ARTICLE
Journal of Cellular Biochemistry 116:1411–1418 (2015)

Journal of Cellular Biochemistry

Progesterone Inhibits Endothelial Cell Migration Through Suppression of the Rho Activity Mediated by cSrc Activation

Tong-Sheng Lee,[1,2] Jhen-Jhe Lin,[1] Yen-Nien Huo,[1] and Wen-Sen Lee[1,3,4]*

[1]*Graduate Institute of Medical Sciences, College of Medicine, Taipei Medical University, Taipei, Taiwan*
[2]*Department of Ophthalmology, Shin Kong Wu Ho-Su Memorial Hospital, Taipei, Taiwan*
[3]*Department of Physiology, School of Medicine, College of Medicine, Taipei Medical University, Taipei, Taiwan*
[4]*Cancer Research Center, Taipei Medical University Hospital, Taipei, Taiwan*

ABSTRACT

We previously showed that progesterone (P4) could inhibit the proliferation of human umbilical venous endothelial cells (HUVECs) through the p53-dependent pathway. In the present study, we further demonstrated that P4 at physiologic levels (5–500 nM) concentration-dependently inhibited migration of HUVECs. This effect was blocked by pre-treatment with the P4 receptor (PR) agonist-antagonist, RU486, suggesting that the P4-induced migration inhibition in HUVECs was through the PR-mediated signaling pathway. Western blot analyses demonstrated that the levels of RhoA and Rac-1 protein were reduced in the P4-treated HUVECs. P4 also inhibited the membrane translocation of RhoA and Rac-1 protein. Moreover, the P4-induced migration inhibition in HUVECs was prevented by over-expression of the constitutively active RhoA construct (RhoA V14). However, pre-treatment with the ROCK (a kinase associated with RhoA for transducing RhoA signaling) inhibitor, Y27632, abolished the over-expression of RhoA-induced prevention effect on the P4-induced migration inhibition in HUVECs. These data suggest that the inhibition of Rho GTPases might account for the P4-induced migration inhibition of HUVECs. Pre-treatment with the cSrc inhibitor, PP2, prevented the P4-induced migration inhibition in HUVEC. The levels of phosphorylated focal adhesion kinase (FAK) and paxillin protein were also decreased by P4 treatment. Taken together, these results suggest that suppression of the Rho-mediated pathway might be involved in the signal transduction leading to the inhibition of cell migration caused by P4 in HUVECs. J. Cell. Biochem. 116: 1411–1418, 2015.

KEY WORDS: ANGIOGENESIS; PROGESTERONE RECEPTOR; RHO PROTEINS

Angiogenesis involves several well-characterized steps including local degradation of the basement membrane of the parent vessel, allowing protrusion of endothelial cells; outward migration of endothelial cells in tandem to form a capillary sprout; proliferation of endothelial cells within the sprout; and the formation of a lumen with subsequent branching [Kalebic et al., 1983; Bussolino et al., 1997; Gupta and Qin, 2003; Bellon et al., 2004]. Angiogenesis is required in many physiological and pathological conditions [Risau, 1997; Papetti and Herman, 2002]. Normally, vascular proliferation occurs only during embryonic development, and is a very slow process in the adult with a few specific exceptions. The ovary and endometrium are two of the few tissues in the adult organism in which angiogenesis is a prominent feature in normal conditions. Studies of the regulation of angiogenesis in the primate endometrium and the presence of estrogen receptor (ER) and progesterone receptor (PR) in vessel walls suggest that physiological angiogenesis is under the control of female sex hormones. It has been demonstrated that physiological concentrations of estradiol could increase vascular endothelial growth factor expression in human endometrial epithelial and stromal cells [Albrecht et al., 2003; Niklaus et al., 2003; Sengupta et al., 2003]. There is a large body of literature describing the important role of estradiol in endometrial angiogenesis, but far less is known about the P4 effect in this aspect. The effect of P4 on proliferation of vascular endothelial cells has been addressed by Vázquez et al. [1999], who showed that P4 could inhibit the proliferation of vascular endothelial cells and the rate of re-endothelialization, and thus impair vascular

The authors declare no conflict of interest.

Grant sponsor: National Science Council and Shin Kong Wu Ho-Su Memorial Hospital-Taipei Medical University; Grant numbers: NSC 100-2320-B-038-011, SKH-TMU-95-09.

*Correspondence to: Wen-Sen Lee, Ph.D., Graduate Institute of Medical Sciences, School of Medicine, Taipei Medical University, 250 Wu-Hsing Street, Taipei 110, Taiwan. E-mail: wslee@tmu.edu.tw

Manuscript Received: 15 May 2014; Manuscript Accepted: 23 January 2015

Accepted manuscript online in Wiley Online Library (wileyonlinelibrary.com): 9 March 2015

DOI 10.1002/jcb.25101 • © 2015 Wiley Periodicals, Inc.

repair processes through the PR dependent pathway. We also demonstrated that P4 inhibited cultured HUVEC proliferation through the p53-dependent mechanism, by which the levels of p21 and p27 protein were increased, and consequently inhibited the CDK2 activity, and finally impairing the transition of HUVEC from the G1 phase to the S phase [Hsu et al., 2008]. Our in vivo study in rats also showed that P4 at physiologic levels exerts an anti-angiogenic action.

Since endothelial cell migration and proliferation are two of the major events essential for angiogenesis, we further examined the effect of P4 on endothelial cell migration. Here, we showed that P4 activated the cSrc signaling pathway, which in turn suppressed the RhoA activity, and finally causing migration inhibition in HUVECs. These experimental findings reported below highlight certain molecular mechanisms of P4-induced inhibition of angiogenesis.

MATERIALS AND METHODS

CELL CULTURES

HUVECs, purchased from ATCC (Manassas, VA), are normal primary human umbilical vein endothelial cells derived from 10 individual donors and von Willebrand factor positive and smooth muscle α-actin negative. The cell was grown in phenol red-free M199 medium (GIBCO, Grand Island, NY) containing charcoal/dextran-treated 10% fetal bovine serum (FBS; GIBCO), endothelial cell growth supplement (ECGS; 0.03 mg mL^{-1}; Biomedical Technologies Inc., Stoughton, MA), sodium heparin (100 units mL^{-1}; Sigma–Aldrich, St. Louis, MO), 10 nM HEPES (Sigma–Aldrich), and kanamycin (10 mg mL^{-1}; GIBCO) in gelatin-coated plates, and incubated at 37°C in 5% CO_2. Cells from passages 5–10 were used.

