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Angiotensin II-Induced Migration of Vascular Smooth Muscle Cells is Mediated by p38-MAPK Activated c-Src Through Spleen Tyrosine Kinase and EGFR Transactivation

Benon E. Mugabe, Fariborz A. Yaghini, ChiYoung Song, Cuneyt K. Buharalioglu, Christopher M. Waters and Kafait U. Malik

Department of Pharmacology, College of Medicine, University of Tennessee Health Science Center, Memphis, Tennessee 38163, USA (BEM, FAY, CS, CKB, KUM)

Department of Physiology, College of Medicine, University of Tennessee Health Science Center, Memphis, Tennessee 38163, USA (CMW)

Running title: Syk and EGFR mediate Ang II-induced migration of VSMCs

Correspondence to: Kafait U. Malik, D.Sc., Ph.D.,
Professor of Pharmacology
College of Medicine
University of Tennessee Health Science Center
Memphis, Tennessee 38163, USA.
Phone: 901-448-6075
Fax: 901-448-7206
E-mail: kmalik@utmem.edu

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Abbreviations: Syk, spleen tyrosine kinase; EGFR, epidermal growth factor receptor; VSMCs, vascular smooth muscle cells; Ang II, angiotensin II; WT, wild type; DN, dominant negative; c-Src, c-terminal non-receptor tyrosine kinase; p38 MAPK, p38 mitogen-activated protein kinase; ERK1/2, extracellular signal-regulated kinase; c-JNK, c-Jun N-terminal kinase; EGF, epidermal growth factor; Pyk2, proline-rich tyrosine kinase; Hsp27, heat shock protein 27; AT_1R, angiotensin II type 1 receptor, Crk, Ca^{2+}-dependent protein-kinase related protein kinase.

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Abstract

Angiotensin II (Ang II) stimulates protein synthesis by activating spleen tyrosine kinase (Syk), and DNA synthesis through epidermal growth factor receptor (EGFR) transactivation in vascular smooth muscle cells (VSMCs). This study was conducted to determine if Syk mediates Ang II-induced migration of aortic VSMCs using a scratch wound approach. Treatment with Ang II (200nM) for 24 h increased by 1.56 ± 0.14 fold VSMC migration. Ang II-induced VSMC migration, and Syk phosphorylation as determined by Western blot analysis, were minimized by Syk inhibitor piceatannol (10μM), and by transfecting VSMCs with dominant negative but not wild type Syk plasmid. Ang II-induced VSMC migration and Syk phosphorylation were attenuated by inhibitors of c-Src (PP2), p38 MAPK (SB202190) and ERK1/2 (U0126). SB202190 attenuated p38 MAPK and c-Src but not ERK1/2 phosphorylation indicating that p38 MAPK acts upstream of c-Src and Syk. c-Src inhibitor PP2 attenuated Syk and ERK1/2 phosphorylation suggesting that c-Src acts upstream of Syk and ERK1/2. Ang II- and EGF-induced VSMC migration and EGFR phosphorylation were inhibited by the EGFR blocker AG1478 (2μM). Neither the Syk inhibitor piceatannol nor the dominant negative Syk mutant altered EGF-induced cell migration or Ang II- and EGF-induced EGFR phosphorylation. c-Src inhibitor PP2 diminished EGF-induced VSMC migration and EGFR, ERK1/2 and p38 MAPK phosphorylation. ERK1/2 inhibitor U0126 (10μM) attenuated EGF-induced cell migration, and ERK1/2 but not EGFR phosphorylation. These data suggest that Ang II stimulates VSMC migration via p38 MAPK activated c-Src through Syk, and via EGFR transactivation through ERK1/2 and partly p38 MAPK.

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Introduction

Ang II, one of the major biologically active components of the renin-angiotensin system (RAS), produces vascular smooth muscle contraction (VSMCs) and contributes to the maintenance of sodium and water homeostasis (Touyz, 2003; Navar, 1997). The increased levels of Ang II contribute to the development of hypertension, heart failure, atherosclerosis and restenosis after vascular injury (Kobori et al, 2007; Weiss et al, 2001; Rakugi et al, 1994; Feng et al, 2001). These pathophysiological effects of Ang II have been attributed to remodeling of cardiovascular tissues consequent to cytoskeleton rearrangement, activation of inflammatory cells, and migration and growth of VSMCs and cardiac cells (de Cavanagh et al, 2009; Suzuki et al, 2003; Gibbons et al, 1994; Weber et al, 1992, Sadoshima and Izumo, 1993). The effects of Ang II on VSMC growth or migration are mediated via angiotensin 1 receptors (AT_1R) through production of reactive oxygen species and activation of one or more serine-threonine and tyrosine kinases including mitogen activated protein kinases (MAPK) (Clempus and Griendling, 2006, Touyz, 2004, Touyz and Schiffrin, 2000), extracellular signal-regulated kinase (ERK1/2), p38 mitogen-activated protein kinase (p38 MAPK) and c-Jun N-terminal kinase (c-JNK) (Servent et al, 1996; Xi et al, 1999; Natarajan et al, 1999; Lee et al, 2007; Kyaw et al, 2004; Ohtsu et al, 2005) and focal adhesion kinase family protein tyrosine kinase, proline-rich tyrosine kinase 2 (Pyk2), and c-Src, respectively (Kyaw et al, 2004; Rocic and Lucchesi, 2001; Eguchi et al, 1999; Touyz et al, 2001; Eguchi et al, 1998; Eguchi et al, 2001; Saito et al, 2002).

Ang II-induced VSMC migration by ERK1/2 activation has been reported to be mediated by c-Src (Kyaw et al, 2004). However, Ang II via c-Src stimulates Ca^{2+}-dependent protein-kinase related protein kinase (Crk) associated substrate (Cas) that promotes VSMC migration by

activating c-JNK (Kyaw et al, 2004). p38-MAPK also contributes to Ang II-induced migration of VSMCs via phosphorylation of Hsp27 (Lee et al, 2007). Involvement of a metalloprotease that stimulates the release of heparin-binding EGF from its precursor has been implicated in Ang II-induced VSMC migration through transactivation of EGFR (Saito et al, 2002). Ang II via transactivation of epidermal growth factor receptor (EGFR) results in increased activity of ERK1/2 and p38 MAPK but not c-JNK activity in VSMCs (Eguchi et al, 2001). Pyk2 activated c-Src has been proposed to promote EGFR transactivation by Ang II in VSMCs (Eguchi et al, 1999). Recently, we have shown that Ang II activates c-Src through p38 MAPK that in turn increases the activity of spleen tyrosine kinase (Syk) and results in increased protein synthesis, whereas Ang II stimulates DNA synthesis via transactivation of EGFR (Yaghini et al, 2007). The present study was conducted to determine if Ang II stimulated VSMC migration is mediated by p38 MAPK activated c-Src via Syk activation and/or EGFR transactivation. The results of this study show that Ang II-induced VSMC migration is mediated by p38 MAPK activated c-Src via both Syk activation and EGFR transactivation.

Methods

Materials. Phospho-Syk (Tyr-525/526), phospho-Src (Tyr-416), phospho-p44/42 MAP kinase, phospho-Tyrosine, Syk, Src, Tyrosine, p44/42 MAP kinase and p38 MAP kinase antibodies were obtained from Cell Signaling (Beverly, MA). Anti-α-actin smooth muscle antibody and tyrphostin AG1478 (4-(3-Chloroanilino)-6,7-dimethoxyquinazoline) were purchased from Sigma-Aldrich (St. Louise, MO). Secondary rabbit or mouse IgG antibodies were obtained from Amersham Biosciences (GE Healthcare, Buckinghamshire, UK). Human Angiotensin II (H-Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-OH) and EGF were purchased from Bachem (King of Prussia, PA). Piceatannol (*trans*-3, 3', 4,5'-Tetrahydroxystilbene), SB202190 (4-(4-Fluorophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl)1H-imidazole, UO126 (1,4-Diamino-2,3-dicyano-1,4-*bis*(2-aminophenylthio)butadiene) and PP2 (4-Amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-d]pyrimidine) were from Calbiochem (San Diego, CA). Fetal bovine serum (FBS), cell medium M-199, penicillin/streptomycin, amphotericin B and trypsin were obtained from Mediatech Inc. (Manassas, VA). Bio-Rad Protein assay dye was from Bio-Rad Laboratories (Hercules, CA) and SuperSignal WestPico chemiluminescent substrate was purchased from Pierce Biotechnology Inc. (Rockford, IL).

Culture of VSMCs. All experiments were performed according to the protocols approved by our Institutional Animal Care and Use Committee in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Male Sprague-Dawley rats (Harlan, Indianapolis, IN) weighing 250–350 g were anesthetized with sodium pentobarbital (Abbott, North Chicago, IL) and their thoracic aortas were rapidly excised. VSMCs were isolated and cultured as described (Uddin et al, 1998). Cultured cells were maintained under

5% CO_2 in medium M-199 with 10% fetal bovine serum, 1% penicillin/streptomycin, and 0.01% amphotericin B at 37 °C. Cells between 4 and 8 passages were made quiescent for 24 hr with serum free medium M-199 before exposure to various inhibitors in all experiments. Trypan blue exclusion assay revealed no changes in viability of VSMCs after treatment with inhibitors, Ang II, EGF or transfection with wild type or dominant negative Syk plasmids.

