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**Signaling Mechanism Underlying the Promotion of Keratinocyte Migration by Angiotensin II**

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**Running title:** Angiotensin II-induced keratinocyte migration

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**Abbreviations:** AT<sub>1</sub>R, angiotensin II type 1 receptor; AT<sub>2</sub>R, angiotensin II type 2 receptor; EGF, epidermal growth factor; HB-EGF, heparin-binding EGF-like growth factor; NHEK, normal human epidermal keratinocyte; PCR, polymerase chain reaction; RNAi, RNA interference; RT, reverse transcription; TGF, transforming growth factor.

## ABSTRACT

Reepithelialization begins early during skin wound healing and is regulated by various growth factors and cytokines. Angiotensin II promotes the migration of keratinocytes and thereby contributes to wound healing. We investigated the mechanism by which angiotensin II stimulates human keratinocyte migration. Angiotensin II-induced keratinocyte migration was inhibited by an angiotensin II type 1 receptor (AT<sub>1</sub>R) antagonist (candesartan) or an AT<sub>2</sub>R antagonist (PD123319) as well as by depletion of AT<sub>1</sub>R or AT<sub>2</sub>R. A biased agonist for AT<sub>1</sub>R, [Sar<sup>1</sup>,Ile<sup>4</sup>,Ile<sup>8</sup>]angiotensin II, induced cell migration, whereas depletion of  $\beta$ -arrestin2 inhibited angiotensin II-induced migration. Angiotensin II-induced migration was blocked by neutralizing antibodies to transforming growth factor- $\beta$  (TGF- $\beta$ ) as well as by the TGF- $\beta$  receptor inhibitor SB431542. The amount of TGF- $\beta$ 1 was increased in the culture medium of angiotensin II-treated cells, and this effect was inhibited by candesartan or PD123319. Both angiotensin II- and TGF- $\beta$ -induced cell migration were inhibited by neutralizing antibodies to the epidermal growth factor (EGF) receptor but not by those to EGF receptor ligands. Angiotensin II induced phosphorylation of the EGF receptor, and this effect was inhibited by candesartan, PD123319, SB431542, or depletion of  $\beta$ -arrestin2 but not by neutralizing antibodies to heparin-binding EGF-like growth factor. Our results indicate that  $\beta$ -arrestin-dependent signaling downstream of AT<sub>1</sub>R as well as AT<sub>2</sub>R signaling are necessary for angiotensin II-induced keratinocyte migration, and that such signaling promotes generation of the active form of TGF- $\beta$ , consequent activation of the TGF- $\beta$  receptor, and trans-activation of the EGF receptor by the TGF- $\beta$  receptor.

## INTRODUCTION

The healing of skin wounds is a dynamic and interactive process mediated by soluble factors, various cell types, and extracellular matrix. Reepithelialization begins at an early stage of wound healing and is associated with the removal of clotted blood and damaged stroma from the wound space (Singer and Clark, 1999). During reepithelialization, epidermal cells undergo a marked phenotypic change compatible with cell movement. Various growth factors and cytokines, in addition to cell-cell and cell-matrix interactions, contribute to this step (Werner and Grose, 2003).

Angiotensin II is a bioactive octapeptide that regulates blood pressure as well as salt and water homeostasis as a circulating hormone. Evidence also indicates that angiotensin II exerts paracrine or autocrine actions that are important for diverse processes in a variety of tissues (Paul et al., 2006). A local renin-angiotensin system has been thought to operate in human skin (Steckelings et al., 2004; Takeda et al., 2004). The topical administration of angiotensin II was found to promote skin wound healing (Rodgers et al., 1997). Up-regulation of proteins related to angiotensin II function has also been detected at wound sites (Steckelings et al., 2005; Viswanathan and Saavedra, 1992), indicative of a role for endogenous angiotensin II in the wound healing process. Furthermore, skin wound healing was found to be delayed in mice deficient in the angiotensin II type 1a receptor (Kurosaka et al., 2009; Yahata et al., 2006). Together, these findings implicate angiotensin II in skin homeostasis and wound healing. Indeed, angiotensin II has been shown to promote the migration of keratinocytes (Takeda et al., 2004; Yahata et al., 2006), although the mechanism of this action has remained unclear.

Angiotensin II exerts its actions through type 1 (AT<sub>1</sub>R) and type 2 (AT<sub>2</sub>R) receptors, both of which are proteins with seven transmembrane domains. AT<sub>1</sub>R activates G protein- or

$\beta$ -arrestin-dependent signaling pathways. The activation of  $G_q$  or  $G_{11}$  proteins by  $AT_1R$  results in the activation of phospholipase C, the release of  $Ca^{2+}$  from intracellular stores, and subsequent activation of various protein kinases including p42/44 extracellular signal-regulated kinase (ERK). The binding of  $\beta$ -arrestin to  $AT_1R$  not only desensitizes the receptor but also provides a scaffold that regulates a large network of signaling pathways (Shenoy and Lefkowitz, 2011). Certain  $AT_1R$  ligands are "biased" in that they selectively activate  $\beta$ -arrestin-dependent signaling.  $[Sar^1, Ile^4, Ile^8]$ angiotensin II is one such biased agonist for  $AT_1R$  that does not activate the G protein-dependent signaling pathway (Holloway et al., 2002). In contrast to  $AT_1R$ , little is known of the biological functions of  $AT_2R$ , although the receptor has been found to be expressed in epidermal cells and wound regions (Steckelings et al., 2005; Steckelings et al., 2004; Takeda et al., 2004; Viswanathan and Saavedra, 1992).

To provide insight into the regulation of skin homeostasis and wound healing by angiotensin II, we have now investigated the mechanism by which angiotensin II promotes human keratinocyte migration. We analyzed cell migration with both the scrape-wound epithelial cell culture assay (scratch assay) and a single-cell migration assay based on time-lapse video microscopy. We found that both  $\beta$ -arrestin-dependent signaling downstream of  $AT_1R$  and signaling from  $AT_2R$  are necessary for angiotensin II-induced migration of keratinocytes. The activation of these signaling pathways induced generation of the active form of transforming growth factor- $\beta$  (TGF- $\beta$ ), resulting in activation of the TGF- $\beta$  receptor, which in turn mediated trans-activation of the epidermal growth factor (EGF) receptor in a ligand-independent manner.

## MATERIALS AND METHODS

### Materials

Human angiotensin II and SB431542 were obtained from Sigma-Aldrich (St. Louis, MO).

Recombinant human TGF- $\beta$ 1, candesartan, PD123319, and CRM197 were from R&D Systems (Minneapolis, MN), Toronto Research Chemicals (North York, Ontario, Canada), Wako Pure Chemical Industries (Osaka, Japan), and Bio Academia (Ibaraki, Japan), respectively.

[Sar<sup>1</sup>,Ile<sup>8</sup>]angiotensin II was from Peptide Institute (Osaka, Japan), and [Sar<sup>1</sup>,Ile<sup>4</sup>,Ile<sup>8</sup>]angiotensin II and CGP42112 were from Bachem (Budendorf, Switzerland). Neutralizing mouse monoclonal antibodies to the EGF receptor were obtained from Millipore (Billerica, MA). Neutralizing antibodies to TGF- $\beta$ 1, - $\beta$ 2, and - $\beta$ 3 (mouse monoclonal, MAB-1835), to EGF (mouse monoclonal, MAB-236), to HB-EGF (mouse monoclonal, MAB-2591), to TGF- $\alpha$  (goat polyclonal, AB-239-NA), and to amphiregulin (goat polyclonal, AB-262-NA) were from R&D Systems. Rabbit monoclonal antibodies to  $\beta$ -arrestin1/2 (#4674) and mouse monoclonal antibodies to  $\alpha$ -tubulin (T9026) were from Cell Signaling Technology (Danvers, MA) and Sigma-Aldrich, respectively. Rabbit monoclonal antibodies to the EGF receptor (#5735) were obtained from Cell Signaling Technology, and horseradish peroxidase-conjugated mouse monoclonal antibodies to phosphotyrosine (PY-20) were from BD Biosciences (San Jose, CA).

