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Deficiency or blockade of angiotensin II type 2 receptor delays tumorigenesis by inhibiting malignant cell proliferation and angiogenesis

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Despite significant expression level in cancer cells, the role of the angiotensin II Type 2 receptor (AT2R) in cancer progression remains poorly understood. We aimed to investigate the involvement of AT2R in tumorigenesis, hypothesizing a role in tumor cell proliferation and/or tumor angiogenesis. Two animal tumor models were used: fibrosarcoma induced by 3-methylcholanthrene (3-MCA) in FVB/N mice invalidated for AT2R (AT2R-KO) and carcinoma LL/2 cells injected in C57BL/6N mice treated with AT2R antagonist PD123,319. Tumor growth was monitored, microvascular density (MVD) evaluated by CD31 staining. Proliferation index of LL/2 and 3-MCA tumor cells was evaluated by expression of Ki-67. Angiogenesis was assessed by aorta ring assay and angiogenic mediators' expression by real-time RT-PCR. Tumor induction by 3-MCA was significantly delayed in AT2R-KO compared to wild-type mice (56 days vs. 28 days). Tumorigenesis following LL/2 cell injection in mice was also significantly reduced by early administration of the antagonist PD123,319. *In vitro*, inactivation or invalidation of AT2R inhibited proliferation of LL/2 and 3-MCA tumor cells, respectively. Tumor MVD was reduced in mice treated early with PD123,319. *Ex vivo* experiments revealed a significant decrease in angiogenesis after PD123,319 treatment or in AT2R-KO mice. Finally, we identified vascular endothelial growth factor (VEGF) as a soluble proangiogenic factor produced by LL/2 cells and we showed that in LL/2 and 3-MCA tumor cells, inhibition or deficiency of AT2R was associated with impaired production of proangiogenic factors included VEGF. This study uncovered novel mechanisms by which AT2R would promote tumor development, favoring both malignant cell proliferation and tumor angiogenesis.

The renin-angiotensin system (RAS) plays a major role in vascular homeostasis, regulating blood pressure and blood flow by modulating vascular tone. Disorders affecting the RAS contribute to the pathophysiology of most cardiovascular diseases. Angiotensin II (AngII), the main effector of the RAS, acting through G-protein-coupled receptors (GPCRs), Type 1 (AT1R) and Type 2 (AT2R).¹ Most of the physiological effects of AngII such as vascular smooth muscle cell contraction, growth and inflammation have been attributed to

Key words: AT2R, tumor growth, angiogenesis, cell proliferation Additional supporting information (supplementary data) may be found in the online version of this article.

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Correspondence to: Daniel Henrion, CNRS UMR 6214, INSERM UMR U771, Faculté de Médecine, Rue Haute de Reculée, 49045 Angers, France, Tel: +332-41-73-58-45, Fax: +332-41-73-58-95, E-mail: daniel.henrion@univ-angers.fr AT1R, which is expressed ubiquitously in adult tissues.² Accumulating evidences also indicate that AT2R, despite being expressed more restrictly, can induce vasodilation, antigrowth and anti-inflammatory actions in adult tissues,³ thereby antagonizing the effects of AT1R. Nevertheless, in various pathologic situations, AT2R was more described as a pro-proliferative factor as it could participate in cardiovascular remodeling following myocardial infarction, hypertension, heart failure and stroke.⁴⁻⁶ Moreover, it was shown in ischemia that AT2R had some pro-angiogenic properties.^{7,8} There is now increasing evidence of implication of the RAS system in cancer biology. In several organs, there exists a local RAS system which may, by paracrin mechanisms, affect cell proliferation, apoptosis and inflammation.⁹ Many studies involved AT1R as the RAS member regulating tumorigenesis. Overexpression of AT1R in cancer tissues is common in a number of cancers (prostate, lung, pancreas, breast, etc.), and may correlate with tumor progression. Recent years, many studies have more precisely investigated the role of AT1R in tumor development and it was shown that AT1R could regulate tumor growth, tumor angiogenesis and metastasis in different tumor animal models.^{10–14}

However, until now the role of the AT2R subtype in tumorigenesis has not been deeply investigated. Nevertheless, AT2R is highly expressed in intratumoral blood vessels in human pituitary adenomas¹⁵ and its upregulation was associated with poor prognosis in astrocytoma.¹⁶ One recent study has described that genetic deletion of AT2R reduced lung tumor growth after tobacco nitrosamine intoxication.¹⁷ These authors demonstrated that AT2R was regulating activation of TGF- β in stromal cells from the tumor microenvironment, suggesting for the first time an oncogenic modulation function of AT2R but without proving any direct effect of AT2R on tumor cells. Importance of the microenvironment is clearly established in tumor development¹⁸ and tumor vasculature, represents a major component of this microenvironment influencing tumorigenesis.¹⁹ We hypothesized that AT2R could regulate directly tumor cell growth properties and could also affect tumor vasculature. Our study was aiming at clarifying precise role of AT2R in tumor progression by exploring blockade effect of AT2R both on tumor cell growth and on tumor angiogenesis from 2 different animal models of tumors.

Material and Methods Reagents

The AT2R blocker PD123,319 was purchased from Tocris (Bristol, United Kingdom), and AngII from Sigma Aldrich (St. Louis, MO). The AT1R blocker candesartan was kindly provided by AstraZeneca (Molndal, Sweden). Trypsin and culture media were obtained from Lonza (Basel, Switzerland). Anti-mouse vascular endothelial growth factor (VEGF) and anti-mouse VEGF-R1 blocker antibodies were obtained from R&D systems (Minneapolis, MN). Methods were summerized in Figure S1.

Mice

Six-week-old female C57BL/6N mice (Charles River laboratories, L'Arbesle, France) and female FVB/N mice (Institut für Experimentelle und Klinische Pharmakologie und Toxikologie, Freiburg, Germany) with deleted AT2R (Agtr2-/-) gene were compared to Agtr2+/+ mice (Ref. 20, #68), were used. All studies involving mice were approved by the regional ethics committee on animal testing and were carried out with permission to conduct experiments on animals in accordance with European guidelines (A49065).

