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Angiotensin II promotes primary tumor growth and metastasis formation of murine TNBC 4T1 cells through the fibroblasts around cancer cells

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ABSTRACT

Angiotensin II (Ang II) reportedly facilitates primary tumor growth and distal hematogenous metastasis formation in various murine intravenous metastasis models. However, it is unclear whether Ang II accelerates the initial processes of metastasis formation that begins in primary tumors surrounded by tumor microenvironment. We examined the effects of Ang II on primary tumors and lung metastasis lesions using a murine spontaneous metastasis model, in which triple negative breast cancer 4T1 cells constitutively expressing luciferase (4T1-Luc cells) were injected into the mammary fat pad of BALB/c mice. Subcutaneous injection of Ang II significantly accelerated primary tumor growth and lung metastasis formation. Ang II increased the protein expression levels of c-Myc, cyclin D1, fibronectin, vimentin, αSMA and Snail, and the treatment with the Ang II type 1 receptor blocker valsartan significantly suppressed the Ang II-induced increases of fibronectin and vimentin. Valsartan also significantly reduced lung metastatic lesions. However, Ang II did not have significant effects on 4T1-Luc cells including the proliferation, migration, invasion, or the expressions of proteins related to cell proliferation and epithelial-to-mesenchymal transition. In contrast, when 4T1-Luc cells were co-cultured with dermal fibroblasts, Ang II significantly accelerated cell migration and increased the expressions of fibronectin, vimentin, αSMA and Snail in 4T1-Luc cells. And moreover, Ang II significantly increased the mRNA expression of IL-6 in fibroblasts co-cultured with 4T1-Luc cells. These results suggested that Ang II accelerates surrounding fibroblasts by soluble factors such as IL-6 to promote epithelial-to-mesenchymal transition, which result in the initiation of cancer metastasis.

1. Introduction

Cancer metastasis is the major cause of cancer-related death. Thus, inhibiting distal hematogenous metastasis is important. Distal hematogenous metastasis is a multiple step process that involves local infiltration of cancer cells into the adjacent tissue, intravasation of cancer cells from the primary site into the bloodstream through vascular endothelial cells, circulation through the bloodstream, adhesion to endothelial cells at another site and extravasation into the target organ, and finally colonization in the target organ (Fidler, 2003). In all steps, the tumor cells themselves and cells that construct the tumor microenvironment play important roles (Quail and Joyce, 2013). Although preventing cancer metastasis is expected to be a beneficial therapy for cancer patients, clinically useable anti-metastatic drugs have not been developed (Anderson et al., 2019; Fontebasso and Dubinett, 2015; Gandaloviĉová et al., 2017; Ishikane and Takahashi-Yanaga, 2018).

The renin-angiotensin system (RAS) plays a key role in controlling vasoconstriction, fluid volume, and electrolyte balance. Upregulation of the RAS causes hypertension. Angiotensin II (Ang II) is a key RAS peptide that induces vasoconstriction, secretion of aldosterone from the

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adrenal gland, and reabsorption of sodium and water. RAS inhibitors, such as angiotensin-converting enzyme inhibitors and Ang II type 1 receptor blockers (ARBs), are used as antihypertensive drugs (Ferrario, 2006). In addition to hypertension, a number of reports have suggested that Ang II is related to cancer progression. For example, Ang II reportedly facilitates progression of cancer metastasis by inducing migration, invasion, and proliferation of cancer cells and accelerating angiogenesis (Suganuma et al., 2005; Xu et al., 2017; Zhao et al., 2014). In animal models, Ang II has been demonstrated to induce distal hematogenous metastasis in various cancer cells, including renal cancer, melanoma, and breast cancer cells (Ishikane et al., 2018; Miyajima et al., 2002; Rodrigues-Ferreira et al., 2012). Consistent with this, RAS inhibitor has been reported to reduce distal hematogenous metastasis (Wen et al., 2013). Furthermore, RAS inhibitors could ameliorate metastatic-free survival rate in renal cancer and upper-tract urothelial carcinoma patients (Miyajima et al., 2015; Tanaka et al., 2012). Thus, RAS inhibitors could be candidate anti-metastatic drugs. However, several aspects remain to be clarified concerning the role of Ang II during metastasis formation, especially the effects of Ang II on the initiation of metastasis in primary tumor lesions.

We previously reported that Ang II exacerbated hematogenous cancer metastasis using an intravenous metastasis model in which the effect of Ang II in metastatic lesions was evaluated (Ishikane et al., 2018). In the present study, we used a spontaneous metastasis model to evaluate the effects of Ang II on the initial steps of metastasis formation in primary tumor lesions. Murine triple negative breast cancer (TNBC) 4T1 cells were used because TNBC often forms lethal metastasis (Dent et al., 2007; Howlader et al., 2018). As epithelial-to-mesenchymal transition (EMT) of cancer cells plays a key role in the initial steps for the metastasis formation (van Zijl et al., 2011), we paid special attention to the effect of Ang II on EMT in primary tumor lesions.

2. Materials and methods

2.1. Chemicals and antibodies

Ang II was purchased from Peptide Institute Inc. (Osaka, Japan). The angiotensin receptor blocker valsartan (Val) was purchased from Novartis Pharma (Basel, Switzerland). Monoclonal anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody and monoclonal anti-Ang II type 1 receptor (AT1R) antibody were obtained from Abcam (Cambridge, UK). Monoclonal anti-α-smooth muscle actin (αSMA) antibody, polyclonal anti-c-Myc antibody, polyclonal anti-vimentin antibody, and polyclonal anti-Snail antibody were obtained from Cell Signaling Technology (Danvers, MA, USA). Polyclonal anti-cyclin D1 antibody, monoclonal anti-cyclin E antibody, and polyclonal anti-fibronectin antibody were obtained from Santa Cruz Biotechnology (Dallas, TX, USA).

2.2. Cell culture

To evaluate cancer metastasis formation in the lung, mouse breast cancer cells stably expressing luciferase (4T1-Luc cells, JCRB Cell Bank, Osaka, Japan) were used. The cells were cultured at 37 °C in an atmosphere of 95% air and 5% CO_2 in RPMI 1640 (FUJIFILM Wako Pure Chemical Co., Osaka, Japan) containing 10% fetal bovine serum (FBS; Nichirei Biosciences, Tokyo, Japan), 100 U/mL penicillin G, and 0.1 μg/mL streptomycin. 4T1-Luc cells in passages 25 to 35 were used for the experiments. Primary dermal fibroblasts (DFBs) were isolated from the dorsal dermis of 8 week old female BALB/c mice obtained from CLEA Japan Inc. (Tokyo, Japan). Briefly, dermis tissues were cut into small pieces and digested with 0.2% collagenase type A (FUJIFILM Wako Pure Chemical Co.) in Hank's balanced salt solution (Nacalai Tesque, Kyoto, Japan) at 37 °C for 30 min and then filtered using a cell strainer (Falcon 100 μm; Corning Inc., Corning, NY, USA). Collected DFBs were cultured at 37 °C in an atmosphere of 90% N_2, 5% O_2, and 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM; FUJIFILM Wako Pure Chemical Co.) containing 10% FBS, 100 U/mL penicillin G, and 0.1 μg/mL streptomycin (Senavirathna et al., 2018). DFBs in passages 2 to 3 were used for the experiments.

2.3. Animal models

Ang II or vehicle was continuously infused at 1.0 μg/kg/min into the female BALB/c mice (8–10 weeks old) by subcutaneously implanted osmotic pumps (model 1004, ALZET, Cupertino, CA, USA) beginning 3 days before cell injection. Blood pressure was measured by the tail-cuff method using a model BP-98A device (Softron Co., Tokyo, Japan) in conscious mice the day of cell injection. 4T1-Luc cells were trypsinized and resuspended in 50% Matrigel (Corning Inc.) in PBS at a concentration of 4×10^6 cells/mL. The suspension (25 μL) was subcutaneously injected into the left #4 mammary fat pad of mice. Val (60 mg/kg/day) or vehicle was orally administered by gastric gavage once a day for 36 days beginning 8 days before cell injection. Twenty-eight days after cell injection, the mice were euthanized by cervical dislocation.

