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Overexpression of angiotensin II type 1 receptor in breast cancer cells induces epithelial–mesenchymal transition and promotes tumor growth and angiogenesis

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A B S T R A C T

The angiotensin II type I receptor (AGTR1) has been implicated in diverse aspects of human disease, from the regulation of blood pressure and cardiovascular homeostasis to cancer progression. We sought to investigate the role of AGTR1 in cell proliferation, epithelial–mesenchymal transition (EMT), migration, invasion, angiogenesis and tumor growth in the breast cancer cell line MCF7. Stable overexpression of AGTR1 was associated with accelerated cell proliferation, concomitant with increased expression of survival factors including poly(ADP-ribose) polymerase (PARP) and X-linked inhibitor of apoptosis (XIAP), as well as extracellular signal-regulated kinase (ERK) activation. AGTR1-overexpressing MCF7 cells were more aggressive than their parent line, with significantly increased activity in migration and invasion assays. These observations were associated with changes in EMT markers, including reduced E-cadherin expression and increased p-Smad3, Smad4 and Snail levels. Treatment with the AGTR1 antagonist losartan attenuated these effects. AGTR1 overexpression also accelerated tumor growth and increased Ki-67 expression in a xenograft model. This was associated with increased tumor angiogenesis, as evidenced by a significant increase in microvessels in the intratumoral and peritumoral areas, and enhanced tumor invasion, with the latter response associated with increased EMT marker expression and matrix metallopeptidase 9 (MMP-9) upregulation. In vivo administration of losartan significantly reduced both tumor growth and angiogenesis. Our findings suggest that AGTR1 plays a significant role in tumor aggressiveness, and its inhibition may have therapeutic implications.

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1. Introduction

Despite advances in clinical management and a growing understanding of tumor biology, breast cancer remains the most frequently diagnosed cancer and the second leading cause of cancer death among women worldwide [1]. Breast cancer growth is frequently governed by activation of the phosphoinositide 3-kinase (PI3K)/Akt pathway, amplification of human epidermal growth factor receptor (HER) family members (such as HER2/ErbB2 and EGFR), and the inactivation of tumor suppressor genes (such as p53, phosphatase and tensin homolog (PTEN) and breast cancer gene (BRCA)) [2–4].

A recent study has reported pathogenic overexpression of the angiotensin II type 1 receptor (AGTR1) in breast cancers [5]. The angiotensin II receptor family members include AGTR1 and AGTR2, and belong to the G-protein-coupled receptor superfamily. These have diverse biological functions including the regulation of blood pressure and cardiovascular homeostasis [6,7]. Increasing evidence demonstrates that AGTR1 is involved in cell proliferation via multiple intracellular signaling pathways including mitogen-activated protein kinase (MAPK), PI3K/Akt and janus kinase/signal transducer and activator of transcription (JAK/STAT3) in solid malignancies of the pancreas, ovary, prostate and breast [5,6,8–11]. In addition, a recent clinical study involving DNA microarray analysis revealed that AGTR1 upregulation is associated with approximately 10–20% of all breast cancer cases that are ER-positive and HER2-negative. More importantly, AGTR1 is overexpressed more than 20-fold in 15.5% of all breast cancers, with some biopsies showing more than 100-fold overexpression in primary and metastatic tumors [5,12]. However, the precise role of AGTR1 overexpression in breast cancer tumor progression has not been clearly elucidated.

EMT is an essential step that occurs during both embryonic development and cancer progression. The initiation of EMT arises from a loss of cell polarity and cell–cell adhesion, which enhances the ability of cells to migrate and invade [13,14]. In breast cancers, EMT can be induced by

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the dysregulation of multiple extracellular signaling factors including TGF-β, Wnt, Notch and receptor tyrosine kinases (RTKs) [15,16]. Over the past several decades, the majority of EMT studies have focused on the TGF-β/Smad signaling pathway [17–19]. The Snail and Smad3/4 transcriptional repressor complex promotes EMT by repressing the junction component E-cadherin [20]. Decreased E-cadherin has been associated with tumor invasiveness and advanced tumor stages, as well as metastasis, leading to poorer clinical outcomes in gastric, colon, lung, prostate and breast cancers [21–28]. Several previous studies have reported that Ang II-AGTR1 regulates TGF-β-mediated EMT in cardiac fibrosis, vascular fibrosis and solid cancers [29–36].

Angiogenesis leads to the formation of new blood vessels and plays an important role in tumor progression [37,38]. AGTR1 is thought to play a major role in tumor angiogenesis in ovary, bladder, lung, and breast cancers [39–41]. Accumulating evidence suggests that the inhibition of AGTR1 by antagonists such as losartan and candesartan can suppress angiogenesis, thereby contributing to the suppression of tumor growth and metastasis [35,42–44]. In the present study, we sought to define the oncogenic role of AGTR1 in cell proliferation, migration, invasion and angiogenesis in breast cancer in vitro and in vivo. We hypothesize that AGTR1 overexpression promotes cell proliferation and EMT, thereby accelerating tumor cell growth and angiogenesis in breast cancer.

2. Materials and methods

2.1. Reagents and antibodies

Triton X-100, propidium iodide (PI), phosphate buffered saline (PBS) tablets, dimethyl sulfoxide (DMSO), losartan potassium and angiotensin II were obtained from Sigma-Aldrich (St. Louis, MO, USA). Phosphatase inhibitor and protease inhibitor cocktail tablets were purchased from Roche Applied Sciences (Penzberg, Germany). The following primary antibodies were used: AGTR1 (Thermo Fisher Scientific Inc., Rockford, IL, USA); total-ERK1/2, phospho-ERK1/2 (Thr202/Tyr204), phospho-Smad3 (Ser423/425), PARP, and MMP-9 (Cell Signaling, Beverly, CA, USA); XIAP, Snail, Ki-67, and CD31 (Abcam, Cambridge, MA, USA); Smad4 and Smad3 (Santa Cruz Biotechnology Inc., CA, USA); E-cadherin and N-cadherin (BD Biosciences, Franklin Lakes, NJ); vimentin (Dako, Glostrup, Denmark); and β-actin (Sigma-Aldrich, St Louis, MO, USA). The secondary antibodies used included horseradish peroxidase (HRP)-conjugated anti-rabbit and mouse IgG (Bio-Rad Laboratories, Hercules, CA, USA); Alexa Fluor-488 or -594 goat anti-mouse and rabbit IgG, and Texas Red-X Phalloidin (Invitrogen, Carlsbad, CA, USA).

2.2. Cell culture

The human breast cancer cell line MCF7 (American Type Culture Collection, Manassas, VA, USA) was cultured in RPMI 1640 containing 10% fetal bovine serum (FBS), streptomycin–penicillin (100 U/ml) and Fungizone (0.625 μg/ml). Cells were incubated at 37 °C in an atmosphere of 5% CO_2.

2.3. Stabilized AGTR1 overexpression in MCF7 cells

AGTR1-overexpressing MCF7 cells were generated using a lentiviral system. The AGTR1 gene was amplified by PCR using specific primers (enzyme site: NheI/NotI), and then inserted into a dual promoter lentivector (CD550A-1, System Biosciences, USA). Viruses were produced with lentiviral packaging, and transfected into HEK293T producer cells. Pseudoviral particles were collected and concentrated via centrifugation, and then transfected into MCF7 target cells. After the infection, puromycin selection was performed, and single colonies were isolated from a dish. The final concentration of puromycin was 3 μg/ml, and no mycoplasmas were detected in the resultant cell lines.

2.4. Si-RNA for Smad4 transfection

Cells were seeded into 6-well plates 24 h prior to transfection. The AGTR1-overexpressing MCF7 cells were transfected with si-Smad4 (Invitrogen, Carlsbad, USA) or control si-RNA (0–200 nM) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) for 48 h according to the manufacturer's instructions. The Smad4 Si-RNA sequences were as follows: 5′-GGA GAG ACG UUU AAG GUC CCU UCA A-3′ and 5′-UUG AAG GGA CCU UAA ACG UCU CUC C-3′.