3-Methylcholanthrene (3-MCA)-induced sarcoma

Female AT2R-KO and wild-type (WT) mice (n = 6 per)group) were inoculated subcutaneously in the right flank with 0.2-ml peanut oil containing 3-MCA (20 mg/kg, Sigma-Aldrich). Tumor growth was monitored weekly and measured using a caliper square along the perpendicular axes of the tumors. Tumor volume was evaluated according to the formula: $V = L \times l^2 \times 0.52$ (where L is the largest superficial diameter and l is the smallest superficial diameter).

Fibrosarcoma-derived cells were extracted from the tumors of AT2R-KO (3-MCA-KO) and WT (3-MCA-WT) mice. Cells were grown in DMEM containing 10% fetal bovine serum (FBS), 1% L-glutamine and 100 UI/ml of penicillin plus streptomycin in a humidified chamber at 37°C under 5% CO2.

Cell culture and cell proliferation

The Lewis lung carcinoma cell line LL/2 was purchased from American Type Culture Collection. These cells were grown in DMEM containing 10% FBS, 1% L-glutamine and 100 UI/ml of penicillin plus streptomycin in a humidified chamber at 37°C under 5% CO₂.

LL/2 or 3-MCA fibrosarcoma-derived cells were seeded onto 6-well plates at a density of 3×10^5 cells/well. Cells were cultured in DMEM media with 10% FBS for 24 hr. Before experiments, cells were starved for 24 hr and thereafter treated with AngII (10^{-8} M) or AngII (10^{-8} M) plus PD123,319 (10⁻⁶ M) in DMEM for 72 hr. After incubation in 5% CO₂ at 37°C, cells were harvested with trypsin and cell number was estimated after staining with Trypan blue.

Cell immunochemistry

Ki-67 immunolabeling was used to determine cell proliferation. LL/2 or 3-MCA-fibrosarcoma-derived cells (3 \times 10⁵) were seeded on glass slides in 6-well plates and treated as previously described. Cells were then fixed for 5 min in acetone at -20° C followed by 5 min in acetone at room temperature. Slides were incubated for 30 min in PBS with 2% BSA and 0.01% Tween 20. Slides were incubated for 60 min at room temperature with mouse anti-Ki-67 antibody (clone TEC-3, Dako, Glostrup, Denmark), then incubated for 30 min with anti-rat secondary biotinylated antibody, and then incubated for 30 min with VECTASTAIN® Elite ABC reagent (Vector Laboratories, Burlingame, CA). Peroxidase activity was detected with DAB® (Roche Diagnostics, Basel, Switzerland) and 0.05% H₂O₂, resulting in the intranuclear deposition of a brown pigment. The number of Ki-67-positive cells was manually counted, for each condition, in 6 randomly selected fields of view using a microscope at $40 \times$ magnification.

Immunoprecipitation and Western blot analysis

LL/2 cells were incubated with AngII or AngII plus PD123,319 for 72 hr. Cells were then lysed in RIPA buffer (50 mM Tris, 150 mM NaCl, 1% Triton X100, 0.5% sodium deoxycholate, 0.1% SDS). After centrifugation at 14,000g for 15 min at 4°C, the supernatant was collected. One hundred micrograms of cell lysate were incubated with 10 µg of anti-AT2R polyclonal antibody (C-18, Santa Cruz Biotechnology, Santa Cruz, CA) at 4°C overnight and precipitated by addition of 10 µl of protein A/G-agarose (Santa Cruz Biotechnology). The immunoprecipitate was resuspended in $2\times$ Laemmli sample buffer and run on a 10% SDS/PAGE. Proteins were then transferred to nitrocellulose membranes (Amersham, Bucks, United Kingdom), blotted with anti-AT2R antibody (H-143, Santa Cruz Biotechnology), and

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proteins were visualized using the SuperSignal West Femto Maximum Sensitivity Substrate Kit (Pierce Biotechnology, Rockford, IL).

For Western blot, 30 μ g of lysate was separated by SDS-PAGE. Proteins were detected with specific antibodies against AT1R (SC-1173, Santa Cruz Biotechnology), VEGF (R&D systems Minneapolis, MN) and β -actin (Sigma Aldrich). Protein expression was visualized using the SuperSignal West Femto Maximum Sensitivity Substrate Kit (Pierce Biotechnology) and normalized for β -actin expression.

In vivo AT2R blockade with PD123,319

Six-week-old female C57BL/6N mice (Charles River laboratories, L'Arbesle, France) were inoculated with LL/2 cells (2.5×10^5 cells per mouse) s.c. in the right flank. Osmotic mini-pumps (Alzet[®] model 2002, Palo Alto, CA) containing sterile saline vehicle, candesartan (5 mg/kg/day) or PD123,319 (20 mg/kg/day) were implanted subcutaneously in the back of the neck 7 or 14 days after LL/2 cell inoculation under anesthesia (5% isoflurane). Tumor growth was monitored weekly. After a treatment for 14 days, mice were sacrificed and tumors were collected and weighed. Tumors were then fixed in 4% paraformaldehyde and embedded in paraffin.

Immunohistochemical analysis and microvascular density

For the measurement of microvascular density (MVD), tumor sections were first deparaffinized. Endogenous peroxide production was blocked with 1% hydrogen peroxidase inhibitor (Sigma Aldrich). Nonspecific protein binding was blocked with 10% rabbit serum and 2% bovine serum for 30 min. Sections were then incubated overnight at 4°C with antimouse CD31 antibody (Dako). Finally, sections were incubated for 30 min with peroxidase-labeled biotinylated anti-rat antibody (Vector Laboratories) at room temperature. Signal was detected with True Blue peroxidase substrate[®] (KPL, Gaithersurbg, MD) following incubation for 10 min and sections counterstained with hematoxylin. Each tumor section was analyzed by three independent observers to determine the MVD/mm².

Mouse aortic ring assay

The abdominal aorta was isolated from WT and AT2R-KO mice. Transverse sections (1-mm thick) were then washed in MCDB131 medium and embedded in BD Matrigel[®] (BD Biosciences, San Jose, CA) in a 96-well plate. Rings were incubated at 37° C in endothelial basal medium supplemented with antibiotics with or without AngII (10^{-8} M) or AngII plus PD123,319 (10^{-8} M and 10^{-6} M, respectively) for 5 days.