Cell Migration Assay (Wound Healing Technique). VSMC migration was determined in 6-well plates using the scratch wound approach as described (Waters and Savla, 1995). Briefly, 80-100 % confluent VSMCs plates were scraped with a sterile plastic pipette tip across the diameter of each well to produce 1-1.5 mm wide wounds. Cells were rinsed twice with serum free M199 medium to remove cellular debris, and images were obtained at the initial time of wounding (0 hr) using a Nikon TE300 inverted microscope equipped with a CoolSnap FX charge-coupled device camera (Roper Scientific, Trenton, NJ), Optiscan ES102 motorized stage system (Prior Scientific, Rockland, MA) and MetaMorph image analysis software (Universal Imaging, Dowingtown, PA). Cells were then treated with various inhibitors or their vehicles for 30 min and then exposed to Ang II (200nM), EGF (100ng/ml) or their vehicle. Images were collected by programming the X, Y, and Z coordinates for each wound location, which enabled the stage to return to the exact location of the original wound throughout the migration experiments. Wound area measurements were averaged from three fields of the same well, using NIH ImageJ 1.6 program and obtained mean values were taken as single data points. Data was presented as the ratio of the difference in the area covered by the cells at 0 and 24 hr to the area at 0 hr as 100 percent (control). The effect of various agents on the areas covered by cells were calculated and presented as percent of change compared to control.

Transfection of VSMC with Plasmids. All transfection experiments were performed according to the procedure previously described (Yaghini et al, 2007). Subconfluent VSMCs were transiently transfected with empty vector (pALTER®MAX) alone, wild type (WT), or dominant negative (DN; Y525F/Y526F) Syk (kindly provided by Dr. H. Band, Harvard Medical School, Boston, MA) using Effectene transfection reagent (Qiagen, Valencia, CA) in a ratio of 25 µl of Effectene to 1 µg of plasmid in M-199 medium containing 5% FBS for 48 hr according to manufacturer's instructions. For migration assays, the transfected cells in 6-well plates were scraped with a sterile plastic pipette tip across the diameter of each well to produce wounds of 1-1.5 mm in width, then the wells were washed and wounded area measured by microscopy at 0 hr and after 24 hr of treatment with Ang II or its vehicle. To determine the activity of various kinases by measuring their phosphorylation, another set of transfected cells were exposed to Ang II (200 nM) or EGF (100ng/ml) or its vehicle for 10 min. Then the cells were scraped, washed with PBS, lysed and subjected to SDS-PAGE and Western blot analysis.

Western blotting. VSMCs were dispersed into lysis buffer [1% IGEPAL CA-630, 1 M Tris, 1 M NaCl, 2.5 mg/ml deoxycholic acid, 1 M EDTA, 1 mM NaF, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 1 mg/ml p-nitrophenyl phosphate, 10 µg/ml aprotinin, and 10 µg/ml leupeptin]. The lysed cells sonicated, centrifuged and protein concentration was determined using Bradford assay. Equal amounts of protein (20-40 µg) were resolved in 8 % Tris-glycine SDS-PAGE and transferred onto nitrocellulose membranes. The blots were then blocked with 5% non-fat milk in TBST (1X) and incubated with primary antibodies (1:500-1:1000) overnight at 4°C. Blots were then washed with TBST (1X), exposed to their respective secondary antibodies (1:1000-1:2000) and developed using Supersignal WestPico

chemiluminescent substrate. Densitometric analysis of phosphorylated and nonphosphorylated protein bands was performed using NIH ImageJ software. The ratios of phosphorylated to nonphosphorylated protein bands from three experiments were determined and then normalized with that obtained with vehicle or inhibitor in the presence or absence of Ang II or EGF.

Statistical Analysis. The data were analyzed by one-way analysis of variance (ANOVA); the unpaired Student's *t* test was used to determine the difference between Ang II and EGF treated and untreated groups in presence or absence of inhibitors. Comparisons between mean values were made using ANOVA and Newman-Keuls multiple comparison test with the values of at least three different experiments for each treatment performed on different batches of cells (prepared from six animals) and expressed as the mean ± S.E, $p < 0.05$ was considered as statistically significant.

Results

Angiotensin II stimulated VSMC migration is mediated by Syk through p38 MAPK activated c-Src. Previously, we reported that Syk is expressed in VSMCs and is activated by Ang II through p38 MAPK activated c-Src and mediates the effect of Ang II on protein synthesis (Yaghini et al, 2007). On the other hand, Ang II-induced DNA synthesis was mediated through EGFR transactivation (Yaghini et al, 2007). To determine if p38 MAPK activated c-Src mediates VSMC migration via Syk and/or EGFR transactivation, we examined the effect of Syk, c-Src and p38 MAPK inhibitors on VSMC migration and on phosphorylation of these kinases on Ang II- and EGF-induced VSMC migration by using wound healing method. Ang II (200 nM) produced a time-dependent increase in wound healing of VSMCs. However, the maximal increase (1.56 ± 0.14 fold) in wound healing of VSMCs was observed at 24 hr and this increase was proportionately similar at 48 and 72 hr compared to basal levels. Therefore, the effect of all inhibitors was determined on Ang II-induced wound healing after 24 hr in subsequent experiments.

Treatment of VSMCs with Syk inhibitor piceatannol (10 μM) significantly reduced Ang II (200 nM) induced wound healing of VSMCs (Figure 1A). Ang II induced increase in phosphorylation of Syk and ERK1/2 (2.64 $\pm$ 0.52- and 3.66 ± 0.69- fold respectively), was inhibited by Piceatannol (10 μM) (1.42 $\pm$ 0.22- and 2.88 ± 0.79- fold, respectively) ($p < 0.05$). However, Ang II induced phosphorylation of c-Src and p38 MAPK (1.74 $\pm$ 0.21- fold and 6.32 $\pm$ 1.44- fold respectively), was not inhibited by Piceatannol (10 μM) (1.73 $\pm$ 0.11-, 5.43 $\pm$ 0.17- fold, respectively) indicating that Syk is downstream of c-Src and p38 MAPK. A representative blot is shown in Figure 1B. To further assess the role of Syk in Ang II induced

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VSMC migration, 60 - 70% confluent quiescent VSMC were transiently transfected with WT or DN Syk mutant for 48 hr using Effectene reagent, as described under "Material and Methods". Transient transfection of VSMCs with DN Syk but not WT Syk inhibited the significant increase ($p < 0.05$) in Ang II-induced wound healing of VSMCs (Figure 2A) and phosphorylation of Syk from 2.67 $\pm$ 0.32- fold from basal (absence of Ang II) to 0.88 $\pm$ 0.19 – fold ($p < 0.05$), but did not alter the significant increase in phosphorylation ($p < 0.05$) of c-Src, ERK1/2 or p38 MAPK (2.76 $\pm$ 0.79-, 2.52 $\pm$ 0.09-, 5.99 $\pm$ 1.44-fold, to 3.13 $\pm$ 1.67-, 2.38 $\pm$ 0.62-, 5.04 $\pm$ 0.91- fold, respectively). A representative blot is shown in Figure 2 B.

The inhibitor of the *Src* family of tyrosine kinases, PP2 (5 and 10 μM), reduced the basal and blocked Ang II-induced wound healing of VSMCs (Figure 3A) and also inhibited the significant increase ($p < 0.05$) in phosphorylation of Syk, c-Src and ERK1/2 from 2.5 $\pm$ 0.3-, 2.51 $\pm$ 0.52-, and 1.75 $\pm$ 0.16-fold above basal (absence of Ang II) to 1.4 $\pm$ 0.25-, 1.18 $\pm$ 0.24-, and 1.18 $\pm$ 0.1 – fold respectively ($p < 0.05$) but not that of p38 MAPK (4.40 $\pm$ 1.37- to 3.66 $\pm$ 0.18- fold), implying that p38 MAPK acts upstream of Src and Syk. A representative blot is shown in Figure 3 B).

The p38 MAPK inhibitor SB202190 (10 and 20 μM) abolished the Ang II induced wound healing of VSMCs (Figure 4A) and attenuated the significant increase ($p < 0.05$) in phosphorylation of Syk, c-Src and p38 MAPK from 2.43 $\pm$ 0.23-, 2.28 $\pm$ 0.42-, 4.7 $\pm$ 0.69-fold above basal (absence of Ang II) to 1.66 $\pm$ 0.23-, 1.57 $\pm$ 0.49-, and 1.97 $\pm$ 0.64-fold, respectively ($p < 0.05$), indicating that Syk and c-Src participate in VSMC migration and are downstream of p38 MAPK. SB202190 did not inhibit the phosphorylation of ERK1/2 elicited by Ang II (2.89 $\pm$ 0.55- to 3.01 $\pm$ 0.29-fold). A representative blot is shown in Figure 4B. The

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ERK1/2 inhibitor, U0126, blocked Ang II-induced wound healing of VSMCs but did not alter phosphorylation of Syk, c-Src or p38 MAPK indicating it acts downstream of these kinases (data not shown).