2.4. Micro-computed tomography imaging

After euthanasia, micro-computed tomography (micro-CT) images of the lungs were obtained using a Cosmo Scan GX device (Rigaku, Tokyo, Japan). The settings were as follows: X-ray voltage, 90 kV; current, 88 μA; field of view (FOV), 25 mm; and irradiation time, 2 min. Metastatic lung tumor was defined as a mass with a diameter >300 μm that could be isolated from lung vessels. The number of tumors and the largest tumor diameter were evaluated.

2.5. Western blot analysis

The primary tumor was dissected and homogenized in RIPA buffer (FUJIFILM Wako Pure Chemical Co.). The protein samples were separated by 10% SDS-PAGE or 7.5% tris-acetate polyacrylamide gel electrophoresis (for high molecular-weight proteins) (Kurien and Scofield, 2012). These samples were transferred to polyvinylidene fluoride (PVDF) membranes using a wet transfer system (Bio-Rad, Hercules, CA, USA). Western blot analysis was performed as described previously (Ishikane et al., 2018). The densities of the bands were quantified by optical densitometric scanning using the ImageJ software program (Ver. 1.46; National Institute of Health, Bethesda, MD, USA).

2.6. Real-time PCR

Total RNA was extracted from the left lung, including the superior, middle, and inferior lobes, using a FastGene RNA Basic Kit (NIPPON Genetics Co., Tokyo, Japan). cDNA was synthesized with the GeneAce cDNA Synthesis Kit according to the manufacturer's instructions (Nippon Gene Co., Tokyo, Japan). Quantitative real-time PCR was performed using GeneAce SYBR qPCR Mix α (Nippon Gene Co.) with a LightCycler Nano System (Roche Molecular Biochemicals, Mannheim, Germany). The mRNA levels of luciferase, transforming growth factor-β1 (TGF-β1), tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), fibroblast growth factor-2 (FGF-2), epidermal growth factor (EGF), and vascular endothelial growth factor-A (VEGF-A) genes were quantified with GAPDH for normalization. The primers are listed in Table 1.

2.7. Cell proliferation assay

4T1-Luc cells were seeded into 96-well plates at 1×10^4 cells/well in RPMI 1640 medium supplemented with 1% FBS. Twenty-four hours after seeding, cells were stimulated with or without Ang II (10^{-10} to 10^{-5} M) for 48 h. Cell proliferation was assessed by WST-8 (Nacalai Tesque) according to the manufacturer's instructions. The results were expressed as the percentage of the absorbance of the control cells.

Table 1
Primer sets used in this study.

Gene	Primer	Primer sequence
luciferase	Forward	ATCCATCTTGCTCCAACACC
	Reverse	TCGCGGTTGTTACTTGACTG
TGF-β1	Forward	CAACAATTCCGGCGTTACC
	Reverse	TCTCCTTGGTTCAGCCACTG
TNF-α	Forward	ACGGCATGGATCTCAAAGAC
	Reverse	AGATAGCAAATCGGCTGACG
IL-6	Forward	CTTCACAAGTCCGGAGAGGA
	Reverse	CAGAATTGCCATTGCACAAC
FGF-2	Forward	CCAACCGGTACCTTGCTATG
	Reverse	AGTGCCACATACCAACTGGAG
EGF	Forward	CGCCGAAGACTTATCCAGAA
	Reverse	CATGCTGCCTTGAAGACGTA
VEGF-A	Forward	GTACCTCCACCATGCCAAGTGGTCC
	Reverse	GGTGAGGTTTHATCCGCATGATCTG
GAPDH	Forward	GACCCCTTCATT GACCTCAACTACA
	Reverse	GCCTTCTCCATGGTGGTGAAGAC

2.8. Migration assay

4T1-Luc cells were seeded into 24-well plates at confluence in RPMI 1640 medium supplemented with 10% FBS. Twenty-four hours after seeding, cells were damaged by scratching with a 200 μL yellow chip in the presence or absence of Ang II (10^{-6} M) in RPMI 1640 medium supplemented with 1% FBS. Each well was imaged at 0 and 12 h after scratching to evaluate the size of the damaged area using an ECLIPSE Ts2 microscope (Nikon, Tokyo, Japan).

2.9. Trans-well invasion assay

The effect of Ang II treatment on the invasion of 4T1-Luc cells was determined using a Boyden Chamber (ThinCert Cell Culture Insets 8 μm pore sizes; Greiner Bio-One GmbH, Frickenhausen, Germany). A polyethylene terephthalate membrane was pre-coated with 10 μg of Matrigel. Cells were suspended at 1×10^4 cells in 100 μL of serum-free RPMI 1640 with or without Ang II (10^{-6} M) and seeded into the upper part of each chamber. The lower compartments were filled with 600 μL of RPMI 1640 supplemented with 1% FBS with or without Ang II (10^{-6} M). After incubation for 24 h at 37 °C, cells that had not invaded were scraped off with a cotton swab. Invasive cells on the lower surface of the membrane were fixed with 4% formaldehyde for 30 min and stained with 4% crystal violet for 5 min. Cell invasion was quantified by counting the number of cells using a BZX-800 microscope (Keyence, Osaka, Japan).

2.10. Trans-well co-culture assay

The Trans-well co-culture assay was performed in a 12-well plate using a Boyden Chamber (ThinCert Cell Culture Insets 0.4 μm pore sizes; Greiner Bio-One GmbH). To evaluate the effect of co-cultured DFBs on 4T1-Luc cells (Fig. 4B), DFBs suspended at 1.5×10^4 cells in 0.5 mL DMEM supplemented with 1% FBS were seeded into the upper part of each chamber. 4T1-Luc cells (2×10^4 cells/mL DMEM supplemented with 1% FBS) were seeded into the lower compartments. After 24 h incubation at 37 °C in an atmosphere of 95% air and 5% CO_2, upper chambers were placed on lower parts and Ang II (10^{-6} M) or vehicle were added to both upper and lower compartments. The incubation was continued for additional 48 h and then upper chambers were removed. 4T1-Luc cells in the lower compartments were subjected to cell proliferation assay or Western blot analysis. Co-cultured medium in the lower compartment was applied to the separately prepared 4T1-Luc cells which were then scratched with a 200 μL yellow chip.

To evaluate the effect of co-cultured 4T1-Luc cells on DFBs (Fig. 5A), 4T1-Luc cells suspended at 1.5×10^4 cells in 0.5 mL DMEM supplemented with 1% FBS were seeded into the upper part of each chamber. DFBs (2×10^4 cells/mL DMEM supplemented with 1% FBS) were seeded into the lower compartments. After 24 h incubation at 37 °C in an atmosphere of 95% air and 5% CO_2, upper chambers were placed on lower parts and Ang II (10^{-6} M) or vehicle were added to both upper and lower compartments. The incubation was continued for additional 48 h and then upper chambers were removed. Total RNA was extracted from DFBs in the lower compartment and subjected to the real-time PCR analysis.

2.11. Statistical analyses

Results are expressed as the mean ± SD. Differences between means were analyzed by Student's *t-test* or one-way ANOVA with the Bonferroni post-hoc test. A P-value < 0.05 was considered significant.

2.12. Ethical information

The study protocol was approved by the Ethics Committee of Animal Care and Experimentation at the University of Occupational and Environmental Health, Japan (approval protocol no. AE17-026). All animal handling and procedures were performed in compliance with the Institutional Guidelines for Animal Experiments and the Law (no. 105) and Notification (no. 6) of the Japanese Government and the NIH guidelines (Guide for the Care and Use of Laboratory Animals). All surgeries were performed under inhaled sevoflurane anesthesia, and all efforts were made to minimize suffering of the experimental animals.

3. Results

3.1. Ang II accelerates formation of metastatic lung tumors and Val attenuates it in vivo

We examined the effect of Ang II and a specific AT1R antagonist Val on the formation of metastatic lung tumors by injecting 4T1-Luc breast cancer cells into mammary fat pads of BALB/c mice. As shown in Fig. 1A, systolic blood pressure (SBP) of Ang II-infused mice was significantly elevated compared with vehicle mice and administration of Val clearly attenuated the elevation of SBP induced by Ang II, indicating that sufficient amounts of Ang II and Val were administrated. None of the control mice, five Ang II-infused mice, and one Ang II plus Val-treated mouse died during the treatment period (Fig. 1B).