Tube formation assay

Abdominal aortic rings were embedded in BD Matrigel (BD Biosciences) in the bottom chambers of 24-well Transwell[®] plates (Corning, NY) and incubated in MCDB131 medium. LL/2 cells (1.5×10^4) were seeded into the top chambers in

Genes	Primers
AT2	Sense 5'-TGCTTTAAACACTGGCAACTAAA-3'
	Antisense 5'-TGCCAGTTGCGTTGAGATTA-3'
Flt1	Sense 5'-GGCCCGGGATATTTATAAGAAC-3'
	Antisense 5'-CCATCCATTTTAGGGGAAGTC-
TGF-β	Sense 5'-TGGAGCAACATGTGGAACTC-3'
	Antisense 5'-CAGCAGCCGGTTACCAAG-
TSP1	Sense 5'-GTTCCTGATGGTGAATGCTG-3'
	Antisense 5'-CACGTTGCTGAATCCATTG-
VEGF	Sense 5'-AAACGAAAGCGCAAGAAATC-3'
	Antisense5'-ATGCTTTCTCCGCTCTGAAC-

Primer were designed using Primer3 software (http://frodo.ui.mit.edu/ cgi-bin/primer3/primer3_www.cgi)

DMEM containing 10% serum or in DMEM supplemented with Ang II (10^{-8} M). Cells were treated for 5 days with anti-mouse VEGF (0.15 µg/ml) or VEGF-R1 (8 µg/ml) anti-bodies. Each ring was scored by three independent observers on a scale of 0 to 5, depending on the extent of outgrowth where 0 equals no growth and 5 represents profuse growth.

Real-time RT-PCR

LL/2 or 3-MCA cells were cultured in DMEM media with 10% FBS for 24 hr. Before experiments, cells were starved for 24 hr. LL/2 cells were thereafter treated with AngII (10^{-8} M) or AngII plus PD123,319 (10⁻⁶ M) in DMEM for 72 hr. Cells were detached using trypsin and reverse transcription was performed using a QuantiTect[®] Reverse transcription kit (Qiagen, Courtaboeuf, France). Real-time PCR was performed on ABI PRISM 7000 Sequence Detection System using the SYBR Green PCR Master Mix (Applied Biosystems®, Carlsbad, CA). The PCR reaction consisted of 7.5 µl of SYBR Green PCR Master Mix, 10 nM of forward and reverse primers (Table 1), and 2.0 µl of 1:20-diluted template cDNA in a total volume of 20 µl. Cycling was performed using the conditions 10 min at 95°C, followed by 40 rounds of 15 sec at 95°C and 1 min at 60°C. To verify that the used primer pair produced only a single product, a dissociation protocol was added after thermocycling, determining dissociation of the PCR products from 65°C to 95°C. We selected the HPRT gene as an endogenous control (Table 1). Finally, the quantification of mRNA was performed according to the ΔCt method.

Statistics

Data represent mean \pm SEM. Statistical tests used were Student's *t*-test or ANOVA as specified in the figure legends. For Kaplan–Meier survival analysis, the end point was the detection of tumor volume >100 mm³. Statistical significance of the difference between the survival curves for different groups of mice was assessed using x² and log-rank tests.

Results

AT2R deficiency delays carcinogen-induced tumorigenesis

To evaluate whether AT2R activation was associated with carcinogen-induced tumorigenesis, AT2R-KO and WT mice were treated with 3-MCA (20 mg/kg). In AT2R-KO mice, tumor development was significantly delayed compared to WT mice (Fig. 1*a*). Fibrosarcomas were observed in 66% of WT mice after 28 days, whereas the same percentage of tumors was observed in AT2R-KO mice after 56 days. Moreover after 35 days, all WT mice had a tumor of at least 100 mm³ while no detectable tumors were found in AT2R-KO mice (Fig. 1*b*). These results suggest that absence of the AT2R significantly delays tumorigenesis.

Early AT2R pharmacological blockade decreases tumor growth

To determine the effect of pharmacological blockade of the AT2R on tumor growth, C57BL/6N female mice were injected with LL/2 cells and were treated with either the AT2R antagonist PD123,319, the AT1R antagonist candesartan or saline 7 or 14 days after cell inoculation. In both cases, detectable tumors were observed 9 days after LL/2 cells injection. In mice treated with PD123,319 from Day 7 onward, tumor growth was significantly retarded compared to mice treated with the vehicle whereas treatment with the AT1R agonist candesartan had no effect on tumor growth (Fig. 2a). Although mice treated with PD123,319 at Day 7 demonstrated a significant delay in tumor growth, withdrawing PD123,319 treatment led to increased tumor growth, confirming the specific and reversible effect of blocking AT2R in tumor development (Fig. 2a). Tumor growth was not significantly affected by either PD123,319 or candesartan when treatment started 14 days after LL/2 cell inoculation (Fig. 2b). These observations were confirmed by the measurement of tumor weights at the end of the protocol: tumor weight was significantly lower only in mice treated 7 days after LL/2 injection with PD123,319, but not with candersartan (Fig. 2c). No differences in tumor weight were observed when PD123,319 or candersartan treatment was initiated at Day 14 post-injection (Fig. 2d). These results confirm that the specific pharmacological blockade of AT2R decreases tumor development, particularly when PD123,319 treatment is initiated at an early stage of tumorigenesis.

Pharmacological blockade of the AT2R with PD123,319 decreases tumor angiogenesis

We hypothesized that AT2R could influence neo-vascularization in tumors. Therefore, we investigated MVD in tumor sections. When AT2R pharmacological blockade by PD123,319 was initiated at Day 7, CD31 (endothelial specific marker)-positive staining was lower in tumors of PD123,319treated mice than of control animals. Nevertheless, when the treatment with PD123,319 was started 14 days after LL/2 cells inoculation, CD31-positive staining was not different



Figure 1. Invalidation of AT2R protects mice from 3-MCA-induced sarcoma. (*a*) Tumor volume in WT or AT2R-KO mice. Data are expressed as tumor volume (mm³) and are presented as mean \pm SEM (n = 6 per group). ***p < 0.001: AT2R-KO vs. WT mice (ANOVA) (*b*) Kaplan-Meier analysis of mice with skin fibrosarcomas in WT and AT2R-KO groups. (AT2R-KO vs. WT mice: χ^2 and log-rank tests). p < 0.002; Hazard ratio: 2.333; 95% Cl of ratio: 1.787–13.44.

from control (Fig. 3). These results show that blockade of AT2R by PD123,319 inhibits tumor angiogenesis only when it was administrated early, implying that AT2R is acting early during tumorigenesis in our model.

