mainrenance of vascular endothelial cells. 第 16 回日本血管生物医学会学術総会, 金沢, 2008.

Sato Y. The significance of vasohibin, an angiogenesis inhibitor expressed in endothelium, in cancers and other pathological conditions. NHRI Conference Series 2009 International Conference on Vascular Biology Zhunan, Taiwan 2009.

Sato Y. Vasohibin expressed in endothelium of tumor vessels regulates angiogenesis. 7th International Symposium on the biology of endothelial cells. Viena, Austria 2009.

宮下浩輝, 佐藤靖史. Vasohibin-1 Prevents Cellular Senescence and Maintains Vascular Endothelial Cells. 10th International Symposium on Mechanisms of Vasodilatation, 松島, 2009.

佐藤靖史. 血管を維持する内因性調節機構. 第 17 回日本血管細胞生物医学会, 東京, 2009.

木村洋, 宮下浩輝, 鈴木康弘, 小林美穂, 渡辺和秀, 園田光, 大田英樹, 藤原隆, 下瀬川徹, 佐藤靖史. Distinctive Localization and Opposed Roles of Vasohibin-1 and Vasohibin-2 in the Reculation of Angiogenesis. 10th International Symposium on Mechanisms of Vasodilatation, 松島, 2009.

小林美穂, 鈴木康弘, 佐藤靖史. Vasohibin-1-induced detyronation of α-tubulin triggers an inhibition of endothelial cell migration. 第 68 回日本癌学会学術総会. 横浜, 2009.

吉永浩介, 伊藤潔, 八重樫伸生, 佐藤靖史, 笹野公伸, 太田英樹, 園田光. The roles of vasohibin, an intrinsic antiogenesis inhibitor, in cervical carcinomas. 第 68 回日本癌学会学術総会. 横浜, 2009.

小林美穂, 鈴木康弘, 佐藤靖史. 血管新生抑制因子 Vasohibin-1 はチューブリンの脱チロシン化を誘導することで内皮細胞の遊走阻害を引き起こす. 第 17 回日本血管細胞生物医学会, 東京, 2009.

高橋詳史, 中村崇宣, 小林美穂, 鈴木康弘, 宮下浩輝, 佐藤靖史. Vasohibin-2 Expressed in Tumors Accelerates Tumor Growth by Promoying Angiogenesis. The 1st International Global COE Symposium, 仙台, 2009.

G. 知的所有権の取得状況
1. 特許取得
特許出願「バソヒビンからなるリンパ管新生抑制剤」
基礎出願 2006-324773
WO PCT/JP2007/072838

特許出願「バソヒビン 1 を認識するモノクローナル抗体および該抗体を用いるバソヒビンの免疫測定方法」
特願 2008-218581

佐藤靖史　固形癌の存在の検出法　特許出願 2009-190830
佐藤靖史、近藤丘　抗バゾヒビンモノクローナル抗体のセット　特許出願 PCT/JP2009/64862
佐藤靖史　血管新生促進因子　特許出願 PCT/JP2009/051359

2. 実用新案登録
なし

3. その他
なし

研究成果の刊行に関する一覧表レイアウト

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ
Sato Y	Endogenous inhibitors of angiogenesis	ME Maragoudakis, E Papadimittiou	Angiogenesis: Basic Science and Clinical Applications	Transworld Research Network	India	2007	1-14
Watabe T, Miyazono K	TGF-β family signaling in stem cell renewal and differentiation	Derynck R, Miyazono K	The TGF-β family	Cold Spring Harbor Laboratory Press	USA	2007	585-612

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Suzuki T, Abe M, Miyashita H, Kobayashi T, Sato Y.	Puromycin insensitive leucyl-specific aminopeptidase (PILSAP) affects RhoA activation in endothelial cells.	J. Cell. Physiol.	211	708-715	2007
Sato Y, Sonoda H.	The vasohibin family: a negative regulatory system of angiogenesis genetically programmed in endothelial cells.	Arterioscler. Thromb. Vasc. Biol.	27	37-41	2007
Huang X, Yamada Y, Kidoya H, Naitoh H, Nagahama Y, Kong L, Katoh S, Li W, Ueno M, Takakura N.	EphB4 overexpression on B16 melanoma cells affects arterial-venous patterning in tumor angiogenesis.	Cancer Res.	67(20)	9800-9808	2007
Yamada Y, Yokoyama S, Wang XD, Fukuda N, Takakura N.	Cardiac stem cells in brown adipose tissue express CD133 and induce bone marrow non-hematopoietic cells to differentiate into cardiomyocytes.	Stem cells	25(5)	1326-1333	2007
Yamada Y, Yokoyama SI, Fukuda N, Kidoya H, Huang XY, Naitoh H, Satoh N, Takakura N.	A novel approach for myocardial regeneration with educated cord blood cells cocultured with cells from brown adipose tissue.	Biochem Biophys Res Commun.	353(1)	182-188	2007
Tonchev AB, Yamashima T, Guo J, Chaldakov GN, Takakura N.	Expression of angiogenic and neurotrophic factors in the progenitor cell niche of adult monkey subventricular zone.	Neuroscience	144(4)	1425-1435	2007

Vachkov IH, Huang X, Yamada Y, Tonchev AB, Yamashima T, Kato S, Takakura N.	Inhibition of Axonal Outgrowth in the Tumor Environment: Involvement of Class 3 Semaphorins.	Cancer Sci.	98(8)	1192-1197	2007
Mishima K, Watabe T, Saito A, Yoshimatsu Y, Imaizumi N, Masui S, Hirashima M, Morisada T, Oike Y, Araie M, Niwa H, Kubo H, Suda T, Miyazono K.	Prox1 induces lymphatic endothelial differentiation via integrin α9 and other signaling cascades.	Molecular Biology of the Cell	18	1421-1429	2007
Ogawa K, Saito A, Matsui H, Suzuki H, Ohtsuka S, Shimosato D, Morishita Y, Watabe T, Niwa H, Miyazono K.	Activin-Nodal signaling is involved in propagation of mouse embryonic stem cells.	Journal of Cell Science	120	55-65	2007
Sato Y.	VEGFR1 for lymphangiogenesis: an alternative signaling pathway?	Arterioscler. Thromb. Vasc. Biol.	28	604-605	2008
Yoshinaga K, Moriya T, Nagase S, Takano T, Niikura H, Ito K, Yaegashi N, Sato Y.	Vasohibin, a novel endothelium-derived angiogenesis inhibitor: its expression on endometrial carcinoma in relation to tumor vascularity.	Cancer Sci.	99	911-919	2008
Wakusawa R, Abe T, Sato H, Yoshida M, Kunitaka H, Sato Y, Nishida K.	Expression of vasohibin, an antiangiogenic factor, in human choroidal neovascular membranes.	Am. J. Ophthalmol.	146	235-243	2008
Kidoya H, Ueno M, Yamada Y, Mochizuki N, Nakata M, Yano T, Fujii R, Takakura N.	Spatial and temporal role of the apelin/APJ system in the caliber size regulation of blood vessels during angiogenesis.	EMBO J.	27(3)	522-534	2008
Moore ML, Teitell MA, Kim Y, Watabe T, Reiter RE, Witte ON, Dubey P.	Deletion of PSCA increases metastasis of TRAMP-Induced prostate tumors without altering primary tumor formation.	Prostate	68	139-151	2008
Fukuhara S, Sako K, Minami T, Noda K, Kim HZ, Kodama T, Shibuya M, Takakura N, Koh GY, Mochizuki N.	Differential function of Tie2 at cell-cell contacts and cell-substratum contacts regulated by angiopoietin-1.	Nat Cell Biol.	10	513-26	2008
Satoh N, Yamada Y, Kinugasa Y, Takakura N.	Angiopoietin-1 alters tumor growth by stabilizing blood vessels or by promoting angiogenesis.	Cancer Sci.	99	2373-2379	2008