The formation of metastatic lung tumors was analyzed by micro-CT 28 days after cell injection. Ang II significantly increased the number and size of metastatic lung tumors (Fig. 1C). Analysis of luciferase expression confirmed that Ang II increased the formation of tumor masses comprised of 4T1-Luc cells in the lung (Fig. 1D). However, there was no significant difference between the vehicle-treated group and Ang II plus Val-treated group in lung metastasis formation (Fig. 1C) and luciferase expression (Fig. 1D), indicating that blockade of AT1R by Val attenuated the effect of Ang II.

3.2. Ang II accelerates growth of primary tumors and Val attenuates it in vivo

We also analyzed the primary tumor lesions. Ang II infusion significantly increased the weight of primary tumor mass compared with the vehicle-treated group. On the other hand, there was no significant difference between the vehicle-treated group and Ang II plus Val-treated group. This result indicates that Val successfully weakened the effect of Ang II (Fig. 2A). Subsequently, we analyzed the expression of cell proliferation-related proteins (c-Myc, cyclin E and cyclin D1) and the EMT-related proteins (fibronectin, vimentin, αSMA and Snail) expression to identify the mechanism by which Ang II accelerated primary tumor growth and metastatic tumor formation. As shown in Fig. 2B, Ang II infusion significantly increased the protein expression levels of c-Myc, cyclin D1, fibronectin, vimentin, αSMA and Snail. On the other hand, there was no significant difference in the protein expression levels of c-

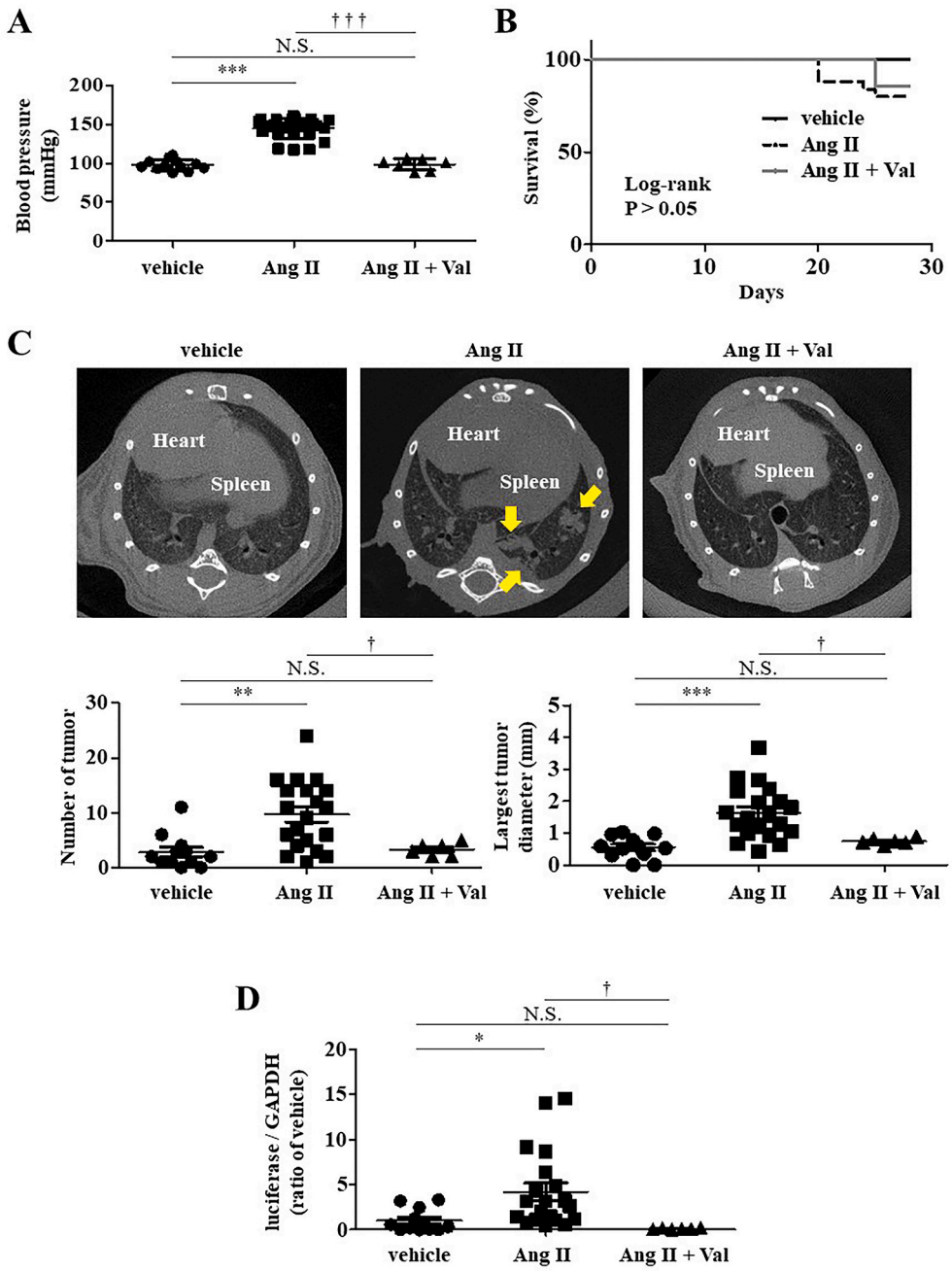

Fig. 1. Ang II accelerates formation of metastatic lung tumors of 4T1-Luc cells and Val attenuates it *in vivo*.
Ang II (1 μg/kg/min) or vehicle was subcutaneously administered using an osmotic pump implanted 3 days before cell injection. Val (60 mg/kg/day) or vehicle was orally administered by gastric gavage once a day for 36 days beginning 8 days before cell injection. 4T1-Luc cells were subcutaneously injected into the mammary fat pads of BALB/c mice 3 days after pump implantation. A. Systolic blood pressure data. Blood pressure was measured 3 days after pump implantation (vehicle-treated group, n = 12; Ang II-treated group, n = 25, Ang II plus Val-treated group, n = 7). B. Survival rate (%) in the vehicle-treated group (n = 12), Ang II-treated group (n = 25), and Ang II plus Val-treated group (n = 7). C. The upper panel displays representative micro-CT images of the lung on day 28 after cell injection. Yellow indicates metastatic tumors. The lower panel displays the number of metastatic lung tumors and the largest metastatic lung tumor diameter in each mouse in the vehicle-treated group (n = 12), Ang II-treated group (n = 20), and Ang II plus Val-treated group (n = 6). D. Luciferase mRNA levels in the right lung. Results normalized to GAPDH are shown as the ratio of the vehicle level in the vehicle-treated group (n = 12), Ang II-treated group (n = 20), and Ang II plus Val-treated group (n = 6). Values are mean ± SD; *p < 0.05 vs vehicle group, **p < 0.01 vs vehicle group, ***$p < 0.001$ vs vehicle group, †p < 0.01 vs Ang II group, †††p < 0.001 vs Ang II group, N.S., not significant.

Myc, cyclin D1, αSMA and Snail between the vehicle-treated group and Ang II plus Val-treated group, indicating that Val significantly attenuated the upregulation of protein expressions caused by Ang II. The collective findings indicate that Ang II, through AT1R, stimulated primary tumor growth by activating tumor cell proliferation and accelerated the formation of metastatic lung tumor by stimulating EMT.

3.3. *Ang II does not affect proliferation, migration, invasion, and protein expression of 4T1-Luc cells in vitro*

The first step of hematogenous metastasis is the local infiltration of cancer cells into the adjacent tissue. This process is involved in the proliferation and EMT of cancer cells in primary tumors. Therefore, we examined the effect of Ang II on the proliferation and EMT of 4T1-Luc cells *in vitro*. The protein expression of AT1R on 4T1-Luc cells was significant, although it was not abundant (Fig. 3A). The proliferation assay showed that Ang II did not affect the proliferation of 4T1-Luc cells, even at a high concentration (10^{-5} M) (Fig. 3B). Subsequently, the effects of Ang II on migration and invasion were analyzed. Ang II did not affect the migration (Fig. 3C) or invasion (Fig. 3D) of 4T1-Luc cells. Next, we analyzed the effects of Ang II on the expression levels of cell proliferation-related proteins (c-Myc, cyclin E and cyclin D1) and EMT-related proteins (fibronectin, vimentin, αSMA and Snail) at different time points. Ang II did not significantly affect the expression of these proteins at any time points (Fig. 3E). These results suggested that Ang II did not have a direct effect on 4T1-Luc cells to stimulate proliferation and/or EMT, although the cells expressed AT1R.