Ang II-induced VSMC migration stimulated by p38 MAPK activated c-Src is also mediated via EGFR transactivation independent of Syk. Ang II stimulates transactivation of EGFR that results in an increase in p38 MAPK and ERK1/2 activity (Eguchi et al, 1998), and EGFR activation results in VSMC migration (Saito et al, 2002). We have shown that Ang II-induced EGFR transactivation is mediated via p38 MAPK (Yaghini et al, 2007) and Ang II is known to promote association of c-Src with EGFR (Eguchi et al, 1999) and c-Src phosphorylates EGFR (Stover et al, 1995). Therefore, we determined if p38 MAPK-activated c-Src promotes VSMC migration through EGFR by a mechanism independent or dependent upon Syk. Ang II-induced VSMC wound healing was blocked by the EGFR inhibitor, AG 1478 (Figure 5A). This agent also inhibited Ang II-induced increase ($p < 0.05$) in phosphorylation of EGFR, ERK1/2 and p38 MAPK from 4.94 $\pm$ 1.96-, 3.82 $\pm$ 1.0-, and 5.24 $\pm$ 1.18- fold above basal (absence of Ang II) to 2.71 $\pm$ 0.86-, 0.89 $\pm$ 0.42-, and 1.56 $\pm$ 0.42-fold, respectively ($p < 0.05$) but not phosphorylation of Syk or c-Src (1.82 $\pm$ 0.35-, 2.79 $\pm$ 0.75-fold, to 1.78 $\pm$ 0.33-, 2.60 $\pm$ 0.30-fold, respectively). A representative blot is shown in Figure 5B. Ang II-induced EGFR transactivation is known to cause activation of ERK1/2 and that results in VSMC migration (Eguchi et al. 2001; Saito et al, 2002 and Kyaw et al, 2004). We confirmed this observation in this study. ERK1/2 inhibitor U0126 abolished Ang II-induced wound healing of VSMCs and inhibited phosphorylation of ERK1/2 but not that of Syk, EGFR, c-Src or p38 MAPK (data not shown).

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To further determine if Ang II-induced VSMC migration is mediated by p38 MAPK activated c-Src via Syk through EGFR transactivation, we examined the effect of Syk inhibitor piceatannol on the action of EGF on wound healing in VSMCs. EGF caused a 2.48 ± 0.49-fold increase in wound healing of VSMCs which was inhibited by AG 1478 (Figure 6A). EGF increased phosphorylation of EGFR, ERK1/2 and p38 MAPK (2.4 ± 0.41-, 2.83 ± 0.51-, and 6.6 ± 1.82-fold above basal (absence of EGF), respectively ($p < 0.05$) but not c-Src and Syk (Yaghini et al, 2007). AG 1478 inhibited EGF-induced phosphorylation of EGFR, ERK1/2 and p38 MAPK to 1.35 ± 0.38-, 1.08 ± 0.09-, and 2.08 ± 0.32-fold, respectively ($p < 0.05$). A representative blot is shown in Figure 6B.

The inhibitor of Syk piceatannol did not alter EGF-induced wound healing (Figure 7A) or the increase ($p < 0.05$) in phosphorylation of EGFR, c-Src, ERK1/2 or p38 MAPK from (2.15 ± 0.37-, 2.44 ± 0.56-, 4.51 ± 0.76-, 7.08 ± 0.35- fold, to 2.16 ± 0.24- 2.27 ± 0.51-, 4.83 ± 1.32-, 7.15 ± 2.11-fold, respectively) above basal (absence of EGF), respectively. A representative blot is shown in Figure 7B. DN Syk mutant also did not alter EGF-induced VSMC wound healing or EGF-induced phosphorylation of p38 MAPK or ERK1/2 (data not shown). These results indicate that VSMC migration by EGF through EGFR is mediated by p38 MAPK and ERK1/2 and is independent of c-Src and Syk.

c-Src inhibitor PP2 abolished EGF-induced wound healing of VSMCs (Figure 8A) and phosphorylation of EGFR from 2.25 ± 0.21-fold to 1.27 ± 0.58-fold ($p < 0.05$). EGF did not increase phosphorylation of c-Src, and PP2 did not alter EGF-induced increase in phosphorylation of p38 MAPK and ERK1/2 (3.37 ± 1.28-, 2.54 ± 0.97-fold to 3.15 ± 1.17-, 2.93 ± 1.37-fold, respectively). A representative blot is shown in Figure 8B. The inhibitor of

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p38 MAPK SB202190 partially inhibited EGF-induced wound healing of VSMCs (Figure 9A) and reduced phosphorylation of p38 MAPK from 10.35 ± 3.88-fold above basal (absence of EGF) to 5.65 ± 1.48-fold ($p < 0.05$) but not that of EGFR, c-Src or ERK1/2 (1.96 ± 0.16-, 1.69 ± 0.51-, 5.47 ± 1.53-fold to 1.81 ± 0.11-, 1.70 ± 0.47-, 4.91 ± 1.09-fold, respectively) caused by EGF. A representative blot is shown in Figure 9B. The ERK1/2 inhibitor U0126 blocked EGFR-induced wound healing of VSMCs and ERK1/2 phosphorylation but not that of Syk, EGFR, c-Src or p38 MAPK (data not shown). These results indicate that p38 MAPK activated c-Src plays a dual role in the action of Ang II to promote wound healing of VSMCs via activation of Syk as well as via EGFR transactivation through ERK1/2 and partly by p38 MAPK activation (Figure 10).

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Discussion

We have previously reported that p38 MAPK activated c-Src promotes VSMC protein synthesis via activation of Syk and DNA synthesis via EGFR transactivation (Yaghini et al, 2007). This study was conducted to determine the contribution of Syk to Ang II-induced VSMC migration, and demonstrates that Ang II promotes VSMC migration through p38 MAPK stimulated c-Src via Syk activation as well as via EGFR transactivation through ERK1/2 and partly by p38 MAPK activation.

Contribution of Syk to Ang II-induced VSMC migration

The conclusion that Syk mediates Ang II-induced VSMC migration, examined by the scratch wound method, was our demonstration that the effect of this peptide to stimulate VSMC migration was minimized by the Syk inhibitor piceatannol and in VSMC transfected with DN Syk but not WT mutant. Since piceatannol and the DN Syk mutant, but not WT Syk, attenuated Syk phosphorylation and not that of p38 MAPK and c-Src, it appears that Syk acts downstream of p38 MAPK and c-Src in stimulating VSMC migration in response to Ang II. Piceatannol reduced Ang II-induced phosphorylation of ERK1/2, suggesting that ERK1/2 is activated by Syk. However, it is unlikely, because in cells transfected with the DN Syk mutant Ang II-induced Syk phosphorylation was not altered, indicating that piceatannol reduces ERK1/2 activity by a nonspecific mechanism. Ang II-induced VSMC wound healing by Syk is mediated through c-Src activation because the c-Src inhibitor PP2 blocked Ang II-induced migration of VSMCs and phosphorylation of Syk. Src-family tyrosine kinases are known to associate with- and activate Syk (Kurosaki et al, 1994). Since the p38 MAPK inhibitor SB202190 blocked

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Ang II-induced wound healing and phosphorylation of c-Src and Syk but not ERK1/2, it appears that p38 MAPK mediates Ang II-induced VSMC migration by c-Src activated Syk. The mechanism by which p38 MAPK activates c-Src is not known. p38 MAPK does not appear to activate c-Src directly because: a) p38 MAPK does not associate with c-Src as determined by co-immunoprecipitation and b) p38 MAPK immunoprecipitated from Ang II stimulated VSMCs with phospho p38 MAPK did not phosphorylate c-Src in an *in vitro* kinase assay (Yaghini et al, our unpublished data). Whether an intermediary kinase is involved in activation of p38 MAPK by c-Src or it requires one or more scaffolding molecules for the productive interaction of p38 MAPK and c-Src remains to be determined. c-Src is known to mediate Ang II-induced VSMC migration via activation of ERK1/2 (Kyaw et al, 2004). In the present study the ERK1/2 inhibitor also blocked Ang II-induced VSMC wound healing, and the c-Src inhibitor PP2 attenuated ERK1/2 phosphorylation. However, the DN Syk mutant did not alter ERK1/2 phosphorylation, indicating that c-Src activated ERK1/2 mediates Ang II-induced VSMC migration by a mechanism independent of Syk.

Contribution of EGFR to Ang II-induced VSMC migration

Ang II is known to cause EGFR transactivation that results in activation of ERK1/2 and p38 MAPK (Eguchi et al, 1998). EGFR transactivation by Ang II is also dependent on c-Src (Eguchi et al, 1999; Touyz et al, 2002). Therefore, it is possible that c-Src might result in activation of Syk and ERK1/2 and p38 MAPK through EGFR transactivation. Our demonstration that the c-Src inhibitor PP2, but not piceatannol or DN Syk mutant, attenuated Ang II-induced EGFR phosphorylation or EGF-induced wound healing of VSMCs and EGFR phosphorylation suggest that c-Src mediates Ang II-induced EGFR

transactivation and VSMC migration independent of Syk. Since the EGFR blocker AG1478, attenuated EGFR phosphorylation and inhibited Ang II- and EGF-induced wound healing of VSMCs, and that EGF did not alter c-Src phosphorylation, suggest that c-Src via EGFR transactivation also mediates Ang II-induced VSMC migration independent of Syk. Our finding that the p38 MAPK inhibitor SB202190 attenuated c-Src and EGFR phosphorylation suggest that p38 MAPK activated c-Src plays a dual role in the action of Ang II to promote VSMC migration via activation of Syk as well as EGFR transactivation. The effect of Ang II to promote VSMC migration through EGFR transactivtion is most likely mediated via ERK1/2 and p38 MAPK because the effect of Ang II as well as EGF to stimulate wound healing of VSMCs and ERK1/2 phosphorylation was inhibited by the ERK1/2 inhibitor U0126. The inhibitor of p38 MAPK SB202190, which blocked Ang II-induced wound healing and phosphorylation of p38 MAPK, only partially reduced EGF-induced wound healing and phosphorylation of p38 MAPK. Therefore, it appears that p38 MAPK that activates Syk via c-Src is more effective than p38 MAPK that is activated through EGFR transactivation via c-Src in stimulating VSMC migration in response to Ang II. Since EGFR blocker AG 1478 that inhibited EGF-induced p38 MAPK phosphorylation did not alter Syk phosphorylation elicited by Ang II, it appears that p38 MAPK activated by EGF is not involved in Syk phosphorylation. Four isoforms of p38 MAPK, p38α, p38β, p38γ and p38δ have been identified in various tissues and are differentially activated depending on the stimulus and signal strength (Conrad et al, 1999; Alonso et al, 2000; Hale et al, 1999). Therefore, it is possible that EGF activates a different isoform of p38 MAPK that is not involved in activation of Syk in response to Ang II VSMCs. Further studies on the activation of

different isoforms of p38 MAPK by Ang II and EGF and their selective inhibition by their respective siRNA on Syk activation would be required to address this issue.