Katoh SY, Ueno M, Takakura N.	Involvement of MDR1 Function In Proliferation of Tumor Cells.	J Biochem.	143	517-524	2008
Hayashi H, Kunisada T, Takakura N, Aoki M, Mizuta K, Ito Y.	Involvement of platelet-derived growth factor receptor-beta in maintenance of mesenchyme and sensory epithelium of the neonatal mouse inner ear.	Hear Res.	245	73-81	2008
Kokudo T, Suzuki Y, Yoshimatsu Y, Yamazaki T, Watabe T, Miyazono K.	Snail is required for TGFβ-induced endothelial-mesenchymal transition of embryonic stem cell-derived endothelial cells.	Journal of Cell Science	121	3317-3324	2008
Nonaka H, Watabe T, Saito S, Miyazono K, Miyajima A	Development of stabilin2+ endothelial cells from mouse embryonic stem cells by inhibition of TGFβ/activin signaling.	Biochemical and Biophysical Research Communications	375	256-260	2008
Kawasaki K, Watabe T, Sase H, Hirashima M, Koide H, Morishita Y, Yuki K, Sasaoka T, Suda T, Katsuki M, Miyazono K, Miyazawa K.	Ras signaling directs endothelial specification of VEGFR2+ vascular progenitor cells.	Journal of Cell Biology	181	131-141	2008
Oka M, Iwata C, Suzuki H, Kiyono K, Morishita Y, Watabe T, Komuro A, Kano MR, Miyazono K.	Inhibition of endogenous TGF-β signaling enhances lymphangiogenesis.	Blood	111	4571-4579	2008
Suzuki Y, Montagne K, Nishihara A, Watabe T, Miyazono K.	BMPs promote proliferation and migration of endothelial cells via stimulation of VEGF-A/VEGFR2 and Angiopoietin-1/Tie2 signals.	Journal of Biochemistry	143	199-206	2008
Tamaki K, Moriya T, Sato Y, Ishida T, Maruo Y, Yoshinaga K, Ohuchi N, Sasano H.	Vasohibin-1 in human breast carcinoma: a potential negative feedback regulator of angiogenesis.	Cancer Sci.	100	88-94	2009
Lefter LP, Dima S, Sunamura M, Furukawa T, Sato Y, Abe M, Chivu M, Popescu I, Horii A.	Transcriptional silencing of ETS-1 efficiently suppresses angiogenesis of pancreatic cancer.	Cancer Gene Ther.	16	137-148	2009

Sato H, Abe T, Wakusawa R, Asai N, Kunikata H, Ohta H, Sonoda H, Sato Y, Nishida K.	Vitreous levels of vasohibin-1 and vascular endothelial growth factor in patients with proliferative diabetic retinopathy.	Diabetologia	52	359-361	2009
Nishida Y, Shibata K, Yamasaki M, Sato Y, Abe M.	A possible role of vimentin on the cell surface for the activation of latent transforming growth factor-β.	FEBS Lett.	583	308-312	2009
Suzuki H, Ohkuchi A, Matsubara S, Takei Y, Murakami M, Shibuya M, Suzuki M, Sato Y.	The effect of recombinant PlGF-2 on hypertension induced by full-length mouse sFlt-1 adenoviral vector in pregnant mice.	Hypertension	54	1129-1135	2009
Taniguchi K, Sasaki K, Watari K, Yasukawa H, Imaizumi T, Ayada T, Okamoto F, Ishizaki T, Kato R, Kohno R, Kimura H, Sato Y, Ono M, Yonemitsu Y, Yoshimura A.	Suppression of Sproutys has a therapeutic effect for a mouse model of ischemia by enhancing angiogenesis.	PLoS ONE	4	e5467	2009
Hosaka T, Kimura H, Heishi T, Suzuki Y, Miyashita H, Ohta H, Sonoda H, Moriya T, Suzuki S, Kondo T, Sato Y.	Vasohibin-1 expressed in endothelium of tumor vessels regulates angiogenesis.	Am. J. Pathol.	175	430-439	2009
Kimura H, Miyashita H, Suzuki Y, Kobayashi M, Watanabe K, Sonoda H, Ohta H, Fujiwara T, Shimosegawa T Sato Y.	Distinctive localization and opposed roles of vasohibin-1 and vasohibin-2 in the regulation of angiogenesis.	Blood	113	4810-4818	2009
Naito H, Kidoya H, Sato Y, Takakura N.	Induction and expression of anti-angiogenic vasohibins in the hematopoietic stem/progenitor cell population.	J. Biochem.	145	653-659	2009
Han Y, Ueno M, Nagahama Y, Takakura N.	Identification and characterization of stem cell-specific transcription of PSF1 in spermatogenesis.	Biochem Biophys Res Commun.	380	609-613	2009
Ueno M, Itoh M, Sugihara K, Asano M, Takakura N.	Both alleles of PSF1 are required for maintenance of pool size of immature hematopoietic cells and acute bone marrow regeneration.	Blood	113	555-562	2009

