

# Conditional expression of type 2 angiotensin II receptor in rat vascular smooth muscle cells reveals the interplay of the angiotensin system in matrix metalloproteinase 2 expression and vascular remodeling

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**Abstract.** Angiotensin II is well implicated in neointimal proliferation and the resulting restenosis, however, the mechanisms involved remain unclear. The type 2 angiotensin II (AT2) receptor, largely unexpressed in the adult vasculature, however, appears at significant levels after vascular injury. To investigate the specific contribution of AT2 receptor and the interplay of the angiotensin system to neointima, we engineered rat vascular smooth muscle cells (VSMCs) to express the AT2 receptor in a tetracycline-regulated system. Several VSMC clones resistant to both hygromycin and G418 were selected, many of which showed high, but regulatable levels of AT2R expression within 48 h of doxycycline (Dox) exposure. In untransfected VSMCs and stable transfectants with no AT2R induction, Ang II significantly increased the expression of matrix metalloproteinase 2 (MMP-2), which is linked to neointimal growth. However, induction of AT2R by Dox addition markedly decreased MMP-2 levels ( $P < 0.01$ ) and this downregulation was further promoted by CV-11974, a specific antagonist of

AT1 receptor. In contrast, the PD123319 compound, which selectively curtails the AT2 receptor, reversed the inhibition caused by CV-11974. We conclude that Ang II enhances the MMP-2 expression via AT1R, and that enforces AT2R inhibited the same. These data confirm that AT2R functions to down-regulate the effects elicited by Ang II + AT1R signaling and point to the role of MMP and extracellular matrix in vascular injury. The findings provide fresh experimental approaches to prevent or control restenosis through transduction of VSMCs expressing optimal levels of AT2R.

## Introduction

Neointima formation plays an important role in the pathogenesis of atherosclerosis, restenosis after angioplasty, and late vein graft failure. After vascular injury, the vascular smooth muscle cells (VSMCs) proliferate and migrate from media to the subendothelial space, secrete a great amount of extracellular matrix, forming a neointima and restenosis (1,2). Matrix metalloproteinases (MMPs) play a key role in extracellular matrix degradation, which is required for cell migration into the intima after arterial injury (3). This is supported by the findings that MMP-2 (gelatinase A) and MMP-9 (gelatinase B) levels are significantly enhanced after arterial injury. Inhibition of MMPs by synthetic inhibitors impairs arterial neointima formation after vascular injury in many animal models (4,5). Matrix metalloproteinases are zinc-dependent proteolytic enzymes involved in stromal wound healing, neovascularization and epithelial regeneration. MMP-2 and -9 are type IV collagenases that remodel the extracellular matrix by cleaving denatured stromal collagen, fibronectin, and basement membranes. The ECM organization under normal and pathological conditions results from a balance between synthesis and degradation of extracellular proteins, a process in which MMPs play a central role (6-8).

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Therefore, MMPs are considered important contributors to intimal growth and restenosis.

Many cytokines and vasoactive substances participate in the development of restenosis. Angiotensin II (Ang II) induces VSMCs to proliferate, migrate, secrete extracellular matrix mainly via Ang II type 1 (AT<sub>1</sub>) receptors and this process is implicated in the development and maintenance of neointima formation and restenosis (9). Most of the biological effects of Ang II are mediated through AT<sub>1</sub> receptors. AT<sub>2</sub> receptor function is less well defined. Studies suggest that many biological effects mediated by AT2R antagonize those of AT1R, showing a significant negative regulatory effect (10). AT1R is stably expressed at high levels throughout the development and growth and AT1R expression is further increased after vascular injury. In contrast, AT2R is slightly expressed after vascular injury (11). Therefore, enhancing AT2R levels may provide a strategy for prevention and/or treatment of restenosis of injured blood vessels. However, under normal conditions, the level of AT2R expression decreases rapidly after birth, and AT2R expression is found only in the adrenal gland, heart, uterus, ovary and certain functional sites of the brain. AT2R is slightly or not expressed in vascular tissues including fibroblasts, VSMCs, and vascular endothelial cells (12,13). If the AT2R gene were introduced into these tissues and its expression was unregulated, serious consequences may result. Therefore, in the present study, a regulatable mammalian expression system of the AT2R gene was developed, and the AT2R gene was introduced into VSMCs using this system to achieve conditional and optimal expression. In this system, the expression of extracellular MMPs in cultured VSMCs was investigated to provide an experimental basis for transplanting the stable VSMC lines into vascular injury sites.