### Cell culture

HaCat cells were obtained from Cell Lines Service (Eppelheim, Germany) and maintained under 5% CO<sub>2</sub> at 37°C in Dulbecco's modified Eagle's medium (high-glucose, DMEM) supplemented with 10% fetal bovine serum. NHEKs isolated from neonatal human foreskin were obtained from Kurabo (Osaka, Japan) and maintained under 5% CO<sub>2</sub> at 37°C in HuMedia-KG2 medium

(Kurabo), which contains growth supplements.

### **Scratch assay**

The assay was performed as described previously (Matsuura et al., 2007) but with some modifications. Confluent HaCat cell monolayers were incubated in serum-free DMEM for 12 h, scratched with the use of a 200- $\mu$ l pipette tip, washed extensively, and cultured for 12 h in DMEM containing various concentrations of angiotensin II. The migration of cells into the wound area was evaluated. A similar assay was also performed with NHEKs cultured in HuMedia-KB2 medium (Kurabo), which does not contain growth supplements.

### **Single-cell migration assay**

HaCat cells were cultured in serum-free DMEM for 8 h, after which the cells were isolated with trypsin-EDTA and transferred to 3.5-cm dishes at a density of  $5 \times 10^4$  cells per dish in serum-free DMEM. The cells were incubated for 4 h and then exposed to various concentrations of angiotensin II and photographed every 2 min for 1 h with the use of an Axiovert S 100 inverted microscope equipped with a CO<sub>2</sub> incubation system (CZI-3) and a heated stage (Carl Zeiss, Jena, Germany). The path length of cell migration was measured with the use of the manual tracking function of NIH ImageJ software (<http://imagej.nih.gov/ij>). A similar assay was also performed with NHEKs cultured in HuMedia-KB2.

### **Knockdown of AT<sub>1</sub>R, AT<sub>2</sub>R, and $\beta$ -arrestin2 in HaCat cells**

Control as well as human AT<sub>1</sub>R, AT<sub>2</sub>R, and  $\beta$ -arrestin2 mRNA-specific siRNAs were obtained as duplexes from Qiagen (Valencia, CA). The sequences targeted by the siRNAs were

5'-AATTCTCCGAACGTGTCACGT-3' for the nonsilencing control,  
5'-CTGGATGTATTGATTCAACTA-3' for AT<sub>1</sub>R siRNA1,  
5'-ACGCACAATGCTTGTAGCCAA-3' for AT<sub>1</sub>R siRNA2,  
5'-AGGGCTAGGAATATAGATTAA-3' for AT<sub>2</sub>R siRNA1,  
5'-TGGAGGTTTATAGTTAATCTA-3' for AT<sub>2</sub>R siRNA2,  
5'-AAGGATGACGACTATGATGAT-3' for  $\beta$ -arrestin2 siRNA1, and  
5'-ACCCGGGACCAGGGTCTTCAA-3' for  $\beta$ -arrestin2 siRNA2. HaCat cells were seeded in a 24-well plate ( $3 \times 10^4$  cells per well) and then transfected for 48 h with 50 nM siRNA with the use of the jetPRIME reagent (Polyplus Transfection, New York, NY). The cells were then transferred to a 3.5-cm dish for migration assays.

### **Immunoblot analysis**

Cells were lysed in a solution containing 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.5% SDS, and a protease inhibitor cocktail (Roche Diagnostics, Tokyo, Japan). Equal amounts of cell lysate protein were subjected to SDS-polyacrylamide gel electrophoresis, and the separated proteins were transferred to a polyvinylidene difluoride membrane. The membrane was incubated first with antibodies to  $\beta$ -arrestin1/2 or to  $\alpha$ -tubulin and then with horseradish peroxidase-conjugated secondary antibodies (Promega, Madison, WI). Immune complexes were detected with the use of a Chemi-Lumi One L detection kit (Nacalai Tesque, Kyoto, Japan) and Amersham Hyperfilm ECL (GE Healthcare/Amersham Biosciences, Piscataway, NJ).

### **RT-PCR analysis**

Total RNA was extracted from HaCat cells or NHEKs with the use of an SV Total RNA Isolation



System (Promega). Semiquantitative RT-PCR analysis was performed with the use of ReverTra Ace reverse transcriptase and KOD FX DNA polymerase (Toyobo, Osaka, Japan) and with the specific primers (sense and antisense, respectively) 5'-CCCAAAGCTGGAAGGCATAATT-3' and 5'-GACGCTGGCTGAAGCAATCTT-3' for AT<sub>1</sub>R, 5'-ACTTCGGGCTTGTGAACATC-3' and 5'-TAAATCAGCCACAGCGAGGT-3' for AT<sub>2</sub>R, and 5'-ACCACAGTCCATGCCATCAC-3' and 5'-TCCACCACCCTGTTGCTGTA-3' for glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

### **Assay of TGF- $\beta$ 1**

Subconfluent HaCat cells in 3.5-cm culture dishes were cultured in serum-free DMEM for 4h or 12 h and then exposed to 30 nM angiotensin II also in serum-free DMEM for 1h or 12 h, respectively. The culture supernatants were then collected, concentrated 10-fold by lyophilization, and assayed for TGF- $\beta$ 1 with the use of a quantitative enzyme-linked immunosorbent assay (Quantikine ELISA kit, R&D Systems).

### **Analysis of EGF receptor phosphorylation**

HaCat cells were washed twice with ice-cold phosphate-buffered saline and then lysed in a solution containing 150 mM NaCl, 25 mM Tris-HCl (pH 7.4), 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 2 mM EDTA, and both phosphatase and protease inhibitor cocktails (Roche Diagnostics). The lysates were subjected to immunoprecipitation for 12 h at 4°C with Sepharose beads conjugated with rabbit monoclonal antibodies to the EGF receptor (Cell Signaling Technology). The beads were then washed five times with lysis buffer, after which the bead-bound proteins were subjected to immunoblot analysis with the antibodies to the EGF

receptor as well as with those to phosphotyrosine. Signals were quantified densitometrically with the use of a Lane & Spot Analyzer (ATTO, Tokyo, Japan), and the intensity of the band corresponding to the phosphorylated EGF receptor was normalized by that of the corresponding EGF receptor band.

### **Statistical analysis**

Data are presented as means  $\pm$  SEM and were compared between two groups with Student's *t* test and among three or more groups with one way ANOVA followed by post hoc Tukey-Kramer test. A *P* value of  $< 0.05$  was considered statistically significant.

## RESULTS

### Angiotensin II-induced migration of keratinocytes

We examined the effect of angiotensin II on skin epithelial cell migration *in vitro* with the use of the scrape-wound epithelial cell culture (scratch) assay and a single-cell migration assay based on time-lapse video microscopy. These assays revealed that angiotensin II not only promoted the collective migration of the human keratinocyte cell line HaCat and normal human epidermal keratinocytes (NHEKs) but also increased the motility of individual cells in a concentration-dependent manner (Fig. 1). The median effective concentration ( $EC_{50}$ ) for angiotensin II determined by the two assays was 0.40 and 0.25 nM, respectively, for HaCat cells, and 1.34 and 0.85 nM, respectively, for NHEKs.

### Roles of $AT_1R$ and $AT_2R$ in angiotensin II-induced keratinocyte migration

To investigate the signaling mechanism responsible for the promotion of cell migration by angiotensin II, we first examined the expression of angiotensin receptors in HaCat cells and NHEKs. The expression of both  $AT_1R$  and  $AT_2R$  genes in these cells was revealed by reverse transcription (RT) and polymerase chain reaction (PCR) analysis (Fig. 2A). Both the scratch assay and the single-cell migration assay revealed that antagonists specific for  $AT_1R$  (candesartan) at 1  $\mu$ M or  $AT_2R$  (PD123319) at 10  $\mu$ M inhibited the stimulatory effect of angiotensin II on the migration of HaCat cells (Fig. 2B, C) and NHEKs (Fig. 2D, E), indicating that both receptors are necessary for the promotion of keratinocyte migration by angiotensin II. The same results were obtained with 1  $\mu$ M PD123319 (data not shown).