3.4. *Ang II induces EMT of 4T1-Luc cells via co-cultured DFBs*

The characteristics of cancer cells do not depend only on themselves. The microenvironment around them, including fibroblasts, is an

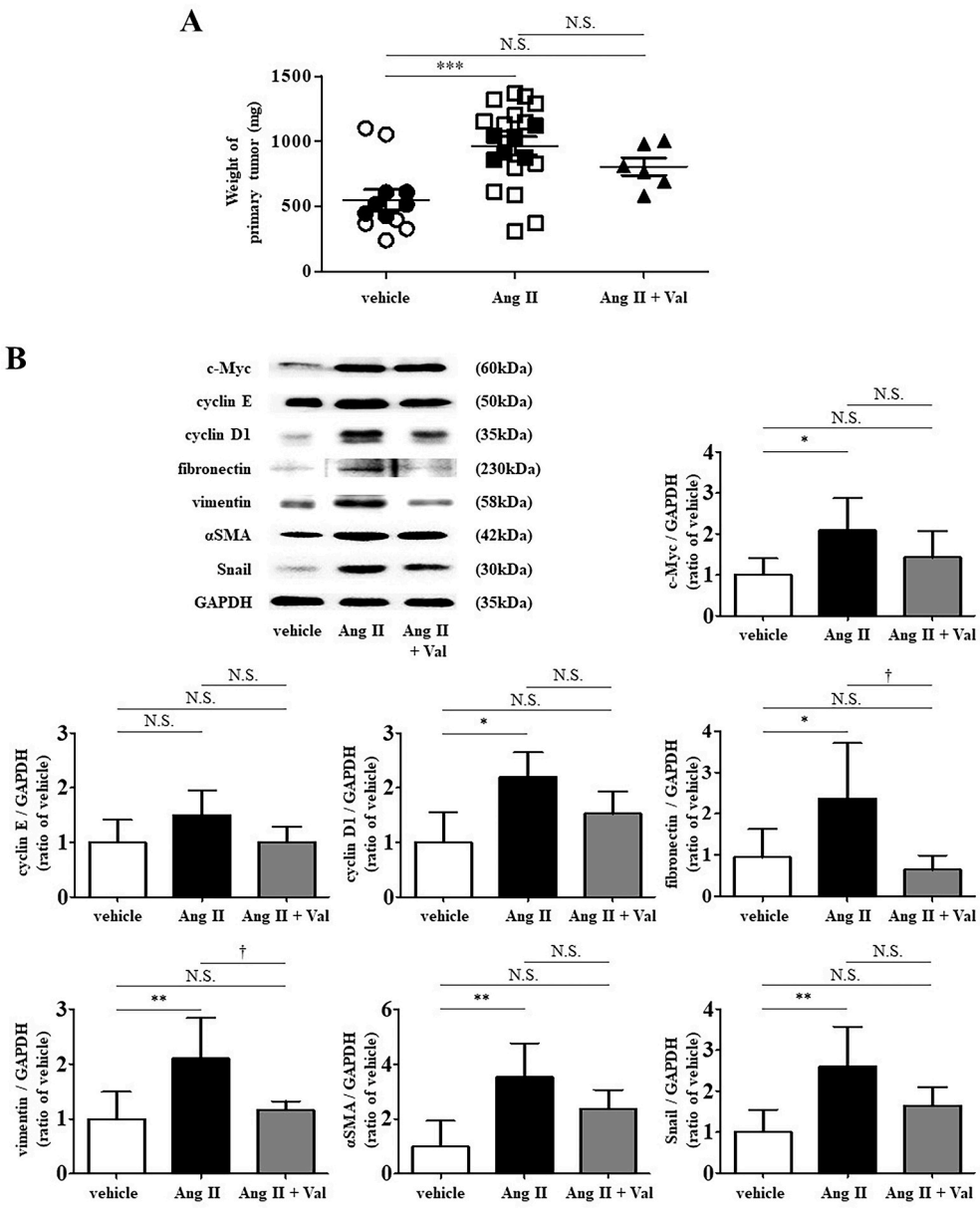

Fig. 2. Ang II accelerates primary tumor growth of 4T1-Luc cells and Val attenuates it *in vivo*.

Primary tumors were dissected on day 28 after cell injection. A. Weight of the primary tumor. Filled symbols in each group show samples used for Western blot analysis. B. Expression of cell proliferation-related proteins (c-Myc, cyclin E and cyclin D1) and EMT-related proteins (fibronectin, vimentin, αSMA and Snail) in primary tumors. Lysates of six tumor masses that have a weight closest to the average in each group were analyzed by Western blotting. The densities of the blots normalized to GAPDH are shown as the ratio of the control level. Values are mean ± SD in the vehicle-treated group (n = 6), Ang II-treated group (n = 6), and Ang II plus Val-treated group (n = 6); $^{*}p < 0.05$ vs vehicle group, $^{**}p < 0.01$ vs vehicle group, $^{***}p < 0.001$ vs vehicle group, $^{\dagger}p < 0.01$ vs Ang II group, N.S., not significant.

influence. To investigate whether Ang II affected 4T1-Luc cells through the fibroblasts, we first examined whether AT1R is expressed in DFBs. As demonstrated in Fig. 4A, DFBs expressed significant amount of AT1R. Then we co-cultured 4T1-Luc cells and DFBs (Fig. 4B). Co-culture with DFBs slightly stimulated 4T1-Luc cell proliferation, although it was not significant. Ang II also slightly but not significantly stimulated 4T1-Luc cell proliferation under the co-culture condition (Fig. 4C). This result suggested that Ang II shows little effect on 4T1-Luc cell proliferation even when co-cultured with DFBs. Subsequently, we performed migration assay of 4T1-Luc cells using co-culture medium. As shown in Fig. 4D, co-culture medium obtained in the presence of Ang II significantly accelerated 4T1-Luc cell migration, although Ang II itself did not have significant effect. Western blot analysis showed that the expressions of c-Myc and cyclin D1 in 4T1-Luc cells were significantly increased under co-culture condition even in the absence of Ang II. On the other hand, Ang II significantly increased the expression levels of fibronectin, vimentin, αSMA and Snail under co-culture condition (Fig. 4E). These results suggested that, in the absence of Ang II, soluble factors secreted from DFBs under co-culture condition could have weak potentiating effect on 4T1-Luc cell proliferation via upregulation of c-Myc and cyclin D1. On the other hand, Ang II-induced soluble factors from DFBs under co-culture condition may accelerate cell migration via upregulation of EMT-related proteins (fibronectin, vimentin, αSMA and Snail).

3.5. *Ang II induced secretion of IL-6 from DFBs co-cultured with 4T1-Luc*

Fibroblasts surrounding cancer cells secrete various cytokines and growth factors including TNF-α1, TGF-β, IL-6, FGF-2, EGF and VEGF-A (Bhowmick et al., 2004; Kalluri, 2016; Sahai et al., 2020). To identify the soluble factor(s) secreted from DFBs under co-culture condition, DFBs were co-cultured with 4T1-Luc cells and the effect of Ang II was examined (Fig. 5A). Although Ang II did not have significant effect on the mRNA expression of TGF- β1, TNF-α, FGF-2, EGF and VEGF-A, the expression of IL-6 was significantly increased by Ang II under the co-culture condition (Fig. 5B). This result suggested that DFBs were also affected by 4T1-Luc cells under the co-culture condition and IL-6 could be a soluble factor secreted from DFBs responsible for the effect of Ang II.