2.5. Cell viability assay

Cell viability was assessed using the CellTiter 96* Aqueous One Solution Cell Proliferation Assay [MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] (Promega, Madison, WI, USA) according to the manufacturer's instructions. The quantity of formazan product was determined by measuring absorbance at 490 nm using a Spectramax Plus 384 microplate analyzer (Molecular Devices, Sunnyvale, CA, USA).

2.6. Western blot analysis

Cells were solubilized in lysis buffer (30 mM NaCl, 0.5% Triton X-100, 50 mM Tris–HCl; pH 7.4) containing a cocktail of phosphatase and protease inhibitors. Supernatant was collected (14,000 × g, 4 °C, 20 min) and protein concentration was determined with a Bradford protein assay kit (Bio-Rad Laboratories). Equal quantities of protein (30 μg) were subjected to SDS-PAGE and electrotransferred onto nitrocellulose membranes (GE Healthcare Life Science, Buckinghamshire, UK). The membranes were incubated overnight at 4 °C with primary antibodies diluted in 5% BSA [AGTR1 (1:1000), t-ERK1/2 (1:2000), p-ERK1/2 (1:2000), PARP (1:2000), XIAP (1:2000), E-cadherin (1:2000), N-cadherin (1:2000), p-Smad3 (1:1000), Smad4 (1:2000), Snail (1:2000) and β-actin (1:5000)], followed by incubation with horseradish peroxidase (HRP)-conjugated rabbit or mouse IgG (1:3000–1:10,000). Signal intensity was detected using an Enhanced Chemiluminescence Kit (Thermo Scientific Inc., Rockford, IL, USA) and x-ray film (Agfa Healthcare, Mortsel, Belgium) and quantitated using AlphaEaseFC software (Alpha Innotech, San Leandro, CA, USA).

2.7. RT-PCR analysis

Total RNA was extracted using an RNeasy mini kit (Qiagen, Valencia, CA, USA), according to the manufacturer's instructions. Amplification of transcripts was achieved by reverse transcriptase-polymerase chain reaction (RT-PCR) using 1 μg/μl total RNA, Molony Murine Leukemia Virus reverse transcriptase (MMLV; Gibco/BRL, Gaithersburg, MD, USA), and oligo-d(T)15 primers (Roche Applied Sciences). PCR amplification was achieved using a Takara PCR Thermal Cycler (Thermo Scientific Inc., Rockford, IL) with the following primers: AGTR1, forward 5′-GATGAT TGTCCCAAAGCTGG-3′ and reverse 5′-TAGGTAATTGCCAAAGGGCC-3′ and actin, forward 5′-ACCCAGATGTTTGAGAC-3 and reverse 5′-GGAG TTGAAGGTAGTTTCGT-3′. The PCR products were separated on 1.2% agarose gels and visualized using a Gel Doc™ XR + System (Bio-Rad Laboratories).

2.8. Immunocytochemistry

Cells on 8-well chamber slides (BD Biosciences, Franklin Lakes, NJ) were fixed with 4% paraformaldehyde, washed with PBS, and incubated with 0.2% Triton X-100 for 10 min. Primary antibodies in antibody-diluent (Dako, Glostrup, Denmark) were incubated overnight at 4 °C and then incubated with fluorescence-conjugated secondary antibodies (Alexa Fluor®-488 or -594). Cells were mounted with ProLong Gold Antifade Reagent with DAPI (Life Technologies, Carlsbad, CA, USA). Images were acquired using a Carl Zeiss confocal microscope (Weimar,

Germany). Intensity of fluorescence was analyzed using fluorescence profiling, as previously described [45].

2.9. Wound healing assay

Cell monolayers (80% confluence) were scratched with a 200 μl pipette tip, and wound closure was monitored over a 48 h period. Images were captured at 0 and 48 h after wounding using a Nikon Eclipse TE300 microscope (Nikon, Tokyo, Japan).

2.10. Invasion assay

Invasion chambers were coated with matrigel matrix (BD Biosciences, Franklin Lakes, NJ) according to the manufacturer's recommendations. Cells (1.5×10^5) were trypsinized, washed and suspended onto the upper chamber with serum-free media. Conditioned media was added to the lower chambers. The chambers were incubated for 48 h at 37 °C in a humidified atmosphere of 5% CO_2. Invaded cells were fixed and stained with Diff-Quik (Sysmex, Kobe, Japan) and counted under a BX51 microscope (Olympus, Tokyo, Japan).

2.11. Xenograft mouse model

All animal procedures were carried out in accordance with animal care guidelines approved by the Korea University Institutional Animal Care and Use Committee (IACUC). Five-week-old female BALB/c nude mice were obtained from the Shizuoka Laboratory Animal Center (Shizuoka, Japan) and housed in a specific pathogen-free environment. The animals were acclimated for 1 week prior to the study and had free access to food and water. MCF7 cells (1×10^7) or AGTR1-overexpressing