In addition to angiotensin II, the partial agonist [Sar<sup>1</sup>,Ile<sup>8</sup>]angiotensin II, which possesses affinities for both  $AT_1R$  ( $K_d = 0.37$  nM) and  $AT_2R$  ( $K_d = 0.56$  nM) similar to those of angiotensin

II (Miura and Karnik, 1999; Wei et al., 2003), promoted the migration of HaCat cells in the scratch assay in a concentration-dependent manner, although the  $EC_{50}$  for [Sar<sup>1</sup>,Ile<sup>8</sup>]angiotensin II was ~10 times that for angiotensin II (Fig. 3A). The stimulatory effect of [Sar<sup>1</sup>,Ile<sup>8</sup>]angiotensin II on HaCat cell migration was also blocked by candesartan and by PD123319 (Fig. 3B). In contrast, CGP42112, a specific ligand for AT<sub>2</sub>R but not for AT<sub>1</sub>R, did not promote the migration of HaCat cells at concentrations up to 1  $\mu$ M (Fig. 3A). Given that the  $K_d$  value of CGP42112 for AT<sub>2</sub>R is in the nanomolar range (Bosnyak et al., 2011), these results suggested that the effect of angiotensin II on cell migration might require activation of AT<sub>1</sub>R in addition to that of AT<sub>2</sub>R. To confirm further the roles of both AT<sub>1</sub>R and AT<sub>2</sub>R in angiotensin II-induced cell migration, we depleted HaCat cells of these receptors by RNA interference (RNAi) with two different small interfering RNAs (siRNAs) for each receptor (Fig. 3C). Angiotensin II did not promote the migration of the AT<sub>1</sub>R- or AT<sub>2</sub>R-depleted cells in the single-cell migration assay (Fig. 3D).

### **Role of $\beta$ -arrestin-dependent signaling in angiotensin II-induced keratinocyte migration**

We next examined the effects of a biased agonist for AT<sub>1</sub>R, [Sar<sup>1</sup>,Ile<sup>4</sup>,Ile<sup>8</sup>]angiotensin II, on the migration of HaCat cells and NHEKs. Given that the  $K_d$  of this ligand for AT<sub>1</sub>R is ~0.3  $\mu$ M (Miura and Karnik, 1999), we performed the experiments with a [Sar<sup>1</sup>,Ile<sup>4</sup>,Ile<sup>8</sup>]angiotensin II concentration of 50  $\mu$ M to ensure full receptor occupancy (Kendall et al., 2011). We found that [Sar<sup>1</sup>,Ile<sup>4</sup>,Ile<sup>8</sup>]angiotensin II promoted the migration of HaCat cells and NHEKs in the scratch assay in a manner sensitive to inhibition by candesartan or by PD123319 (Fig. 4A, B). Given that [Sar<sup>1</sup>,Ile<sup>4</sup>,Ile<sup>8</sup>]angiotensin II is incapable of activating G proteins (Miura and Karnik, 1999) and that it has a high affinity for AT<sub>2</sub>R ( $K_d$  = 2 nM) (Miura and Karnik, 1999), these results suggested that  $\beta$ -arrestin-dependent signaling from AT<sub>1</sub>R together with signaling from AT<sub>2</sub>R might be

necessary for the stimulatory effect of angiotensin II on cell migration. Indeed, depletion of  $\beta$ -arrestin2 by RNAi with two different siRNAs (Fig. 4C) markedly attenuated the promotion of HaCat cell migration by angiotensin II in the single-cell migration assay, whereas it had no effect on the basal level of cell migration (Fig. 4D).

### **TGF- $\beta$ mediates angiotensin II-induced cell migration**

Angiotensin II increases production of the active form of TGF- $\beta$  in various cell types. We therefore next examined the effect of neutralizing antibodies to TGF- $\beta$  on angiotensin II-induced HaCat cell migration in the scratch assay. The antibodies completely inhibited the stimulatory effect of angiotensin II on cell migration (Fig. 5A). An inhibitor of the TGF- $\beta$  receptor, SB431542, also blocked angiotensin II-induced cell migration (Fig. 5A). Essentially identical results were obtained with the single-cell migration assay (data not shown). Furthermore, TGF- $\beta$ 1 promoted HaCat cell migration in the scratch assay in a concentration-dependent manner (Fig. 5B). This effect of TGF- $\beta$ 1 was not inhibited by candesartan or PD123319 (Fig. 5C). When we measured the amounts of TGF- $\beta$ 1 in the culture medium of HaCat cells, TGF- $\beta$ 1 significantly increased in the medium of angiotensin II-treated cells at 1 h and 12 h after addition of angiotensin II (Fig. 5D). We also found that this effect was inhibited by candesartan or PD123319 (Fig. 5D). On the other hand, the amounts of TGF- $\beta$ 1 mRNAs in HaCat cells were not significantly affected by exposure of the cells to angiotensin II (Supplemental Fig. 1). These results thus suggested that activation of angiotensin II receptors induces generation of the active form of TGF- $\beta$  without its transcription enhancement, and consequent activation of the TGF- $\beta$  receptor in keratinocytes.

### **Role of the EGF receptor in angiotensin II-induced cell migration**

We next explored signaling downstream of TGF- $\beta$  in the promotion of HaCat cell migration by angiotensin II. Activation of the TGF- $\beta$  receptor has previously been found to result in trans-activation of the EGF receptor in a ligand-dependent or -independent manner in HaCat cells (Joo et al., 2008; Koivisto et al., 2006). We found that neutralizing antibodies to the EGF receptor abolished the effect of angiotensin II on HaCat cell migration in the scratch assay (Fig. 6A). To determine whether activation of the EGF receptor induced by angiotensin II is ligand dependent, we examined the effects of neutralizing antibodies to EGF receptor ligands including amphiregulin, EGF, TGF- $\alpha$ , and heparin-binding EGF-like growth factor (HB-EGF) on angiotensin II-induced HaCat cell migration. None of these antibodies inhibited the effect of angiotensin II on cell migration (Fig. 6A), although they all inhibited (at the concentration used in the migration assay) phosphorylation of the EGF receptor induced by the corresponding ligand (Supplemental Fig. 2). An inhibitor of HB-EGF, CRM197, also had no effect on angiotensin II-induced cell migration (Fig. 6A), although it also inhibited HB-EGF-induced phosphorylation of the EGF receptor (Supplemental Fig. 2). The TGF- $\beta$ -induced migration of HaCat cells was also abolished by neutralizing antibodies to the EGF receptor but not by those to HB-EGF or by CRM197 (Fig. 6B). These results thus suggested that trans-activation of the EGF receptor induced by the TGF- $\beta$  receptor downstream of angiotensin II in HaCat cells is ligand independent.

To confirm the signaling mechanism responsible for angiotensin II-induced cell migration, we monitored phosphorylation of the EGF receptor. The extent of EGF receptor phosphorylation in HaCat cells was increased 5 min after the addition of angiotensin II and remained increased at 30 min (Fig. 7A). Candesartan and PD123319 each inhibited angiotensin II-induced

phosphorylation of the EGF receptor (Fig. 7B, C), as did RNAi-mediated depletion of  $\beta$ -arrestin2 (Fig. 7D). Furthermore, the TGF- $\beta$  receptor inhibitor SB431542 attenuated angiotensin II-induced phosphorylation of the EGF receptor (Fig. 7E), whereas neutralizing antibodies to HB-EGF had no such effect (Fig. 7F).

## DISCUSSION

Among various actions of angiotensin II during skin wound healing, we focused on the promotion of keratinocyte migration and its underlying mechanism. We found that activation of both AT<sub>1</sub>R and AT<sub>2</sub>R is necessary for this effect of angiotensin II. Furthermore, our observations that a biased agonist for AT<sub>1</sub>R, [Sar<sup>1</sup>,Ile<sup>4</sup>,Ile<sup>8</sup>]angiotensin II, also promoted cell migration and that angiotensin II-induced migration was inhibited by depletion of  $\beta$ -arrestin2 suggested that  $\beta$ -arrestin-dependent signaling by AT<sub>1</sub>R as well as signaling downstream of AT<sub>2</sub>R are responsible for the stimulatory effect of angiotensin II on keratinocyte migration. Our results further indicated that activation of both AT<sub>1</sub>R and AT<sub>2</sub>R in keratinocytes increased production of the active form of TGF- $\beta$ , resulting in activation of the TGF- $\beta$  receptor and consequent trans-activation of the EGF receptor in a ligand-independent manner. These angiotensin II-induced signaling pathways in keratinocytes were confirmed by monitoring phosphorylation of the EGF receptor. On the basis of these pharmacological, biochemical, and molecular biological observations, we propose a model for the mechanism of angiotensin II-induced migration of keratinocytes (Fig. 8).