TRANSWELL MIGRATION ASSAY

Migration assay was performed as described previously with minor modifications [Hou et al., 2013]. To assess the migration potential of HUVECs, the lower face of Transwell (8 μm pore size) was pre-coated with Type-1 collagen at a concentration of 1 mg mL^{-1} for 1 h at 37°C. The Transwell was assembled in a 24-well plate; the upper chambers were filled with DMEM containing charcoal/dextran-treated 2% FBS, whereas the lower chambers were filled with phenol red-free DMEM containing charcoal/dextran-treated 10% FBS. Two hundred microliter of cells (10^5 cells mL^{-1}) were inoculated onto the upper chamber of each Transwell. P4 (5–500 nM) was added into the medium of lower and upper chambers. The plate was then placed at 37°C in 5% CO_2/95% air for 13 h. After removing the non-migrating cells with a cotton swab, cells that had migrated to the lower surface of the filters were fixed and stained with 0.1% crystal violet/20% (v/v) methanol. All assays were performed in triplicate. Three random fields were chosen in each insert, and the cells were counted and photographed under a light microscope (200×).

WOUND HEALING ASSAY

Wound healing assay was performed as previously described [Rodriguez et al., 2005; Hou et al., 2013]. Briefly, HUVECs were grown in 24-well plates. White tips for 10 μL micropipette were used to scrape a "wound" in a cell monolayer. The images captured at the beginning were compared to the images after 6–8 h in a humidified 37°C, CO_2 incubator. The cells migrating to close the wound were counted to quantify the migration rate of the cells.

VIABILITY ASSAY

Cell viability was estimated by a modified MTT [3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide] (Sigma) assay as previously described [Lee et al., 1997]. Four samples were analyzed in each experiment.

ADHESION ASSAY

Adhesion assays were performed as previously described [Ho et al., 2006]. Briefly, HUVECs were plated onto a collagen (0.1 mg mL^{-1})-coated 24-well plate, grown in 10% FBS with P4 (50 nM) or vehicle for 13 h at 37°C, and then washed with phosphate-buffered saline (PBS). The cell number was counted under a light microscope.

LAMELLIPODIA ASSAY

Lamellipodia assay was performed as previously described [Hou et al., 2013]. Briefly, HUVECs were seeded on coverslips and incubated in growth medium containing vehicle or P4 (50 nM) for 3 h, fixed in 4% paraformaldehyde, and then permeabilized with 1% triton X-100. To detect actin polymerization in lamellipodia, HUVECs were stained with rhodamine-phalloidin (Cytoskeleton Inc., Denver, CO), and cell nuclei were stained with Hoechst 333342 (Invitrogen, Carlsbad, CA). Cells were viewed under a laser confocal spectral microscope imaging system (Leica, TCS SP5; Mannheim, Germany).

CELL TRANSFECTION

The constitutively active RhoA construct, RhoA V14, was transfected into HUVECs using jetPEI-HUVEC transfection reagent (Poly-plus Transfection, Illkirch, France) following the manufacturer's protocol with minor modification. Briefly, a jetPEI-HUVEC/DNA mixture was added drop-wise onto the M199M + GlutamaxTM I medium (GIBCO) containing 2% FBS, mixed gently, and incubated in a humidified 37°C incubator for 4 h. The growth medium was then replaced and the cells were incubated further for 24 h.

SUBCELLULAR FRACTIONATION

The cells were washed with cold PBS and lysed by Dounce homogenizer in lysis buffer (20 mM Tris, pH 8.0, 3 mM $MgCl_2$, 1 mM PMSF), and centrifuged at 12,000*g* for 30 min at 4°C. The supernatant was collected as the cytosolic fraction. Pellets were washed with cold PBS, and then homogenized in the lysis buffer (Tris 50 mM, pH 7.5, NaCl 150 mM, PMSF 1 mM, NP-40 1%, 0.1% SDS) on ice, and centrifuged at 12,000*g* for 30 min at 4°C. The supernatant was collected as the particulate (membrane) fraction. Proteins of cytosolic and particulate fractions were detected for RhoA, RhoB, RhoC, and Rac-1 by Western blot analysis.

WESTERN BLOT ANALYSIS

To determine the expression levels of cSrc, p-cSrc, FAK, paxillin, RhoA, RhoB, RhoC, Rac-1, cadherin, and G3PDH in HUVECs, the total proteins were extracted and Western blot analyses were performed as previously described [Lin et al., 2002]. Briefly, HUVECs were cultured in 10 cm Petri dishes. After reaching subconfluence, the cells were pre-treated for 21 h in DMEM containing 0.04% FBS, and challenged

with 10% FBS and various concentrations of P4 (50 or 500 nM), and then incubated at 37°C. At different time points, the cells were washed with PBS (pH 7.4), incubated with extraction buffer (Tris 50 mM, pH 7.5, NaCl 150 mM, PMSF 1 mM, NP-40 1%, 0.1% SDS) on ice, and then centrifuged at 12,000*g* for 30 min. The cell extract was then boiled in a ratio of 3:1 with sample buffer (Tris-HCl 250 mM, pH 6.8, glycerol 40%, dithiothreitol 400 mM, SDS 8% and bromophenol blue 0.2%). Electrophoresis was carried out using a 12% SDS-polyacrylamide gel (4 h, 60 V, 50 μg protein per lane). Separated proteins were transferred to PVDF membranes (20 h, 80 mA), treated with 1% BSA/0.02% $NaNO_3$ to block the nonspecific IgGs, and incubated for 1 h with specific antibody for FAK, p-FAK, paxillin, p-paxillin, RhoA, RhoB, RhoC, Rac-1, cadherin, or G3PDH at a concentration of 0.2 $\mu g\,mL^{-1}$ (Jackson ImmunoResearch Laboratories, West Grove, PA). The blot was then incubated with anti-mouse, anti-rabbit, or anti-goat IgG (Jackson ImmunoResearch Laboratories) linked to horseradish peroxidase (HRP; 1:10,000) for 1 h. Subsequently, the blot was developed using the ECL (enhanced chemiluminescence) system (Amersham). The intensity of each band was quantified by densitometry analysis using Image Pro-Plus 4.5 Software.