Our results clearly demonstrate two independent pathways for Ang II-induced migration of VSMCs mediated by p38 MAPK activated c-Src pathways, one mediated through Syk and another through EGF pathway. Since inhibition of each pathway blocked Ang II-induced VSMC migration, it appears that these are redundant pathways that do not appear to converge. Further studies on the transcription level could allow determining the point of convergence of these distinct pathways that lead to Ang II-induced VSMC migration.

In conclusion, this study demonstrates that Ang II stimulates VSMC migration via p38 MAPK activated c-Src through two different redundant pathways, one through activation of Syk and another via EGFR transactivation through activation of ERK1/2 and partly through p38 MAPK (Figure 10).

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References

Alonso G, Ambrosino C, Jones M, and Nebreda**,** AR (2000) Differential activation of p38 mitogen-activated protein kinase isoforms depending on signal strength. *J Biol Chem* **275**: 40641-40648.

Brilla CG, Maisch B, Rupp H, Funck R, Zhou G, and Weber KT (1995) Pharmacological modulation of cardiac fibroblast function. *Herz* **20**: 127-134.

Clempus RE, and Griendling KK (2006) Reactive oxygen species signaling in vascular smooth muscle cells. *Cardiovasc Res* **71**: 216-225.

Conrad PW, Rust R, Han J, Millhorn DE, and Beitner-Johnson D (1999) Selective activation of p38 alpha and p38 gama by hypoxia: Role in regulation of cyclin D1 by hypoxia in PC12 cells. *J Biol Chem* **274**, 23570 –23576.

de Cavanagh EM, Ferder M, Inserra F, and Ferder L (2009) Angiotensin II, mitochondria, cytoskeletal and extracellular matrix connections: an integrating viewpoint. *Am J Physiol Heart Circ Physiol* **296**: H550-558.

Eguchi S, Dempsey PJ, Krank GD, Motley ED, and Inagami T (2001) Activation of MAP kinases by angiotensin II in vascular smooth muscle cells: Metalloprotease-dependent EGF receptor activation is required for activation of ERK and p38 MAP kinase but not JNK. *J Biol Chem* **276**: 7957-7962.

Eguchi S, Iwasaki H, Inagami T, Numaguchi K, Yamakawa T, Motley ED, Owada KM, Marumo F, and Hirata Y (1999) Involvement of PYK2 in angiotensin II signaling of vascular smooth muscle cells. *Hypertension* **33**: 2010-2016.

Eguchi S, Numaguchi K, Iwasaki H, Matsumoto T, Yamakawa T, Utsunomiya H, Motley ED, Kawakatsu H, Owada KM, Hirata Y, Marumo F, and Inagami T (1998) Calcium-dependent epidermal growth factor receptor transactivation mediates the angiotensin II-induced mitogen-activated protein kinase activation in vascular smooth muscle cells. *J Biol Chem* **273**: 8890-8896.

Feng TC, Ying WY, Hua RJ, Ji YY, and de Gasparo M (2001) Effect of valsartan and captopril in rabbit carotid injury. Possible involvement of bradykinin in the antiproliferative action of the renin-angiotensin blockade. *J Renin Angiotensin Aldosterone Syst* **2**: 19-24.

Gibbons GH, Pratt RE, and Dzau VJ (1992) Vascular smooth muscle cell hypertrophy vs. hyperplasia: autocrine transforming growth factor-1 expression determines growth response to angiotensin II. *J Clin Invest* **90**: 456 – 461.

Hale KK, Trollinger D, Rihanek M, and Manthey C L (1999) Differential expression and activation of p38 mitogen-activated protein kinase α, β, γ, and δ in inflammatory cell lineages. *J Immunol* **162**: 4246-4252.

Kobori, H, Nangaku M, Navar, LG, and Nishiyama A (2007) The intrarenal renin-angiotensin system: From physiology to the pathophysiology of hypertension and kidney disease. *Pharmacological Rev* **59**: 251-287.

Kurosaki T, Takata M, Yamanashi Y, Inazu T, Taniguchi T, Yamamoto T, and Yamamural, H (1994) Syk activation by the Src-family tyrosine kinase in the B cell receptor signaling. *J Exp Med.* **179**: 1725-1729.

Kyaw M, Yoshizumi M, Tsuchiya K, Kagami S, Izawa Y, Fujita Y, Ali N, Kanematsu Y, Toida K, Ishimura K, and Tamaki T (2004) Src and Cas are essentially but differentially involved in angiotensin II-stimulated migration of vascular smooth muscle cells via extracellular signal-regulated kinase 1/2 and c-Jun NH2-terminal kinase activation. *Mol*

Pharmacol **65**: 832-841.

Lee HM, Lee C-K, Lee SH, Roh HY, Bae YM, Lee K-Y, Lim J, Park P-J, Park T-K, Lee YL, Won K-J, and Kim B (2007) p38 Mitogen-activated protein kinase contributes to angiotensin II-stimulated migration of rat aortic smooth muscle cells. *J Pharmacol Sci* **105**: 74-81.

Natarajan R, Scott S, Bai W, Yerneni KK, and Nadler J (1999) Angiotensin II signaling in vascular smooth muscle cells under high glucose conditions. *Hypertension* **33**: 378-884.

Navar LG (1997) The kidney in blood pressure regulation and development of hypertension. *Med Clin North Am* **81**: 1165-1198.

Ohtsu H, Mifune M, Frank GD, Saito S, Inagami T, Kim-Mitsuyama S, Takuwa Y, Sasaki T, Rothstein JD, Suzuki H, Nakashima H, Woolfolk EA, Motley ED, and Eguchi S (2005) Signal-crosstalk between Rho/ROCK and c-Jun NH2-terminal kinase mediates migration of vascular smooth muscle cells stimulated by angiotensin II. *Arterioscler Thromb Vasc Biol* **25**: 1831-1836.

Rakugi H, Wang DS, Dzau VJ, and Pratt RE (1994) Potential importance of tissue angiotensin-converting enzyme inhibition in preventing neointima formation. *Circulation* **90**: 449-455.

Rocic P and Lucchesi PA (2001) Down-regulation by antisense oligonucleotides establishes a role for the proline-rich tyrosine kinase PYK2 in angiotensin II-induced signaling in vascular smooth muscle. *J Biol Chem* **276**: 21902-21906.

Sadoshima J, and Izumo S (1993) Molecular characterization of angiotensin II-induced hypertrophy of cardiac myocytes and hyperplasia of cardiac fibroblasts: critical role of the AT1 receptor subtype. *Circ Res* **73**: 413-423.

Saito S, Frank GD, Motley ED, Dempsey PJ, Utsunomiya H, Inagami T, and Eguchi S (2002) Metalloprotease inhibitor blocks angiotensin II-induced migration through inhibition of epidermal growth factor receptor transactivation. *Biochemical and Biophysical Research Communications* **294**: 1023-1029.

Servant MJ, Giasson E, and Meloche S (1996) Inhibition of growth factor-induced protein synthesis by a selective MEK inhibitor in aortic smooth muscle cells. *J Biol Chem* **271**: 16047-16052.

Stover DR, Becker M, Liebetanz J, and Lydon NB (1995) Src phosphorylation of the epidermal growth factor receptor at novel sites mediates receptor interaction with Src and P85α. J *Biol Chemi* **270**: 15591-15597.

Suzuki Y, Ruiz-Ortega M, Lorenzo O, Ruperez M, Esteban V, and Egido J (2003) Inflammation and angiotensin II. *Int J Biochem. Cell Biol* **35**: 881-900.

Touyz RM (2003) The role of angiotensin II in regulating vascular structural and functional changes in hypertension. *Curr Hypertens Rep* **5**: 55-164.

Touyz RM (2004) Reactive oxygen species and angiotensin II signaling in vascular cells - implications in cardiovascular disease. *Braz J Med Biol Res* **37**: 1263-1273.

Touyz RM, He G, Wu XH, Park JB, Mabrouk ME, and Schiffrin EL (2001) Src is an important mediator of extracellular signal-regulated kinase 1/2-dependent growth signaling by angiotensin II in smooth muscle cells from resistance arteries of hypertensive patients. *Hypertension* **38**: 56-64.

Touyz RM and Schiffrin EL (2000) Signal transduction mechanisms mediating the physiological and pathophysiological actions of angiotensin II in vascular smooth muscle cells. *Pharmacol Rev* **52**: 639-672.

Touyz RM, Wu XH, He G, Salomon S, and Schiffrin EL (2002) Increased angiotensin II-mediated Src signaling via epidermal growth factor receptor transactivation is associated with decreased C-terminal Src kinase activity in vascular smooth muscle cells from spontaneously hypertensive rats. *Hypertension* **39**: 479-85.

Uddin MR, Muthalif MM, Karzoun NA, Benter IF, and Malik KU (1998) Cytochrome P-450 metabolites mediate norepinephrine-induced mitogenic signaling. *Hypertension* **31**: 242-247.

Waters CM and Savla U (1991) Keratinocyte growth factor accelerates wound closure in airway epithelium during cyclic mechanical strain. *J Cel Physio* **181**: 424-432.