We showed that activation of both AT<sub>1</sub>R and AT<sub>2</sub>R is necessary for angiotensin II-induced keratinocyte migration. A role for AT<sub>1</sub>R has previously been demonstrated in the angiotensin II-induced migration of various cell types including vascular smooth muscle cells (Dubey et al.,

1995), monocytes (Kintscher et al., 2001), and keratinocytes (Takeda et al., 2004; Yahata et al., 2006). We now show that  $\beta$ -arrestin-dependent signaling, but not G protein-dependent signaling, downstream of AT<sub>1</sub>R mediates the stimulatory effect of angiotensin II on keratinocyte migration. Forced expression of mouse AT<sub>1</sub>R (AT<sub>1a</sub>R) in heterologous cells previously implicated  $\beta$ -arrestin in AT<sub>1a</sub>R-dependent cellular motility (Hunton et al., 2005; Simard et al., 2013).

The role of AT<sub>2</sub>R in cell migration has been less clear. PD123319 was previously shown to enhance the angiotensin II-induced migration of keratinocytes in the scratch assay, suggestive of an inhibitory action of AT<sub>2</sub>R in cell migration (Takeda et al., 2004). In contrast, we found that PD123319 inhibited the angiotensin II-induced migration of HaCat cells as well as NHEKs in both the scratch assay and the single-cell migration assay. We also showed that specific depletion of AT<sub>2</sub>R in HaCat cells prevented the stimulatory effect of angiotensin II on cell migration. No substantial effect of PD123319 on angiotensin II-induced migration of vascular smooth muscle cells (Kohno et al., 1997) or monocytes (Kintscher et al., 2001) was detected previously with a transwell culture assay. Other studies with different agents or methods have also suggested that AT<sub>2</sub>R does not contribute to the effect of angiotensin II on the migration of vascular smooth muscle cells (Dubey et al., 1998; Dubey et al., 1995; Liu et al., 1997). On the other hand, a recent study showed that PD123319 inhibited the angiotensin-induced migration of porcine vascular smooth muscle cells in both the scratch assay and the transwell assay (Louis et al., 2011). This latter study pointed out that the role of AT<sub>2</sub>R in rodent vascular smooth muscle cells has been difficult to elucidate because of the down-regulation of the receptor apparent in the culture systems examined (Louis et al., 2011). Although the reason for these apparent discrepancies concerning the role of AT<sub>2</sub>R in keratinocytes is unclear, the results of the latter study appear consistent with our present data.



Our examination of the effects of an AT<sub>2</sub>R-specific ligand and of AT<sub>1</sub>R depletion indicated that activation of AT<sub>2</sub>R alone is not sufficient for angiotensin II-induced keratinocyte migration. Depletion of AT<sub>2</sub>R also revealed that AT<sub>1</sub>R alone is not sufficient for this effect of angiotensin II. Given that AT<sub>1</sub>R and AT<sub>2</sub>R have been shown to form a heterodimeric complex (AbdAlla et al., 2001), such direct interaction of the receptors might play an important role in signaling responsible for the promotion of cell migration by angiotensin II.

Angiotensin II has been shown to induce the release of TGF- $\beta$  from various cell types—including vascular smooth muscle cells (Gibbons et al., 1992; Koibuchi et al., 1993), renal proximal tubular cells (Wolf et al., 1993), glomerular mesangial cells (Kagami et al., 1994), cardiac fibroblasts and myofibroblasts (Campbell and Katwa, 1997; Fisher and Absher, 1995), and dermal fibroblasts (Tang et al., 2009)—in a paracrine manner and thereby to regulate various cellular activities. We have now shown that this is also the case in the regulation of keratinocyte migration by angiotensin II. Our observations that both neutralizing antibodies to TGF- $\beta$  and an inhibitor of the TGF- $\beta$  receptor prevented angiotensin II-induced cell migration whereas antagonists of AT<sub>1</sub>R and AT<sub>2</sub>R did not inhibit TGF- $\beta$ -induced migration thus indicate that activation of the TGF- $\beta$  receptor occurs downstream of that of AT<sub>1</sub>R and AT<sub>2</sub>R by angiotensin II. Furthermore, angiotensin II induces the release of the active form of TGF- $\beta$  form in HaCat cells. These observations indicate that angiotensin II induces the release of the active form of TGF- $\beta$  from keratinocytes, which results in activation of the TGF- $\beta$  receptor and consequent promotion of cell migration.

Trans-activation of the EGF receptor by the TGF- $\beta$  receptor has been observed in various cell types. TGF- $\beta$ -induced shedding of HB-EGF or TGF- $\alpha$  has thus been shown to result in activation of the EGF receptor in association with angiogenesis by endothelial cells (Vinals and

Pouyssegur, 2001), fibronectin expression by mesangial cells (Uchiyama-Tanaka et al., 2002), hypertrophy of renal proximal tubular cells (Chen et al., 2006), growth of gastric cancer cells (Ebi et al., 2010), and wound repair by airway epithelial cells (Ito et al., 2011). TGF- $\beta$  was also previously found to promote HaCat cell migration through trans-activation of the EGF receptor mediated by autocrine HB-EGF signaling (Koivisto et al., 2006), whereas another study demonstrated ligand-independent trans-activation of the EGF receptor by TGF- $\beta$  in these cells (Joo et al., 2008). Furthermore, AT<sub>1</sub>R has been proposed to induce trans-activation of the EGF receptor through shedding of HB-EGF and thereby to promote migration of keratinocytes (Yahata et al., 2006). Although the reason for these discrepancies is unclear, we have now shown that neither neutralizing antibodies to HB-EGF nor an inhibitor of HB-EGF (the diphtheria toxin mutant CRM197) had an effect on angiotensin II- or TGF- $\beta$ -induced migration of HaCat cells or on angiotensin II-induced phosphorylation of the EGF receptor, indicating that angiotensin II promotes the migration of these cells in a manner dependent on EGF receptor activation but not on the shedding of HB-EGF. Consistent with those of a previous study (Joo et al., 2008), our results thus suggest that TGF- $\beta$  receptor-dependent trans-activation of the EGF receptor in a ligand-independent manner mediates the stimulatory effect of angiotensin II on keratinocyte migration.

In conclusion, our study suggests that  $\beta$ -arrestin-dependent signaling downstream of AT<sub>1</sub>R as well as AT<sub>2</sub>R signaling in keratinocytes induce production of the active form of TGF- $\beta$  and consequent activation of the TGF- $\beta$  receptor, which in turn induces trans-activation of the EGF receptor in a ligand-independent manner, and that this signaling cascade underlies the regulation of keratinocyte migration by angiotensin II. Elucidation of these signaling events responsible for angiotensin II-induced migration of keratinocytes sheds light on the molecular mechanisms of

skin wound healing and may provide a basis for the development of new approaches to the treatment of skin wounds.

## **AUTHORSHIP CONTRIBUTIONS**

*Participated in research design:* Sakai, Matsuura, Nishida, and Inui

*Conducted experiments:* Sakai, Matsuura, Tanaka, and Honda

*Contributed new reagents or analytical tools:* Honda, and Nishida

*Performed data analysis:* Sakai, Matsuura, and Inui

*Wrote or contributed to the writing of the manuscript:* Sakai, and Inui

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MOL #96461

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## FOOTNOTES

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## FIGURE LEGENDS

**Fig. 1.** Effects of angiotensin II on the migration of HaCat cells and NHEKs. In the scratch assay (closed circles), a monolayer of HaCat cells (A) or NHEKs (B) was scratched with the use of a pipette tip and then incubated for 12 h at 37°C with various concentrations of angiotensin II. In the single-cell migration assay based on time-lapse video microscopy (open circles), the cells were photographed every 2 min during incubation for 1 h at 37°C in the presence of various concentrations of angiotensin II. Data are means  $\pm$  SEM from six independent experiments (scratch assay) or for a total of 38 to 44 cells in four independent experiments (single-cell migration assay).  $**P < 0.01$ ,  $***P < 0.001$  versus corresponding control value.