MATRIX METALLOPROTEASES ACTIVITY ASSAY

The activities of matrix metalloproteases (MMPs) in HUVECs were measured using gelatin zymography protease assays as described previously [Chien et al., 2012]. Briefly, the extracted proteins were subjected to 0.1% gelatin-8% SDS-PAGE electrophoresis. After electrophoresis, gels were washed with 2.5% Triton X-100, incubated in reaction buffer (40 mM Tris-HCl, pH 8.0; 10 mM $CaCl_2$ and 0.01% NaN_3) for 12 h at 37°C, and then stained with Coomassie brilliant blue R-250 (J.T. BAKER, Center Valley, PA).

STATISTICS

All data were expressed as the mean value ± SEM. Comparisons were subjected to one way analysis of variance (ANOVA) followed by Fisher's least significant difference test. Significance was accepted at $P < 0.05$.

RESULTS

EFFECT OF P4 ON MIGRATION OF HUVECS

Previously, we demonstrated that P4 exerts an anti-angiogenic activity through inhibiting the growth of vascular endothelial cells [Hsu et al., 2008]. To examine whether inhibition of vascular endothelial cell migration is also involved in the P4-induced anti-angiogenesis, the Transwell migration assay was performed in HUVECs. As illustrated in Figure 1A, P4 (5–500 nM) concentration-dependently inhibited the migration of HUVECs. Treatment with the PR agonist-antagonist, RU486 (100 nM), alone partially inhibited the migration of HUVECs. Pre-treatment of HUVCEs with RU486 prevented the P4-induced migration inhibition (Fig. 1B).

Since lamellipodia formation is necessary for cell migration, we also examined the effect of P4 on the lamellipodia formation of HUVECs. As shown in Figure 1C, treatment with P4 reduced the formation of both stress fibers and lamellipodia in HUVECs.

INVOLVEMENT OF RHOA IN THE P4-INDUCED MIGRATION INHIBITION IN HUVECS

Since RhoA have been indicated to be involved in regulating the actin cytoskeleton in the formation of stress fibers and in growth factors-stimulated cell migration and cytoskeletal organization, and membrane trafficking, the involvement of RhoA in the P4-induced HUVEC migration inhibition was explored. In the P4 (500 nM)-treated HUVEC, the levels of RhoA protein in total cell lysate (Supplementary Fig. 1A, Fig. 2A) and the membranous fraction (Supplementary Fig. 1B, Fig. 2B) were significantly decreased, suggesting that the RhoA activity in HUVEC was decreased by P4 treatment. We also examined the effect of P4 on the Rac-1 activity, which is generally associated with the lamellipodia formation. Treatment with P4 (500 nM) for 13 h also reduced the levels of Rac-1 protein in total cell lysate (Supplementary Fig. 1A, Fig. 2A) and the membranous fraction (Supplementary Fig. 1A, Fig. 2B). These results suggest that interference of prenylation might be involved in the inhibition of HUVEC migration caused by P4. To confirm the involvement of RhoA reduction and inactivation in the P4-induced migration inhibition in HUVECs, we transfected HUVECs with RhoA V14 followed by P4 treatment. As illustrated in Figure 2C, transfection with RhoA V14 caused an increase of RhoA expression, which prevented the P4-induced migration inhibition in HUVECs (Fig. 2D). However, pre-treatment of HUVECs with a ROCK (a kinase associated with RhoA for transducing RhoA signaling) inhibitor, Y27632, abolished the over-expression of RhoA-induced prevention effect on the P4-induced migration inhibition.

EFFECT OF P4 ON THE LEVELS OF P-FAK AND P-PAXILLIN

Since the focal adhesion complex is one of the important components promoting cell motility, the adhesive interactions between cells and extracellular matrix can influence the attachment and transmigration across the surrounding cells [Hood and Cheresh, 2002]. We evaluated the effects of P4 on the formation of focal adhesion complexes by examining the levels of p-FAK and p-paxillin, two elementary proteins for forming stable adhesion complexes and transducing the survival/motility signals, in the P4-treated HUVECs using Western blot analysis. As shown in Figure 3 and Supplementary Figure 2, treatment with P4 (500 nM) for 5 and 10 min, the ratios of both p-FAK/FAK and p-paxillin/paxillin were decreased in the P4-treated HUVEC as compared with vehicle-treated HUVECs (control).

EFFECT OF A LOW CONCENTRATION OF P4 ON HUVEC MIGRATION

We further examined the effect of P4 at a lower concentration on endothelial cell migration. As shown in Figure 4A, P4 at a concentration of 50 nM reduced the cell number migrated through the collagen. Wound healing assay demonstrated that P4 (50 nM) inhibited the motility of HUVECs (Fig. 4B). However, P4 (50 nM) did not affect the cell viability (Fig. 4C), cell adhesion to collagen (Fig. 4D) and MMPs activity (4E).

INVOLVEMENT OF CSRC ACTIVATION ON THE P4-INDUCED MIGRATION INHIBITION IN HUVEC

Since PR interacts with the tyrosine kinase c-Src [Boonyaratana-kornkit et al., 2001; Hsu and Lee, 2011] and our previous study