Weber H, Taylor DS, and Molloy CJ (1994) Angiotensin II induces delayed mitogenesis and cellular proliferation in rat aortic smooth muscle cells: correlation with the expression of specific endogenous growth factors and reversal by suramin. *J Clin Invest* **93**: 788 –798.

Weiss D, Sorescu D, and Taylor WR (2001) Angiotensin II and atherosclerosis. *Am J Cardiol* **87**: 25C-32C.

Xi XP, Graf K, Goetze S, Fleck E, Hsueh WA, and Law RE (1999) Central role of the MAPK pathway in ang II-mediated DNA synthesis and migration in rat vascular smooth muscle cells. *Arterioscler Thromb Vasc Biol* **19**: 73-82.

Yaghini AF, Li F, and Malik KU (2007) Expression and mechanism of spleen tyrosine kinase activation by angiotensin II and its implication in protein synthesis in rat vascular smooth muscle cells. *J Biol Chem* **282**: 16878-16890.

Footnotes

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Legends for figures

Figure 1. Piceatannol inhibits angiotensin II (Ang II)-stimulated VSMC migration. A. Subconfluent VSMCs were washed twice with serum-free M199 medium, wounded by scraping, images taken at the initial time of wounding (0 hr) using a microscope followed by treatment with piceatannol (PIC) (5 and 10 μM) for 30 min. Cells were then treated with Ang II (200nM) and after 24 hr, images were again taken with the same microscope equipped with a camera and MetaMorph image analysis software. The remaining wound area was quantified and the results are represented as relative cell migration versus control from three different batches of cells (prepared from 6 animals). Values are means $\pm$ SE. * Denotes value significantly different from the corresponding value obtained in absence of Ang II ($p < 0.05$). † Denotes value significantly different from the corresponding value obtained in presence of vehicle (Veh) ($p < 0.05$). **B.** Quiescent VSMCs were treated with and without the Syk inhibitor PIC (5 and 10 μM) for 30 min and then stimulated with Ang II (200nM) for 10 min. An equal amount of protein from each treatment was analyzed by Western blotting for p-Syk, p-Src, p-ERK1/2 and p-p38 MAPK using their phospho- and nonphospho-specific antibodies. The representative blots of three experiments are shown.

Figure 2. Transient transfection of VSMCs with DN Syk plasmid inhibits Ang II-induced VSMC migration. A. Confluent VSMCs (60-80%) were transiently transfected with wild type (WT) or dominant negative (DN) Syk for 48 hr, and then starved overnight before treatment with Ang II. Plasmid transfected VSMCs were washed twice with serum-free M199 media, wounded by scraping, and images taken at the initial time of wounding (0 hr) using a microscope. Cells were then stimulated with Ang II (200nM) and after 24 hr; images were

taken and quantitated as described in the legend of Figure 1. * Denotes value significantly different from the corresponding value obtained in presence of Ang II ($p < 0.05$). **B.** Quiescent VSMCs were transiently transfected with WT or DN Syk mutant and were treated with Ang II and analyzed by Western immunoblotting for p-Syk, p-Src, p-ERK1/2 and p-p38 MAPK using their phospho- and nonphospho-specific antibodies as described in legend of Figure 1. The representative blots of three experiments are shown.

Figure 3. Angiotensin II-induced VSMC migration is c-Src dependent. A. Confluent VSMCs grown in 6-well dishes starved for 24 hr, were wounded and images taken as described above in the legend of Figure 1, and treated with the c-Src inhibitor PP2 (5 and 10 μM) for 30 min and then treated with Ang II (200nM) and after 24 hr, images were again taken and the remaining wound area was quantitated and results presented as described in Legend for Figure 1. Values are means ± SE. * Denotes value significantly different from the corresponding value obtained in presence of Ang II ($p < 0.05$). † Denotes values significantly different from the corresponding value obtained in presence of vehicle (Veh) ($p < 0.05$). **B.** Quiescent VSMCs were treated with and without the c-Src inhibitor PP2 (5 and 10 μM) for 30 min and then stimulated with Ang II (200nM) for 10 min. An equal amount of protein from each treatment was analyzed by Western blotting for p-Tyr (p-EGFR), p-Syk, p-Src, p-ERK1/2 and p-p38 MAPK using their phospho- and nonphospho-specific antibodies. The representative blots of three experiments are shown.

Figure 4. Angiotensin II-induced VSMC migration is mediated by p38 MAPK. A. Confluent VSMCs were wounded and field images were taken as described in Legend for Figure 1 and then treated with p38 MAPK inhibitor SB202190 (SB, 10 and 20 μM) for 30

min. Cells were then exposed to Ang II (200nM) and after 24 hr, images were again and the wounded area quntitated as described in Methods section Values are means ± SE. * Denotes value significantly different from the corresponding value obtained in presence of Ang II ($p < 0.05$). † denote values significantly different from the corresponding value obtained in presence of vehicle (Veh) ($p < 0.05$). **B.** Quiescent VSMCs were treated with and without p38 MAPK inhibitor SB202190 (SB, 10 and 20 μM) for 30 min and then stimulated with Ang II (200nM) for 10 min and analyzed by Western blotting for p-Syk, p-Src, p-ERK1/2 and p-p38 MAPK using their phospho- and nonphospho-specific antibodies as shown in the representative blots of three experiments.

Figure 5. Angiotensin II-induced migration of VSMCs is dependent on c-Src induced EGFR transactivation. A. Confluent VSMCs prepared and wounded and field images were taken as described in Legend for Figure 1. Cells were then treated with EGFR blocker Tryphostin AG 1478 (1 and 2 μM) for 30 min and then stimulated with Ang II (200nM) for 24 hr, after which images were again taken and the remaining wound area was quantitated as described in Methods section. Values are means ± SE. * Denotes value significantly different from the corresponding value obtained in the presence of Ang II ($p < 0.05$). **B.** VSMCs were treated with and without EGFR blocker Tryphostin AG1478 (1 and 2 μM) for 30 min and then stimulated with Ang II (200nM) for 10 min. Whole cell lysates with equal amount of protein from each treatment were analyzed by Western blotting for p-Tyr (p-EGFR), p-Syk, p-Src, p-ERK1/2 and p-p38 MAPK using their phospho- and nonphospho-specific antibodies. Representative blots of three experiments are shown.

Figure 6. EGF induced migration of VSMCs is dependent on EGFR transactivation. A. Images of the wounded confluent VSMCs were taken as described in Legend for Figure 1 and treated with EGFR blocker Tryphostin AG 1478 (1 and 2 μM) for 30 min and then stimulated with EGF (100ng/ml) for 24 hr, after which images were again taken and quantitated as described in Methods section. Values are means ± SE. * denotes value significantly different from the corresponding value obtained in presence of EGF ($p < 0.05$). B. VSMCs were treated with and without EGFR blocker Tryphostin AG1478 (1 and 2 μM) for 30 min and then stimulated with EGF (100ng/ml) for 10 min and were analyzed by Western blotting for p-Tyr (p-EGFR), p-Src, p-ERK1/2 and p-p38 MAPK using their phospho- and nonphospho-specific antibodies. Representative blots of three experiments are shown.

Figure 7. Effect of piceatannol on EGF-induced VSMC migration. A. Images of wounded confluent VSMCs were aken as described in Legend for Figure 1 followed by treatment with piceatannol (5 and 10 μM) for 30 min and then stimulated with EGF (100ng/ml). After 24 hr, images were again taken and quantitated as described in Methods section. Values are means ± SE. * Denotes value significantly different from the corresponding value obtained in presence of EGF ($p < 0.05$). **B.** Quiescent VSMCs were treated with and without piceatannol (5 and 10 μM) for 30 min and then stimulated with EGF (100ng/ml) for 10 min. and lysates were subjected to Western blott analysis for p-Tyr (p-EGFR), p-Src, p-ERK1/2, p-p38 MAPK using their phospho- and nonphospho-specific antibodies as shown in the representative blots of three experiments.

Figure 8. EGF induced VSMC migration is c-Src dependent. A. Images of wounded confluent VSMCs were taken as described in Legend for Figure 1 followed by treatment with the c-Src inhibitor PP2 (5 and 10 μM) for 30 min and then treated with EGF (100ng/ml) and after 24 hr, images were again taken and quantitated as described in Methods section. Values are means ± SE. * Denotes value significantly different from the corresponding value obtained in presence of EGF ($p < 0.05$). † denotes values significantly different from the corresponding value obtained in presence of vehicle (Veh) ($p < 0.05$). **B.** VSMCs were treated with and without the c-Src inhibitor PP2 (5 and 10 μM) for 30 min and then stimulated with EGF (100ng/ml) for 10 min. And lysates were analyzed by Western blotting for p-Tyr (p-EGFR), p-Src, p-ERK1/2 and p-p38 MAPK using their phospho- and nonphospho-specific antibodies. Representative blots of three experiments.

Figure 9. EGF-induced VSMC migration is partially dependent on p38 MAPK. A. Images of wounded confluent VSMCs were taken as indicated in Legend for Figure 1 and treated with p38 MAPK inhibitor SB202190 (SB, 10 and 20 μM) for 30 min. Cells were then stimulated with EGF (100ng/ml) and after 24 hr, images were again taken and quantitated as described in Methods section. Values are means ± SE. * Denotes value significantly different from the corresponding value obtained in presence of EGF ($p < 0.05$). † denotes value significantly different from the corresponding value obtained in presence of EGF and vehicle (Veh) of SB ($p < 0.05$). B. Quiescent VSMCs were treated with and without SB202190 (SB, 10 and 20 μM) for 30 min and then stimulated with EGF (100ng/ml) for 10 min and the lysates were analyzed by Western blotting for p-Tyr (p-EGFR), p-Src, p-ERK1/2 and p-p38

MAPK using their phospho-specific and nonphospho-specific antibodies as shown in the representative blots of three experiments.