AT2R has direct effects on LL/2 carcinoma cells and on 3-MCA-fibrosarcoma-derived cells

As our *in vivo* analysis implicated the AT2R in tumorigenesis, we chose to characterize malignant tumor cells from our 2 models: LL/2 cells used for injection and 3-MCA-induced fibrosarcoma cells isolated from WT or AT2R-KO mice. First, we examined expression of AT2R and AT1R and found high expression of each angiotensin receptor in LL/2 cells, whether treated or not with AngII plus PD123,319 (Fig. 4*a*). In 3-MCA fibrosarcoma cells, AT2R expression was absent in 3-MCA-AT2R KO whereas expression of AT1R was high in both WT and AT2R KO (Fig. 4*a*). Results of AT1R and AT2R protein expression were confirmed at mRNA level by



Figure 2. Early AT2R pharmacological blockade reduces *in vivo* tumor growth. LL/2 cells (2.5×10^5) were injected s.c. into the flank of C57BL/6N female mice. Mice were chronically treated with PD123,319 (20 mg/kg/day), candesartan (5 mg/kg/day) or their vehicle (saline). The treatment was initiated 7 (*a*) or 14 (*b*) days after LL/2 cells implantation and tumor dimensions were measured weekly. Data are expressed as tumor volume (mm³) and are presented as mean \pm SEM (n = 6 per group) ***p < 0.0001: PD123,319 vs. vehicle (ANOVA). Tumor weights were determined 14 days after pump implantation at the sacrifice of animal, that is, at Day 21 for mice treated 7 days after cell injection (*c*) or Day 28 for mice treated 14 days after cell injection (*d*). Data are expressed as tumor weight (g) and are presented as mean \pm SEM (n = 6 per group) ** p < 0.05: PD123,319 vs. vehicle (ANOVA).

real time PCR (data not shown). We then analyzed the proliferation rates of LL/2 and 3-MCA fibrosarcoma cells in order to determine if the AT2R could directly affect the proliferation status of these cells. Thus, using cell counting and Ki-67 immuno-staining, we found that in LL/2 cells, AngII induces a 35% increase in cell number whereas treatment with AngII and the AT2R antagonist PD123,319 decreases the cell number by 50% (Fig. 4*b*). A clear decrease (45%) of Ki-67-positive LL/2 cells was observed when these cells were treated with AngII and PD123,319. No difference was observed between control cells and AngII treated-LL/2 cells (Fig. 4*c*). In 3-MCA-fibrosarcoma derived cells, treatment with PD123,319 induced a 65 and 47% decrease in cell counting, respectively in AT2R-KO and in WT cells (Fig. 4*d*). Moreover, a significant decrease of Ki-67 positive cells was found in 3-MCA-KO cells or in 3-MCA-WT cells treated with AngII and PD123,319 for 72 hr (Fig. 4*e*). To show that AT2R role in tumor cell proliferation was not only limited to lung carcinoma derived cells LL/2 and 3-MCA fibrosarcomas, two other cell lines CT-26²¹ and ID8,²² derived respectively from BALB/c mouse colon carcinoma and C57BL/6N mouse ovarian cancer and both expressing

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Figure 3. AT2R blockade with PD123,319 reduces tumor-associated angiogenesis. Photographs were taken with $20 \times$ objectives. A representative frozen section of tumor from each group (n = 6) is shown and revealed CD31 staining in blue. d7: PD123,319 treatment initiated 7 days after LL/2 cell injection. d14: PD123,319 treatment initiated 14 days after LL/2 cell injection. Mean MVD (number of vessels/mm²) from tumors treated with PD123,319 or saline are graphically represented. * p < 0.05. PD123,319-treated mice *vs.* vehicle-treated group (ANOVA). Horizontal bar = 50 µm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

AT2R, were subjected to the same treatments for 72 hr. As in LL/2 and 3-MCA cells, a significant decrease in proliferation was observed in AngII plus PD123,319-treated cells after 72 hr in both cell lines CT26 and ID8 cells (data not shown).

These observations demonstrate that AT2R is directly involved in the control of malignant cell proliferation.

AT2R blockade or AT2R gene deletion inhibits angiogenesis ex vivo

As we have shown that pharmacological blockade of AT2R with PD123,319 correlated with decreased MVD, we assessed the role of the AT2R in angiogenesis in our animal models. Using the aortic ring assay (Fig. 5a), we showed in rings

from WT mice that treatment with AngII did not affect the sprouting formation whereas treatment with PD123,319 significantly decreased it. In rings from AT2R-KO mice, the sprouting was significantly reduced in aortic control rings compared with rings from WT mice. We also noticed that the treatment with AngII increased the sprouting index of AT2R-KO rings compared to untreated KO rings. The specificity of the AT2R antagonist PD123,319 was further confirmed, as no difference in sprout formation was observed in AngII plus PD123,319-treated aortic rings from AT2R-KO mice compared with untreated AT2R-KO rings (Fig. 5*b*). According to these observations, we conclude that the AT2R can directly modulate angiogenesis.