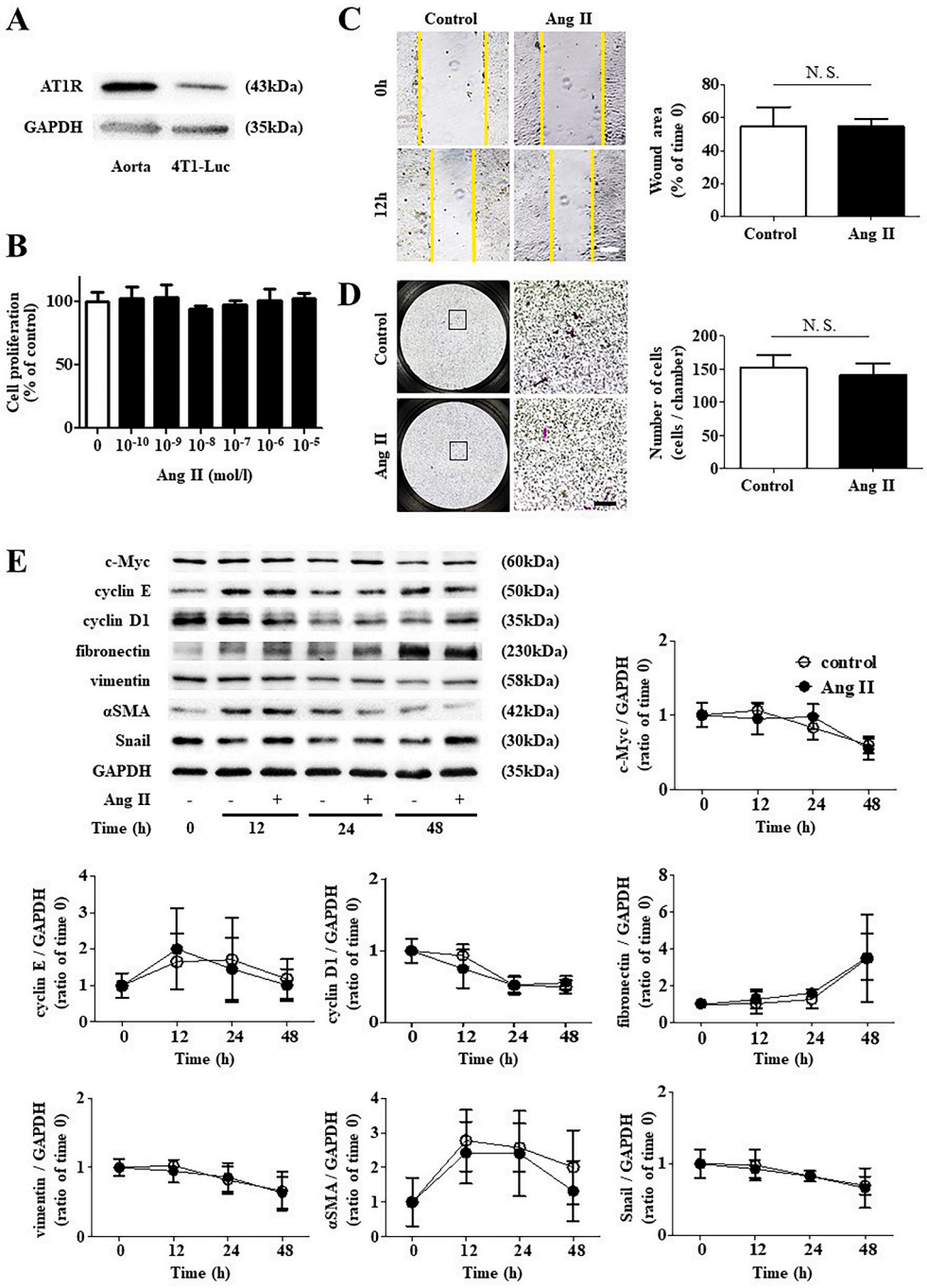

Fig. 3. Ang II does not significantly affect 4T1-Luc cells *in vitro*.

A. AT1R protein expression. B. Proliferation assay. 4T1-Luc cells were incubated with various concentration of Ang II (10^{-10} to 10^{-5} M) or vehicle for 48 h. Data are shown as % of vehicle (each group, n = 4). C. Migration assay. Cells were incubated with Ang II (10^{-6} M) or vehicle for 12 h. The reduction of wound area was evaluated (n = 4). Scale bar = 200 μm. D. Cell invasion assay. 4T1-Luc cells were incubated with Ang II (10^{-6} M) or vehicle for 24 h. Infiltrating cells were stained with crystal violet and the total number was counted (n = 4). Left panel shows the phots of whole chamber. Right panel displays the amplification of indicated area shown in left panel. Scale bar = 200 μm. E. Effect of Ang II on protein expression levels. 4T1-Luc cells were incubated with Ang II (10^{-6} M) or vehicle for indicated periods in RPMI 1640 medium supplemented with 1% FBS. Cell lysates were analyzed by Western blotting (n = 4). Densities of the blots normalized to GAPDH are shown as ratio of the control level at time 0. Values are mean ± SD; N.S., not significant.

4. Discussion

In the present study, we demonstrated that Ang II accelerated distal metastatic lesion formation by promoting EMT and subsequent migration and infiltration of primary tumor cells through AT1R-mediated alteration of fibroblasts closely located with cancer cells.

Distal metastasis of cancer has been assessed *in vivo* by "experimental metastasis models" or "spontaneous metastasis models" (Gómez-Cuadrado et al., 2017; Pillar et al., 2018). In the former, cancer cells are directly injected into the bloodstream. Therefore, only the steps of metastasis after circulation can be evaluated. On the other hand, in the latter, cancer cells are injected orthotopically to form primary tumors. Thus, initial changes that occur within primary tumor sites, including the microenvironment, can also be evaluated. We adopted a spontaneous metastasis model for the present study, to assess the role of Ang II in the initiation of cancer cell metastasis.

Ang II clearly stimulated primary tumor growth and increased the expressions of c-Myc and cyclin D1. Since several reports have shown that Ang II can stimulate cancer cell proliferation by upregulating c-Myc expression in hepatocellular carcinoma cells (Ji et al., 2016; Qi et al., 2019) and cyclin D1 expression in breast cancer cells (Du et al., 2012), these cell proliferation-related proteins may be involved in Ang II-stimulated primary tumor growth in our model.

The initial step of distal metastasis is local infiltration of cancer cells that acquired properties of mesenchymal cells via EMT. Oh et al. reported that the overexpression of AT1R induced EMT in breast cancer cell lines followed by migration and invasion (Oh et al., 2016). Okamoto et al. reported that Ang II directly enhanced the migratory capability and