Takakura N, Kidoya H	Maturation of blood vessels by haematopoietic stem cells and progenitor cells : involvement of apelin/APJ and Angiopoietin/Tie2 interactions in vessel caliber size regulation.	Thrombosis and Haemostasis	101	999-1005	2009
Katoh SY, Kamimoto T, Yamakawa D, Takakura N.	Lipid rafts serve as signaling platforms for Tie2 receptor tyrosine kinase in vascular endothelial cells.	Exp Cell Res.	315	2818-2823	2009
Yamazaki T, Yoshimatsu Y, Morishita Y, Miyazono K, Watabe T	COUP-TFII regulates the functions of Prox1 in lymphatic endothelial cells through direct interaction.	Genes to Cells	14	425-434	2009
Harada K, Yamazaki T, Iwata C, Yoshimatsu Y, Sase H, Mishima K, Morishita Y, Hirashima M, Oike Y, Suda T, Miura N, Watabe T, Miyazono K	Identification of targets of Prox1 during in vitro vascular differentiation from embryonic stem cells: functional roles of HoxD8 in lymphangiogenesis.	Journal of Cell Science	122	3923-3930	2009
Yamamizu K, Kawasaki K, Katayama S, Watabe T, Yamashita JK	Enhancement of vascular progenitor potential by protein kinase A through dual induction of Flk-1 and Neuropilin-1.	Blood	114	3707-3716	2009
Sase H, Watabe T, Kawasaki K, Miyazono K, Miyazawa K	VEGFR2-PLCgamma1 axis is essential for endothelial specification of VEGFR2+ vascular progenitor cells.	Journal of Cell Science	122	3303-3311	2009
Saito RA, Watabe T, Horiguchi K, Kohyama T, Saitoh M, Nagase T, Miyazono K	TTF-1 inhibits TGF-β-mediated epithelial-to-mesenchymal transition in lung adenocarcinoma cells.	Cancer Research	69	2783-2791	2009
Miura S, Mitsui K, Heishi T, Shukunami C, Sekiguchi K, Kondo J, Sato Y, Hiraki Y.	Impairment of VEGF-A-stimulated lamellipodial extensions and motility of vascular endothelial cells by Chondromodulin-I, a cartilage-derived angiogenesis inhibitor.	Exp Cell Res.	316	775-788	2010
Komi Y, Sogabe Y, Ishibashi N, Sato Y, Moriwaki H, Shimokado K, Kojima S.	Acyclic retinoid inhibits angiogenesis via suppressing MAPK pathway.	Lab. Invest.	90	52-60	2010

Nagahama Y, Ueno M, Miyamoto S, Morii E, Minami T, Mochizuki N, Saya H, Takakura N.	PSF1, a DNA replication factor expressed widely in stem and progenitor cells, drives tumorigenic and metastatic properties.	Cancer Res.	70	1215-1224	2010
Nagahama Y, Ueno M, Haraguchi N, Mori M, Takakura N.	PSF3 marks malignant colon cancer and has a role in cancer cell proliferation.	Biochem Biophys Res Commun.	392	150-154	2010
Heishi T, Hosaka T, Suzuki Y, Miyashita H, Oike Y, Takahashi T, Nakamura T, Arioka S, Mitsuda Y, Takakura T, Hojo K, Matsumoto M, Yamauchi C, Ohta H, Sonoda H, Sato Y.	Endogenous angiogenesis inhibitor vasohibin1 exhibits a broad-spectrum anti-lymphangiogenic activity and suppresses lymph node metastasis.	Am. J. Pathol.	176	1950-1958	2010
Tamaki K, Sasano H, Maruo Y, Takahashi Y, Miyashita M, Moriya T, Sato Y, Hirakawa H, Tamaki N, Watanabe M, Ishida T, Ohuchi N.	Vasohibin-1 as a potential predictor of aggressive behavior of ductal carcinoma in situ of the breast.	Cancer Sci.	101	1051-1058	2010
Yoshida T, Sato Y, Morita I, Abe M.	Pigpen, a nuclear coiled body component protein is involved in angiogenesis.	Cancer Sci.	(Epub ahead of print)		2010
Murakami A, Takasugi H, Ohnuma S, Koide Y, Sakurai A, Takeda S, Hasegawa T, Sasamori J, Konno T, Hayashi K, Watanabe Y, Mori K, Sato Y, Takahashi A, Mochizuki N, Takakura N.	Sphingosine 1-Phosphate Regulates Vascular Contraction via S1P3 Receptor: Investigation Based on a New S1P3 Receptor Antagonist.	Mol Pharmacol.	In press		
Kidoya H, Naito H, Takakura N.	Apelin induces enlarged and non-leaky blood vessels for functional recovery from ischemia.	Blood	In press		
Suzuki Y, Ohga N, Morishita Y, Hida K, Miyazono K, Watabe T	BMP-9 induces proliferation of multiple types of endothelial cells in vitro and in vivo.	Journal of Cell Science	in press		2010

Journal of Cellular Physiology

Puromycin Insensitive Leucyl-Specific Aminopeptidase (PILSAP) Affects RhoA Activation in Endothelial Cells

TAKAHIRO SUZUKI,[1,2] MAYUMI ABE,[1] HIROKI MIYASHITA,[1] TOSHIMITSU KOBAYASHI,[2] AND YASUFUMI SATO[1]*

[1]*Department of Vascular Biology, Institute of Development, Aging and Cancer, Tohoku University, Sendai, Japan*

[2]*Department of Otolaryngology-Head and Neck Surgery, Tohoku University Graduate School of Medicine, Sendai, Japan*

Puromycin insensitive leucyl-specific aminopeptidase (PILSAP) expressed in endothelial cells (ECs) plays an important role in angiogenesis due to its involvement in migration, proliferation and network formation. Here we examined the biological function of PILSAP with respect to EC morphogenesis and the related intracellular signaling for this process. When mouse endothelial MSS31 cells were cultured, a dominant negative PILSAP mutant converted cell shape to disk-like morphology, blocked stress fiber formation, and augmented membrane ruffling in random directions. These phenotypic changes led us to test whether PILSAP affected activities of Rho family small G-proteins. Abrogation of PILSAP enzymatic activity or its expression attenuated RhoA but not Rac1 activation during cell adhesion. This attenuation of RhoA activation was also evident when G-protein coupled receptors such as proteinase-activated receptor or lysophosphatidic acid receptor were activated in ECs. These results indicate that PILSAP affects RhoA activation and that influences the proper function of ECs.

J. Cell. Physiol. 211: 708–715, 2007.

Angiogenesis is the formation of new blood vessels through endothelial cell (EC) proliferation and migration in combination with tubular morphogenesis. Angiogenesis is indispensable for various physiological and pathological processes, such as embryonic development, wound healing, diabetic retinopathy, and solid tumor growth. A number of molecules regulate angiogenesis both positively and negatively. However, the molecular mechanism of angiogenesis is not yet completely understood.

We searched for novel molecules involved in angiogenesis regulation, and isolated puromycin insensitive leucyl-specific aminopeptidase (PILSAP) with the use of subtraction strategy whose expression was augmented during the in vitro differentiation of murine embryonic stem (ES) cells to ECs (Miyashita et al., 2002). The expression of PILSAP in ECs is evident at the site of angiogenesis in vivo, and is regulated, at least in part, by a transcription factor polyomavirus enhancer-binding protein 2 (PEBP2) (Miyashita et al., 2002; Niizeki et al., 2004). Interestingly, endoplasmic reticulum aminopeptidase associated with antigen processing (ERAAP) was found to be identical to PILSAP, which was involved in the cleavage of various peptides for antigen presentation by MHC class I molecules (Serwold et al., 2002). Nevertheless, our analyses have revealed that PILSAP plays an important role in angiogenesis by its involvement in migration, proliferation and network formation (Akada et al., 2002; Miyashita et al., 2002). Aminopeptidases catalyze the sequential removal of amino acids from unblocked N-termini of peptides and proteins and play important roles in various biological processes, such as maturation, activation, modulation, degradation of bioactive peptides (Taylor, 1993). PILSAP belongs to the M1 aminopeptidase family, which contains an HEXXH(18X)E motif and a central Zn^{2+} ion essential for enzymatic activity. We examined the mechanism by which PILSAP regulates vascular endothelial growth factor (VEGF)-stimulated proliferation of ECs. As PILSAP is an aminopeptidase, PILSAP is expected to modulate cell function by catalyzing its physiological substrates. Our analysis have revealed that PILSAP binds to phosphatidylinositol-dependent kinase1 (PDK1) and removes 9 amino acids from the PDK1 N-terminus, which subsequently allows S6 kinase (S6K) to associate with PDK1 and PILSAP upon VEGF stimulation (Yamazaki et al., 2004).