Figure 10. Proposed mechanism of Ang II-induced VSMC migration. Ang II induced VSMC migration is mediated by p38-MAPK activated c-Src through two distinct but redundant pathways, one via Syk, and another via EGFR transactivation through ERK1/2 and partially through p38 MAPK.

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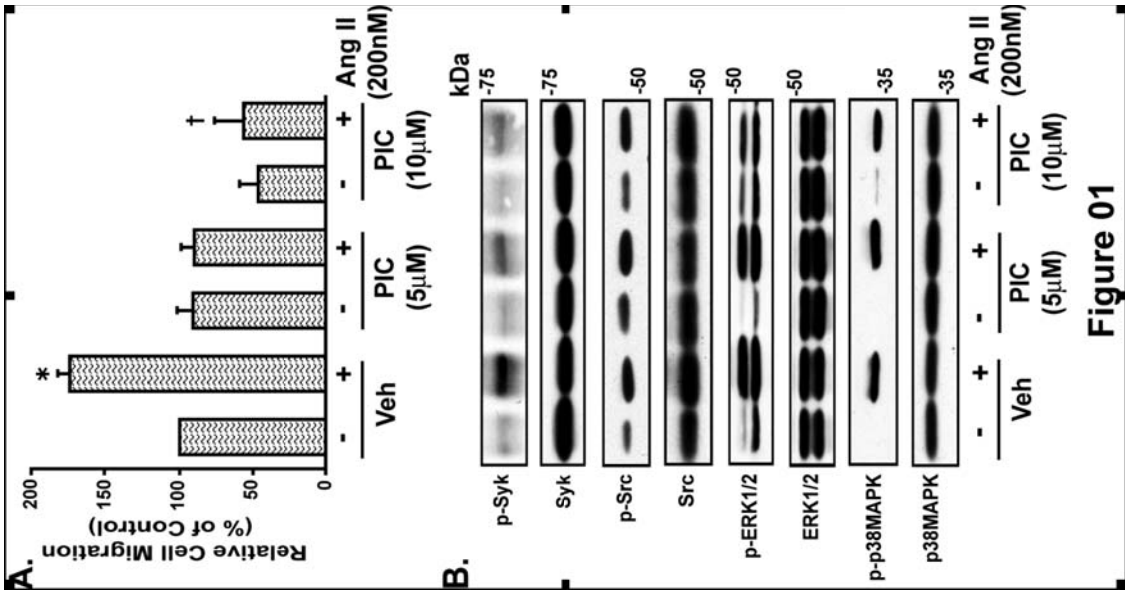

Figure 01

Figure 02

Figure 03

Figure 04

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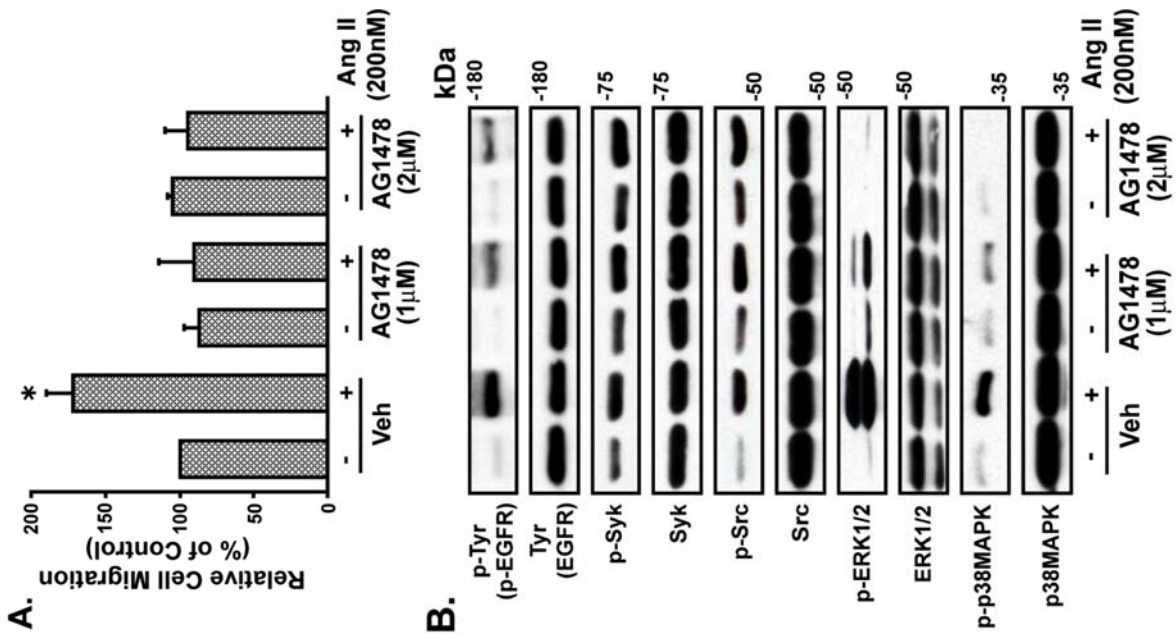

Figure 05

Figure 06

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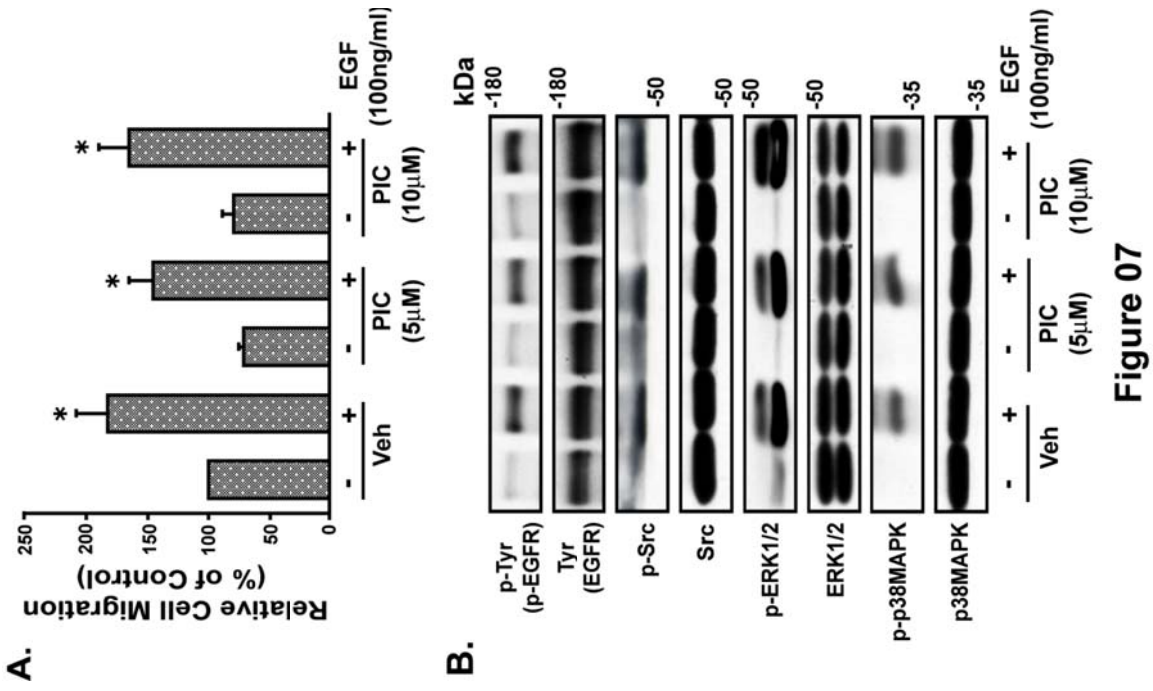