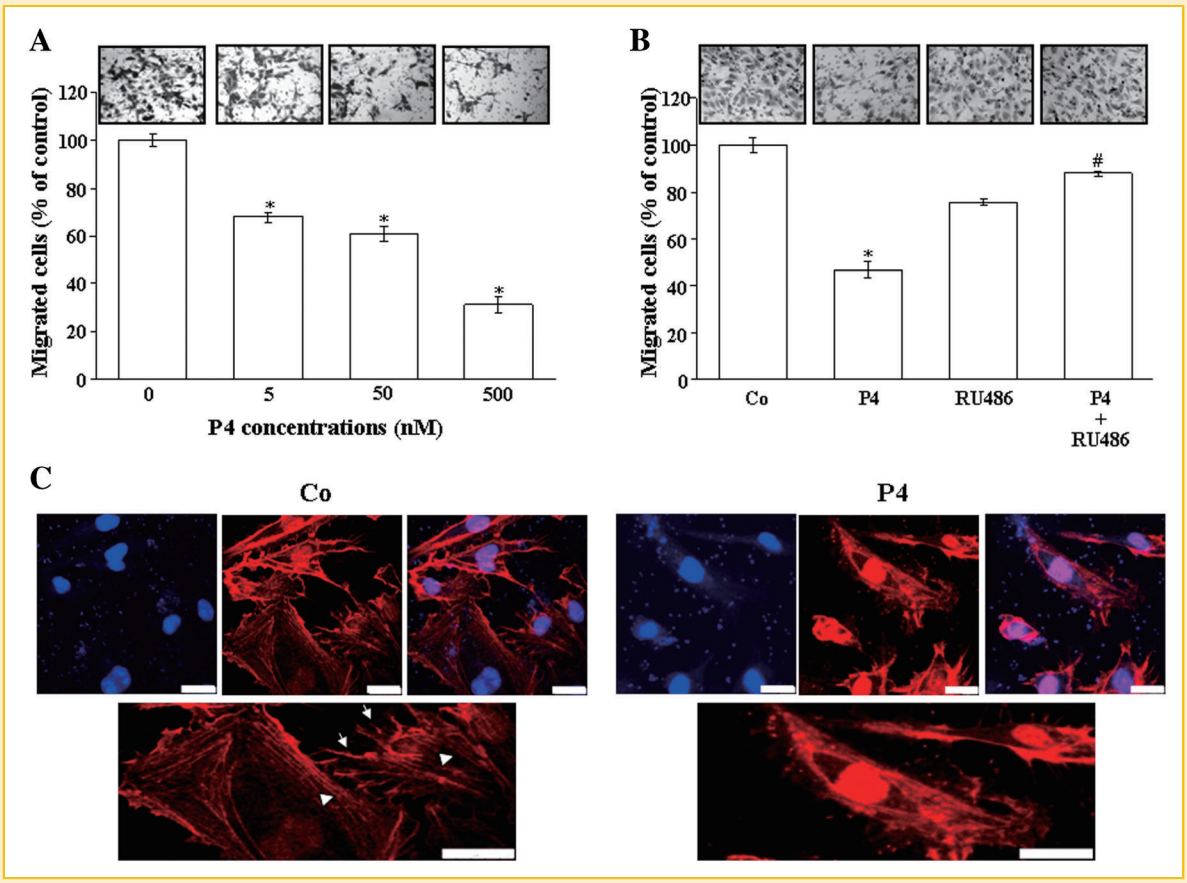

Fig. 1. Inhibitory effects of P4 on HUVEC migration. A: P4 (5–500 nM) concentration-dependently inhibited HUVEC migration through collagen (1 mg mL^{-1}). B: P4 inhibited HUVEC migration through the PR-mediated pathway. HUVECs were pre-incubated with RU486 (100 nM) followed by P4 (500 nM) or DMSO (control) for 20 h. Values represent the means ± SEM. (n = 4). $^{*}P < 0.05$ different from control. $^{\#}P < 0.05$ different from P4-treated group. C: P4 inhibited the formation of lamellipodia (arrows) and stress fibers (arrowheads) in HUVECs. HUVECs were treated with DMSO or P4 (500 nM) for 1 h. Phalloidin staining of HUVECs was performed as described in the Materials and Methods. Nuclei were stained with Hoechst 333342. Scale bar 25 μm; Co, control.

demonstrated that P4 can increase the formation of PR-cSrc complex and activate cSrc [Hsu and Lee, 2011], we thus explored the role of cSrc activation on P4-induced migration inhibition in HUVECs. As shown in Figure 5A and Supplementary Figure 3A, treatment of P4 (50 nM) for 2 min increased the level of p-cSrc protein. However, pre-treatment with the cSrc inhibitor, PP2 (400 nM), prevented the P4-induced reductions of membranous translocation of RhoA from the cytosol (Supplementary Fig. 3B, Fig. 5B) and migration inhibition in HUVECs (Fig. 5C).

DISCUSSION

Previously, we demonstrated that P4 inhibits angiogenesis in vivo and suppresses the proliferation of vascular endothelial cells through the PR-mediated pathway in vitro [Hsu et al., 2008]. Here, we further demonstrated that P4 at physiologic concentrations (5–500 nM) also concentration-dependently inhibited migration of vascular endothelial cells. The effect of P4 on endothelial cell migration is blocked by pre-treatment of the cell with the PR agonist-antagonist, RU486, suggesting that P4-induced migration inhibition is mediated through the PR-mediated signaling pathway. The findings of the present study suggest that the Rho-mediated signaling pathway might be involved in the migration inhibition caused by P4 in cultured HUVECs. To our knowledge, this is the first demonstration that P4 inhibits endothelial cell migration through the Rho-dependent pathway mediated by PR activation.

Transwell migration assay demonstrated that P4 (5–500 nM) concentration-dependently inhibited the endothelial cell migration (Fig. 1A). Although cell adhesion is one of the principal requirements for migration [Keledjian et al., 2005], our data indicated that the adhesion of HUVECs on the collagen-coated plate, which was used for the migration assay, was not significantly affected by P4 treatment (Fig. 4D), suggesting that HUVEC migration inhibition induced by P4 was not due to reduction of adhesion. Moreover, P4 at a concentration of 50 nM did not cause death in HUVECs (Fig. 4C), indicating that the P4-induced migration inhibition in HUVECs was not due to cytotoxicity. Proteolytic breakdown of the basement membrane, which results from secretion and activation of MMPs in response to exogenous signals such as cytokines, growth factors and cell-matrix interactions, is also required for endothelial cell migration during angiogenesis [Bellon et al., 2004]. During angiogenesis, the