Figure 4. Direct effects of AT2R on LL/2 and 3-MCA-fibrosarcoma-derived cells proliferation. (*a*) AT1R and AT2R expression in LL/2 cells treated with PD123,319 (PD) or its vehicle (saline) and in wild-type (WT) or AT2R-KO (KO) fibrosarcoma-derived cells. (*b*) Effect of AnglI (10^{-8} M) or AnglI plus PD123,319 $(10^{-8} \text{ M} \text{ and } 10^{-6} \text{ M}, \text{ respectively})$ treatments on LL/2 cell number after 72 hr. Data are graphically expressed as mean (n = 6) \pm SEM (ANOVA). * p < 0.05: AnglI-treated vs. untreated cells. # p < 0.05: AnglI-treated vs. AnglI plus PD123,319-treated cells. § p < 0.05: AnglI plus PD123,319-treated vs. untreated cells. (*c*) Ki-67 expression was analyzed in LL/2 by immunochemistry. Data are graphically expressed as mean of percentage of labelled cells (n = 6) \pm SEM. * p < 0.05: AnglI plus PD123,319-treated cells vs. untreated cells vs. untreated cells vs. untreated cells vs. untreated cells vs. anglI plus PD123,319-treated cells vs. untreated cells (ANOVA). (*d*) Influence of AT2R invalidation and PD123,319 treatment on malignant cell number after 72 hr. Data are graphically expressed as mean (n = 6) \pm SEM (ANOVA). ** p < 0.01: KO vs. WT cells. * p < 0.05: KO vs. AnglI plus PD123,319-treated WT cells. (*e*) Ki-67 expression was analyzed in 3-MCA-fibrosarcoma-derived cells by immunochemistry. Data are graphically expressed as mean (n = 6) \pm SEM. * p < 0.05: KO vs. AnglI plus PD123,319-treated WT cells. (*e*) Ki-67 expression was analyzed in 3-MCA-fibrosarcoma-derived cells by immunochemistry. Data are graphically expressed as mean of percentage of labelled cells or WT cells treated with AnglI plus PD123,319 vs. 3-MCA-WT cells (ANOVA).



Figure 5. Influence of PD123,319 treatment or AT2R invalidation in the regulation of *ex vivo* angiogenesis. (*a*) Abdominal aortic ring segments from AT2R-KO or WT mice were embedded in Matrigel[®] and incubated with Angll (10^{-8} M), AnglI plus PD123,319 (10^{-8} M and 10^{-6} M, respectively), or vehicle (saline) for 5 days. Panel shows representative photomicrographs of tube formation in each condition after 5 days in culture. (*b*) Effect on the sprouting vessels from *ex vivo* aortic rings embedded in Matrigel. Bars represent mean of 3 independent experiments ± SEM. * p < 0.05: each ring *vs*. WT untreated ring; # p < 0.05: KO-rings treated with AnglI *vs*. KO-untreated rings (ANOVA).



Figure 6. Increase of sprouting in mouse aortic rings cocultured with LL/2 cells. Effect on the sprouting vessels from coculture of LL/2 cells and aortic rings. Bars represent the mean of 3 independent experiments \pm SEM. ** p < 0.01 : WT + LL2 vs. WT - LL/2 (Student's *t*-test).

Soluble proangiogenic factors, and particularly VEGF, produced by LL/2 tumor cells favor angiogenesis in aortic ring assays

To determine whether the AT2R could be involved in tumor-regulated angiogenesis, we first set up a coculture model wherein sprout formation was studied using aortic rings embedded in Matrigel in the bottom chambers of Transwell plates and cocultured with LL/2 cells seeded in upper chambers. In rings cocultured with LL/2 cells, sprouting increased significantly (Fig. 6). These data strongly suggest that soluble factor(s) produced by LL/2 cells, is (are) required to fully optimize the angiogenic process.

VEGF is known to be an essential proangiogenic factor. Accordingly, to determine if this proangiogenic factor was involved in tumor-regulated angiogenesis, sprouting was studied in Transwell plates, as previously described. Wild-type aortic rings, cocultured with LL/2 cells, were treated with VEGF or VEGF-R1 blocker antibodies. Sprout formation was significantly reduced in aortic rings treated with VEGF or VEGF-R1 blocker antibodies compared to WT-untreated rings (Fig. 7). These observations clearly indicate that VEGF produced by LL/2 cells is required to promote angiogenesis in our model of *ex vivo* angiogenesis.

Influence of the AT2R in the regulation of proangiogenic molecules expression in tumor cells

We then investigated if the modulation of AT2R may influence the production of soluble proangiogenic molecules by



Figure 7. Influence of soluble proangiogenic factors produced by tumor cells on *ex vivo* angiogenesis. The effect on the sprouting vessels from *ex vivo* aortic rings embedded in Matrigel[®] are shown by bars representing the mean of 3 independent experiments \pm SEM. * p < 0.05: each treated ring *vs*. WT untreated ring (ANOVA). VEGF Ab: treated with VEGF blocker antibodies; VEGF-R1 Ab: treated with VEGF-R1 blocker antibodies.

tumor cells (Table S1). A significant decrease of VEGF and VEGF-R1 transcripts and in VEGF protein expression was found in 3-MCA-KO cells compared with 3-MCA-WT cells. Moreover, deletion of the AT2R was associated with an increase of TGF-β and TSP-1 transcripts expression (Fig. 8a and Inset). In LL/2 cells, levels of VEGF, VEGF-R1 and TGF-B transcripts were increased upon AngII stimulation but this AngII-induced increase of pro-angiogenic factors was inhibited by PD123,319. To the opposite, pharmacological blockade of AT2R with PD123,319 (i.e., in cells treated simultaneously with AngII and PD123,319) induced a significant increase of anti-angiogenic factor TSP-1 (Fig. 8b). Study of VEGF protein expression showed a significant increase of VEGF in AngII-treated cells and a significant decrease in cells treated simultaneously with AngII and PD123,319 (Fig. 8b and Inset). Our data clearly show that blockade of AT2R can modulate production of angiogenic molecules by tumor cells.