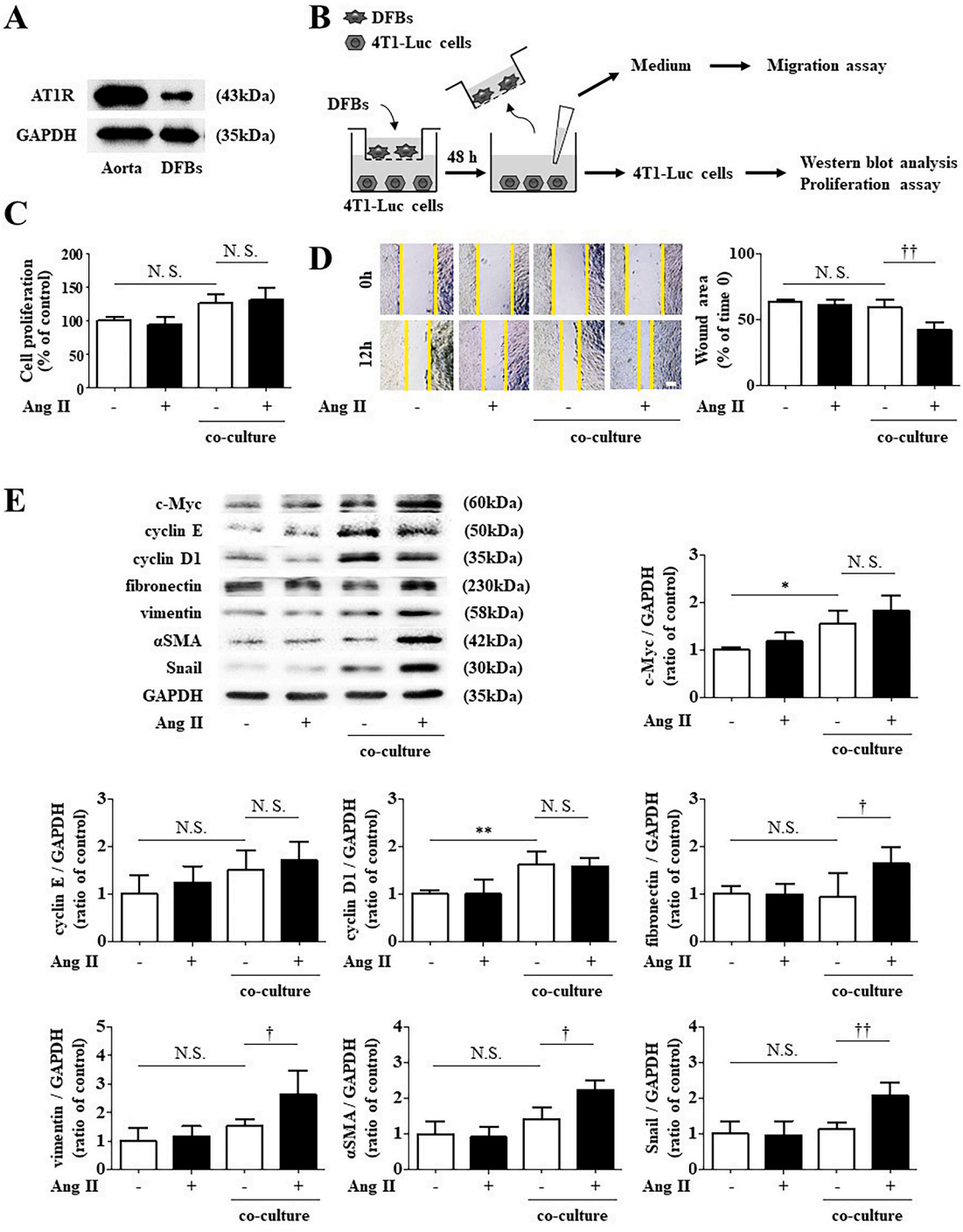

Fig. 4. Co-cultured DFBs mediate Ang II-induced EMT of 4T1-Luc cells *in vitro*.
Effect of Ang II on proliferation, migration and protein expression levels of 4T1-Luc cells with or without DFBs co-culture. A. AT1R protein expression. B. Experimental schema. Co-culture was performed using a Trans-well system. DFBs were seeded into the upper chambers and 4T1-Luc cells were seeded in the lower compartments. Both cells were incubated with or without Ang II (10^{-6} M) for 48 h 4T1-Luc cells in the lower compartments were subjected to proliferation assay and Western blotting analysis. Culture media from the lower compartments were used for migration assay. C. Proliferation assay (each group, n = 4). D. Migration assay. The reduction of wound area was evaluated (n = 4). Scale bar = 200 μm. E. Protein expression levels (n = 4). The densities of the blots normalized to GAPDH are shown as the ratio of the control level. Values are mean ± SD; *$p < 0.05$ vs control, **$p < 0.01$ vs control, $^{\dagger}p < 0.01$ vs co-culture without Ang II, $^{\dagger\dagger}p < 0.01$ vs co-culture without Ang II.

expression of EMT markers in intrahepatic cholangiocarcinoma cell lines (Okamoto et al., 2012). In the present study, we showed that Ang II significantly increased the expressions of fibronectin, vimentin, αSMA and Snail to induce EMT of primary tumor 4T1-Luc cells *in vivo*.

To determine the mechanisms by which Ang II promoted cancer cell proliferation and EMT, we first examined the direct effect of Ang II on 4T1-Luc cells *in vitro*. However, unexpectedly, Ang II had no effect on the proliferation, migration, infiltration, and expressions of the related proteins. These results made us think that Ang II-induced primary tumor growth and EMT *in vivo* was not direct but indirect effects of Ang II on 4T1-Luc cells.

Tumor growth and metastatic lesion formation do not only depend

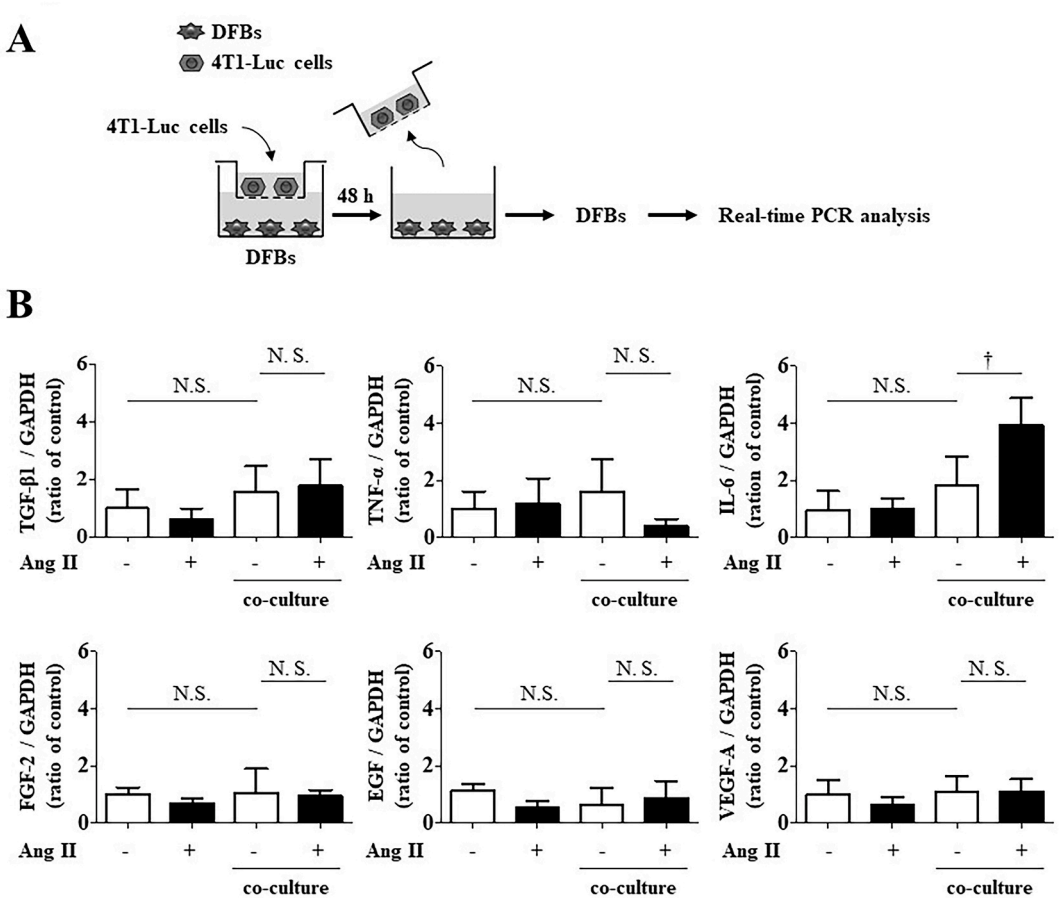

Fig. 5. Co-cultured 4T1-Luc cells mediate Ang II-induced IL-6 secretion of DFBs *in vitro*.
Effect of Ang II on mRNA expression levels of DFB with or without 4T1-Luc cells co-culture. A. Experimental schema. Co-culture was performed using a Trans-well system. 4T1-Luc cells were seeded into the upper chambers and DFBs cells were seeded in the lower compartments. Both cells were incubated with or without Ang II (10^{-6} M) for 48 h. DFBs in the lower compartments were subjected to real-time PCR analysis. B. mRNA expression in DFBs (each group: n = 4). Gene expressions were normalized to GAPDH are shown as the ratio of the control level. Values are mean ± SD; $^{\dagger}p < 0.01$ vs co-culture without Ang II.

on the properties of cancer cells, but also depend on the surrounding tumor microenvironment, consisting of immune cells, vascular endothelial cells, extracellular matrix (ECM), and fibroblasts (Quail and Joyce, 2013). Stromal fibroblasts surrounding a tumor differentiate into cancer-associated fibroblasts (CAFs) by being exposed to physiological stress, activated ECM, and various other signals. They modulate tumor growth and metastasis through ECM remodeling and secretion of cytokines and growth factors such as TGF-β1, TNF-α, IL-6, FGF-2, EGF, and VEGF-A, which affects immune cells and influences angiogenesis (Bhowmick et al., 2004; Kalluri, 2016; Sahai et al., 2020).