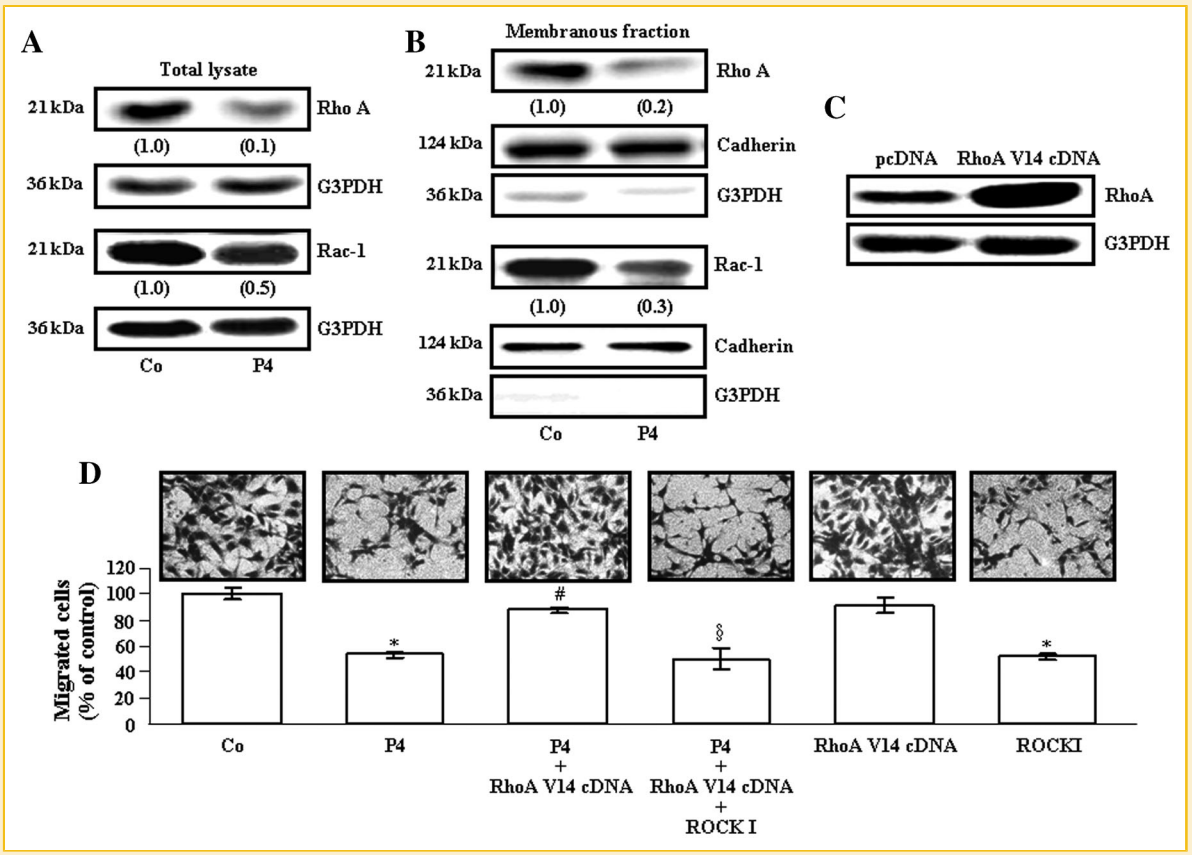

Fig. 2. Involvement of the Rho/ROCK signaling pathway in the P4-induced inhibition in HUVEC migration. A: Treatment of HUVEC with P4 (500 nM) for 20 h inhibited the total protein levels of RhoA and Rac-1. Data are representative of two independent experiments with similar results. Values shown in parentheses represent the quantified results of RhoA and Rac-1 protein levels after adjusted with their own G3PHD protein levels and expressed by fold of control. B: P4 inhibited membrane translocation of RhoA and Rac-1 protein from cytosol. Cadherin and G3PDH were used as a membrane and cytosolic protein marker, respectively, to confirm the purities of isolation. Data are representative of two independent experiments with similar results. Values shown in parentheses represent the quantified results of RhoA and Rac-1 protein levels after adjusted with their own cadherin protein levels and expressed by fold of control. C: Transfection with RhoA V14 cDNA induced increases of RhoA protein level in HUVECs as compared with transfection with pcDNA3.1+. D: P4 (500 nM) inhibited migration of HUVECs. However, over-expression RhoA V14 completely prevented the P4-induced migration inhibition. Treatment with Y-27632 (5 μM), a ROCK inhibitor, inhibited HUVEC migration and abolished the over-expression RhoA-induced prevention effect on P4-induced inhibition of HUVEC migration. Top panel shows the representative photographs of the results. Values represent the means ± SEM (n = 3). $^{*}P < 0.05$ different from control. $^{\#}P < 0.05$ different from P4-treated group. $^{\S}P < 0.05$ different from P4 + RhoA-treated group. Co, control.

extracelluar proteolytic activity in the endothelial pericellular environment was significantly increased. It was demonstrated that MMPs might mediate the basement membrane degradation, and the catalytic activity of secreted MMPs is tightly regulated. In the present study, however, treatment of HUVECs with P4 (50 nM) for 13 h did not significantly affect the MMPs activity (Fig. 4E), suggesting that reduction of the MMPs activity might not contribute to the P4-induced migration inhibition in HUVECs. Taken together, these data suggest that P4 inhibited endothelial cell migration mainly through suppressing the cell motility. This notion was supported by our data showing that P4 inhibited the formation of stress fibers and lamellipodia in HUVECs (Fig. 1C). It has been indicated that the lamellipodia is generally associated with Rac activation, whereas the stress fibers are with Rho activation [Hall, 1998]. Our data showed that P4 inhibited the activation of both RhoA and Rac-1 (Fig. 2B) in HUVECs. These data confirm that the formation of stress fibers and lamellipodia in HUVECs was reduced by P4 treatment.

Cell migration, a highly integrated multi-step process that is crucial for development and plays an important role in the process of various diseases, is initiated by an initial protrusion at the leading edge of the cell or lamellipodium followed by the establishment of new adhesion sits at the front, cell body contraction, and detachment of adhesions at the cell rear. It has been suggested that a key element in cell migration is the reciprocal regulation of Rac and Rho. Rac is thought to be required at the leading edge of the cell to regulate actin polymerization and membrane protrusion, whereas Rho appears to regulate the contraction and retraction forces required in the cell body and at the rear. During angiogenesis, dynamic changes in adhesive structures of endothelial cells are very important for cellular remodeling. It has been indicated that Vascular Endothelial (VE)-cadherin, an endothelial-specific cell–cell adhesion protein of the adherens junction complex plays an important role in all branches of endothelial cell biology (Harris and Nelson, 2010). VE-cadherin-signaling could activate Rho kinase (ROCK) and myosin light-chain 2

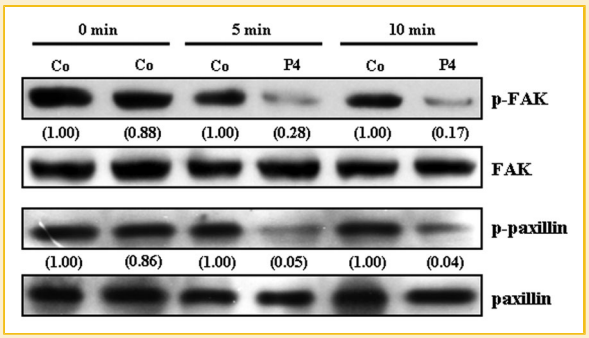

Fig. 3. P4 inhibits focal adhesion complex formation. HUVECs were treated with or without P4 (500 nM). After treatment with P4 or PBS for 5 or 10 min followed by total protein extraction and Western blot analysis. The levels of phosphorylated paxillin (p-paxillin) and FAK (p-FAK) were decreased after P4 treatment. Data are representative of two independent experiments with similar results. Values shown in parentheses represent the quantified results of p-FAK and p-paxillin protein levels after adjusted with their own total protein levels and expressed by fold of control. Co, control.

phosphorylation. Previous studies also demonstrated VE-cadherin signaling enhances actomyosin-mediated cell contraction through stimulating RhoA-ROCK [Nelson and Chen, 2003; Nelson et al., 2004]. VE-cadherin, ROCK, and actomycin act in a positive feedback loop [Abraham et al., 2009]. Disruption of this loop induces sprouting through reversing the suppression of VEGF receptor-mediated signaling to Rac-1. The interaction among VE-cadherin, RhoA, ROCK and Rac-1 is a very important issue for understanding the molecular mechanism underlying P4-induced anti-angiogenesis and deserves further investigation.