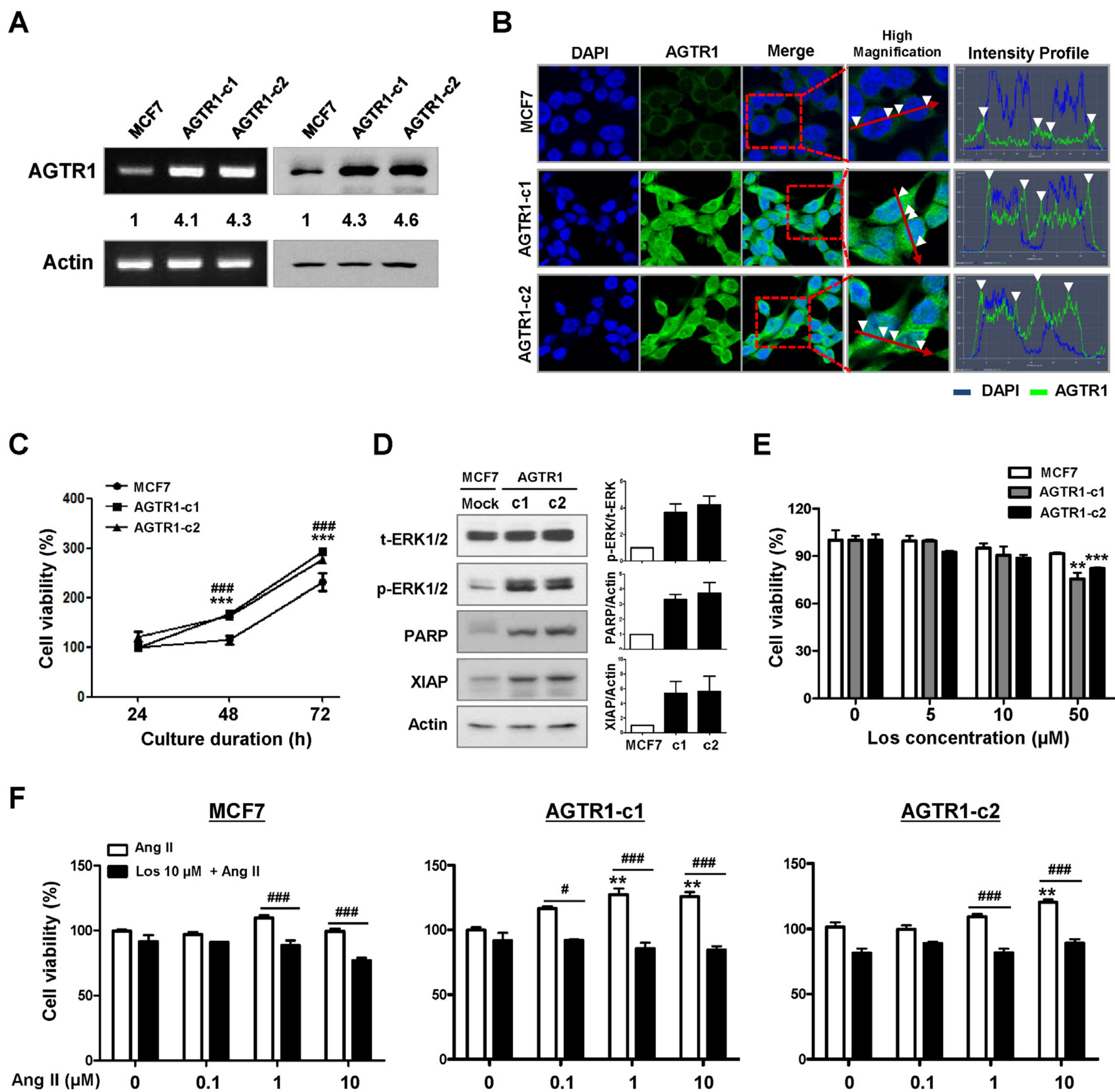

Fig. 1. AGTR1 overexpression increases cell proliferation and upregulates survival factors. (A) AGTR1 mRNA and protein expression normalized to β-actin. (B) Subcellular localization of AGTR1. Cells were immunostained for AGTR1 (green) and nuclei were counter-stained with DAPI (blue). Selected areas from merged images are shown at high magnification. AGTR1 intensity (green) and subcellular localization (white arrows) were analyzed by confocal microscopy using the intensity profile tool (straight red line with arrow). (C) Effect of AGTR1 overexpression on cell proliferation. Cell proliferation was assessed using MTS assay and data were analyzed by two-way ANOVA followed by Bonferroni's post-hoc test (MCF7 vs AGTR1-c1, $^{***}p < 0.001$; MCF7 vs AGTR1-c2, $^{###}p < 0.001$). (D) Total-ERK1/2 and phospho-ERK1/2, as well as total PARP and XIAP protein expression in MCF7 cells and the AGTR1-c1, -c2 overexpression cell lines as determined by Western blot analysis. Quantification of the phospho-ERK/ERK ratio and levels of PARP and XIAP are shown in the right panels. β-Actin was used as loading control. (E) Effect of losartan on cell viability. Cells were treated with losartan (0–50 μM) for 48 h and cell viability was determined by MTS assay in MCF7 and AGTR1-overexpressing cells ($^{**}p < 0.01$; DMSO control vs losartan treatment groups). (F) Influence of losartan on cell viability in the presence of Ang II in MCF7 and AGTR1-overexpressing cells. Cells were treated with Ang II (0–10 μM) and/or losartan (10 μM) for 48 h and cell viability was analyzed by MTS assay. Results are expressed as mean ± SEM (n = 3 independent experiments) and were analyzed by two-way ANOVA followed by Bonferroni's post-hoc test ($^{**}p < 0.01$; DMSO control vs Ang II; $^{\#}p < 0.05$, $^{###}p < 0.001$, Ang II alone vs Ang II + losartan treatment).

MCF7 cells (1×10^7) in 100 μl of culture medium were mixed with 100 μl of matrigel and implanted subcutaneously in the left or right flank of 6-week-old BALB/c nude female mice (n = 10/each group). Mice were inoculated with 17β-estradiol pellets (1.72 mg, Innovative Research of America, USA) 48 h before injection of cells. Tumor volumes were measured using a caliper and calculated using the formula $V = (\text{Length} \times \text{Width}^2) / 2$.

2.12. Mammary fat pad xenograft model

5×10^6 AGTR1-overexpressing cells were injected into the fourth mammary fat pads in six-week-old female BALB/c nude mice. After 9 days, the animals were randomized into 2 groups (n = 5/each group) and vehicle control (PBS) or losartan potassium (90 mg/kg, 3 times/week) were administered intraperitoneally. Tumor volumes (V) were measured by caliper (3 times/week) and calculated using the following formula; $V = (\text{Length} \times \text{Width}^2) / 2$.

2.13. Immunohistochemistry

At sacrifice, tumor tissues were removed, fixed in 10% neutral-buffered formalin, and paraffin embedded. Tissue sections of 4-μm thickness were mounted on positively-charged glass slides and then deparaffinized with xylene and dehydrated through a graded alcohol