To our knowledge, only one report has addressed the effect of Ang II on CAFs. Nakamura et al. reported that CAFs isolated from ARB-treated mice displayed lower expression of immunosuppressive factors, such as chemokine ligand 12 and nitric oxide synthase 2 (Nakamura et al., 2018). However, the effect of Ang II on CAFs leading to proliferation and/or metastasis formation of primary cancer cells remains unexplored. Since many cells in the tumor microenvironment express AT1R (Wen et al., 2013; Nakamura et al., 2018; Egami et al., 2003), Ang II can induce proliferation and EMT of cancer cells, even if they do not express AT1R, by activating fibroblasts in the tumor microenvironment.

In the present study, we showed for the first time that Ang II significantly increased the mRNA expression levels of IL-6 in DFBs when co-cultured with 4T1-Luc cells. It has been reported that IL-6 induces EMT of breast cancer cells (Sullivan et al., 2009; Xie et al., 2012; Gyamfi et al., 2018). Among cells constituting tumor microenvironment, CAFs and adipocytes have shown to secret IL-6 to induce EMT of cancer cells (Gyamfi et al., 2018; Goulet et al., 2019; Shintani et al., 2016). Thus, Ang II-induced IL-6 secretion from CAFs could facilitate EMT of 4T1-Luc cells to promote the initiation of metastasis.

Ang II did not promote the proliferation of 4T1-Luc cells or the expression of proliferation-related proteins (c-Myc, cyclin E or cyclin D1) in 4T1-Luc cells even under the co-culture condition with DFBs. However, since co-culture condition itself induced slight but significant stimulation of 4T1-Luc cell proliferation and the expression of proliferation-related proteins, some soluble factors secreted from CAFs could facilitate the proliferation of 4T1 cells independent of Ang II. Further, although we could not investigate it in the present study, direct contact of 4T1-Luc cells with CAFs and/or other cells in the tumor microenvironment could promote the Ang II-induced primary tumor growth. Further studies are required to clarify these points.

Hypertension, which is common among cancer patients, has been reported to accelerate cancer progression (Chow et al., 2000; Chow et al., 2010; Häggström et al., 2013; Radišauskas et al., 2016). The present study suggested that Ang II could be involved in cancer progression in patients with cancer and hypertension. Therefore, RAS inhibitors, especially ARBs, could be a better choice for them to prevent cancer growth and metastasis. In conclusion, our study revealed that Ang II accelerates tumor growth and metastatic lesion formation of 4T1-Luc cells mainly by promoting EMT via soluble factors such as IL-6 secreted from surrounding fibroblasts.

CRediT authorship contribution statement

Tomohiro Takiguchi: Conceptualization, Methodology,

Investigation, Formal analysis, Writing – original draft. **Fumi Takahashi-Yanaga:** Conceptualization, Writing – original draft, Writing – review & editing, Project administration, Funding acquisition. **Shin Ishikane:** Conceptualization, Methodology, Investigation. **Fumi Tetsuo:** Methodology. **Hiroshi Hosoda:** Conceptualization. **Masaki Arioka:** Methodology. **Takanari Kitazono:** Supervision. **Toshiyuki Sasaguri:** Writing – review & editing, Supervision, Project administration.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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References

Anderson, R.L., Balasas, T., Callaghan, J., Coombes, R.C., Evans, J., Hall, J.A., Kinrade, S., Jones, D., Jones, P.S., Jones, R., Marshall, J.F., Panico, M.B., Shaw, J.A., Steeg, P.S., Sullivan, M., Tong, W., Westwell, A.D., Ritchie, J.W.A., 2019. A framework for the development of effective anti-metastatic agents. Nat. Rev. Clin. Oncol. 16, 185–204.

Bhowmick, N.A., Neilson, E.G., Moses, H.L., 2004. Stromal fibroblasts in cancer initiation and progression. Nature 432, 332–337.

Chow, W.H., Dong, L.M., Devesa, S.S., 2010. Epidemiology and risk factors for kidney cancer. Nat. Rev. Urol. 7, 245–257.

Chow, W.H., Gridley, G., Fraumeni Jr., J.F., Järvholm, B., 2000. Obesity, hypertension, and the risk of kidney cancer in men. N. Engl. J. Med. 343, 1305–1311.

Dent, R., Trudeau, M., Pritchard, K.I., Hanna, W.M., Kahn, H.K., Sawka, C.A., Lickley, L. A., Rawlinson, E., Sun, P., Narod, S.A., 2007. Triple-negative breast cancer: clinical features and patterns of recurrence. Clin. Canc. Res. 13, 4429–4434.

Du, N., Feng, J., Hu, L.J., Sun, X., Sun, H.B., Zhao, Y., Yang, Y.P., Ren, H., 2012. Angiotensin II receptor type 1 blockers suppress the cell proliferation effects of angiotensin II in breast cancer cells by inhibiting AT1R signaling. Oncol. Rep. 27, 1893–1903.

Egami, K., Murohara, T., Shimada, T., Sasaki, K., Shintani, S., Sugaya, T., Ishii, M., Akagi, T., Ikeda, H., Matsuishi, T., Imaizumi, T., 2003. Role of host angiotensin II type 1 receptor in tumor angiogenesis and growth. J. Clin. Invest. 112, 67–75.

Ferrario, C.M., 2006. Role of angiotensin II in cardiovascular disease therapeutic implications of more than a century of research. J. Renin-Angiotensin-Aldosterone Syst. JRAAS 7, 3–14.

Fidler, I.J., 2003. The pathogenesis of cancer metastasis: the 'seed and soil' hypothesis revisited. Nat. Rev. Canc. 3, 453–458.

Fontebasso, Y., Dubinett, S.M., 2015. Drug development for metastasis prevention. Crit. Rev. Oncog. 20, 449–473.

Gandalovičová, A., Rosel, D., Fernandes, M., Veselý, P., Heneberg, P., Čermák, V., Petruželka, L., Kumar, S., Sanz-Moreno, V., Brábek, J., 2017. Migrastatics-Anti-metastatic and anti-invasion drugs: promises and challenges. Trends Canc. 3, 391–406.

Gómez-Cuadrado, L., Tracey, N., Ma, R., Qian, B., Brunton, V.G., 2017. Mouse models of metastasis: progress and prospects. Dis. Model. Mech. 10, 1061–1074.

Goulet, C.R., Champagne, A., Bernard, G., Vandal, D., Chabaud, S., Pouliot, F., Bolduc, S., 2019. Cancer-associated fibroblasts induce epithelial-mesenchymal transition of bladder cancer cells through paracrine IL-6 signalling. BMC Canc. 19, 137.

Gyamfi, J., Lee, Y.H., Eom, M., Choi, J., 2018. Interleukin-6/STAT3 signalling regulates adipocyte induced epithelial-mesenchymal transition in breast cancer cells. Sci. Rep. 8, 8859.

Häggström, C., Rapp, K., Stocks, T., Manjer, J., Bjørge, T., Ulmer, H., Engeland, A., Almqvist, M., Concin, H., Selmer, R., Ljungberg, B., Tretli, S., Nagel, G., Hallmans, G., Jonsson, H., Stattin, P., 2013. Metabolic factors associated with risk of renal cell carcinoma. PLoS One 8, e57475.

Howlader, N., Cronin, K.A., Kurian, A.W., Andridge, R., 2018. Differences in breast cancer survival by molecular subtypes in the United States. Canc. Epidemiol. Biomarkers Prev. 27, 619–626.

Ishikane, S., Hosoda, H., Nojiri, T., Tokudome, T., Mizutani, T., Miura, K., Akitake, Y., Kimura, T., Imamichi, Y., Kawabe, S., Toyohira, Y., Yanagihara, N., Takahashi-Yanaga, F., Miyazato, M., Miyamoto, K., Kangawa, K., 2018. Angiotensin II promotes pulmonary metastasis of melanoma through the activation of adhesion molecules in vascular endothelial cells. Biochem. Pharmacol. 154, 136–147.