**Fig. 2.** Roles of AT<sub>1</sub>R and AT<sub>2</sub>R in angiotensin II-induced migration of HaCat cells and NHEKs. (A) RT-PCR analysis of AT<sub>1</sub>R and AT<sub>2</sub>R mRNAs. GAPDH mRNA was examined as a control. (B-E) HaCat cells (B, C) or NHEKs (D, E) were incubated in the absence or presence of 30 nM angiotensin II (Ang II) or of 1  $\mu$ M candesartan (B, D) or 10  $\mu$ M PD123319 (C, E). The extent of cell migration was determined with the scratch assay (closed bars) and the single-cell migration assay (open bars). Data are means  $\pm$  SEM from 6 to 10 independent experiments (scratch assay) or for a total of 20 to 24 cells in two independent experiments (single-cell migration assay).  $***P < 0.001$ .

**Fig. 3.** Effects of angiotensin II analogs and of AT<sub>1</sub>R or AT<sub>2</sub>R depletion on HaCat cell migration. (A, B) The scratch assay was performed with various concentrations of angiotensin II (closed circles), [Sar<sup>1</sup>,Ile<sup>8</sup>]angiotensin II (open circles), or CGP42112 (closed triangles) (A) or with 100 nM [Sar<sup>1</sup>,Ile<sup>8</sup>]angiotensin II ([S1,I8]) and either 1  $\mu$ M candesartan or 10  $\mu$ M PD123319 (B).

Data are means  $\pm$  SEM from four to six independent experiments. (C, D) HaCat cells transfected with control, AT<sub>1</sub>R, or AT<sub>2</sub>R siRNAs were subjected to RT-PCR analysis of AT<sub>1</sub>R and AT<sub>2</sub>R mRNAs (C) as well as to the single-cell migration assay with 30 nM angiotensin II (D).

Migration data are means  $\pm$  SEM for a total of 41 to 63 cells in four to six independent experiments. \**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.001 versus corresponding control value or for the indicated comparisons.

**Fig. 4.** Effects of an AT<sub>1</sub>R-biased ligand and  $\beta$ -arrestin2 depletion on cell migration. (A, B) The scratch assay was performed with HaCat cells (A) or NHEKs (B) and with or without 50  $\mu$ M [Sar<sup>1</sup>,Ile<sup>4</sup>,Ile<sup>8</sup>]angiotensin II ([S1,I4,I8]), 1  $\mu$ M candesartan, or 10  $\mu$ M PD123319. Data are means  $\pm$  SEM from six independent experiments. (C, D) HaCat cells transfected with control or  $\beta$ -arrestin2 siRNAs were subjected to immunoblot analysis with antibodies to  $\beta$ -arrestin and to tubulin (C) or to the single-cell migration assay with 30 nM angiotensin II (D). Two isoforms of  $\beta$ -arrestin,  $\beta$ -arrestin1 (upper band) and  $\beta$ -arrestin2 (lower band), were detected by the antibodies, and the siRNAs for  $\beta$ -arrestin2 specifically depleted  $\beta$ -arrestin2 but not  $\beta$ -arrestin1. Migration data are means  $\pm$  SEM for a total of 20 to 24 cells in two independent experiments. \**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.001.

**Fig. 5.** Role of TGF- $\beta$  in angiotensin II-induced HaCat cell migration. (A-C) The scratch assay was performed in the presence either of 30 nM angiotensin II, neutralizing antibodies to TGF- $\beta$  (5  $\mu$ g/ml), or 10  $\mu$ M SB431542 (A); of various concentrations of TGF- $\beta$ 1 (B); or of TGF- $\beta$ 1 (5 ng/ml), 1  $\mu$ M candesartan, or 10  $\mu$ M PD123319 (C). Data are means  $\pm$  SEM from four to six independent experiments. (D) Cells were incubated in the absence or presence of 30 nM

angiotensin II, 1  $\mu$ M candesartan, or 1  $\mu$ M PD123319 for 1 h or 12 h, after which the amount of TGF- $\beta$ 1 in the culture supernatants was determined. Data are means  $\pm$  SEM from five independent experiments. \* $P$  < 0.05, \*\* $P$  < 0.01, \*\*\* $P$  < 0.001 versus corresponding control values or for the indicated comparisons.

**Fig. 6.** Role of the EGF receptor in angiotensin II-induced migration of HaCat cells. (A) The scratch assay was performed in the presence of 30 nM angiotensin II with or without CRM197 (50  $\mu$ g/ml) or neutralizing antibodies to the EGF receptor (EGFR) (0.1  $\mu$ g/ml), to amphiregulin (Amph) (50  $\mu$ g/ml), to EGF (20  $\mu$ g/ml), to TGF- $\alpha$  (10  $\mu$ g/ml), or to HB-EGF (20  $\mu$ g/ml). (B) The scratch assay was performed in the presence of TGF- $\beta$ 1 (5 ng/ml) and with or without CRM197 (50  $\mu$ g/ml) or neutralizing antibodies to the EGF receptor (0.1  $\mu$ g/ml) or to HB-EGF (20  $\mu$ g/ml). All data are means  $\pm$  SEM from six independent experiments. \* $P$  < 0.05, \*\* $P$  < 0.01, \*\*\* $P$  < 0.001.

**Fig. 7.** Roles of AT<sub>1</sub>R, AT<sub>2</sub>R,  $\beta$ -arrestin2, and the TGF- $\beta$  receptor in angiotensin II-induced activation of the EGF receptor in HaCat cells. (A) Lysates of HaCat cells prepared at the indicated times after exposure of the cells to 30 nM angiotensin II were subjected to immunoprecipitation with antibodies to the EGF receptor, and the resulting precipitates were subjected to immunoblot analysis with antibodies to phosphotyrosine (P-EGFR) and to the EGF receptor (Total EGFR). (B, C, E, F) HaCat cells were incubated for 15 min in the absence or presence of 30 nM angiotensin II and with or without 1  $\mu$ M candesartan (B), 1  $\mu$ M PD123319 (C), 10  $\mu$ M SB431542 (E), or neutralizing antibodies to HB-EGF (20  $\mu$ g/ml) (F). Cell lysates were then subjected to immunoprecipitation and immunoblot analysis (upper panels) as in (A).

The normalized abundance of the phosphorylated EGF receptor (lower panels) was also determined as means  $\pm$  SEM from 6 to 12 independent experiments. (D) HaCat cells transfected with control or  $\beta$ -arrestin2 siRNAs were incubated with or without 30 nM angiotensin II for 15 min, after which cell lysates were subjected to immunoprecipitation and immunoblot analysis (upper panel) as in (A). The normalized abundance of the phosphorylated EGF receptor (lower panel) was also determined as means  $\pm$  SEM from 11 independent experiments.  $*P < 0.05$ ,  $**P < 0.01$ ,  $***P < 0.001$ .