Discussion

Increasing evidence suggests that AngII plays an important role in tumorigenesis. Although the involvement of AT1R has been well explored,^{11,14,23,24} the role of the AT2R in tumorigenesis remained controversial and has been suggested mainly by correlative studies showing important expression or overexpression of AT2R in tumor tissues.^{15,16,25–27} This study was conducted in an attempt to elucidate AT2R



Figure 8. Genetic invalidation or pharmacological blockade of AT2R modulates expression of various proangiogenic factors. (*a*) Influence of AT2R invalidation on VEGF, VEGF-R1, TSP-1 and TGF- β mRNA expression. (Inset) Influence of AT2R invalidation on VEGF expression. Data are graphically expressed as mean of relative values ± SEM (Student's *t*-test). * *p* < 0.05 : KO vs. WT cells; ** *p* < 0.01: KO vs. WT cells. (*b*) Influence of AngII or AngII plus PD123,319 treatments on VEGF, FIt-1, TSP-1 and TGF- β mRNA expression. (Inset) Influence of AngII or AngII plus PD123,319 treatments on Data are graphically expressed as mean of relative values ± SEM (ANOVA). * *p* < 0.05 : AngII plus PD123,319-treated vs. untreated cells.

implication in tumorigenesis. We used 2 different *in vivo* tumor models (FVB/N AT2R-KO mice in which fibrosarcomas were induced by 3-MCA injection and C57BL/6 mice with LL/2-xenografted carcinoma) to demonstrate that AT2R has a key role in tumorigenesis by modulating both malignant cell proliferation and tumor angiogenesis.

In mice lacking AT2R, 3-MCA-induced tumorigenesis was significantly delayed, suggesting a role for the AT2R in tumor growth. These results are in agreement with two previous studies in AT2R KO mice using chemical carcinogens azoxymethane or nitrosamine as inducers of colon²⁸ and lung tumors,¹⁷ respectively. In both studies, AT2R deficiency attenuates mice susceptibility to tumorigenesis by different mechanisms: carcinogen detoxification²⁸ or modulation of active TGF- β levels by tumor stroma.¹⁷ Besides these effects on detoxification and on stromal cells, our results now established a direct and specific role of AT2R on tumor growth and tumor angiogenesis. Thus, the specificity of AT2R in tumorigenesis was shown using a FVB/N mice model invalidated for AT2R in which no compensation of AT1R expression was described.²⁰ In our study, we did not want to use another mouse model,²⁹ due to conclusions about the significant involvement of AT1R in various physiological processes, including regulation of the blood pressure.

Many studies have identified a role for AT1R in tumor development mainly by the use of antagonists in animal models at different concentrations.^{14,23,30} Our results showed no effect of candersartan on tumor growth in a mice model of LL/2 pulmonary carcinoma cells. Discrepancy between our study and studies cited above could be explained first by differences in tumor histology. Moreover, doses of AT1R antagonist usually used were much higher (40-80 mg/kg/day) than the dose we used (5 mg/kg/day). We have shown that this latter lower dose is significantly blocking AT1R effect on contraction of mesenteric arteries (D. Henrion, personal communication). Although the dose of AT1R blocker used in our study does not reproduce the antigrowth effects of AT1R previously described, we would argue that the use of lower doses of sartans is probably more compatible with chronic treatment, not reducing dramatically blood pressure. Using high doses of AT1R blocker in long-term experiments would make more difficult to interpret the data as regard to direct effect of AT1R and AT2R if blood pressure is too different in treated and not treated group. Furthermore, a study conducted by Uemura et al.²⁴ demonstrated that in a mouse model of prostate xenograft, AT1R blocker candesartan, used at the same concentration (5 mg/kg/day) as in our study, was not affecting tumor development in absence of specific microenvironnment stromal cells.

Our work described a specific key role of AT2R in tumor growth and particularly a direct effect on malignant cell proliferation. Decreased expression of Ki-67, specific marker of cell proliferation, in LL/2 carcinoma cells treated with AngII plus PD123,319 or in AT2R-KO-fibrosarcoma-derived cells revealed a proproliferative role of AT2R in these two



Figure 9. Proposed model of AT2R implication in tumorigenesis. In tumor cells, stimulation of AT2R led to an increase of malignant cell proliferation and pro-angiogenic factors production. As AT2R is commonly expressed on endothelial cells, we can propose that AT2R activation would stimulate endothelium proliferation, potentiated also by tumor produced-VEGF stimulation. AT2R by acting on both tumor cell and angiogenesis might now be considered as an important actor in tumorigenesis.

different tumor cell lines. This was quite surprising as AT2R was essentially described as an antiproliferative and proapoptotic mediator in different in vitro cell systems such as in neuronal PC12 cells, fibroblasts or renal mesenglial cells.³¹ Despite not in a tumor background, AT2R has been described as mediating trophic and proliferative effects of AngII in mesenteric vasculature,³² proximal tubular epithelial cells³³ or in smooth muscle cells.³⁴ In the cardiovascular context, where AT2R functions have been deeply explored, dual effects of AT2R on proliferation have also been described as it has been associated with proliferative effects in cardiac pathologies such as myocardial infarction,³⁵ hypertension⁷ and heart stroke.⁴ These studies confirmed that one particularity of AT2R is to function in a microenvironment context, but also in a cell type-specific manner.⁶ Although proproliferative effects of AT2R were observed mainly in various nontumor cells, we showed here that AT2R can also have progrowth properties in malignant cells.

Angiogenesis is critical for tumor development¹⁹ and neovascularisation is now known as a prerequisite to the rapid expansion of tumor cells associated with formation of macroscopic tumors and required induction and maintenance of angiogenesis, the so-called event "angiogenic switch."¹⁸ Our data revealed that detection of macroscopic LL2-derived tumor (*i.e.*, >100 mm³) in mice treated with the AT2R antagonist, PD123,319, was significantly delayed when it was given 7 days and not 14 days after LL/2 inoculation, suggesting involvement of AT2R in early events of tumorigenesis. Thus, we showed a decrease of microvascular density in tumor from mice treated with PD123,319 7 days after LL/2 cell inoculation. Altogether, these in vivo results strongly support an effect of AT2R in regulating tumor development via an effect on tumor angiogenesis. Our findings are consistent with previous studies describing a proangiogenic effect of AT2R in tumors: in pituitary adenocarcinoma, blockade of the AT2R was associated with inhibition of angiogenesis¹⁵ and in an in vivo model of tumor angiogenesis, the absence of AT2R was associated with inhibition of angiogenesis in a mice alginate implant angiogenesis model.³⁶ As in non-tumor context such as ischemia³⁷ or in diabetic retinopathy³⁸ AT2R was described as having some antiangiogenic properties, we would argue once again that AT2R behavior depends strongly on cell context.