Ishikane, S., Takahashi-Yanaga, F., 2018. The role of angiotensin II in cancer metastasis: potential of renin-angiotensin system blockade as a treatment for cancer metastasis. Biochem. Pharmacol. 151, 96–103.

Ji, Y., Wang, Z., Li, Z., Zhang, A., Jin, Y., Chen, H., Le, X., 2016. Angiotensin II enhances proliferation and inflammation through AT1/PKC/NF-κB signaling pathway in hepatocellular carcinoma cells. Cell. Physiol. Biochem. 39, 13–32.

Kalluri, R., 2016. The biology and function of fibroblasts in cancer. Nat. Rev. Canc. 16, 582–598.

Kurien, B.T., Scofield, R.H., 2012. Protein Electrophoresis Methods and Protocols. Humana Press: Springer, NY, pp. 205–213, 2012.

Miyajima, A., Kosaka, T., Asano, T., Seta, K., Kawai, T., Hayakawa, M., 2002. Angiotensin II type I antagonist prevents pulmonary metastasis of murine renal cancer by inhibiting tumor angiogenesis. Canc. Res. 62, 4176–4179.

Miyajima, A., Yazawa, S., Kosaka, T., Tanaka, N., Shirotake, S., Mizuno, R., Kikuchi, E., Oya, M., 2015. Prognostic impact of renin-angiotensin system blockade on renal cell carcinoma after surgery. Ann. Surg Oncol. 22, 3751–3759.

Nakamura, K., Yaguchi, T., Ohmura, G., Kobayashi, A., Kawamura, N., Iwata, T., Kiniwa, Y., Okuyama, R., Kawakami, Y., 2018. Involvement of local renin-angiotensin system in immunosuppression of tumor microenvironment. Canc. Sci. 109, 54–64.

Oh, E., Kim, J.Y., Cho, Y., An, H., Lee, N., Jo, H., Ban, C., Seo, J.H., 2016. Overexpression of angiotensin II type 1 receptor in breast cancer cells induces epithelial-mesenchymal transition and promotes tumor growth and angiogenesis. Biochim. Biophys. Acta 1863, 1071–1081.

Okamoto, K., Tajima, H., Nakanuma, S., Sakai, S., Makino, I., Kinoshita, J., Hayashi, H., Nakamura, K., Oyama, K., Nakagawara, H., Fujita, H., Takamura, H., Ninomiya, I., Kitagawa, H., Fushida, S., Fujimura, T., Harada, S., Wakayama, T., Iseki, S., Ohta, T., 2012. Angiotensin II enhances epithelial-to-mesenchymal transition through the interaction between activated hepatic stellate cells and the stromal cell-derived factor-1/CXCR4 axis in intrahepatic cholangiocarcinoma. Int. J. Oncol. 41, 573–582.

Pillar, N., Polsky, A.L., Weissglas-Volkov, D., Shomron, N., 2018. Comparison of breast cancer metastasis models reveals a possible mechanism of tumor aggressiveness. Cell Death Dis. 9, 1–11. https://www.nature.com/articles/s41419-018-1094-8.

Qi, R., Lei, C.G., Bai, Y.X., Tang, N., Xing, X., 2019. The AT1/Raf/ERK1/2 signaling pathway is involved in Angiotensin II-enhanced proliferation of hepatic carcinoma cells. Neoplasma 66, 83–91.

Quail, D.F., Joyce, J.A., 2013. Microenvironmental regulation of tumor progression and metastasis. Nat. Med. 19, 1423–1437.

Radišauskas, R., Kuzmickienė, I., Milinavičienė, E., Everatt, R., 2016. Hypertension, serum lipids and cancer risk: a review of epidemiological evidence. Medicina 52, 89–98.

Rodrigues-Ferreira, S., Abdelkarim, M., Dillenburg-Pilla, P., Luissint, A.C., di-Tommaso, A., Deshayes, F., Pontes, C.L., Molina, A., Cagnard, N., Letourneur, F., Morel, M., Reis, R.I., Casarini, D.E., Terris, B., Couraud, P.O., Costa-Neto, C.M., Di Benedetto, M., Nahmias, C., 2012. Angiotensin II facilitates breast cancer cell migration and metastasis. PLoS One 7, e35667.

Sahai, E., Astsaturov, I., Cukierman, E., DeNardo, D.G., Egeblad, M., Evans, R.M., Fearon, D., Greten, F.R., Hingorani, S.R., Hunter, T., Hynes, R.O., Jain, R.K., Janowitz, T., Jorgensen, C., Kimmelman, A.C., Kolonin, M.G., Maki, R.G., Powers, R. S., Puré, E., Ramirez, D.C., Scherz-Shouval, R., Sherman, M.H., Stewart, S., Tlsty, T. D., Tuveson, D.A., Watt, F.M., Weaver, V., Weeraratna, A.T., Werb, Z., 2020. A framework for advancing our understanding of cancer-associated fibroblasts. Nat. Rev. Canc. 20, 174–186.

Senavirathna, L.K., Huang, C., Yang, X., Munteanu, M.C., Sathiaseelan, R., Xu, D., Henke, C.A., Liu, L., 2018. Hypoxia induces pulmonary fibroblast proliferation through NFAT signaling. Sci. Rep. 8, 2709.

Shintani, Y., Fujiwara, A., Kimura, T., Kawamura, T., Funaki, S., Minami, M., Okumura, M., 2016. IL-6 secreted from cancer-associated fibroblasts mediates chemoresistance in NSCLC by increasing epithelial-mesenchymal transition signaling. J. Thorac. Oncol. 11, 1482–1492.

Suganuma, T., Ino, K., Shibata, K., Kajiyama, H., Nagasaka, T., Mizutani, S., Kikkawa, F., 2005. Functional expression of the angiotensin II type 1 receptor in human ovarian carcinoma cells and its blockade therapy resulting in suppression of tumor invasion, angiogenesis, and peritoneal dissemination. Clin. Canc. Res. 11, 2686–2694.

Sullivan, N.J., Sasser, A.K., Axel, A.E., Vesuna, F., Raman, V., Ramirez, N., Oberyszyn, T. M., Hall, B.M., 2009. Interleukin-6 induces an epithelial-mesenchymal transition phenotype in human breast cancer cells. Oncogene 28, 2940–2947.

Tanaka, N., Miyajima, A., Kikuchi, E., Matsumoto, K., Hagiwara, M., Ide, H., Kosaka, T., Masuda, T., Nakamura, S., Oya, M., 2012. Prognonstic impact of renin-angiotensin system blockade in localised upper-tract urothelial carcinoma. Br. J. Canc. 106, 290–296.

van Zijl, F., Krupitza, G., Mikulits, W., 2011. Initial steps of metastasis: cell invasion and endothelial transmigration. Mutat. Res. 728, 23–34.

Wen, S.W., Ager, E.I., Neo, J., Christophi, C., 2013. The renin angiotensin system regulates Kupffer cells in colorectal liver metastases. Canc. Biol. Ther. 14, 720–727.

Xie, G., Yao, Q., Liu, Y., Du, S., Liu, A., Guo, Z., Sun, A., Ruan, J., Chen, L., Ye, C., Yuan, Y., 2012. IL-6-induced epithelial-mesenchymal transition promotes the generation of breast cancer stem-like cells analogous to mammosphere cultures. Int. J. Oncol. 40, 1171–1179.

Xu, Z.W., Yan, S.X., Wu, H.X., Chen, J.Y., Zhang, Y., Li, Y., Wei, W., 2017. The influence of TNF-α and Ang II on the proliferation, migration and invasion of HepG2 cells by regulating the expression of GRK2. Canc. Chemother. Pharmacol. 79, 747–758.

Zhao, Y., Wang, H., Li, X., Cao, M., Lu, H., Meng, Q., Pang, H., Li, H., Nadolny, C., Dong, X., Cai, L., 2014. Ang II-AT1R increases cell migration through PI3K/AKT and NF-κB pathways in breast cancer. J. Cell. Physiol. 229, 1855–1862.