## Materials and methods

**Construction of the Dox-On induced rat AT2R gene expression system.** An AT2R gene fragment was cloned into the expression vector containing TetO repeat sequence, i.e., pUHD10-3, by homologous recombination (14) to establish Doxycycline-regulated mammalian expression system (Dox-On system) containing regulatory plasmid pUHD 17-1hyg, mammalian expression plasmid pUHD 10-3/AT2R, fluorescence reporter plasmid pUHC 13-3 and neomycin screening-resistant simple plasmid pSV2 neo (15,16). pUHD 17-1hyg, 6,484 bp in length, was derived from pBR322 and contained human cytomegalovirus (hCMV) promoter and chimeric tetracycline induced transactivator (rtTA) encoding sequence. The plasmid was a gift from Dr L. Ronnstrand (Ludwig Institute for Cancer Research, Sweden). pUHD 10-3 plasmid contained tetracycline regulated promoter (tRP) which has a regulatable domain comprising seven tetracycline operator sequence (TetO) repeats. There was an hCMV promoter downstream of the domain. There were polyclonal sites downstream of tRP. The length of the plasmid was 3,150 bp. This plasmid was a gift from Dr C. Lindon (Cancer Research UK Institute, Cambridge, UK). The fluorescence reporter plasmid pUHC13-3 contained the TetO repeat sequence and the luciferase gene, with a length of 5,157 bp; the plasmid was also a gift from Drs Lindon and Ronnstrand.

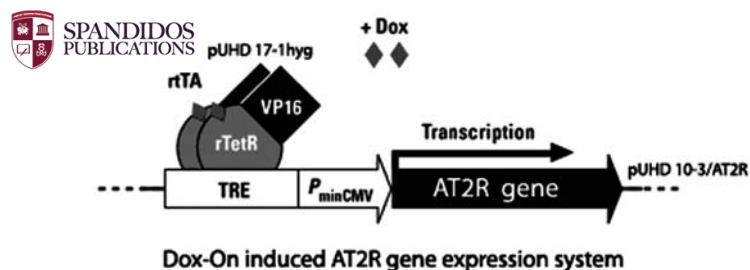
The screening plasmid pSV2neo contained neomycin-resistant gene sequence, which provided neomycin resistance at the second round screening after transfection. This DNA was a gift from Dr H.Y. Park (Boston University Medical School, USA). pACCMV/AT2R plasmid (9.7 kb) contained full-length rat AT2R cDNA sequence. The identity of recombinant pUHD 10-3/AT2R was confirmed by DNA sequencing. (Fig. 1, Dox-On induced AT2R gene expression system).

**Primary culture of rat aortic VSMCs.** Rat aortic VSMCs were derived from the thoracic aortas of male Wistar rats by using standard enzymatic dissociation techniques (17). Cells were plated and grown in DMEM supplemented with 10% (vol/vol) fetal bovine serum, and cultured at 37°C in air containing 5% CO<sub>2</sub>. VSMCs were immunohistochemically identified using specific mouse  $\alpha$ -smooth muscle actin monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA; Cat. No. sc-130616). Early cell subcultures (first or second) were used for experiments described below.

**Determination of optimal antibiotic concentrations and optimal inoculation density of rat VSMCs (18).** Determination of optimal drug concentration, 1x10<sup>5</sup> cells were plated, and hygromycin at 0, 15, 25, 50, 100, 200, or 0-800  $\mu$ g/ml of G418 were added. The VSMCs were grown for 10-14 days. The endpoint of observation was the lowest antibiotic concentration that gives massive cell death in 5 days and kills all cells within two weeks. The results indicated that 100  $\mu$ g/ml hygromycin and 200  $\mu$ g/ml G418 were the optimal concentrations.

Determination of optimal inoculation density, VSMCs were plated in 6-well plates at densities of 1x10<sup>3</sup>, 2x10<sup>3</sup>, 5x10<sup>3</sup>, 1x10<sup>4</sup>, 5x10<sup>4</sup>, and 1x10<sup>5</sup> cells/ml, and were cultured for 10 days in the presence of 100  $\mu$ g/ml hygromycin or 200  $\mu$ g/ml G418. The plating density that allowed the cells to reach 80% confluency before massive cell death began (~day 5) was determined. The results demonstrated that 5x10<sup>3</sup> cells/ml was the inoculation density of VSMCs at which hygromycin or G418 exhibited its optimal screening effect on VSMCs.