It has been recognized that the post-translational modification of proteins by the addition of isoprenoids is a key physiological process for facilitating cellular protein–protein interactions and membrane-associated protein trafficking [McTaggart, 2006]. To be functionally active, Rho proteins must be localized to the cell membrane by post-translational modification through addition of isoprenyl groups from isoprenoid pyrophosphate substrates [Seabra, 1998]. To test whether P4 suppressed HUVEC migration through regulating the activity of Rho proteins, we examined the effect of P4 on the expression of Rho proteins. P4 at a concentration of 50 nM decreased the protein levels of Rho A and Rac-1 (Fig. 2A), but not RhoB and RhoC protein (data

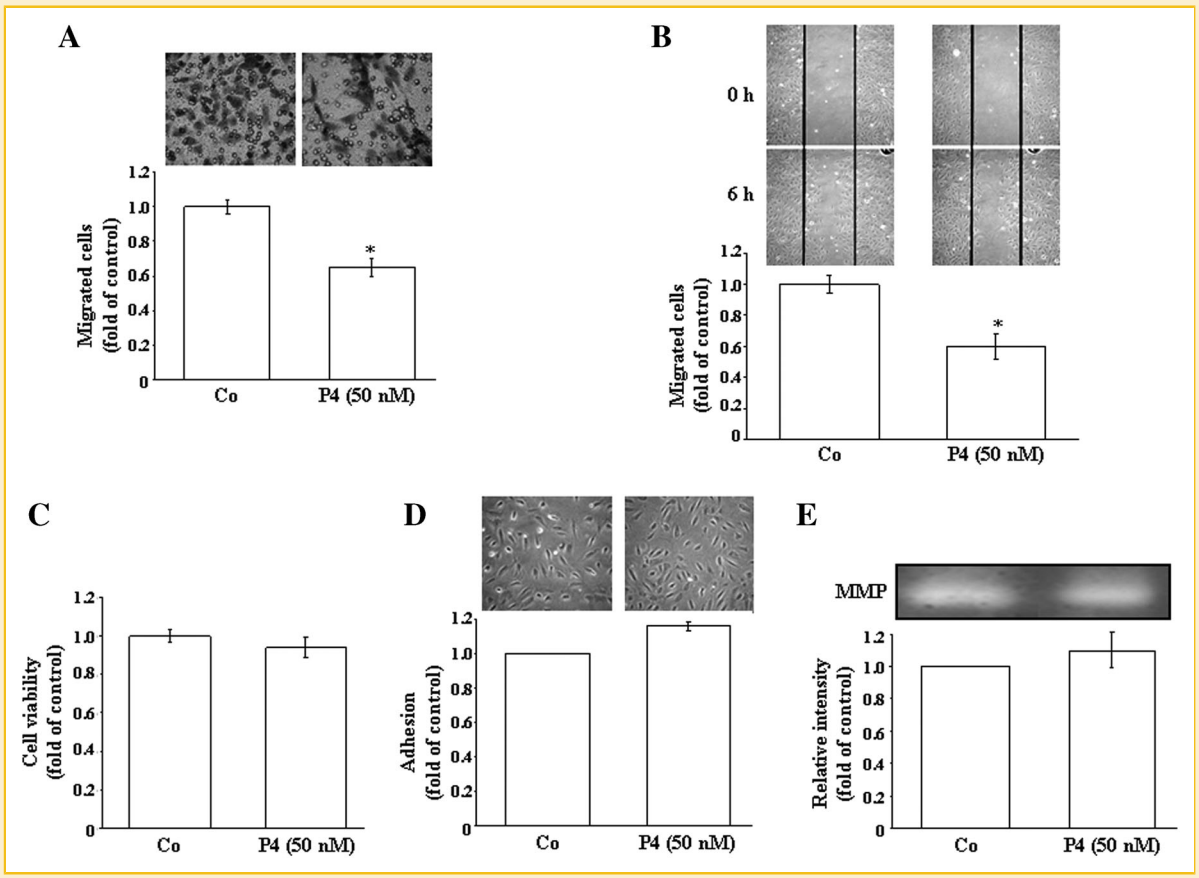

Fig. 4. P4 at a lower concentration inhibits migration of HUVECs. A: P4 at a concentration of 50 nM inhibited HUVEC migration examined using the Transwell migration assay (A) or the wound healing assay (B), but did not affect the cell viability (C), cell adhesion onto the collagen (D), or the MMPs activity (E). For adhesion assay, 24-well plates were coated with collagen (0.1 mg ml^{-1}). HUVECs were pre-incubated with P4 (50 nM) or DMEM for 24 h. The P4-treated HUVECs were seeded on the pre-coated plates and allowed to attach for 13 h. Cell numbers were assessed by MTT assay. Values represent the means ± SEM (n = 4). $^{*}P < 0.05$ different from control. Co, control.