Cell migration is a mechanically integrated molecular process that involves dynamic, coordinated changes in cell adhesions and cytoskeletal reorganization. The migration process includes protrusion of the leading edge, formation of new adhesions at the front, cell contraction, and the release of adhesions at the rear (Lauffenburger and Horwitz, 1996; Sheetz et al., 1998; Li et al., 2005). Reorganization of actin cytoskeleton generates locomotive force, and this process is regulated by Rho family small GTPases such as RhoA, Rac, and Cdc42. Rho family small GTPases act as molecular switches by cycling between GTP- and GDP-bound states and transmit extracellular chemotactic signals to downstream effectors. Activated Rac and Cdc42 induce reorganization of actin cytoskeleton at the leading edge. This reorganization of actin

Contract grant sponsor: Scientific Research on Priority Areas of the Japanese Ministry of Education, Science, Sports and Culture;
Contract grant number: 16022205.
Contract grant sponsor: Ministry of Education, Science, Sports and Culture of Japan;
Contract grant number: 17014006.

Mayumi Abe's present address is Department of Nanomedicine, Tokyo Medical and Dental University Graduate School, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8519, Japan.

*Correspondence to: Yasufumi Sato, Department of Vascular Biology, Institute of Development, Aging and Cancer, Tohoku University, 4-1 Seiryo-machi, Aoba-ku, Sendai 980-8575, Japan.
E-mail: y-sato@idac.tohoku.ac.jp

Received 12 May 2006; Accepted 14 November 2006

Published online in Wiley InterScience (www.interscience.wiley.com.), 26 March 2007.
DOI: 10.1002/jcp.20980

cytoskeleton induces the formation of membrane protrusion such as membrane ruffling (Small et al., 2002). In contrast, RhoA regulates the assembly of contractile acto-myosin filaments. This RhoA-mediated acto-myosin contractile force promotes locomotion of the cell body and the trailing edge (Nobes and Hall, 1999). These organized activities of Rho family small GTPases make the proper cell polarity.

We previously showed that PILSAP did not affect integrin expression, but was involved in adhesion to extracellular matrix proteins (Akada et al., 2002). Here, we extended our analysis to elucidate the molecular mechanism as to how PILSAP regulates adhesion of ECs. Our present analysis revealed that PILSAP takes part in the activation of RhoA in ECs.

Materials and Methods

Materials

The following materials were used: growth factor-reduced Matrigel (Collaborative Research, Bedford, MA); α-minimum essential medium (αMEM), Opti-MEM, Lipofectamine, Superscript II reverse transcriptase, oligo(dT)12–18 primer, and oligofectamine (Gibco BRL, Rockville, MD); anti-RhoA Ab and anti-Rac1 Ab (Santa Cruz Biotechnology, Santa Cruz, CA); Isogen (Nippon Gene, Toyama, Japan); PAR1 agonist peptide (PAR-1 AP) (TFLLR-NH_2, Tocris Cookson, Bristol, UK); Y27632 (EMD Bioscience, San Diego, CA); lysophosphatidic acid (LPA; Biomol Laboratories, Plymouth Meeting, PA); nitrocellulose membranes (Amersham Biosciences, Buckinghamshire, UK); FuGENE 6 Reagent (Roche Diagnostics, Mannheim, Germany); C3 exoenzyme (Calbiochem, La Jolla, CA). Other chemicals were purchased from Sigma (St. Louis, MO).

Cell culture

Mouse endothelial MSS31 cells isolated from mouse spleen microfossils (Yanai et al., 1991) were routinely cultured in αMEM containing 5% fetal bovine serum (5% FBS/αMEM; Oda et al., 1999). MSS31 cells were stably transfected with pcDNA4A (Invitrogen, Carlsbad, CA) empty vector (Mock), Wt-PILSAP or Mut-PILSAP (Yamazaki et al., 2004). Established transfectants in bulk were maintained in 5% FBS/αMEM containing 100 μg/ml of zeocin (Invitrogen). Mut-PILSAP acts as a dominant negative molecule in which glutamate residue 343 is substituted with an alanine (E343A) in the aminopeptidase motif of PILSAP.

Immunofluorescence staining

For the inhibition of RhoA, cells were treated with C3 exoenzyme according to the method described by Minambres et al. (2006). Briefly, C3 exoenzyme (2.5 μg) was precomplexed with FuGENE 6 Reagent (5 μl) in 100 μl of opti-MEM, and C3 exoenzyme complex was added to the cultures. For the inhibition of Rho kinase, cells were incubated in 5% FBS/αMEM on type-I collagen coated dishes for 8 or 10 h with or without Y27632. In addition, cells were cultured in 1% FBS/αMEM prior to inoculation onto type-I collagen coated dishes for 10 h and then stimulated with or without PAR-1 AP (40 μM) or LPA (10 μM).

Thereafter, cells were fixed with 3.8% formaldehyde for 10 min at room temperature and permeabilized with 0.1% Triton X-100 in phosphate buffered saline (PBS). Non-specific binding sites were blocked with 1% bovine serum albumin (BSA) in PBS. Filamentous actin (F-actin) was detected by rhodamine phalloidine (Molecular Probes, Eugene, OR) and focal adhesion complexes were detected by indirect immunofluorescence by using anti-paxillin Ab (Transduction Laboratories, Lexington, KY) and FITC-labeled secondary Ab (Jackson ImmunoResearch, West Grove, PA). Then, cells were observed by confocal microscopy (LSM410, Carl Zeiss Jena GmbH, Jena, Germany).

Cell morphology during movement

Transfected cells were plated onto type-I collagen coated 35 mm dishes for 10 h in 5% FBS/αMEM at a sparse density, so that cells did not affect the movement of each other. Cells were cultured at 37°C in 5% CO_2. Next, cells were photographed by phase-contrast time lapse microscopy in random high-power (200×) fields after additional incubation of 2–6 min.