Our present work clearly established, in aortic ring assays, that AT2R modulates angiogenesis because invalidation or pharmalogical blockade of AT2R leads to decrease *ex vivo* formation of vessels. Direct effect of AT2R on capillary microvessels surrounding the tumor has not been undoubt-edly proven as we did not work on purified microvessels. Nevertheless, use of *ex vivo* aortic ring assay performed on AT2R-KO aorta suggested that AT2R was strongly implied as a regulator of angiogenesis.

On a more mechanistic approach, we showed that the pharmacological blockade or the genetic invalidation of the AT2R in tumor cells was associated with decreased expression of the proangiogenic factor VEGF and increased expression of thrombospondin-1, a major antiangiogenic factor. These results indicated that AT2R was able to regulate tumor angiogenesis by modulating expression of pro-angiogenic and antiangiogenic factors. Until now, studies highlighting role of AT2R in VEGF regulation were only performed in non-tumor angiogenesis models.^{38,39} In tumor cells, expression of

VEGF is subjected to multiple levels of regulation, which hypoxia being a major inducer of VEGF *via* hypoxia-inducible factor-1 (for review, Ref. 40). Nevertheless, mechanisms of regulation of VEGF independently of hypoxia but implying GPCR, like AT2R have been described.⁴¹ These findings imply that understanding precisely by which mechanisms AT2R could modulate VEGF mRNA and protein expression, especially in tumor models, requires further studies that are currently under investigation in our laboratory.

Altogether these results described a role for AT2R in the control of tumorigenesis *via* pro-proliferative effects on both tumor cells and *via* regulation of pro-angiogenic factors associated with an increase of the tumor vasculature development. Of our work, we can propose an hypothetical model where in malignant cells, AT2R stimulated cell proliferation and participated to the control of VEGF expression, whereas in vessels it induced increase proliferation, which can even be potentiated by VEGF secreted by tumor cells (Fig. 9).

Epidemiologic studies provide evidence that components of the RAS system could influence tumor progression. Different retrospective cohort studies on patients treated with drugs targeting RAS (ACE inhibitors/AT1R blockers) led to variable conclusions, associating RAS system inhibition with either decreasing risk of developing cancer or with no incidence on cancer progression (for review, Ref. 42). As AT2R, described as expressed in many tumor cell types,⁹ affects tumor proliferation and angiogenesis, we can speculate that this RAS component could also be a potential interesting target in a cancer therapy.

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References

- Dzau VJ, Mukoyama M, Pratt RE. Molecular biology of angiotensin receptors: target for drug research? J Hypertens Suppl 1994;12:S1–S5.
- Inagami T. Molecular biology and signaling of angiotensin receptors: an overview. J Am Soc Nephrol 1999;10 (Suppl 11):S2–S7.
- Horiuchi M, Lehtonen JY, Daviet L. Signaling mechanism of the AT2 angiotensin II receptor: crosstalk between AT1 and AT2 receptors in cell growth. *Trends Endocrinol Metab* 1999;10:391–6.
- Faure S, Bureau A, Oudart N, Javellaud J, Fournier A, Achard JM. Protective effect of candesartan in experimental ischemic stroke in the rat mediated by AT2 and AT4 receptors. J Hypertens 2008;26:2008–15.
- Kaschina E, Unger T. Angiotensin AT1/ AT2 receptors: regulation, signalling and function. *Blood Press* 2003;12:70–88.

- Widdop RE, Jones ES, Hannan RE, Gaspari TA. Angiotensin AT2 receptors: cardiovascular hope or hype? Br J Pharmacol 2003;140:809–24.
- Henrion D, Kubis N, Levy BI. Physiological and pathophysiological functions of the AT(2) subtype receptor of angiotensin II: from large arteries to the microcirculation. *Hypertension* 2001;38: 1150–7.
- Tamarat R, Silvestre JS, Durie M, Levy BI. Angiotensin II angiogenic effect in vivo involves vascular endothelial growth factorand inflammation-related pathways. *Lab Invest* 2002;82:747–56.
- Deshayes F, Nahmias C. Angiotensin receptors: a new role in cancer? *Trends Endocrinol Metab* 2005;16:293–9.
- 10. Egami K, Murohara T, Shimada T, Sasaki K, Shintani S, Sugaya T, Ishii M, Akagi T,

Ikeda H, Matsuishi T, Imaizumi T. Role of host angiotensin II type 1 receptor in tumor angiogenesis and growth. *J Clin Invest* 2003;112:67–75.

- Fujita M, Hayashi I, Yamashina S, Itoman M, Majima M. Blockade of angiotensin AT1a receptor signaling reduces tumor growth, angiogenesis, and metastasis. *Biochem Biophys Res Commun* 2002;294: 441–7.
- Huang W, Wu YL, Zhong J, Jiang FX, Tian XL, Yu LF. Angiotensin II type 1 receptor antagonist suppress angiogenesis and growth of gastric cancer xenografts. *Dig Dis Sci* 2008;53:1206–10.
- Rivera E, Arrieta O, Guevara P, Duarte-Rojo A, Sotelo J. AT1 receptor is present in glioma cells; its blockage reduces the growth of rat glioma. *Br J Cancer* 2001;85: 1396–9.