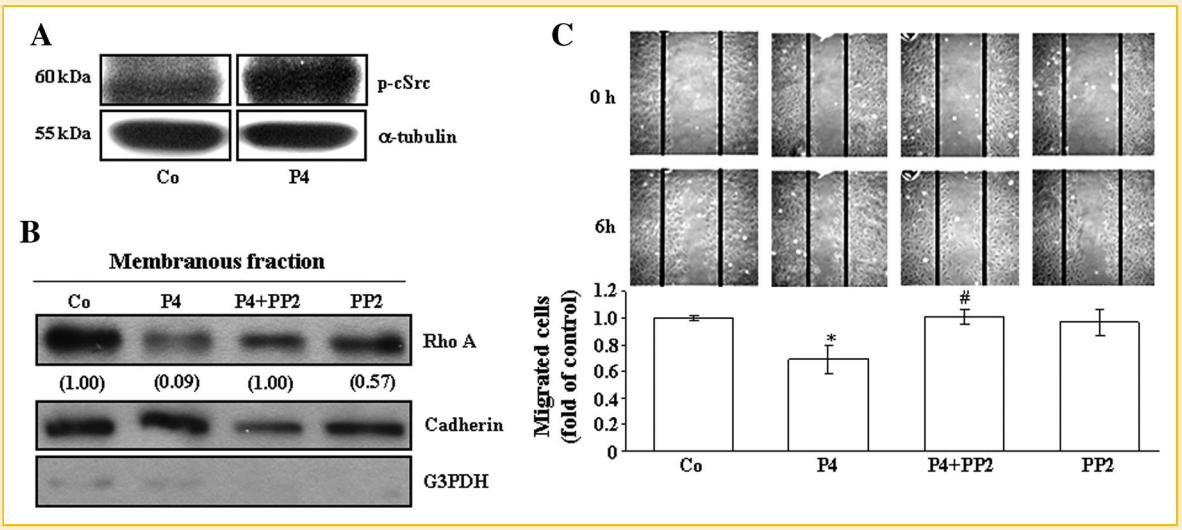

Fig. 5. Involvement of cSrc activation on the P4-induced migration inhibition in HUVECs. A: Treatment of HUVECs with P4 (50 nM) for 2 min induced an increase of cSrc activation. B: Pre-treatment with the cSrc inhibitor, PP2 (400 nM), for 1 h prevented the P4-induced decreases of membranous translocation of RhoA protein from the cytosol in HUVECs. The membranous proteins were extracted after 20 h treatment with Vehicle, P4 (50 nM), PP2 (400 nM), or PP2 + P4. Cadherin and G3PDH were used as a membrane and cytosolic protein marker, respectively, to confirm the purities of isolation. Data are representative of two independent experiments with similar results. Values shown in parentheses represent the quantified results of RhoA protein levels after adjusted with their own cadherin protein levels and expressed by fold of control. C: Pre-treatment with PP2 (400 nM) for 1 h prevented the P4-induced migration inhibition in HUVECs. Values represent the means ± SEM (n = 4). $^{*}P < 0.05$ different from control. $^{\#}P < 0.05$ different from P4-treated group. Co, control.

not shown). On the other hand, P4 decreased the levels of membrane-bound RhoA and Rac-1 (Fig. 2B). These data suggested that P4 might interfere with cell migration via suppressing the prenylation of RhoA and Rac-1. Although various Rho GTPase family members (such as RhoA, RhoB and RhoC) are highly homologous, our present data suggest that inhibition of RhoA/ROCK signaling is critical for suppressing migration activity in the P4-treated HUVECs. Unlike RhoA protein, which is located in plasma membrane for regulating actin stress fiber formation and integrin signaling, RhoB is located in the endosome and nuclear membranes with a unique function in intracellular trafficking of growth factor receptors such as the epidermal growth factor receptor [Wherlock et al., 2004]. RhoB plays an inhibitory role during cell cycle regulation and is up-regulated in response to stress stimuli [Fritz and Kaina, 1997]. It has been indicated that RhoA and RhoC have overlapping functions including promotion of cell motility, cytoskeletal alterations, and metastasis [Wheeler and Ridley, 2004]. Treatment of HUVECs with P4 did not significantly change the level of RhoC protein, suggesting that RhoC might not be involved in the P4-induced inhibition of HUVEC migration. The notion of involvement of RhoA inactivation in the P4-induced migration inhibition was supported by the evidence that over-expression of RhoA V14 completely prevented the P4-induced migration inhibition and this prevention effect was abolished by pre-treatment of the cell with a ROCK inhibitor, Y27632 (Fig. 2D).

It has been suggested that phosphorylation of FAK and paxillin is able to affect cellular events either that require modulation of cell adhesion such as cell migration, or that are dependent on cell adhesion such as cell proliferation and survival [Cohen and Guan, 2005]. In the present study, we demonstrated that the levels of both p-FAK and p-paxillin were reduced after 5 min treatment with P4 (Fig. 3). Tyrosine phosphorylation of FAK triggers downstream signaling events including phosphorylation of paxillin, which is necessarily and sufficiently for regulation of Rho-family GTPases (Rho, Rac and Cdc42) and Pak (a downstream effector of Rac and Cdc42) [Abedi and Zachary, 1995]. Therefore, our data suggest that suppression of FAK and paxillin activity might contribute to P4-induced inhibition of the RhoA activity.

Previously, we have demonstrated that P4 can induce proliferation inhibition of HUVECs through activating cSrc, a non-receptor tyrosine kinase that is essential for the regulation of a wide range of cellular functions including cell migration [Arthur et al., 2000], mediated by the extra-nuclear PR [Hsu and Lee, 2011]. In cultured HUVECs, cSrc and PR form the complex in the presence or absence of P4 treatment, and P4 can induce increases of p-cSrc through increasing the formation of cSrc-PR complex [Hsu and Lee, 2011]. We also showed that cSrc activation also plays an important role in folic acid-induced migration inhibition in HUVECs through RhoA inactivation mediated by cSrc activation [Hou et al., 2013]. The involvement of cSrc activation in the P4-induced HUVEC migration inhibition was supported by the evidence that P4 treatment induced cSrc activation (Fig. 5A) and pre-treatment with the cSrc inhibitor, PP2, prevented the P4-induced reductions of membranous translocation of RhoA from the cytosol (Fig. 5B), and migration inhibition in HUVECs (Fig. 5C).

In conclusion, this study provides the evidence that P4 inhibited endothelial cell migration through suppression of the Rho activity. Based on the results from the present study, we propose a model of the molecular mechanism underlying P4-induced migration inhibition in HUVECs as shown in Figure 6. Taken together, our previous and current findings strongly suggest the potential applications of P4 as

an anti-angiogenesis agent because it is capable of inhibiting the proliferation and migration of endothelial cells. This study also provides new insights into the regulation of vascular endothelial cell behaviors by P4.

ACKNOWLEDGMENTS

We thank Professor Meei-Jyh Jiang for providing RhoA V14 cDNA. This work was supported by grant from NSC 100-2320-B-038-011– and SKH-TMU-95-09 to Dr. Wen-Sen Lee.

REFERENCES

Abedi H, Zachary I. 1995. Signalling mechanisms in the regulation of vascular cell migration. Cardiovasc Res 30:544–556.

Abraham S, Yeo M, Montero-Balaguer M, Paterson H, Dejana E, Marshall CJ, Mavria G. 2009. VE-cadherin-mediated cell-cell interaction suppresses sprouting via signaling to MLC2 phosphorylation. Curr Biol 19:668–674.