**Establishment of VSMCs lines with Dox-inducible stable expression of AT2R gene.** The protocols used for establishing the double stable expression cells with Dox were described previously (19). Briefly, transfection of VSMCs with regulatable plasmid pUHD 17-1hyg was performed using the standard calcium phosphate method (20,21). Transfected cells were cultured in selective medium containing 100  $\mu$ g/ml hygromycin. Thirty separate, large, healthy cell clones were selected. Induction of luciferase expression in hygromycin-resistant VSMC clones was analyzed by transient transfection. Next, the fluorescence reporter plasmid pUHC13-3 was introduced into hygromycin-resistant VSMCs clones. Dox (1  $\mu$ g/ml) was added after transfection and the VSMCs were allowed to grow for 48 h. The lysates were then mixed with isovolumetric Steady-Glo™ analysis reagent, followed by scintillation counting. VSMC clones with highest Dox induced expression and lowest background expression were considered Dox-On VSMC lines. In the second round of transfection, cotransfection of Dox-on VSMCs with response



**Dox-On: rTA binds TRE and activates transcription in the presence of Dox**

Figure 1. The Dox-On induced AT2R gene expression system.

plasmid pUHD10-3/AT2R and pSV2neo was carried out by the standard calcium phosphate method, and AT2R mRNA expression was detected by RT-PCR (22). Rat AT2R primers (765 bp), sense 5'-AAT CTG GCT GTG GCT GAC-3', antisense 5'-CCA AGT AAT GGG AAC TCT AAA C-3'. GAPDH primers (452 bp), sense 5'-ACC ACA GTC CAT GCC ATC AC-3', antisense 5'-TCC ACC ACC CTG TTG CTG TA-3'. RNA expression was quantitated by densitometry and expressed in arbitrary units. Then, 30 separate, large, healthy clones were successfully selected to express the AT2R gene. Clones with low background AT2R mRNA expression and high Dox induced expression were selected and designated as double stable VSMCs. They were treated with 1  $\mu\text{g/ml}$  Dox for 48 or 72 h, or with 0,  $1 \times 10^{-3}$ ,  $1 \times 10^{-2}$ , 0.1, 1, 10,  $10^2$ ,  $10^3$ ,  $10^4$  ng/ml Dox for 72 h, or with 1  $\mu\text{g/ml}$  Dox for 72 h at 1, 2, 4, 6, 8, 10, 12 W after transfection. AT2R mRNA expression was determined in all these cells.

**Western blot analysis of AT2R protein expression in Dox induced double stable VSMC lines** (23). Total protein was extracted using TriPure™ kit and protein contents were estimated. Cell extracts (50  $\mu\text{g}$  protein) were subjected to SDS-PAGE followed by transfer to nitrocellulose membranes. The blots were blocked with 2% BSA in PBS, and incubated with polyclonal antibodies specific to AT2R (Santa Cruz Biotechnology; Cat. No. sc-7420) at a dilution 1:500 overnight at 4°C. Enhanced chemiluminescence (ECL) was performed to detect the antigen and protein bands were quantitated by densitometry.

**Effect of AT2R expression on MMP-2 expression in double stable VSMCs.** The untransfected and stably transfected VSMCs were treated with Ang II ( $10^{-7}$  M) or Doxycycline (1  $\mu\text{g/ml}$  72 h) or CV-11974 ( $10^{-6}$  M) or PD123319 ( $10^{-6}$  M) either singly or in combination and MMP-2 mRNA levels were determined by RT-PCR. RNA was extracted using TriPure™ kits, and specific primers were designed using the premier primer 5.0 program. MMP-2 primers, (490 bp) annealing temperature 50°C, sense 5'-TCA ACG GTC GGG AAT ACA-3', antisense 5'-GCT GCC ACG AAT AGG-3'. GAPDH primers, (294 bp), annealing temperature 50-60°C, sense 5'-GTG ACT TCA ACA GCA ACT CCC ATT C-3', antisense 5'-GTT ATG GGG TCT GGG ATG GAA TTG TG-3'. PCR amplification products were electrophoresed on 1.2% agarose gels. The ethidium bromide stained gels were photographed and the band intensities quantified by densito-

metry. Western blot analysis of MMP-2 expressed in response to AT2R was performed as described above (23). Antibodies to MMP-2 (Santa Cruz Biotechnology; Cat. No. sc-6838) were used at a dilution 1:400.