- 14. Suganuma T, Ino K, Shibata K, Kajiyama H, Nagasaka T, Mizutani S, Kikkawa F. 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 Cancer Res* 2005;11:2686–94.
- 15. Pawlikowski M. Immunohistochemical detection of angiotensin receptors AT1 and AT2 in normal rat pituitary gland, estrogen-induced rat pituitary tumor and human pituitary adenomas. *Folia Histochem Cytobiol* 2006;44:173–7.
- 16. Arrieta O, Pineda-Olvera B, Guevara-Salazar P, Hernandez-Pedro N, Morales-Espinosa D, Ceron-Lizarraga TL, Gonzalez-De la Rosa CH, Rembao D, Segura-Pacheco B, Sotelo J. Expression of AT1 and AT2 angiotensin receptors in astrocytomas is associated with poor prognosis. Br J Cancer 2008;99:160–6.
- 17. Kanehira T, Tani T, Takagi T, Nakano Y, Howard EF, Tamura M. Angiotensin II type 2 receptor gene deficiency attenuates susceptibility to tobacco-specific nitrosamine-induced lung tumorigenesis: involvement of transforming growth factorβ-dependent cell growth attenuation. *Cancer Res* 2005;65:7660–5.
- 18. Hanahan D, Weinberg RA. The hallmarks of cancer. *Cell* 2000;100:57–70.
- Folkman J. Tumor angiogenesis: therapeutic implications. N Engl J Med 1971;285:1182–6.
- Hein L, Barsh GS, Pratt RE, Dzau VJ, Kobilka BK. Behavioural and cardiovascular effects of disrupting the angiotensin II type-2 receptor in mice. *Nature* 1995;377:744–7.
- Brattain MG, Strobel-Stevens J, Fine D, Webb M, Sarrif AM. Establishment of mouse colonic carcinoma cell lines with different metastatic properties. *Cancer Res* 1980;40:2142–6.
- 22. Sylvain V, Lafarge S, Bignon YJ. Molecular pathways involved in response to ionizing radiation of ID-8 mouse ovarian cancer cells expressing exogenous full-length Brca1 or truncated Brca1 mutant. *Int J Oncol* 2001;19:599–607.
- Arrieta O, Guevara P, Escobar E, Garcia-Navarrete R, Pineda B, Sotelo J. Blockage of angiotensin II type I receptor decreases

the synthesis of growth factors and induces apoptosis in C6 cultured cells and C6 rat glioma. *Br J Cancer* 2005;92:1247–52.

- 24. Uemura H, Ishiguro H, Nagashima Y, Sasaki T, Nakaigawa N, Hasumi H, Kato S, Kubota Y. Antiproliferative activity of angiotensin II receptor blocker through cross-talk between stromal and epithelial prostate cancer cells. *Mol Cancer Ther* 2005;4:1699–709.
- 25. De Paepe B, Verstraeten VM, De Potter CR, Bullock GR. Increased angiotensin II type-2 receptor density in hyperplasia, DCIS and invasive carcinoma of the breast is paralleled with increased iNOS expression. *Histochem Cell Biol* 2002;117: 13–19.
- Fogarty DJ, Sanchez-Gomez MV, Matute C. Multiple angiotensin receptor subtypes in normal and tumor astrocytes in vitro. *Glia* 2002;39:304–13.
- Juillerat-Jeanneret L, Celerier J, Chapuis Bernasconi C, Nguyen G, Wostl W, Maerki HP, Janzer RC, Corvol P, Gasc JM. Renin and angiotensinogen expression and functions in growth and apoptosis of human glioblastoma. *Br J Cancer* 2004;90: 1059–68.
- Takagi T, Nakano Y, Takekoshi S, Inagami T, Tamura M. Hemizygous mice for the angiotensin II type 2 receptor gene have attenuated susceptibility to azoxymethane-induced colon tumorigenesis. *Carcinogenesis* 2002;23:1235–41.
- Ichiki T, Labosky PA, Shiota C, Okuyama S, Imagawa Y, Fogo A, Niimura F, Ichikawa I, Hogan BL, Inagami T. Effects on blood pressure and exploratory behaviour of mice lacking angiotensin II type-2 receptor. *Nature* 1995;377: 748–50.
- 30. Uemura H, Ishiguro H, Nakaigawa N, Nagashima Y, Miyoshi Y, Fujinami K, Sakaguchi A, Kubota Y. Angiotensin II receptor blocker shows antiproliferative activity in prostate cancer cells: a possibility of tyrosine kinase inhibitor of growth factor. *Mol Cancer Ther* 2003;2: 1139–47.
- Unger T. The angiotensin type 2 receptor: variations on an enigmatic theme. J Hypertens 1999;17:1775–86.
- 32. Cao Z, Dean R, Wu L, Casley D, Cooper ME. Role of angiotensin receptor subtypes

in mesenteric vascular proliferation and hypertrophy. *Hypertension* 1999;34:408–14.

- 33. Cao Z, Kelly DJ, Cox A, Casley D, Forbes JM, Martinello P, Dean R, Gilbert RE, Cooper ME. Angiotensin type 2 receptor is expressed in the adult rat kidney and promotes cellular proliferation and apoptosis. *Kidney Int* 2000;58:2437–51.
- 34. Levy BI, Benessiano J, Henrion D, Caputo L, Heymes C, Duriez M, Poitevin P, Samuel JL. Chronic blockade of AT2-subtype receptors prevents the effect of angiotensin II on the rat vascular structure. *J Clin Invest* 1996;98:418–25.
- Janiak P, Pillon A, Prost JF, Vilaine JP. Role of angiotensin subtype 2 receptor in neointima formation after vascular injury. *Hypertension* 1992;20:737–45.
- 36. Walther T, Menrad A, Orzechowski HD, Siemeister G, Paul M, Schirner M. Differential regulation of in vivo angiogenesis by angiotensin II receptors. *FASEB J* 2003;17:2061–7.
- 37. Silvestre JS, Tamarat R, Senbonmatsu T, Ichiki T, Ebrahimian T, Iglarz M, Besnard S, Duriez M, Inagami T, Levy BI. Antiangiogenic effect of angiotensin II type 2 receptor in ischemia-induced angiogenesis in mice hindlimb. *Circ Res* 2002;90:1072–9.
- Zhang X, Lassila M, Cooper ME, Cao Z. Retinal expression of vascular endothelial growth factor is mediated by angiotensin type 1 and type 2 receptors. *Hypertension* 2004;43:276–81.
- Rizkalla B, Forbes JM, Cooper ME, Cao Z. Increased renal vascular endothelial growth factor and angiopoietins by angiotensin II infusion is mediated by both AT1 and AT2 receptors. J Am Soc Nephrol 2003;14: 3061–71.
- Mukhopadhyay D, Datta K. Multiple regulatory pathways of vascular permeability factor/vascular endothelial growth factor (VPF/VEGF) expression in tumors. *Semin Cancer Biol* 2004;14: 123–30.
- Richard DE, Vouret-Craviari V, Pouyssegur J. Angiogenesis and G-proteincoupled receptors: signals that bridge the gap. Oncogene 2001;20:1556–62.
- 42. Ager EI, Neo J, Christophi C. The reninangiotensin system and malignancy. *Carcinogenesis* 2008;29:1675–84.