Albrecht ED, Aberdeen GW, Niklaus AL, Babischkin JS, Suresch DL, Pepe GJ. 2003. Acute temporal regulation of vascular endothelial growth/permeability factor expression and endothelial morphology in the baboon endometrium by ovarian steroids. J Clin Endocr Metab 88:2844–2852.

Arthur WT, Petch LA, Burridge K. 2000. Integrin engagement suppresses RhoA activity via a c-Src-dependent mechanism. Curr Biol 10:719–722.

Bellon G, Martiny L, Robinet A. 2004. Matrix metalloproteinases and matrikines in angiogenesis. Crit Rev Oncol Hematol 49:203–220.

Boonyaratanakornkit V, Scott MP, Ribon V, Sherman L, Anderson SM, Maller JL, Miller WT, Edwards DP. 2001. Progesterone receptor contains a proline-rich motif that directly interacts with SH3 domains and activates c-Src family tyrosine kinases. Mol Cell 28:269–280.

Bussolino F, Mantovani A, Persico G. 1997. Molecular mechanisms of blood vessel formation Trends Biochem Sci 22:251–256.

Chien MH, Hsieh YS, Yeh CM, Lee WS, Chang JH, Yang SF. 2012. *Dioscorea nipponica* Makino inhibits migration and invasion of human oral cancer HSC-3 cells by transcriptional inhibition of matrix metalloproteinase-2 through modulation of CREB and AP-1 activity. Food Chem Toxicol 50:558–566.

Cohen LA, Guan JL. 2005. Mechanisms of focal adhesion kinase regulation. Curr Cancer Drug Targets 5:629–643.

Fritz G, Kaina B. 1997. RhoB encoding a UV-inducible Ras-related small GTP-binding protein is regulated by GTPases of the Rho family and independent of JNK, ERK, and p38 MAP kinase. J Biol Chem 272:30637–30644.

Gupta MK, Qin RY. 2003. Mechanism and its regulation of tumor-induced angiogenesis World J Gastroenterol 9:1144–1155.

Hall A. 1998. Rho GTPases and the actin cytoskeleton. Science 279:509–514.

Harris ES, Nelso WJ. 2010. VE-cadherin: At the front, center, and sides of endothelial cell organization and function. Curr Opin Cell Biol 22:651–658.

Ho PY, Zhong WB, Ho YS, Lee WS. 2006. Terbinafine inhibits endothelial cell migration through suppression of the Rho-mediated pathway. Mol Cancer Ther 5:3130–3138.

Hood JD, Cheresh DA. 2002. Role of integrins in cell invasion and migration. Nat Rev Cancer 2:91–100.

Hou TC, Lin JJ, Wen CH, Chen LC, Hsu SP, Lee WS. 2013. Folic acid inhibits endothelial cell migration through inhibiting the RhoA activity mediated by activating the folic acid receptor/cSrc/p190RhoGAP-signaling pathway. Biochem Pharmacol 85:376–384.

Hsu SP, Lee WS. 2011. Progesterone receptor activation of extra-nuclear signaling pathways in regulating p53 expression in vascular endothelial cells. Mol Endocrinol 25:421–432.

Hsu SP, Ho PY, Juan SH, Liang YC, Lee WS. 2008. Progesterone inhibits human endothelial cell proliferation through a p53-dependent pathway. Cell Mol Life Sci 65:3839–3850.

Kalebic T, Garbisa S, Glaser B, Liotta LA. 1983. Basement membrane collagen: Degradation by migrating endothelial cells. Science 221:281–283.

Keledjian K, Garrison JB, Kyprianou N. 2005. Doxazosin inhibits human vascular endothelial cell adhesion, migration, and invasion. J Cell Biochem 94:374–388.

Lee WS, Harder JA, Yoshizumi M, Lee ME, Haber E. 1997. Progesterone inhibits arterial smooth muscle cell proliferation. Nat Med 3:1005–1008.

Lin SY, Yeh SD, Chang HC, Lin CH, Liu JD, Lee WS. 2002. Magnolol suppresses proliferation of cultured human colon and liver cancer cells by inhibiting DNA synthesis and activating apoptosis. J Cell Biochem 84:532–544.

McTaggart SJ. 2006. Isoprenylated proteins. Cell Mol Life Sci 63:255–267.

Nelson CM, Chen CS. 2003. VE-cadherin simultaneously stimulates and inhibits cell proliferation by altering cytoskeletal structure and tension. J Cell Sci 116:3571–3581.

Nelson CM, Pirone DM, Tan JL, Chen CS. 2004. Vascular endothelial-cadherin regulates cytoskeletal tension, cell spreading, and focal adhesions by stimulating RhoA. Mol Biol Cell 15:2943–2953.

Niklaus AL, Aberdeen GW, Babischkin JS, Pepe GJ, Albrecht ED. 2003. Effect of estrogen on vascular endothelial growth/permeability factor expression by glandular epithelial and stromal cells in the baboon endometrium. Biol Reprod 68:1997–2004.

Papetti M, Herman IM. 2002. Mechanisms of normal and tumor-derived angiogenesis. Am J Physiol Cell Physiol 282:C947–970.

Risau W. 1997. Mechanisms of angiogenesis. Nature 386:671–674.

Rodriguez LG, Wu X, Guan JL. 2005. Wound-healing assay. Methods Mol Biol 294:23–29.

Seabra MC. 1998. Membrane association and targeting of prenylated Ras like GTPases. Cell Signal 10:167–172.

Sengupta K, Banerjee S, Saxena N, Banerjee SK. 2003. Estradiol-induced vascular endothelial growth factor-A expression in breast tumor cells is biphasic and regulated by estrogen receptor-alpha dependent pathway. Int J Oncol 22:609–614.

Vázquez F, Rodriguez-Manzaneque JC, Lydon JP, Edwards DP, O'Malley BW, Iruela-Arispe ML. 1999. Progesterone regulates proliferation of endothelial cells. J Biol Chem 274:2185–2192.

Wheeler AP, Ridley AJ. 2004. Why three Rho proteins? RhoA, RhoB, RhoC, and cell motility. Exp Cell Res 301:43–49.

Wherlock M, Gampel A, Futter C, Mellor H. 2004. Farnesyltransferase inhibitors disrupt EGF receptor traffic through modulation of the RhoB GTPase. J Cell Sci 117:3221–3231.

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article at the publisher's web-site.