RhoA and Rac1 activities

Pull-down assay kits (Rho activation assay kit and Cdc42 activation kit, Upstate Biotechnology, Lake Placid, NY) were used to measure RhoA, Rac1 and Cdc42 activities in stable transfectants, leucinethiol (LT), a specific inhibitor of leucine aminopeptidase, treated parental cells or siRNA transfected parental cells. The Cdc42 activation kit includes p21 activated kinase 1 (PAK-1) binding domain agarose. PAK-1 binds both Rac1 and Cdc42 thus; it can measure both Rac1 and Cdc42 activity. Cells were cultured in 1% FBS/αMEM for 24 h and replated in 1% FBS/αMEM on type-I collagen coated dishes for 10 h. In LT treatment experiments, parental cells were plated onto type-I collagen coated dishes for 10 h in 1% FBS/αMEM with or without LT. In some experiments, cells were treated with or without 1% FBS/αMEM containing PAR-1 AP (40 μM) or LPA (10 μM) for 5 min. Cells were extracted with lysis buffer A (25 mM HEPES pH 7.5, 150 mM NaCl, 1% Igepal CA-630, 10 mM $MgCl_2$, 1 mM EDTA, and 10% glycerol). The pull-down of activated RhoA, Rac1, or Cdc42 was performed according to the manufacturer's protocol. A protein assay was performed to equalize the protein amount of each treatment group.

Western blot analysis

The proteins extracted by lysis buffer A or the samples obtained by pull-down assay were separated by SDS–polyacrylamide gel electrophoresis on a 10% gel and then transferred to nitrocellulose membranes (Iwasaka et al., 1996). The membranes were blocked for 1 h at room temperature with Tris-HCl-buffered saline (TBS), pH 7.4, containing 5% skim milk, and then incubated for 1 h at room temperature in TBS containing 0.05% Tween-20 (T-TBS), 1% BSA, and anti-RhoA Ab (1:1,000) or anti-Rac1 Ab (1:1,000). The filters were washed three times with T-TBS and incubated for 1 h with horseradish peroxidase-conjugated protein G (Bio-Rad, Hercules, CA) diluted 1:3,000 in T-TBS. After the filters were washed with T-TBS three times, signal was detected by an enhanced chemiluminescence method with the ECL Western blotting detection kit (Amersham Bioscience). The results were visualized with LAS-1000 (Fuji Film, Tokyo, Japan).

siRNA transfection

RNA interference of the ERAAP gene (identical to PILSAP) was described by Serwold et al. (2002). We generated another siRNA to strengthen the siRNA-mediated knock down of PILSAP. The coding strands of the two pair of siRNA oligonucleotide directed to the 5′ end of mouse PILSAP messenger RNA were 5′-AGCUAGUAAUGGAGACUCATT-3′, 5′-UGAGUCUCCAUUACUAGCUTT-3′ and 5′-CCUCAGCACUCGACUUUCTT-3′, 5′-GAAAGUCAGAGUGCUGAGGTT-3′ and scramble RNAs, the negative control of siRNA, were 5′-AAGAUUCGACGAGCUAUAGTT-3′, 5′-CUAUAGCUCGUCGAAUCUUTT-3′ and 5′-UUAGCCCGUCUACGAAUUUTT-3′, 5′-AAAUUCGUAGACGGGCUAATT-3′. RNA duplexes were denatured in annealing buffer (100 mM NaCl, 50 mM Tris-HCl pH 7.5) at 90°C for 1 min and subsequently annealed at 60°C for 1 h. MSS31 cells were cultured in 5% FBS/αMEM for 24 h, and then oligonucleotides were transfected into cells using oligofectamine according to the manufacturer's instructions. Twenty-four hours

after transfection, MSS31 cells were cultured in 5% FBS/αMEM for 24 h. The gene silencing effect was confirmed by a quantitative real time RT-PCR.

Quantitative real time RT-RCR

Quantitative real time RT-PCR was performed using a Light Cycler System (Roche Diagnostics) as described previously (Shibuya et al., 2006). Total RNA isolated from parental MSS 31 cells was extracted by ISOGEN according to the manufacturer's instructions. RNA was reverse transcribed with AMV reverse transcriptase (Roche Diagnostics) and oligo(dT)12–18 primer according to the manufacturer's instructions. PCR conditions consisted of an initial denaturation step at 95°C for 10 min, followed by 40 cycles of 15 sec at 95°C, 5 sec at 60°C and 15 sec at 72°C. The primer pairs used were: PILSAP 5′-GATGATGGATGGGCTTCTCT-3′ (forward primer) and 5′-GGCTTTTCTCAGTACTAGAC-3′ (reverse primer); mouse β-actin 5′-TCGTGCGTGACATCAAAGAG-3′ (forward primer) and 5′-TGGACAGTGAGGCCAGGATG-3′ (reverse primer). Each mRNA level was measured as a fluorescent signal corrected according to the signal for β-actin.

Network formation

The transfected cells were harvested with 0.25% trypsin and 1 mM EDTA, resuspended in 5% FBS/αMEM with or without Y27632 (10 μM) in a final volume of 1 ml, replated (2×10^5 cells per dish) onto 35 mm dishes coated with Matrigel (700 μl per dish), and incubated at 37°C for 12 h. Cells were observed by phase-contrast microscopy. The length of network structures was quantified with Soft Imaging System Analysis.

Calculations and statistical analysis

The statistical significance of differences in the data was evaluated by the use of unpaired analysis of variance. *P* values were calculated by the unpaired Student *t*-test. $P < 0.05$ was accepted as statistically significant.

Results

PILSAP is involved in F-actin formation during cell adhesion

The consensus HEXXH(18X)E motif is defined as a unique signature for zinc metalloproteinase and glutamate residues are essential for catalytic activity (Hooper, 1994). We previously established a plasmid in which glutamate residue 343 is substituted with an alanine (E343A, namely, HAXXH(18X)E) in the aminopeptidase motif of PILSAP, and this mutant PILSAP acts as a dominant negative molecule (Yamazaki et al., 2004). To investigate the biological function of PILSAP in ECs, we transfected MSS31 cells with empty vector (Mock), wild-type PILSAP (Wt-PILSAP) expressing vector or mutant PILSAP (Mut-PILSAP) expressing vector, and established the respective stable transfectants. Cells were routinely cultured on type-I collagen coated dishes. The most significant change that we found was cell morphology. As shown in Figure 1A, Mut-PILSAP transfectants showed disk-like morphology and lost cell polarity in sparse to subconfluent conditions. Because of this phenotypic change, we examined the actin cytoskeleton organization. Stress fibers were scarcely formed in Mut-PILSAP transfectants (Fig. 1B). In contrast, formation of membrane ruffling was increased but in random directions in Mut-PILSAP transfectants (Fig. 1C).

ECs use at least a type I collagen receptor integrin α2β1, a fibronectin receptor α5β1, and vitronectin receptors αvβ3 and αvβ5 for angiogenesis (Brooks et al., 1994; Collo and Pepper, 1999). Expression of these integrins was unaltered, and identical phenotypic changes were observed when transfectants were plated on fibronectin or vitronectin coated dishes (data not shown).