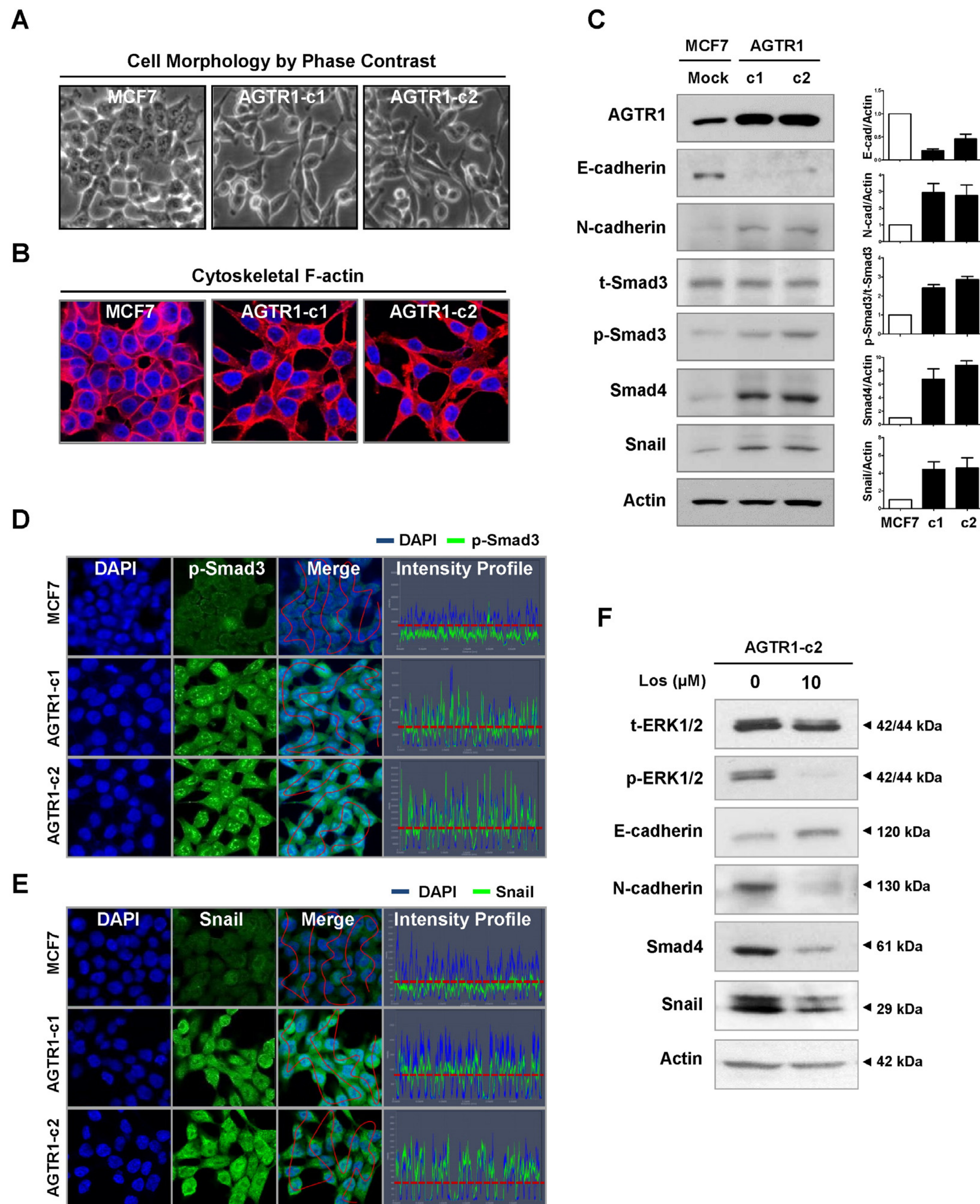

Fig. 2. Overexpression of AGTR1 induces EMT in MCF7 cells.(A) Effect of AGTR1 overexpression on MCF7 cell morphology (×200 magnification). (B) Differential expression patterns of cytoskeletal F-actin in MCF7 and AGTR1-c1 and -c2 cell lines. Cells were stained with fluorescent phalloidin (F-actin, red) and nuclei were stained with DAPI (blue). (C) Effect of AGTR1 overexpression on levels of EMT-related markers. Quantitative graphs of E-cadherin, N-cadherin, Smad4 and Snail expression, and the ratio of phospho-Smad3/total-Smad3 are shown in the right panel. (D–E) Influence of AGTR1 on p-Smad3/Snail expression and subcellular localization. Cells were immunostained for p-Smad3 (green, D) or Snail (green, E) and nuclei were stained with DAPI (blue). Immunofluorescence analysis reveals increased nuclear accumulation of p-Smad3 and Snail. Images are shown at ×500 magnification. Intensity profiles are represented in arbitrary units as defined by the software and scaled on the y-axis. (F) Effect of losartan treatment on AGTR1-induced EMT. AGTR1-c2 cells were treated with losartan (10 μM) for 6 days and total-ERK1/2, phospho-ERK1/2, E-cadherin, N-cadherin, Smad4 and Snail protein levels were determined by Western blot analysis.

series to water. For antigen retrieval, sections were boiled in citric acid buffer (pH 6.0) for immunofluorescence analysis. Tissue sections with primary antibodies (Ki-67; 1:200 or CD31; 1:100, E-cadherin; 1:100 and vimentin; 1:200, MMP-9; 1:100) in antibody-diluent were incubated overnight at 4 °C. For secondary antibody reactions, the sections were incubated with fluorescent Alexa Fluor® 594 or 488-conjugated secondary antibody (Invitrogen, Carlsbad, CA, USA) at RT for 2 h, and then mounted with ProLong gold anti-fade reagent with DAPI. Images were acquired using a Carl Zeiss confocal microscope and fluorescence intensity was analyzed using a histogram tool in the Carl Zeiss software package.

2.14. Statistical analysis

All data were analyzed using GraphPad Prism 5.0 statistical software (San Diego, CA, USA). The results are presented as mean ± SEM of at least three independent experiments. Data were analyzed by Student's t-test and two-way ANOVA, as appropriate. A two-way ANOVA was used to assess the effects and interactions of two variables and multiple comparisons were achieved using Bonferroni's post hoc test. Statistical significance was defined at $p < 0.05$.

3. Results

3.1. AGTR1 overexpression promotes cell proliferation and upregulates survival-related factors

To investigate the role of AGTR1 in breast cancer, MCF7 cells were stably transfected with an expression construct carrying cDNA for human full-length AGTR1, and named AGTR1 clone number 1 (AGTR1-c1) and clone number 2 (AGTR1-c2). Western blot and RT-PCR analyses were performed to confirm the stable overexpression of AGTR1. AGTR1 levels were confirmed to be more than 4- to 5-fold higher in comparison to the parental MCF7 cells (Fig. 1A). Subcellular localization of AGTR1 was also confirmed by immunocytochemistry using confocal microscopy. Distribution of AGTR1 was localized to the plasma membrane, with green fluorescent intensity markedly higher in the AGTR1-c1 and -c2 cells (Fig. 1B).

MTS assays revealed that AGTR1-c1 and -c2 cells proliferated significantly faster than their parental MCF7 cells ($^{###}p < 0.001$, $^{***}p < 0.001$, Fig. 1C). This response was associated with the increased expression of survival factors such as PARP and XIAP as well as ERK activation (Fig. 1D).

To explore the effect of losartan on cell viability, cells were treated with various concentrations of losartan (0–50 μM) for 48 h. A significant effect on cell viability was observed at 50 μM of losartan in AGTR1-c1 and -c2 cells. However, losartan had no significant inhibitory effect in the parental MCF7 cells (Fig. 1E). We next examined the effect of AGTR1 overexpression on cell viability upon stimulation with angiotensin II (Ang II), a bioactive ligand of AGTR1, and the antagonist losartan for 48 h. Ang II significantly increased cell proliferation in AGTR1-c1 and -c2 cells in a dose-dependent manner (CTL vs Ang II $^{**}p < 0.01$), whereas the parental MCF7 cells were not statistically different. Ang II-induced cell proliferation was suppressed by losartan challenge (10 μM) in AGTR1-c1 and -c2 cells (Ang II alone vs combination treatment with Ang II + losartan, $^{#}p < 0.05$, $^{###}p < 0.001$, Fig. 1F).

3.2. AGTR1 overexpression induces EMT

Luminal-type MCF7 cells exhibited typical features of epithelial cells, whereas the AGTR1 overexpressing AGTR1-c1 and-c2 cells were observed to exhibit a mesenchymal-like phenotype (Fig. 2A), including re-arrangement of cytoskeletal F-actin with elongated spindles (Fig. 2B). We sought to further confirm whether these changes in cell morphology were indicative of EMT. Western blot analysis revealed that AGTR1-c1 and-c2 cells exhibited a loss of E-cadherin and a marked increase in N-cadherin expression, as well as increased EMT-related transcription factors such as phospho-Smad3, Smad4 and Snail, when compared with the parental MCF7 cells. There were no differences in protein expression of total-Smad3 between the MCF7- and AGTR1-

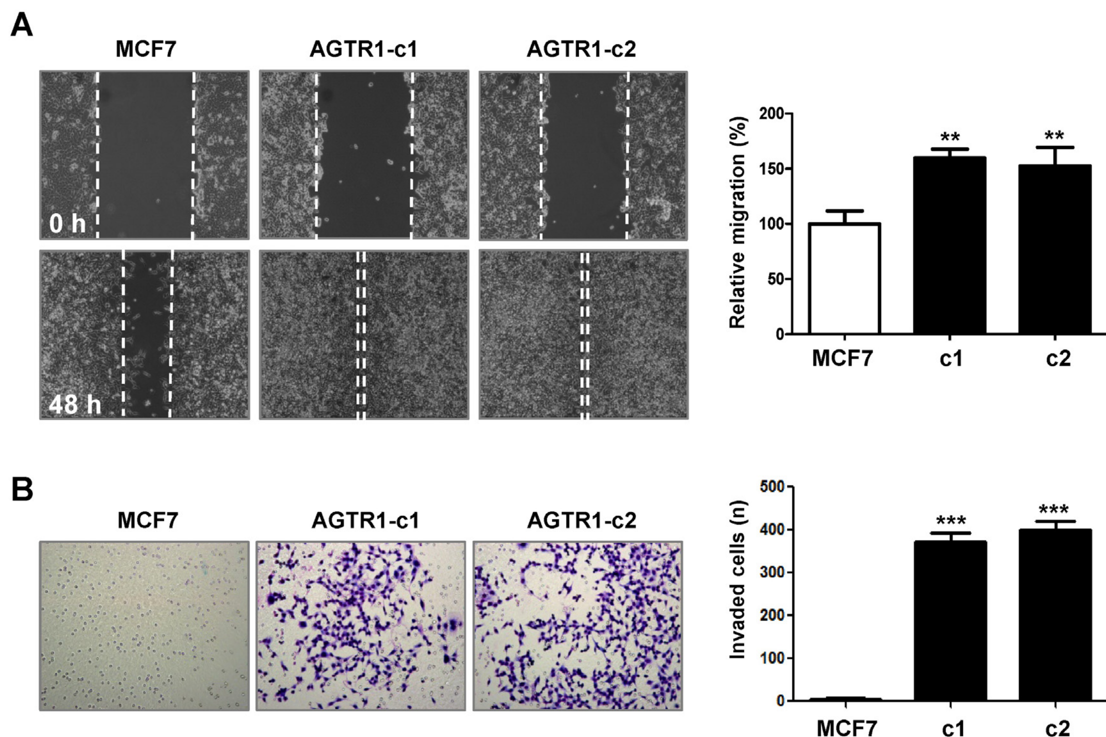

Fig. 3. AGTR1 accelerates cell migration and invasion. (A) Cell migration was assessed over 48 h by wound healing assay. Images were captured by phase contrast microscopy (×100 magnification). Graph values represent the average relative migratory distance (right panel, $^{**}p < 0.01$). (B) Effect of AGTR1 overexpression on cell invasion. Images were captured with an inverted microscope (×200 magnification). The total number of invading cells in the fields were counted and are shown in the right panel ($^{***}p < 0.001$).