Figure 07

Figure 08

Figure 09

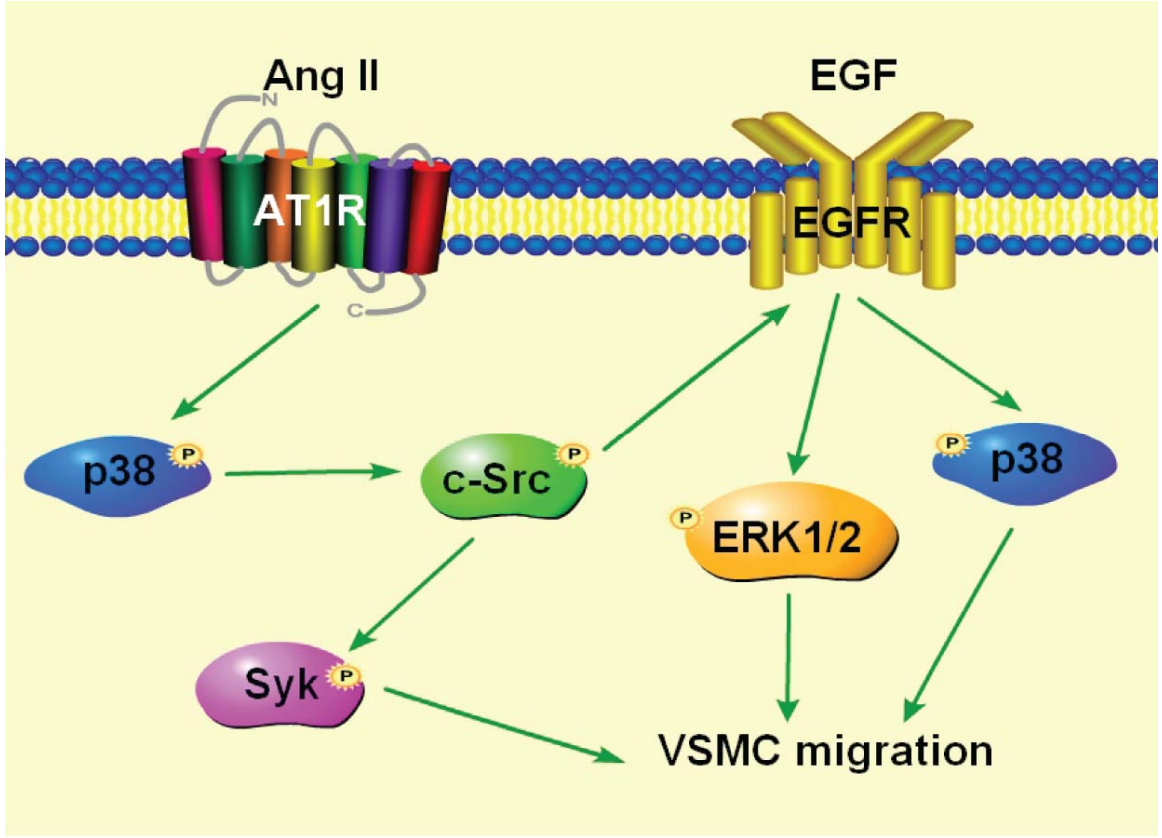
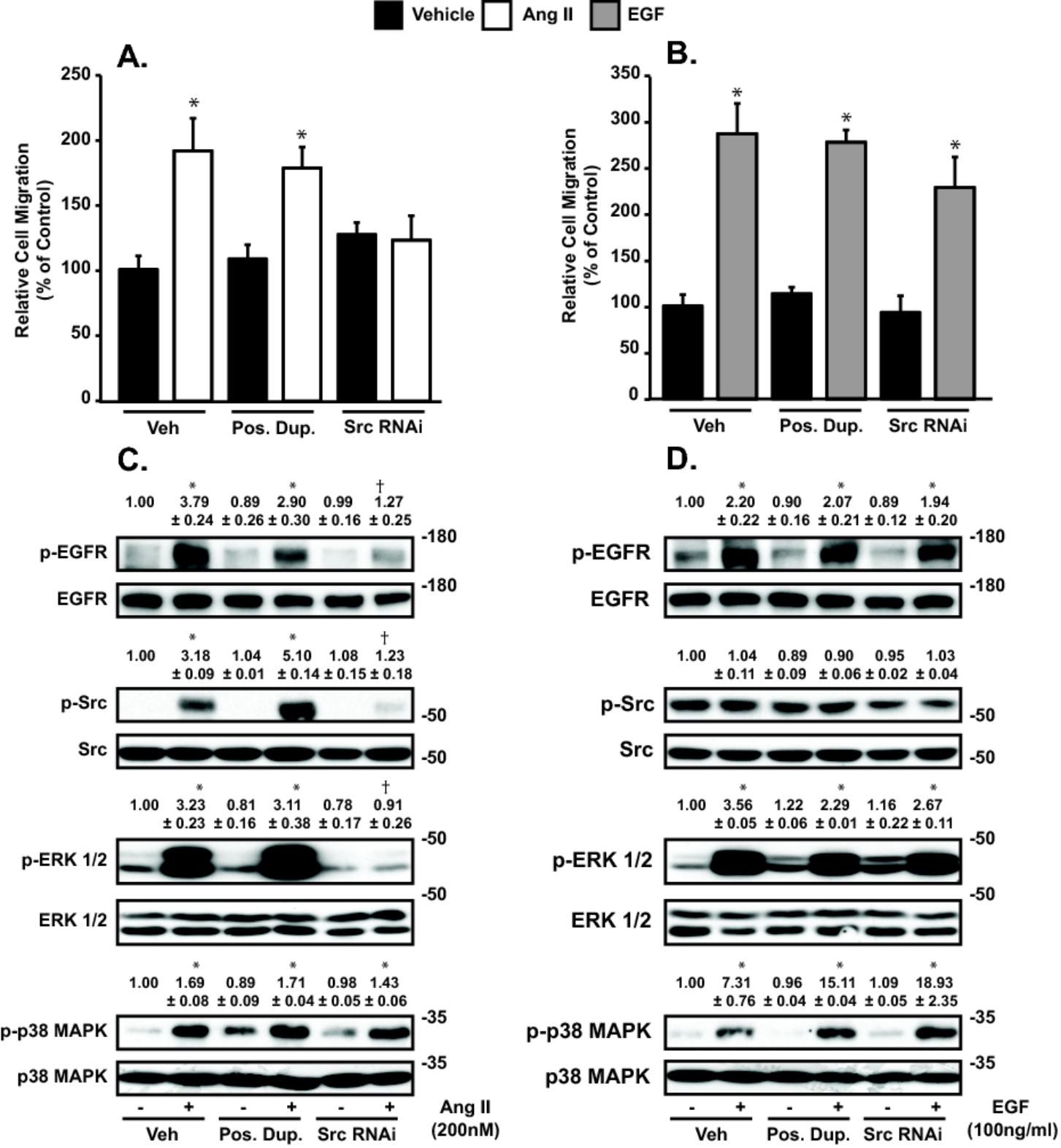


Figure 10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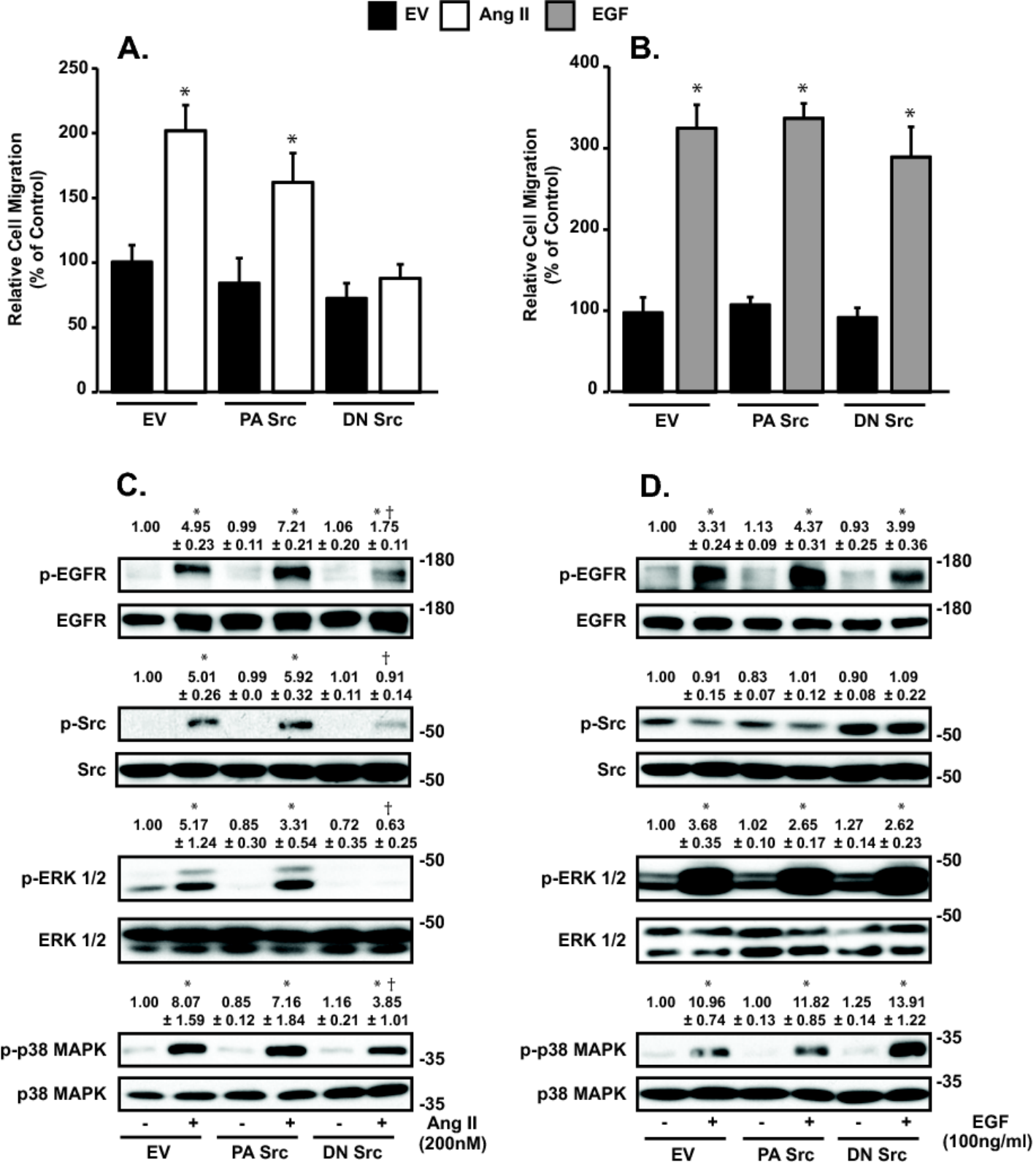
EGF
A.
B.
Relative Cell Migration (% of Control)
Veh
Pos. Dup.
Src RNAi
C.
D.
p-EGFR
EGFR
p-Src
Src
p-ERK 1/2
ERK 1/2
p-p38 MAPK
p38 MAPK
Ang II (200nM)
EGF (100ng/ml)