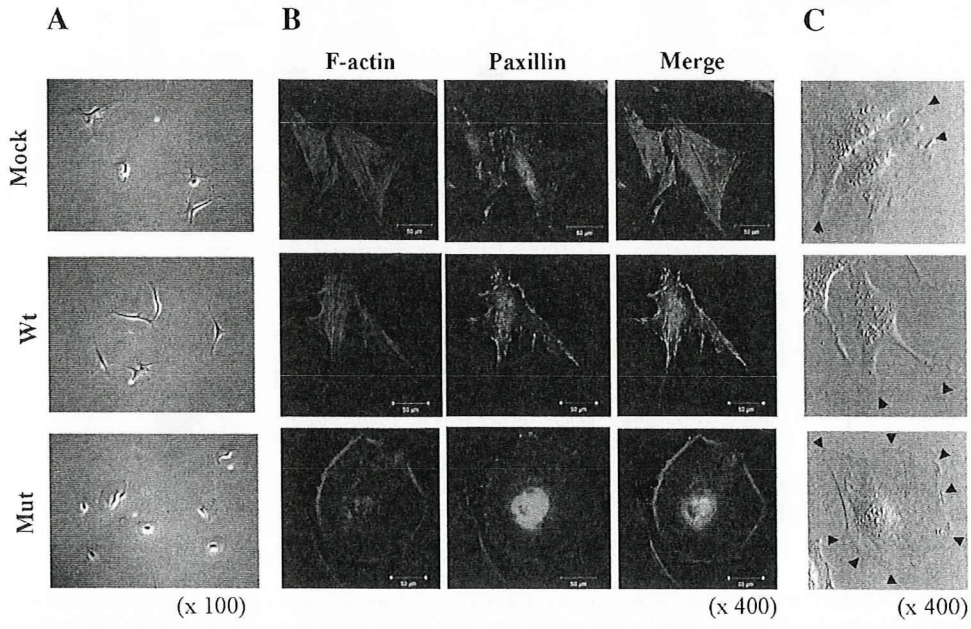

Fig. 1. Involvement of PILSAP in cell shape change, actin organization and membrane ruffling. A: Transfectants were plated onto type-I collagen coated dishes and incubated for 8 h in 5% FBS/αMEM. Cell shape was observed by a phase-contrast microscopy (upper part, 40×, lower part, 100×). B: Transfectants were plated onto type-I collagen coated dishes, and incubated for 8 h in 5% FBS/αMEM, and then stained with rhodamine-phalloidin (red) and anti-paxillin Ab (green). A scale bar indicates 50 μm. C: Cell morphology during movement was observed by time lapse microscopy. Typical pictures are shown here. Arrow heads indicate membrane ruffling.

PILSAP is involved in RhoA activation for EC morphogenesis

As Rho family small GTPases play a pivotal role in actin cytoskeleton reorganization, we examined the activities of RhoA, Rac1 and Cdc42 in transfectants upon cell adhesion. We observed that RhoA activity was attenuated, while Rac1 activity was augmented in Mut-PILSAP transfectants (Fig. 2A). Cdc42 activity was hardly detected (data not shown). Involvement of PILSAP in RhoA activation was confirmed by two additional treatments. We previously showed that PILSAP was highly sensitive to LT, but was insensitive to puromycin (Miyashita et al., 2002). LT inhibited spreading of MSS31 cells upon extracellular matrix such as type1 collagen, fibronectin and vitronectin (Akada et al., 2002). Here we examined whether LT influenced RhoA activity. As shown in Figure 2B, LT inhibited RhoA activation in parental MSS31 cells when added to the medium. Moreover PILSAP siRNAs, which knocked down to 16% of the control level of PILSAP mRNA (Fig. 2D), decreased RhoA activity of parental MSS31 cells (Fig. 2C).
RhoA regulates cell contractility through its downstream Rho kinase (Alblas et al., 2001). To further confirm the involvement of RhoA activity in EC morphogenesis, we employed specific RhoA inhibitor, C3 exoenzyme, or a Rho kinase inhibitor, Y27632. When parental MSS31 cells were treated with C3 exoenzyme or Y27632, cells exhibited cell-shape changes identical to that in Mut-PILSAP transfectants (Fig. 3A,B).
ECs form network-like structures when plated on Matrigel. This network formation was aborted in Mut-PILSAP transfectants, or by the treatment of Wt-PILSAP transfectants with Y27632 (Fig. 4). These results indicate that PILSAP is involved in RhoA activation for proper morphogenesis and organization of ECs.

PILSAP is involved in RhoA activation via G-protein coupled receptors

During the maintenance of transfectants in culture, we noticed that Mut-PILSAP transfectants showed delayed cell shrinkage upon trypsin/EDTA treatment for cell harvest. We reasoned this delay of cell shrinkage was due to impaired RhoA activation. Indeed, when transfectants were pretreated with Y27632, cell shrinkage upon trypsin/EDTA treatment was inhibited in Mock or Wt-PILSAP transfectants (data not shown). Trypsin activates proteinase-activated receptors (PARs). PARs belong to the G-protein coupled receptors (GPCRs), and ECs express PAR1 and PAR2 (Brass and Molino, 1997). Western blot analysis revealed that protein levels of PAR1 and PAR2 were identical between the three transfectants (data not shown). It was previously reported that PAR1 or PAR2 agonist induced RhoA activation in HUVEC (Vouret-Craviari et al., 2003). Here we used the PAR1 AP to induce RhoA activity in our system. There was no significant difference in the basal level of RhoA activity between transfectants. However when transfectants were treated with the PAR1 AP, RhoA activation was almost completely abolished in Mut-PILSAP transfectants (Fig. 5A). Moreover, with respect to actin reorganization, PAR1 AP