overexpressing cells (Fig. 2C). Furthermore, immunofluorescence intensity profiling revealed that p-Smad3 and Snail expression was considerably upregulated and accumulated in the nuclei of AGTR1-overexpressing cells, whereas their distribution was primarily confined to the cytoplasm in the parental MCF7 cells (Fig. 2D and E). Importantly, challenge with the AGTR1 antagonist losartan notably downregulated N-cadherin, Smad4 and Snail expression, while upregulating E-cadherin. ERK phosphorylation was also decreased in the AGTR1-overexpressing cells by losartan treatment (Fig. 2F).

3.3. AGTR1 overexpression promotes breast cancer cell migration and invasion

Cell migration and invasion are two hallmarks of EMT in human mammary epithelial cells [46]. To investigate the effect of AGTR1 overexpression on cell migration and invasion, wound healing and matrigel invasion assays were performed. As shown in Fig. 2A, MCF7 cells exhibited a typical epithelial phenotype with an absence of invasive or migratory behavior. In contrast, the AGTR1-overexpressing cells acquired both migratory (**$p < 0.01$, Fig. 3A) and invasive properties (***$p < 0.001$, Fig. 3B).

3.4. Inhibition of Smad4 reverses AGTR1-induced EMT

To examine whether AGTR1-induced EMT was mediated by Smad action, knockdown of Smad4, as a major Smad signaling component, was undertaken. AGTR1-c1 and-c2 cells were transiently transfected with si-Smad4 or si-CTL for 48 h to reduce Smad4 activity, before cell morphology was assessed. Phase contrast analysis revealed that RNA interference-mediated downregulation of Smad4 induced morphological changes in the AGTR1-overexpressing cells from a spindle-shaped mesenchymal phenotype to a cobblestone-shaped epithelial phenotype (Fig. 4A). To further support this observation, we examined the effect of Smad4 knockdown on E-cadherin expression in AGTR1-overexpressing cells. E-cadherin level was completely restored by blockage of Smad4 in both AGTR1-c1 and -c2 cells (*$p < 0.05$, ***$p < 0.001$, Fig. 4B). Immunofluorescence analysis also revealed that si-Smad4-transfected cells exhibited a predominant epithelial-like phenotype with a marked increase in E-cadherin levels (Fig. 4C). These results suggest that induction of EMT by AGTR1 is mediated by the Smad signaling pathway.

3.5. AGTR1 promotes tumor growth in vivo

To confirm the physiological relevance of our in vitro observations, we examined the effects of AGTR1 on tumor growth using xenografted mice harboring both MCF7 and AGTR1-overexpressing MCF7 cells. Parental MCF7 and AGTR1-overexpressing MCF7 cells were subcutaneously injected into the left and right flanks of 6-week Balb/c nude female mice. Tumor volumes were measured every other day for three weeks with a caliper. Over the course of 21 days, we observed that the AGTR1 tumors exhibited a significant increase in volume (*$p < 0.05$, ***$p < 0.001$, Fig. 5A and B) and weight (***$p < 0.001$, Fig. 5C) as well as a marked increase in the number of Ki-67-positive cells (***$p < 0.001$, Fig. 5D) compared to the MCF7 tumors. To support our in vitro observations, EMT markers were assessed by immunohistochemical analysis. AGTR1-overexpressing

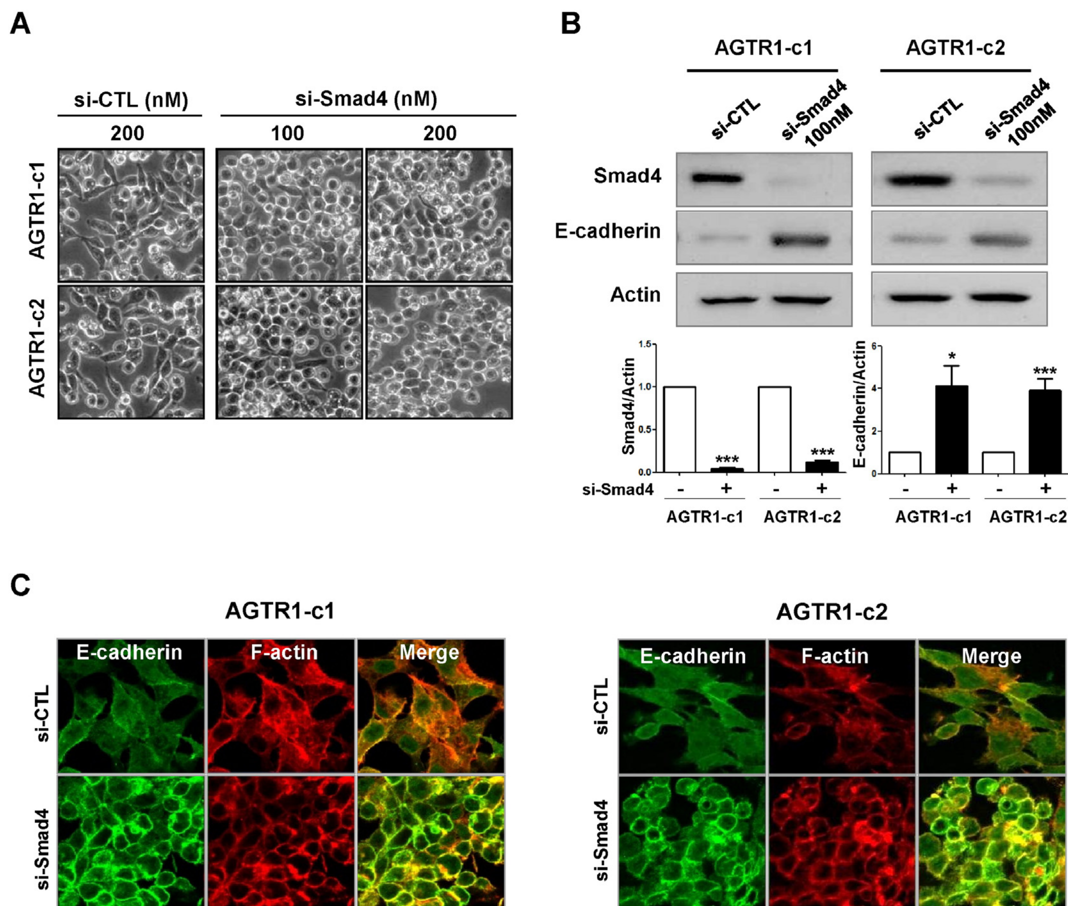

Fig. 4. Knockdown of Smad4 suppresses AGTR1-induced EMT. (A–C) AGTR1-c1 and-c2 cells were transiently transfected with si-Smad4 or si-CTL (0–200 nM) for 48 h. (A) Effect of Smad4 knockdown on cell morphology in AGTR1-c1 and -c2 cells. (B) Smad4 knockdown upregulates E-cadherin protein levels, as determined by Western blot assay. Quantitative graphs of signal intensities of Smad4 and E-cadherin are shown (bottom panel, *$p < 0.05$, ***$p < 0.001$). (C) Influence of Smad4 knockdown on E-cadherin expression and organization of cytoskeletal F-actin. Cells were immunostained for E-cadherin (green) or F-actin (red) and images were captured with a confocal microscope.

tumors exhibited downregulated E-cadherin levels (**p < 0.01, Fig. 5E) and an upregulation of vimentin expression (**p < 0.01, Fig. 5F). Furthermore, we observed that AGTR1 tumor cells had invaded into the marginal adipose tissue and muscle layer (Fig. 5G).