**Statistical analysis.** Measurement data were expressed as mean  $\pm$ SD. Student's t-test or ANOVA was adopted to compare group difference using the software SPSS 11.0.

## Results

**Induced expression and analysis of luciferase in various hygromycin-resistant VSMC clones.** After transfection of VSMCs with pUHD 17-1hyg plasmid, 30 separate, large, healthy clones were selected, and fluorescence reporter plasmid pUHC13-3 were introduced by calcium phosphate method into these hygromycin-resistant clones. Transient luciferase expression was analyzed 48 h after transfection. VSMC clones with low background expression and high Dox induced AT2R expression were deemed Dox-on VSMC lines (data shown partly in Table I).

The results showed that of hygromycin-resistant VSMC clones, numbered 1-30, clone 22 VSMCs showed 4,973 pulses/min<sup>-1</sup> without Dox treatment, and after treatment with 1  $\mu\text{g/ml}$  Dox and luciferase, 378,689 fluorescence pulses/min<sup>-1</sup>, a 76-fold enhancement, revealing a highly inducible expression with little background. Therefore, clone 22 was considered a Dox-On VSMC line, which was amplified and frozen.

**Luciferase expression induced by various concentrations of Dox in Dox-On VSMC line.** As expected, a very low background expression of luciferase was detected in the control group. Very low luciferase activity was detected in the Dox treated group after transfection, suggesting very small 'leaks' of luciferase expression before Dox treatment ( $P < 0.01$ , versus the control group). Within a certain concentration range ( $1 \times 10^{-3}$ - $10^3$  ng/ml), Dox dose-dependently upregulated luciferase expression in Dox-On VSMC line ( $P < 0.01$ , versus the group transfected, but not treated with Dox). In Dox-On VSMC line, luciferase expression increased at  $1 \times 10^{-3}$  ng/ml Dox, compared to the group not treated with Dox (induction multiple, 1.23,  $P < 0.01$ ),  $1 \times 10^2$  ng/ml Dox significantly increased the amplitude of induced luciferase expression increase, (induction multiple, 74.30,  $P < 0.01$ ), and  $1 \times 10^3$   $\mu\text{g/l}$  Dox induced a peak of luciferase expression increase (induction multiple, 76.58,  $P < 0.01$  versus the group not treated with Dox) (Fig. 2). These results indicate that Dox dose-dependently increases the luciferase expression in Dox-on VSMC line.

**Establishment of VSMC line with Dox-induced double stable expression of AT2R gene**

**Determination of AT2R mRNA expression in G418-resistant VSMC clones (RT-PCR).** After Dox-on VSMCs were co-transfected with response plasmid pUHD10-3/AT2R and pSV2neo, 30 separate, large, healthy clones expressing the AT2R gene were successfully selected. The results showed that No. 1 VSMC clone had the lowest AT2R mRNA expression without Dox treatment, and that after 1  $\mu\text{g/ml}$  Dox treatment, induced AT2R mRNA expression increased markedly. AT2R mRNA expression was induced 162-fold



Table I. Induced luciferase expression in hygromycin-resistant VSMC clones (min<sup>-1</sup>, x ±s, n=5).

Clone number	Control	16	17	18	19	20	21	22	23
Dox(-)	1991±257.2	33072±2176.1 <sup>d</sup>	29952±1568.4 <sup>d</sup>	11408±823.4 <sup>d</sup>	32276±1479.2 <sup>d</sup>	9152±848.8 <sup>d</sup>	29700±2071.3 <sup>d</sup>	4973±139.5 <sup>d</sup>	30812±2286.3 <sup>d</sup>
Dox(1 mg/l)	2182±266.4 <sup>a</sup>	156234±3586.4 <sup>b</sup>	338376±3013.7 <sup>b</sup>	238663±2504.0 <sup>b</sup>	434182±3456.7 <sup>b</sup>	128259±1278.6 <sup>b</sup>	204501±2373.7 <sup>b</sup>	378689±7528.7 <sup>b</sup>	275879±3194.0 <sup>c</sup>
Induction multiple		4.72	11.30	20.92	13.45	14.01	6.89	76.15	8.95

The control group-untransfected VSMCs, 16-23, hygromycin-resistant VSMC clones transfected with pUHD 17-lhyg plasmid; Dox (-), not treated with DOX; Dox, treated 72 h with DOX (1 μg/ml).<sup>a</sup>P>0.05, (-Dox) vs (+Dox) in a group; <sup>b</sup>P<0.05, (-Dox) vs (+Dox) in a group; <sup>c</sup>P<0.01, (-Dox) vs (+Dox) in a group; <sup>d</sup>P<0.05, vs control. The results indicate that 4, 973 electric pulses/min<sup>-1</sup> were detected in No. 22 VSMC clone not treated with Dox, and after treatment with 1 μg/ml Dox and luciferase, 378, 689 fluorescence pulses/min<sup>-1</sup> were detected in VSMCs, i.e., the fluorescence intensity differed 76 times.