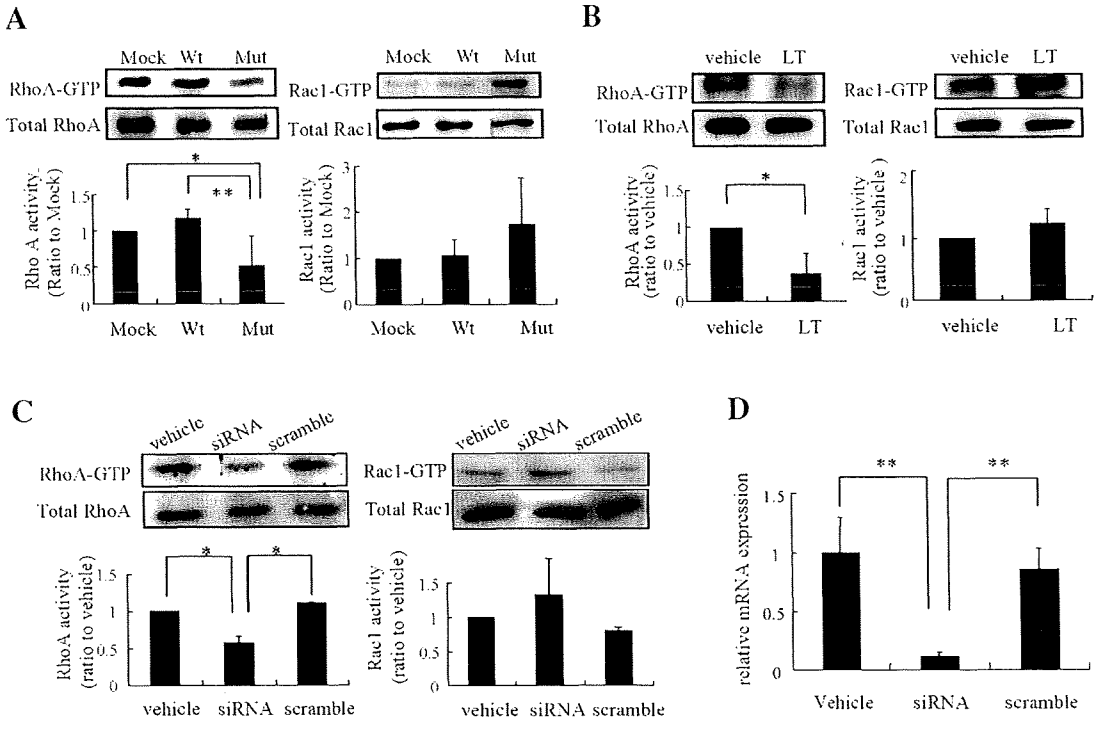

Fig. 2. Involvement of PILSAP in RhoA activation. A: Transfectants were incubated on type-1 collagen for 10 h in 1% FBS/αMEM. Then, RhoA and Rac1 activities were determined. B: Parental MSS31 cells were incubated on type-1 collagen for 10 h in 1% FBS/αMEM with or without LT (10 μmol/L). Next, RhoA and Rac1 activities were determined. GTP-RhoA or GTP-Rac1 was quantified by density and normalized to that of total RhoA or total Rac1. The values are expressed as the mean ± SD from three independent experiments; $^{*}P < 0.05$, $^{**}P < 0.01$. C: Parental MSS31 cells were transfected with PILSAP siRNA and incubated in 1% FBS/αMEM for 24 h. Then, transfectants were plated onto type-1 collagen coated dishes and incubated for 10 h. Next, RhoA and Rac1 activities were determined. The values are expressed as the mean ± SD from two independent experiments; $^{*}P < 0.05$. D: The gene silencing effect of PILSAP siRNA was confirmed by quantitative real time RT-PCR. The values are expressed as the mean ± SD from three samples; $^{**}P < 0.01$.

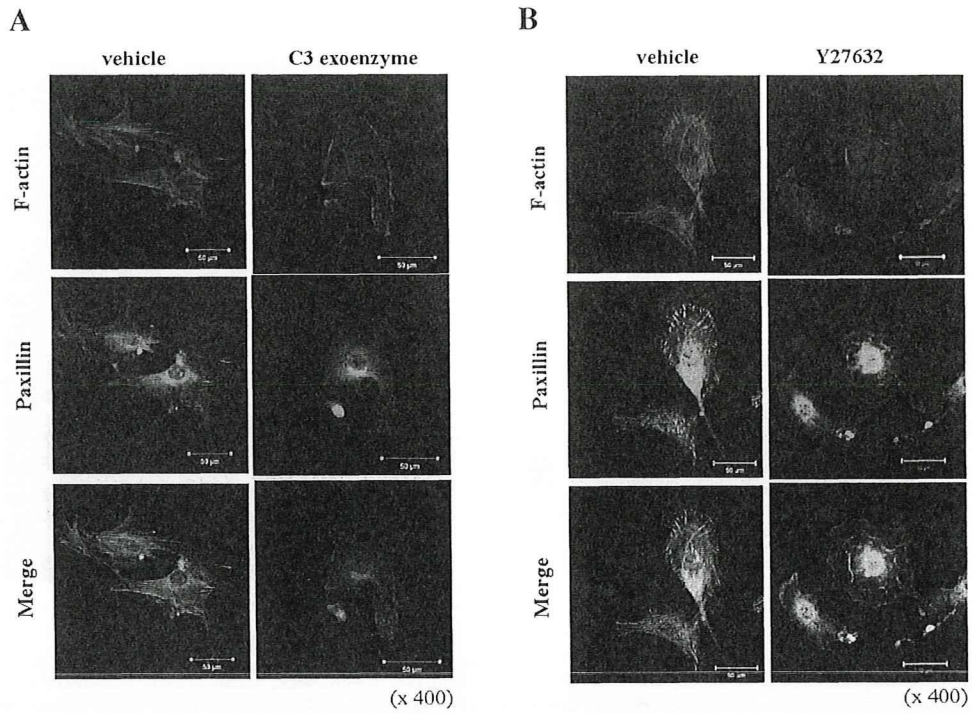

Fig. 3. Effects of RhoA and Rho kinase inhibitors on cell morphology. A: Parental MSS31 cells were plated onto type-I collagen coated dishes and incubated for 1 h in 5% FBS/αMEM. Next, cells were treated with or without C3 exoenzyme precomplexed with FuGENE 6 Reagent for additional 7 h. Cells were then stained with rhodamine-phalloidin (red) and anti-paxillin Ab (green). B: Parental MSS31 cells were plated onto type-I collagen coated dishes and incubated for 8 h in 5% FBS/αMEM. Next, cells were treated with or without Y27632 (10 μmol/L) for 30 min. Cells were then stained with rhodamine-phalloidin (red) and anti-paxillin Ab (green).

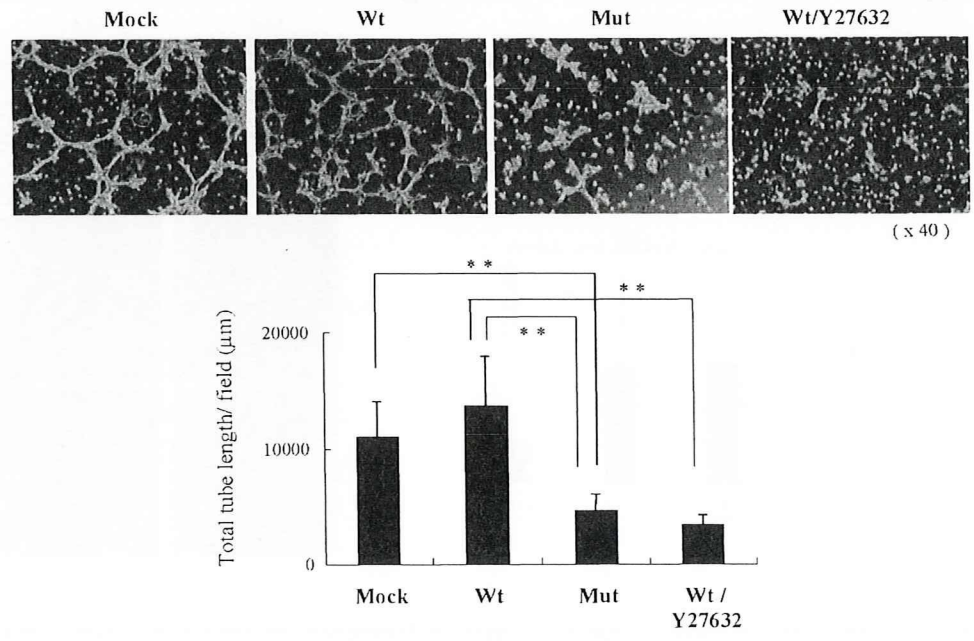

Fig. 4. Role of Rho kinase in alteration of network formation mediated by PILSAP. Transfectants were plated onto Matrigel and incubated in 5% FBS/αMEM for 12 h. In some experiments, cells were incubated in the presence of Y27632 (10 μmol/L). The total length of network structures per field (×40 magnification) was quantified with Soft Imaging System Analysis. The values are expressed as the mean ± SD of four fields; $^{**}P < 0.01$.