Matrix metalloproteinase MMP-9 is extensively expressed in aggressive malignant tumors, and the degradation of extracellular matrix (ECM) by MMPs contributes toward tumor invasion and angiogenesis [47,48]. Recent studies have shown that Ang II-AGTR1 increases MMP-9 expression, which accelerates cell migration and invasion, whereas knockdown of MMP-9 reduces these events in breast cancer cells [49,50]. To examine whether AGTR1-induced invasiveness is associated with MMP-9 expression, immunohistochemical analysis of the xenograft tumor samples for MMP-9 was performed. A significant upregulation of MMP-9 expression

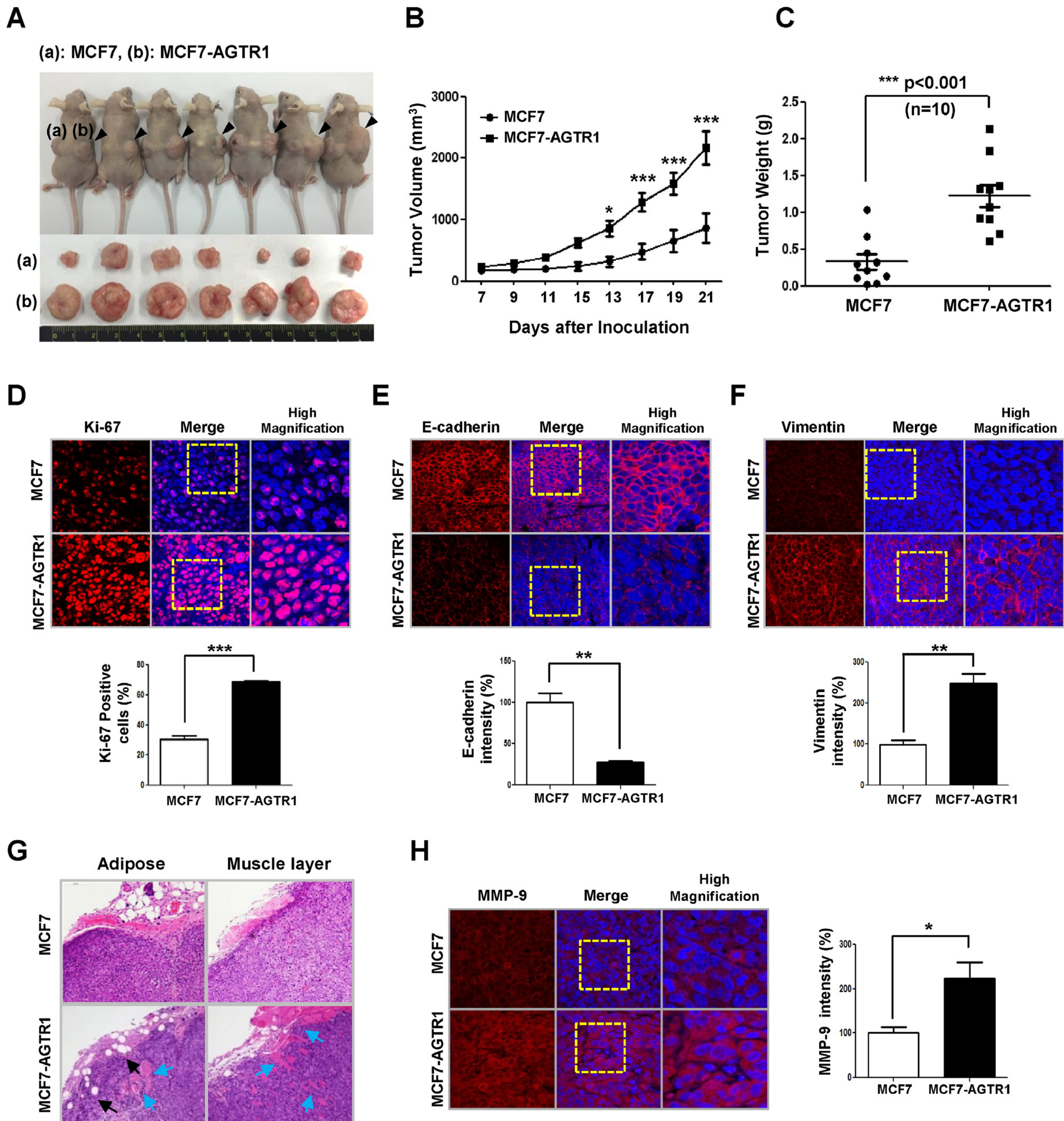

Fig. 5. Overexpression of AGTR1 increases tumor growth in a mouse xenograft model. (A–D) Effect of AGTR1 on tumor growth in vivo. (A) Images of tumors formed by subcutaneous insertion of MCF7 cells (left flanks, a) or AGTR1-overexpressing MCF7 cells (right flanks, b) in female 6-week old Balb/c nude mice (n = 10). (B–C) AGTR1 tumors exhibited statistically significant increases in tumor growth (B, *p < 0.05, ***p < 0.001) and tumor weight (C, ***p < 0.001) when compared to MCF7 tumors. (D) Expression of Ki-67 in MCF7 and MCF7-AGTR1 tumors. Tumor tissues were immunostained with Ki-67 (red) and nuclei were stained with DAPI (blue). The number of Ki-67-positive cells was higher in the AGTR1 tumors when compared to the control groups. The selected areas in merged images are shown at high magnification (×1000). Quantitative graph of the number of Ki-67-positive cells is shown in the bottom panel (***p < 0.001). (E–F) Expression of E-cadherin and vimentin in MCF7 and MCF7-AGTR1 tumors. Tissue sections were immunostained for E-cadherin (E, red) or vimentin (F, red) with DAPI (**p < 0.01). (G) Representative H&E images from MCF7 and MCF7-AGTR1 tumors. AGTR1 tumor cells invaded into both the adipose tissue and muscle layer. Black arrows and blue arrows indicate adipose and muscle tissue invasion, respectively. (H) Expression of MMP-9 in MCF7 and MCF7-AGTR1 tumors. Tumor tissue sections were immunostained with MMP-9 (red) and nuclei were stained with DAPI (nuclei, blue). Quantitation of MMP-9 signal intensity is shown in the right panel (*p < 0.05).

was observed in AGTR1-overexpressing tumors when compared to their control counterparts (*$p < 0.05$, 5H).

3.6. AGTR1 promotes angiogenesis in vivo

Tumor growth is thought to be in part reliant upon the stimulation of tumor angiogenesis [51]. To investigate the effect of AGTR1 overexpression on angiogenesis, blood vessel growth was observed after the experimental animals were sacrificed. AGTR1 overexpression promoted angiogenesis, with an increase in the number and size of the blood vessels present between the tumors and the surrounding tissue (Fig. 6A). Microvessel density (MVD) assays were used for the examination of tumor angiogenesis with the specific vascular endothelial cell marker CD31 [52,53]. The number of CD31-positive microvessels in the peritumoral and intratumoral areas was significantly increased in the AGTR1-overexpressing tumors when compared to the control tumors (**$p < 0.01$, ***$p < 0.001$, Fig. 6B).