**Fig. 8.** Model for the signaling events underlying the promotion of keratinocyte migration by angiotensin II.

Figure 1

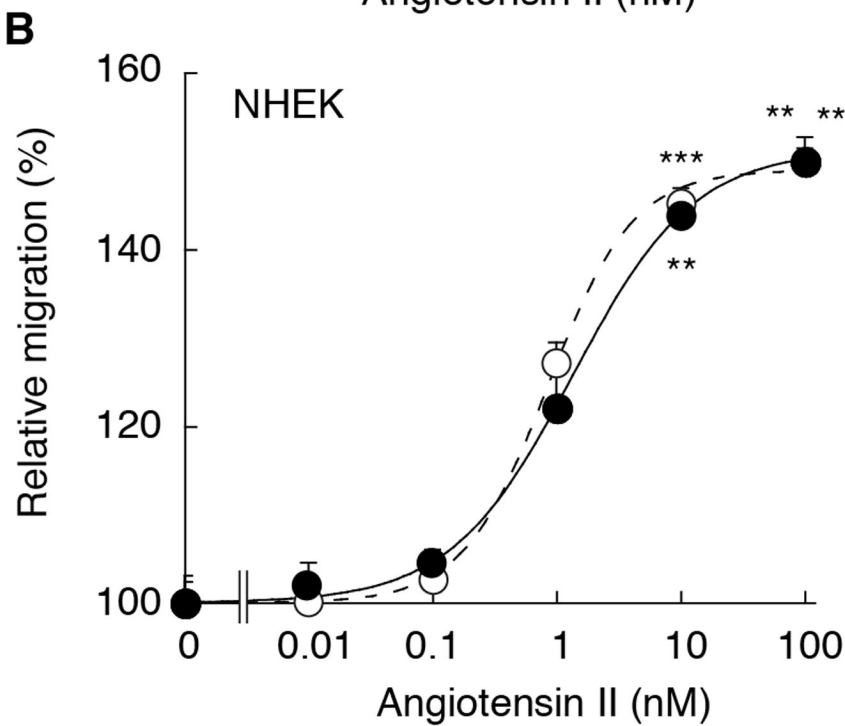
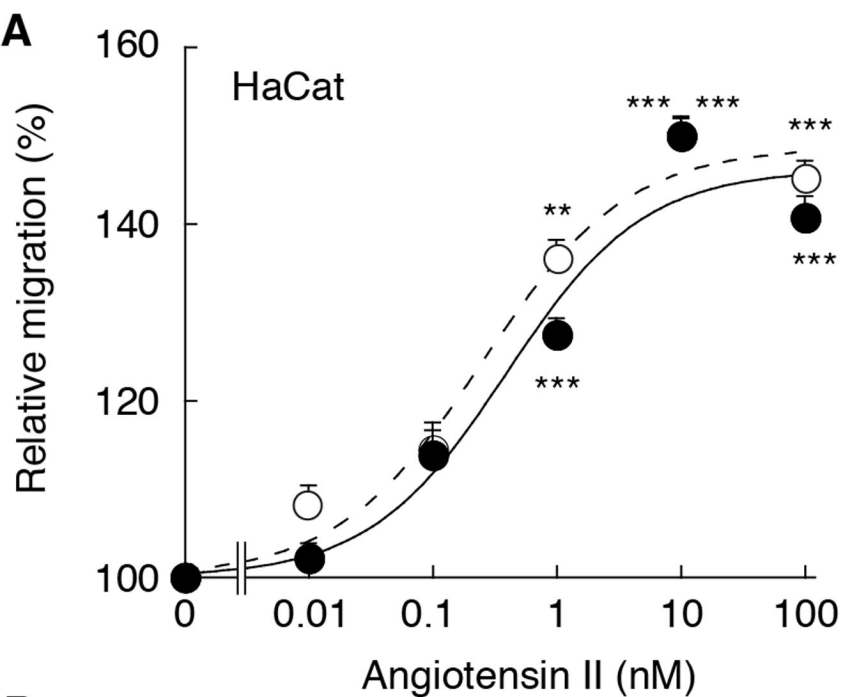




Figure 2

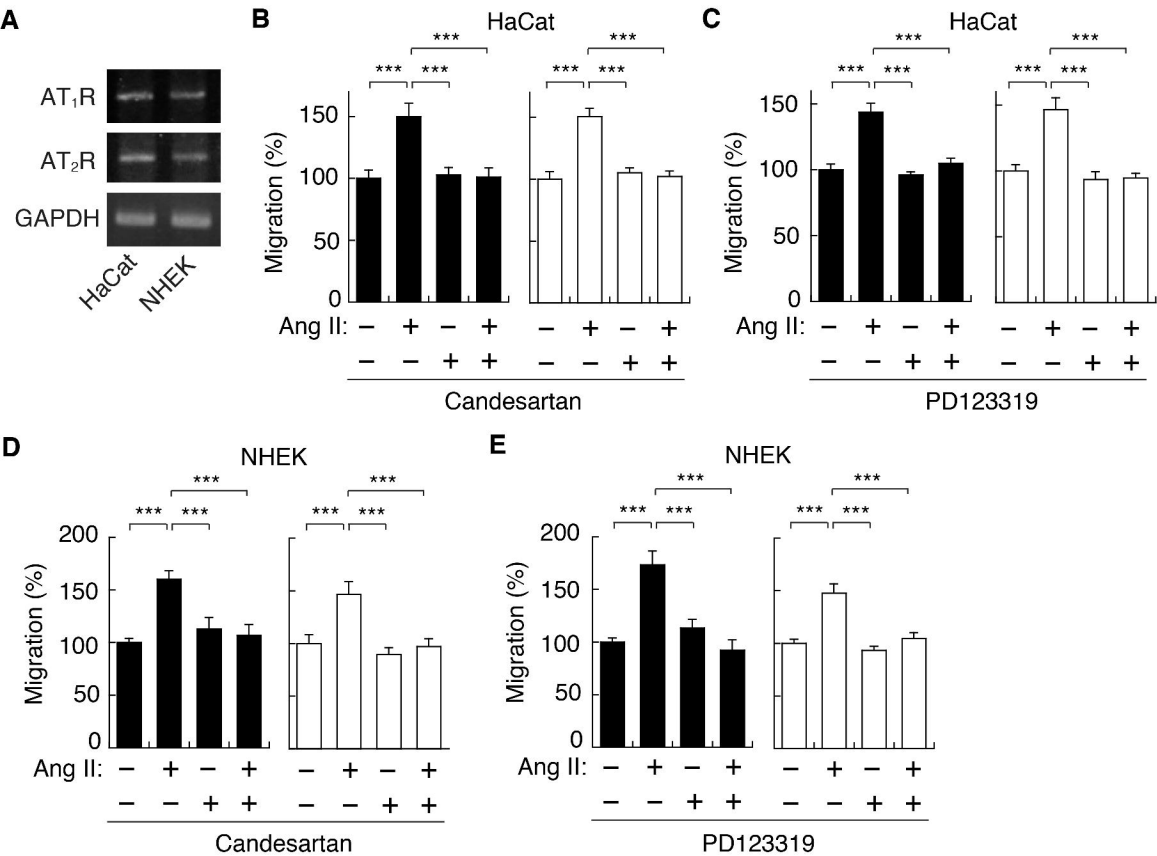
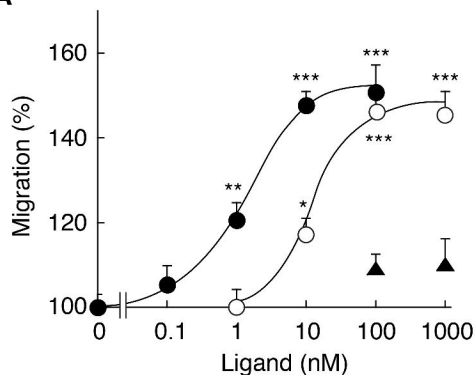
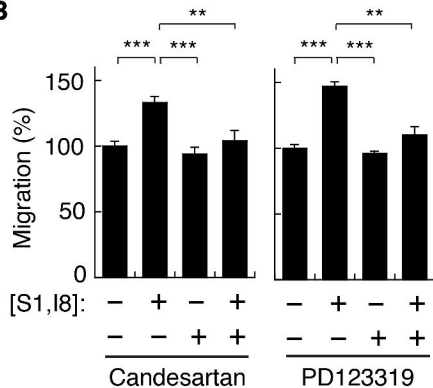