Figure S1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Ang II
EGF
A.
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250
200
150
100
50
0
EV
PA Src
DN Src
B.
Relative Cell Migration (% of Control)
400
300
200
100
0
EV
PA Src
DN Src
C.
1.00 4.95 ± 0.23 0.99 ± 0.11 7.21 ± 0.21 1.06 ± 0.20 1.75 ± 0.11
p-EGFR
EGFR
-180
-180
1.00 5.01 ± 0.26 0.99 ± 0.0 5.92 ± 0.32 1.01 ± 0.11 0.91 ± 0.14
p-Src
Src
-50
-50
1.00 5.17 ± 1.24 0.85 ± 0.30 3.31 ± 0.54 0.72 ± 0.35 0.63 ± 0.25
p-ERK 1/2
ERK 1/2
-50
-50
1.00 8.07 ± 1.59 0.85 ± 0.12 7.16 ± 1.84 1.16 ± 0.21 3.85 ± 1.01
p-p38 MAPK
p38 MAPK
-35
-35
- + - + - +
Ang II (200nM)
EV
PA Src
DN Src
D.
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EGFR
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-180
1.00 0.91 ± 0.15 0.83 ± 0.07 1.01 ± 0.12 0.90 ± 0.08 1.09 ± 0.22
p-Src
Src
-50
-50
1.00 3.68 ± 0.35 1.02 ± 0.10 2.65 ± 0.17 1.27 ± 0.14 2.62 ± 0.23
p-ERK 1/2
ERK 1/2
-50
-50
1.00 10.96 ± 0.74 1.00 ± 0.13 11.82 ± 0.85 1.25 ± 0.14 13.91 ± 1.22
p-p38 MAPK
p38 MAPK
-35
-35
- + - + - +
EGF (100ng/ml)
EV
PA Src
DN Src

Figure S2

Correction to "Angiotensin II-Induced Migration of Vascular Smooth Muscle Cells Is Mediated by p38 Mitogen-Activated Protein Kinase-Activated c-Src through Spleen Tyrosine Kinase and Epidermal Growth Factor Receptor Transactivation"

In the above article [Mugabe BE, Yaghini FA, Song CY, Buharalioglu CK, Waters CM, and Malik KU (2010) *J Pharmacol Exp Ther* **332:**116–124], a reader raised the question regarding the data in Fig. 8, which shows that the c-Src inhibitor PP2 abolished the wound healing of vascular smooth muscle cells (VSMCs) and phosphorylation of the epidermal growth factor receptor (EGFR) elicited by epidermal growth factor (EGF). Given that EGF does not cause c-Src phosphorylation, one would expect that it should not alter EGF-induced cell migration and EGFR phosphorylation unless PP2 has some nonspecific effect on EGF-induced VSMC migration and EGFR phosphorylation. Unfortunately, these data escaped attention of the authors, and the discrepancy in the article was not discovered prior to its publication.

After reviewing all of the data, the authors discovered an error in the analysis of the effect of PP2 on EGF-induced wound healing and EGFR phosphorylation. Additional experiments were conducted to examine the effect of the c-Src inhibitor PP2 on Ang II- and EGF-induced VSMC migration and EGFR phosphorylation. These experiments confirmed the authors' original observation that PP2 inhibits Ang II-induced VSMC migration and phosphorylation of EGFR, c-Src, and extracellular signal-regulated kinase 1/2 but not p38 mitogen-activated protein kinase (Fig. 3 in the original article); however, the authors neglected to observe an inhibitory effect of PP2 on EGF-induced VSMC migration and on the basal or EGF-induced EGFR phosphorylation, although previous data on the lack of effect of EGF and PP2 on c-Src phosphorylation and that of PP2 on EGF-induced ERK1/2 and p38 MAPK phosphorylation were confirmed as shown in Fig. 8 of the original article. The data from the new experiments are shown in the revised version of Fig. 8 in the online version of the corrected article. The revised figure shows the lack of inhibitory effect of PP2 on EGF-induced VSMC migration and EGFR phosphorylation. Moreover, the authors have confirmed these observations by performing additional series of experiments by using small interfering Src RNA and dominant negative Src mutant containing adenoviruses (Figs. 1 and 2 here; also available as Supplemental Data online).

The online versions of the article and table of contents will be corrected in departure from the print version.

The authors regret these errors and apologize for any confusion or inconvenience they may have caused.

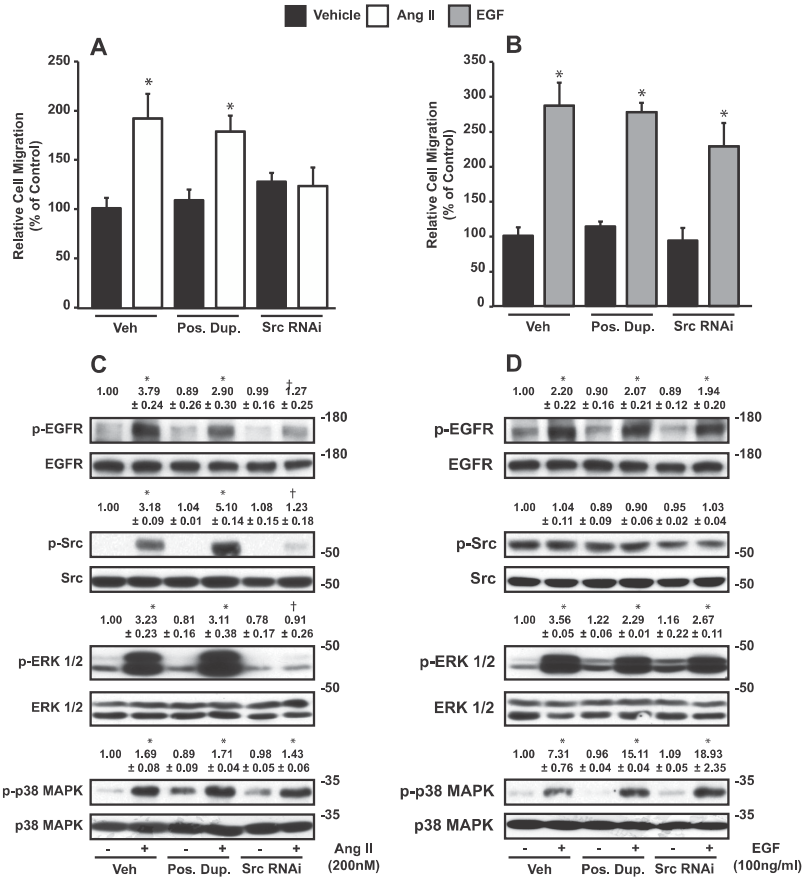
Vehicle
Ang II
EGF
A
B
Relative Cell Migration (% of Control)
Veh
Pos. Dup.
Src RNAi
C
D
p-EGFR
EGFR
p-Src
Src
p-ERK 1/2
ERK 1/2
p-p38 MAPK
p38 MAPK
Ang II (200nM)
EGF (100ng/ml)

Fig. 1.

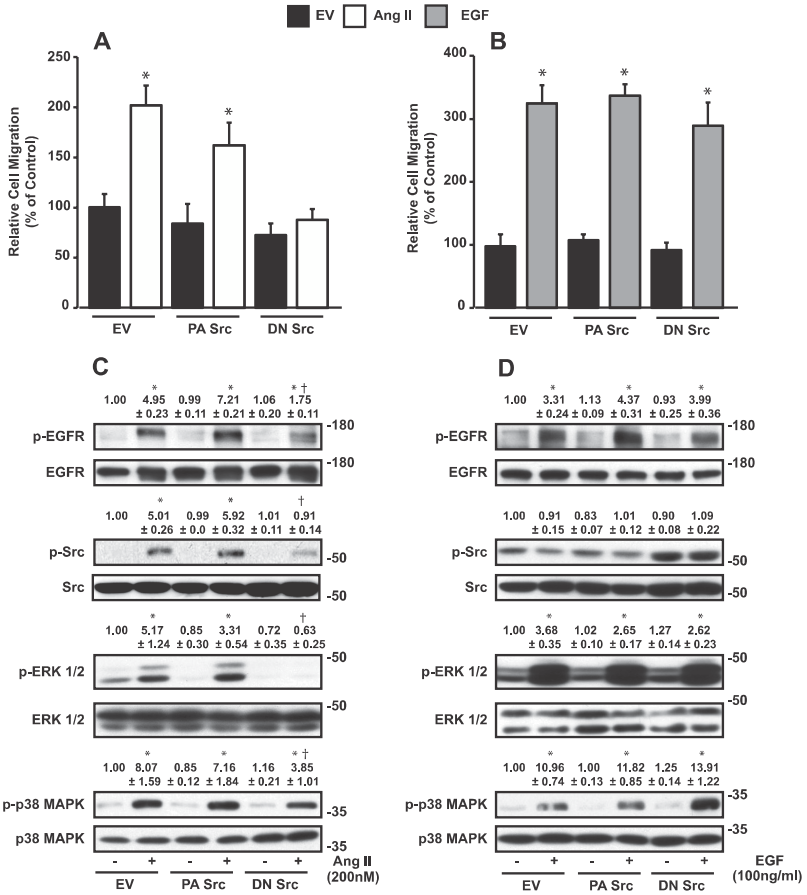
EV
Ang II
EGF
A
B
Relative Cell Migration (% of Control)
EV
PA Src
DN Src
C
D
p-EGFR
EGFR
p-Src
Src
p-ERK 1/2
ERK 1/2
p-p38 MAPK
p38 MAPK
Ang II (200nM)
EGF (100ng/ml)

Fig. 2.