3.7. Losartan inhibits tumor growth and angiogenesis upregulated by AGTR1 overexpression

Finally, we examined the effect of losartan on tumor growth using a mammary fat pad xenograft model with the AGTR1-overexpressing cells. 5×10^6 cells were orthotopically implanted into the mammary fat pads of Balb/c female nude mice, and after 9 days, control vehicle (PBS) or losartan (90 mg/kg, body weight) was administered every other day. Losartan administration resulted in a significant reduction in tumor growth (**$p < 0.01$, Fig. 7A) and a significant decrease in the number of Ki-67-positive cells in the AGTR1-overexpressing xenograft tumors (**$p < 0.01$, Fig. 7B). Losartan administration notably upregulated E-cadherin (***$p < 0.001$, Fig. 7C) and suppressed vimentin expression (**$p < 0.01$, Fig. 7D). Furthermore, microvessel density analysis revealed that the number of CD31-positive microvessels in both the peritumoral and intratumoral areas was significantly decreased in the losartan-treated groups (***$p < 0.001$, Fig. 7E).

4. Discussion

Our observations suggest that AGTR1 overexpression promotes cell growth, EMT, cell migration and invasion in vitro, while stimulating tumor growth in vivo. AGTR1-overexpression resulted in an aggressive phenotype characterized by increased levels of the mesenchymal marker vimentin and enhanced tumor invasiveness, as well as increased microvessel density. These in vitro and in vivo events were notably attenuated by the inhibition of AGTR1 by losartan.

AGTR1 activation is primarily regulated by its ligand, angiotensin II (Ang II) as well as antagonists including angiotensin receptor blockers (ARBs) such as losartan, telmisartan, valsartan and candesartan [54,55]. In this study, the AGTR1 activation observed in the MCF7 cells appears to be Ang II-independent. AGTR1 overexpression promoted cell growth, concomitant with increases in PARP and XIAP protein content, but in the absence of Ang II stimulation in vitro. Recent studies have demonstrated that renin–angiotensin system (RAS) components including angiotensin I/II, angiotensinogen, renin, and angiotensin converting enzyme (ACE) are expressed in MCF7, MDA-MB-468 and T47D human breast cancer cell lines and breast cancer tissue [56,57]. It is therefore possible that interplay between higher levels of AGTR1 and the local Ang II/RAS components activates the AGTR1 signaling pathway, enhancing breast cancer cell proliferation.

Inhibitor of apoptosis proteins (IAPs) are broad regulators of survival signaling relevant to malignant neoplasms. In particular, XIAP is the most commonly overexpressed in breast cancer, and promotes cell survival, migration and invasion, as well as evasion of apoptosis via the inactivation of caspases 3, 7 and 9 [58,59]. PARP is essential for DNA repair and recombination, as well as genomic stability. Its upregulation helps tumor cells to evade apoptosis, thereby promoting cancer progression [60]. In this context, we examined whether AGTR1-induced cell proliferation was associated with XIAP and PARP expression. The considerable upregulation of these factors in AGTR1-c1 and -c2 cells observed may contribute to an acceleration of breast cancer cell proliferation.

Recent studies have shown that ERK1/2 phosphorylation is mediated by AGTR1 activation via an Ang II-independent pathway [5,61]. Forced expression of AGTR1 is known to increase ERK phosphorylation, regardless of Ang II stimulation in human mammary epithelial HME cells [5]. Moreover, mechanical factors such as stretch and shear stress induce AGTR1 activation in the absence of Ang II stimulation and subsequently increases ERK and P13K activation. These responses can be blocked by candesartan treatment in cardiomyocytes and Chinese hamster ovary CHO cells [61]. In agreement with these observations, we found that AGTR1 overexpression increased the phospho-ERK/total-ERK ratio, whereas its effect was diminished following losartan challenge. Exposure to Ang II significantly promoted cell proliferation in AGTR1-overexpressing MCF7 cells, but no statistically significant difference between Ang II and the control groups was observed in the parental MCF7 cells. Losartan also had a greater inhibitory effect on Ang II-induced cell viability in the AGTR1-overexpressing MCF7 cells compared to the parental line.

To examine whether AGTR1 is indeed involved in breast cancer proliferation, mice were xenografted with both MCF7 and AGTR1-overexpressing MCF7 cells. Our in vitro observations were in agreement with the in vivo observations showing that AGTR1-overexpressing tumors exhibited a markedly higher tumor growth rate and larger tumor burden, as indicated by the considerable upregulation of Ki-67. Blockade of AGTR1 significantly retarded tumor growth as well as significantly reduced the number of Ki-67-positive cells. These findings suggest that the targeting of AGTR1 could be a potential anticancer strategy for the treatment of breast cancer.

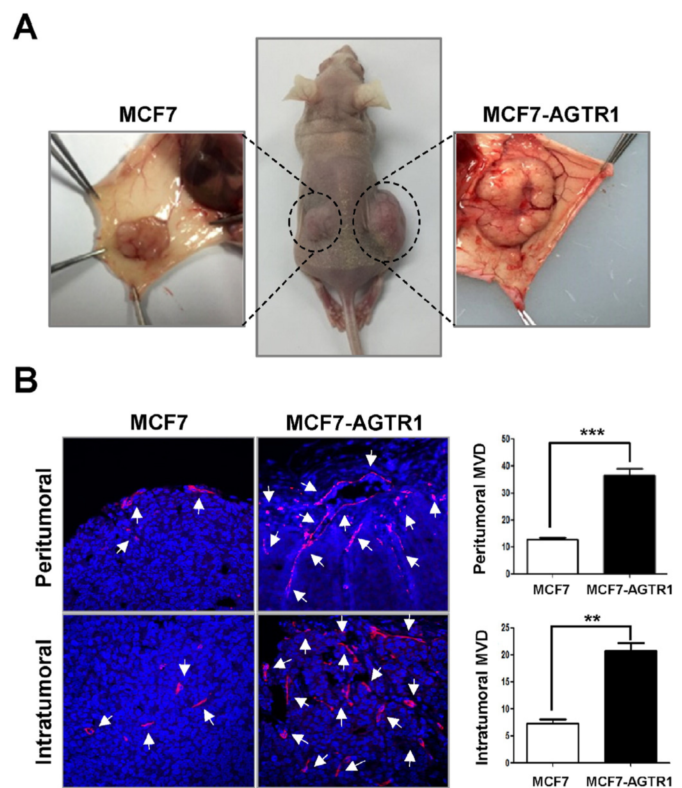

Fig. 6. AGTR1 enhances tumor angiogenesis in vivo. (A) Images of blood vessels between MCF7 and MCF7-AGTR1 tumors and surrounding tissue. (B) AGTR1 tumors exhibited a significant increase in microvessel density (MVD). Tumor tissues were immunostained with a specific endothelial cell marker CD31 (red) and nuclei were stained with DAPI (blue). Quantitative graphs represent the number of CD31-positive microvessels in peritumoral (***$p < 0.001$) and intratumoral areas (**$p < 0.01$).

The role of AGTR1 in the regulation of EMT in breast cancer has yet to be fully elucidated. AGTR1-overexpressing cells exhibited a mesenchymal-like phenotype, together with an increase in nuclear accumulation of phospho-Smad3 and Snail, increased Smad4 and N-cadherin levels and a loss of E-cadherin. The transcription factor Smad4 is a major determinant of EMT, and operates via the Smad/TGF-β pathway [17–19]. Upon TGF-β1 stimulation, the Smad3/4-Snail complex forms, which can then bind to Smad binding element (SBE) and the promoter of E-cadherin, resulting in the induction of EMT due to E-cadherin suppression [20]. Inhibition of Smad4 has been shown to suppress TGF-β-induced responses associated with EMT in mammary epithelial NMuMG cells in vitro, and prevents bone metastasis of MDA-MB-231 cells in vivo [62]. Knockdown of either Smad2 or Smad3 also inhibits TGF-β-induced EMT in pulmonary epithelial cells [63]. Furthermore, recent studies have reported that AGTR1 stimulation by Ang II induces EMT via the Smad signaling pathway in renal epithelial cells and vascular smooth muscle cells in vitro [33,34]. In contrast, AGTR1 antagonism inhibits Smad activation, which suppresses the induction of EMT [29,30,32]. Our results showed that knockdown of Smad4 in the AGTR1-overexpressing cells induced the upregulation of E-cadherin, followed by the induction of mesenchymal to epithelial transition (MET). Losartan treatment was observed to attenuate these AGTR1-induced responses by increasing mesenchymal markers and decreasing E-cadherin levels in vitro and in vivo.