Figure 3

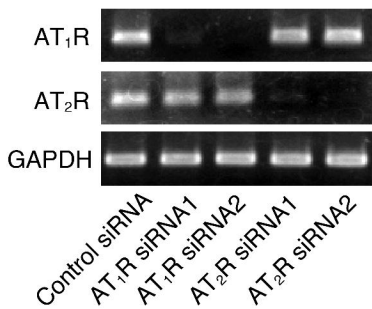
**A**



**B**



**C**



**D**

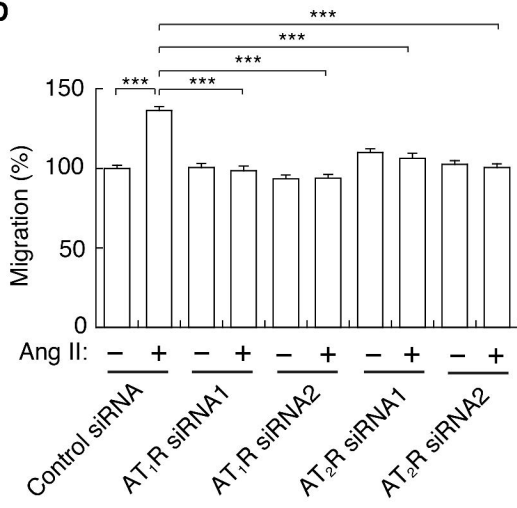


Figure 4

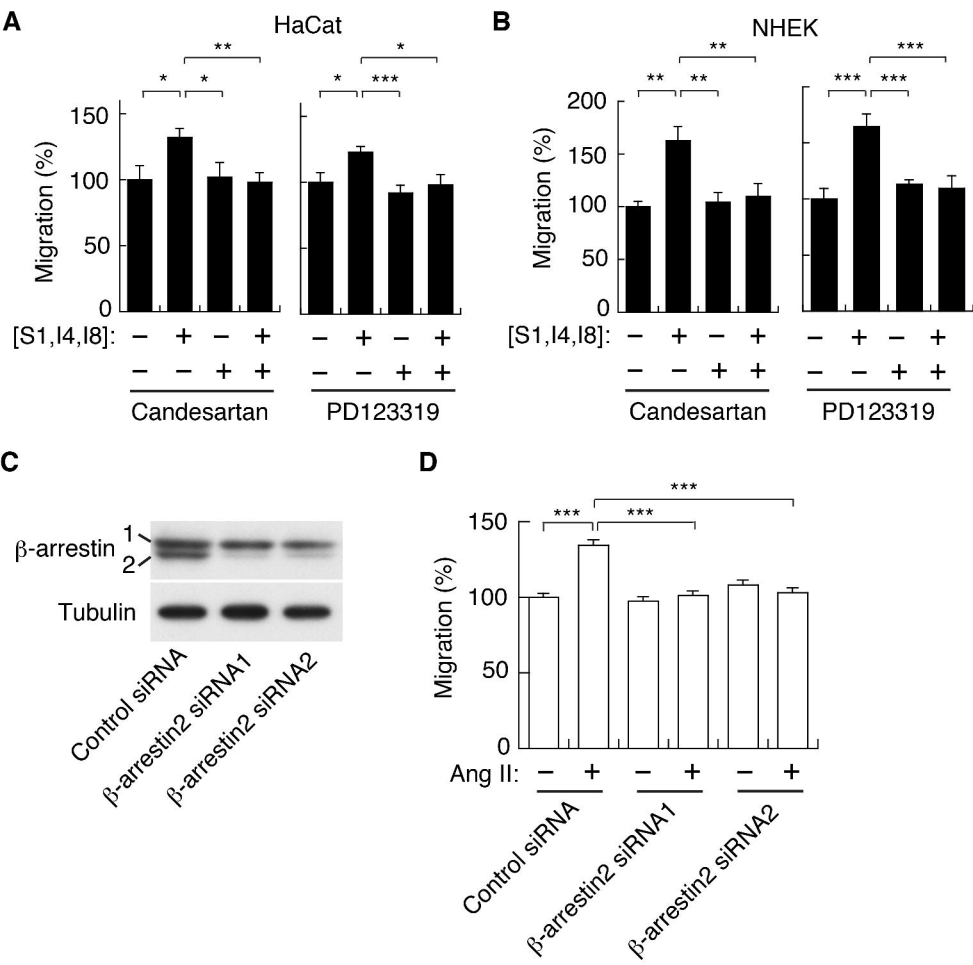


Figure 5