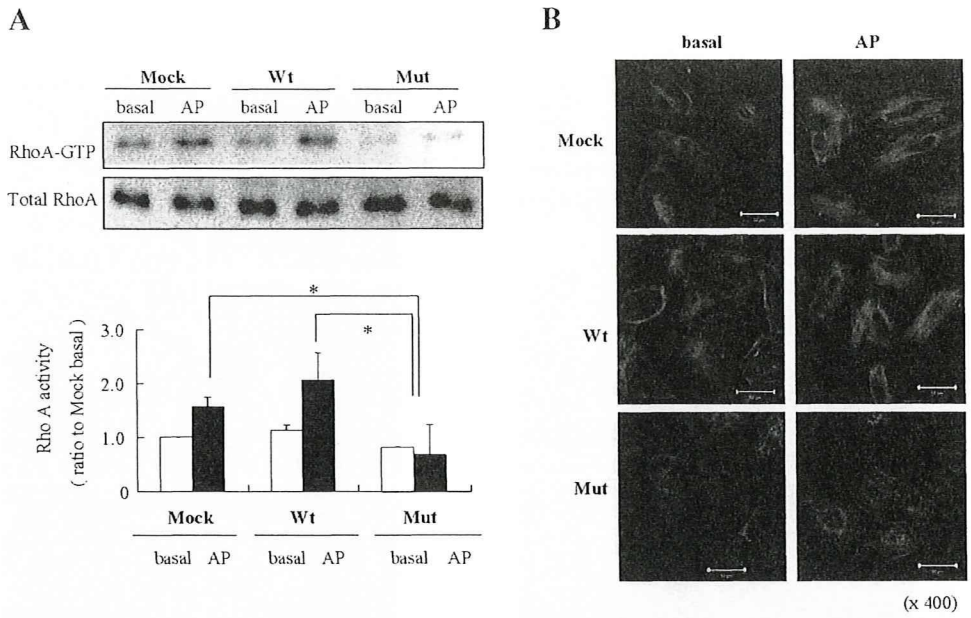

Fig. 5. Involvement of PILSAP in RhoA activation upon stimulation through PARs. A: Transfectants were incubated on type-I collagen for 10 h in 1% FBS/αMEM, and then treated with or without PAR-1 AP (AP) (40 μmol/L) for 5 min. Next, RhoA activity was determined. GTP-RhoA was quantified by density and normalized to that of total RhoA. The values are expressed as the mean ± SD from four independent experiments; *$P < 0.05$. B: Transfectants were incubated on type-I collagen for 10 h in 1% FBS/αMEM, and then treated with or without PAR-1 AP (AP) (40 μmol/L) for 5 min. Subsequently, they were fixed and stained with rhodamine-phalloidine. A scale bar indicates 50 μm.

induced stress fiber formation in Mock or Wt-PILSAP transfectants but not in Mut-PILSAP transfectants (Fig. 5B). LPA binds to the LPA receptor and activates RhoA for F-actin formation in ECs (Panetti, 2002). When transfectants were treated with LPA, RhoA activation was significantly lower in Mut-PILSAP transfectants (Fig. 6A). Stress fiber formation was not induced by LPA in Mut-PILSAP transfectants (Fig. 6B). Moreover, LT or PILSAP siRNA inhibited LPA-stimulated activation of RhoA in parental MSS31 cells (Fig. 7A,B). These results indicate that the involvement of PILSAP in RhoA

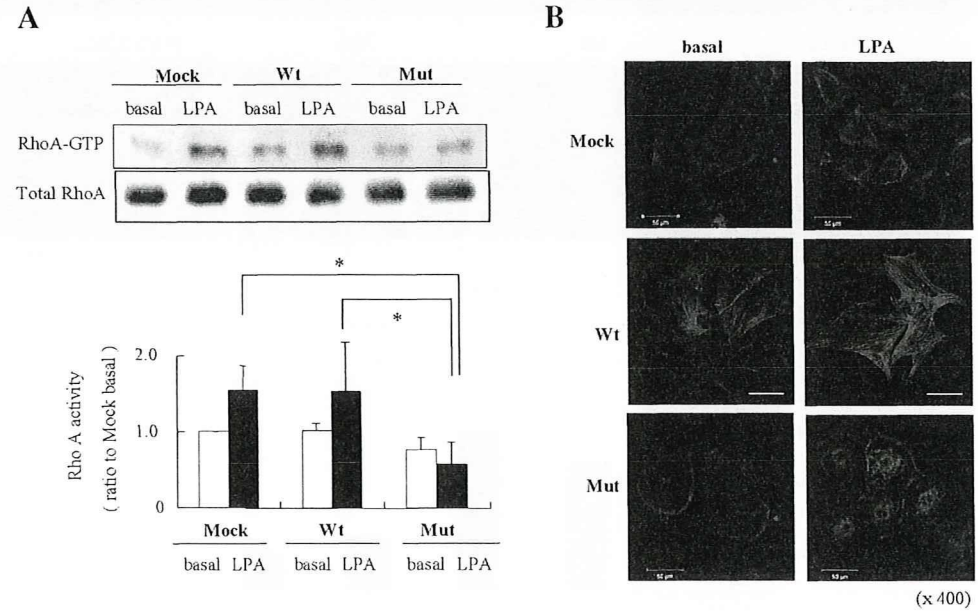

Fig. 6. Involvement of PILSAP in RhoA activation upon LPA stimulation. A: Transfectants were incubated on type-I collagen for 10 h in 1% FBS/αMEM, and then treated with or without LPA (10 μmol/L) for 5 min. Next, RhoA activity was determined. GTP-RhoA was quantified by density and normalized to that of total RhoA. The values are expressed as the mean ± SD from four independent experiments; *$P < 0.05$. B: Transfectants were incubated on type-I collagen for 10 h in 1% FBS/αMEM, and then treated with or without LPA (10 μmol/L) for 5 min. Subsequently, they were fixed and stained with rhodamine-phalloidine. A scale bar indicates 50 μm.