EMT is often associated with aggressive and invasive phenotypes and malignant tumor progression [64,65]. Clinical evidence demonstrates that adipose tissue invasion (ATI) of cancer cells at the tumor margin is a biological indicator of tumor aggressiveness in early-stage breast cancer, and is correlated with both axillary lymph node metastasis and a lower survival rate [66]. The AGTR1-overexpressing cells overall had a more aggressive phenotype and invaded into the adipose tissue and penetrated the muscle layers at the tumor margin. This invasiveness was associated with a significant upregulation of MMP-9 expression. We found that MMP-9 levels were considerably higher in the AGTR1-overexpressing tumors, an observation in agreement with previous findings in gastric and breast cancers [50,67].

Clinical and preclinical data have revealed that intratumoral MVD is positively correlated with elevated AGTR1 expression in the advanced stages of ovarian and prostate cancer, resulting in poorer clinical outcomes [39,40,43,68]. We observed that AGTR1 overexpression increased the number of CD31-positive microvessels in the peritumoral and intratumoral areas, and this response was strongly suppressed by

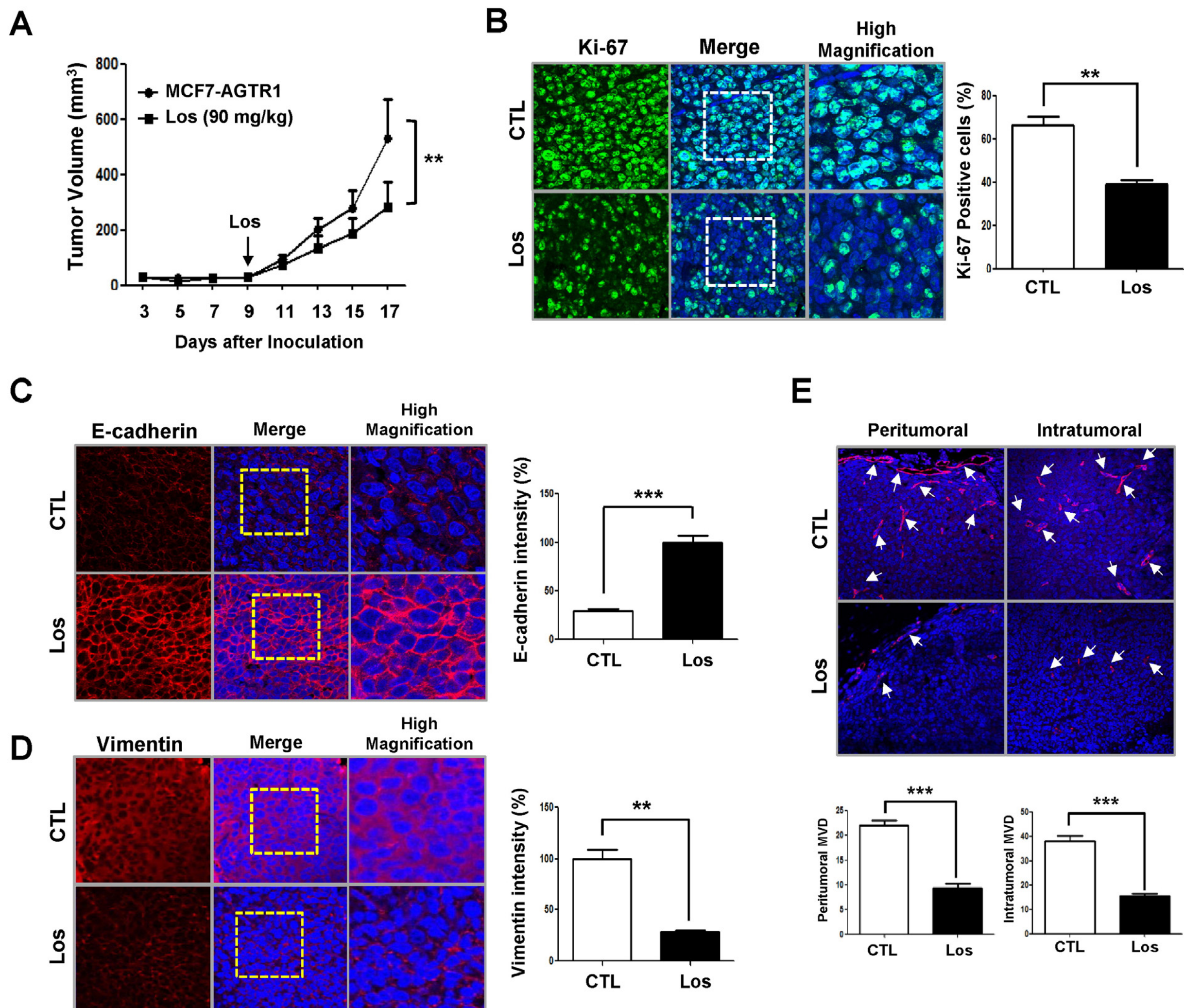

Fig. 7. Losartan administration suppresses tumor growth and MVD. (A) Effect of losartan on AGTR1-overexpressing xenograft tumor growth. 5×10^6 cells were injected into the mammary fat pads (right and left) of BALB/c nude mice. After 9 days, losartan (90 mg/kg, body weight) or a vehicle control (PBS) was injected intraperitoneally. Tumor volumes were measured for the indicated time durations. Losartan treatment reduced tumor volume (A, $^{**}p < 0.01$) in AGTR1-overexpressing xenografts. (B) Effect of losartan on Ki-67 expression. Tumor tissue sections were immunostained for Ki-67 (1:100, green) with DAPI (blue). The selected areas in merged images are shown at high magnification (×1000). The graph represents the percentage of Ki-67-positive cells ($^{**}p < 0.01$). (C–D) Losartan induced E-cadherin downregulation ($^{***}p < 0.001$) and vimentin upregulation ($^{**}p < 0.01$) in vivo. (E) Losartan administration significantly reduced peritumoral-MVD ($^{***}p < 0.001$) and intratumoral-MVD ($^{**}p < 0.01$).

losartan administration, suggesting that AGTR1 plays a crucial role in breast cancer angiogenesis.

In summary, we have observed that AGTR1 overexpression promotes cell proliferation and upregulates the expression of survival factors in vitro, and that AGTR1-induced EMT appears to be mediated by the Smad signaling pathway. AGTR1 overexpression also promotes a more aggressive phenotype with increased cell migration and invasion in vitro and in vivo, and increased tumor microvessel density indicative of angiogenesis. Our findings support the notion that AGTR1 is a potentially useful diagnostic marker and its inhibition may provide an effective therapeutic strategy for breast cancer treatment.

Author contributions

Conceived and designed the experiments: JYK, EHO and JHS. Performed the experiments: EHO, JYK, YKC, HSA and NHL. Analyzed the data: EHO and JYK. Contributed reagents/materials/analysis tools: EHO, YKC, HSA, NHL, HHJ, CIB, JYK and JHS. Wrote the paper: EHO and JYK.

Conflict of interest

The authors declare no conflicts of interest.

Transparency document

The Transparency document associated with this article can be found in the online version.

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