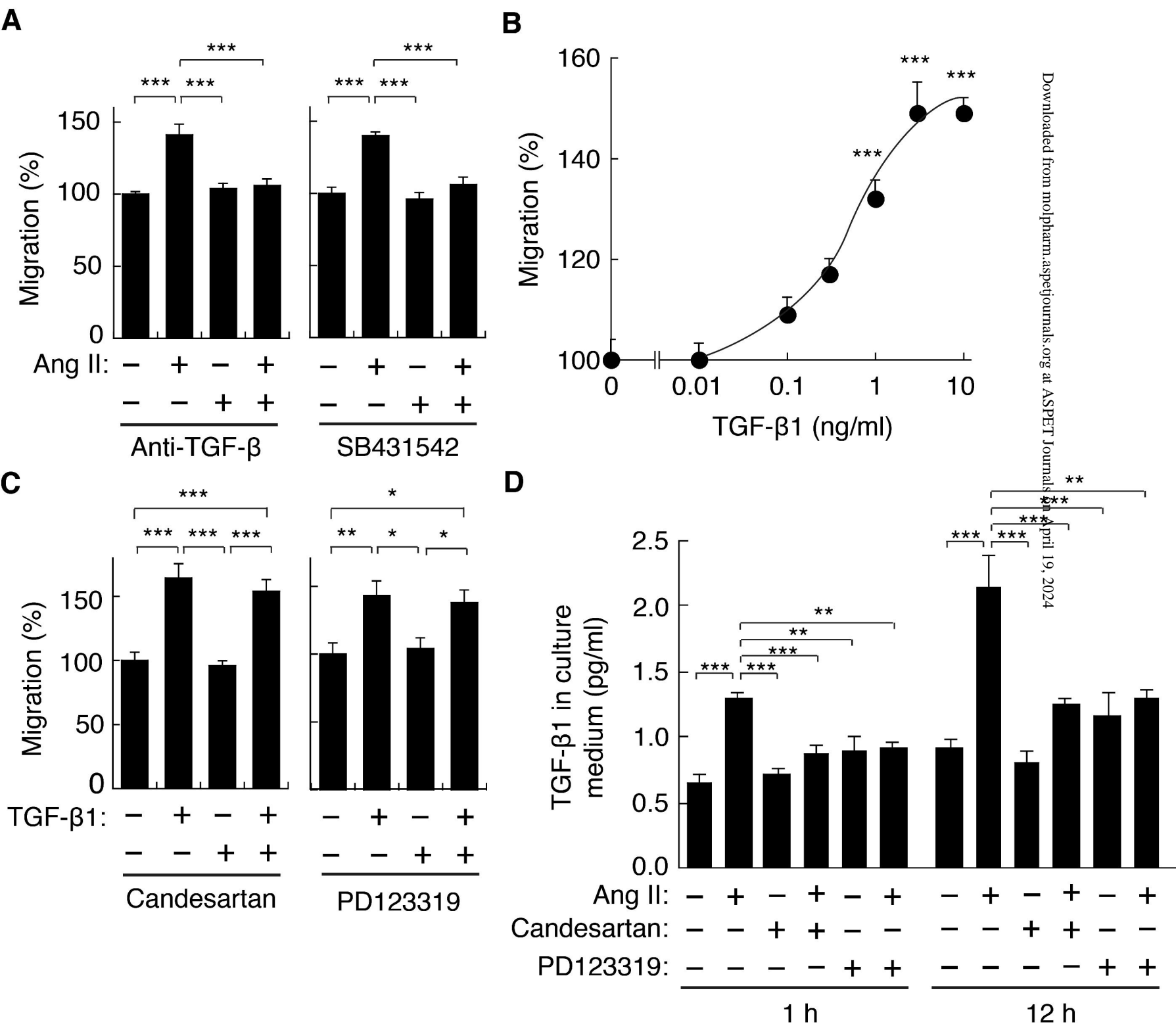


Figure 6

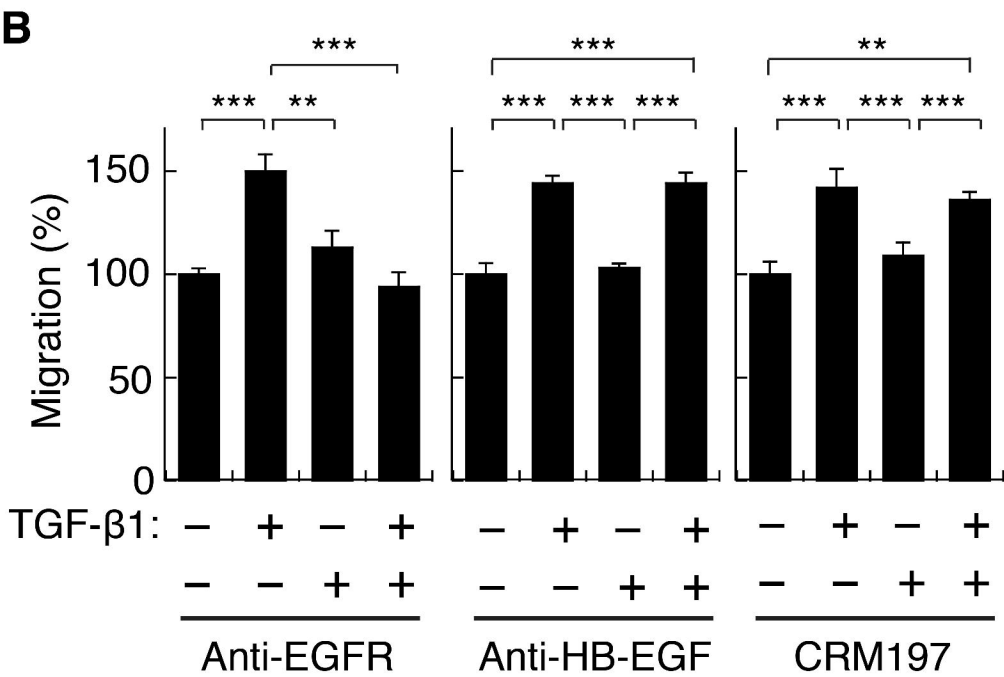
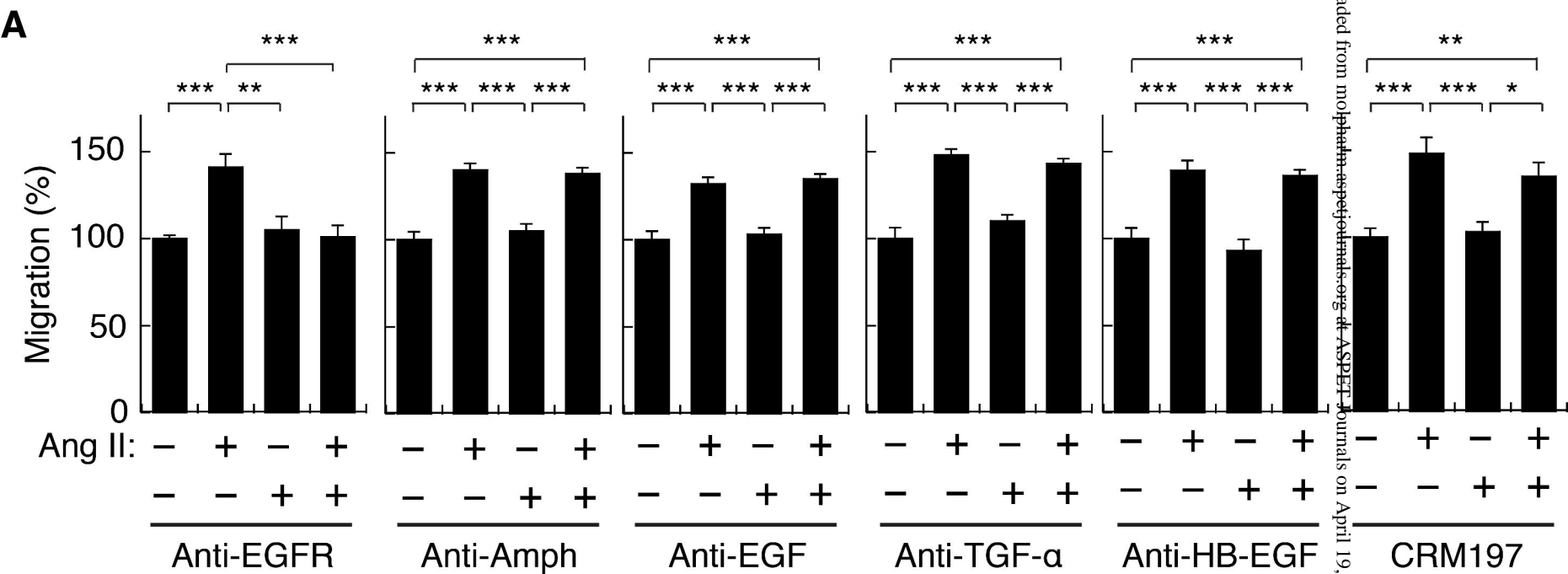


Figure 7

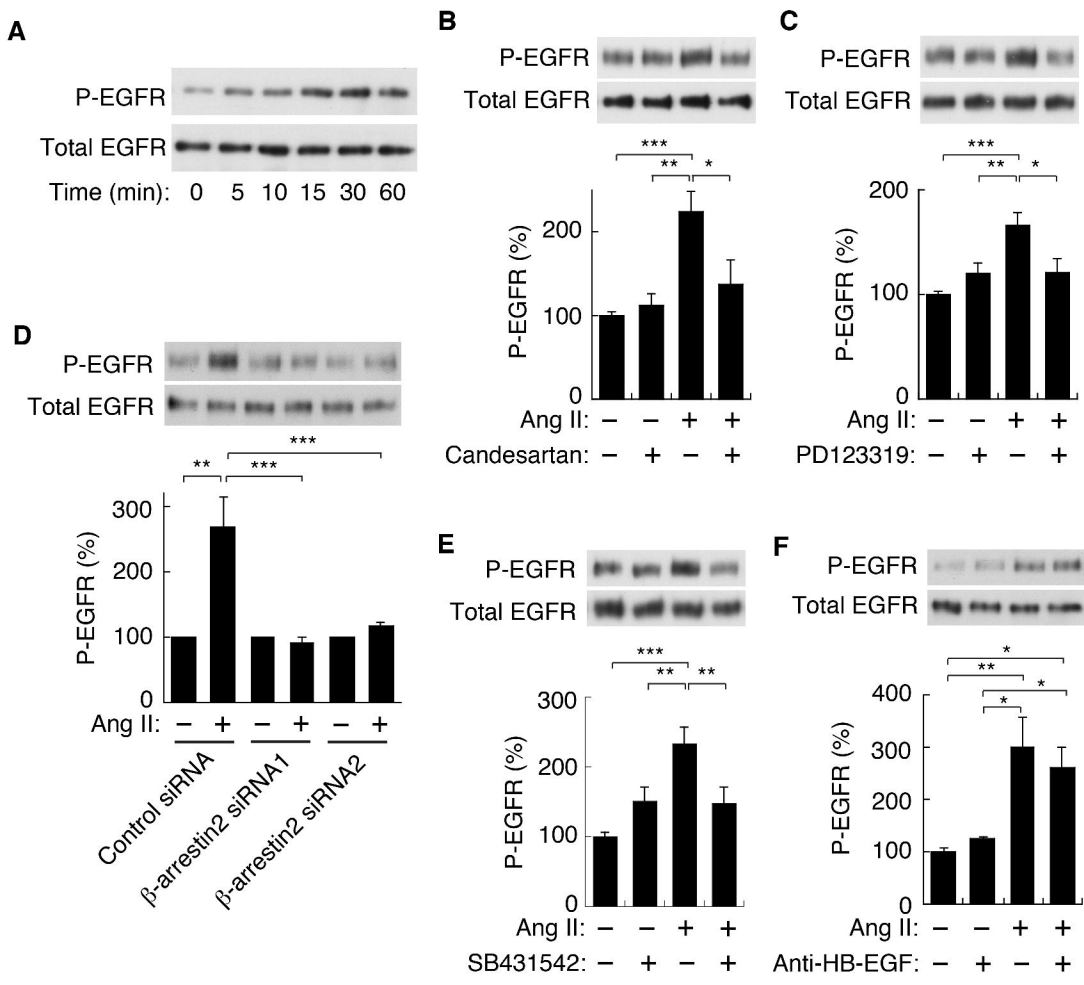


Figure 8