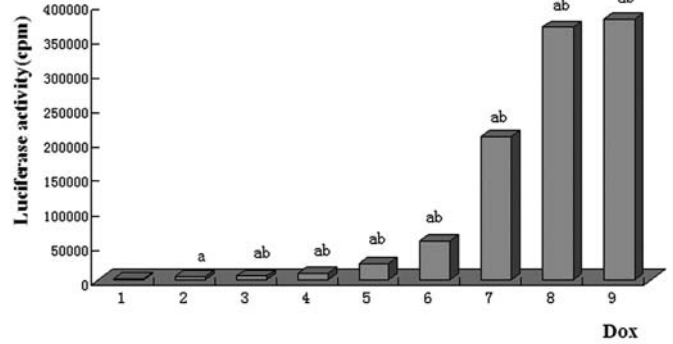


Figure 2. Luciferase expression induced by various concentrations of Dox in Dox-on VSMC line (min<sup>-1</sup>, x ±s, n=5). 1, Untransfected VSMCs control group; 2, Dox-on VSMCs line not treated with DOX; 3-9, Dox-on VSMC line treated 72 h with 1x10<sup>-3</sup>-10<sup>3</sup> ng/ml Dox. <sup>a</sup>P<0.01 versus control group; <sup>b</sup>P<0.01 versus (-Dox) group. The results indicate very little luciferase expression in the control group. 1x10<sup>-3</sup>-10<sup>3</sup> ng/ml Dox treatment dose-dependently induced luciferase expression increase in Dox-on VSMC line (P<0.01, versus the transfected group not treated with Dox). In Dox-on VSMC line, luciferase expression increased at 1x10<sup>-3</sup> ng/ml Dox, compared to the group not treated with Dox (induction multiple, 1.23, P<0.01), 1x10<sup>-2</sup> ng/ml Dox significantly increased the amplitude of induced luciferase expression increase (induction multiple, 74.30, P<0.01), and 1x10<sup>3</sup> μg/l Dox induced a peak of luciferase expression increase (induction multiple, 76.58, P<0.01 versus the group not treated with Dox).

after Dox treatment (data not shown). The data also indicated a varied degree of leaky expression of AT2R in these clones. Through clone screening, the ‘leaks’ were well controlled, and did not affect the ability of Dox to regulate AT2R expression. Hence, No. 1 G418-resistant VSMC clone was used as the VSMC line with Dox inducible double stable expression of the AT2R gene. This clone was amplified and the stocks frozen.

Next, changes in AT2R mRNA expression induced by various concentrations of Dox were determined in the VSMC line. The results demonstrated that Dox within 1x10<sup>-3</sup>-10<sup>4</sup> ng/ml dose-dependently increased AT2R mRNA. Induced AT2R mRNA expression reached a peak at 1x10<sup>2</sup> ng/ml Dox, and decreased at higher Dox concentrations (P<0.01) (Fig. 3, Table II).

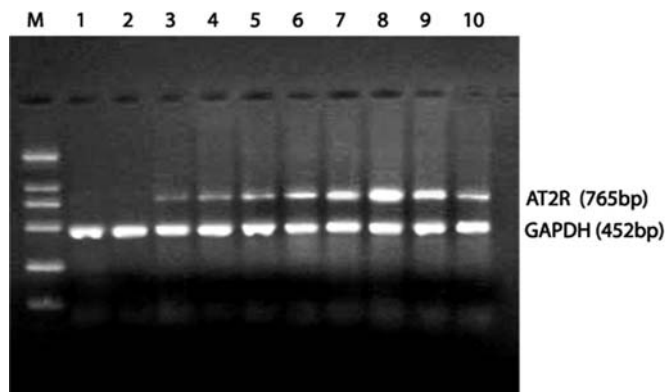
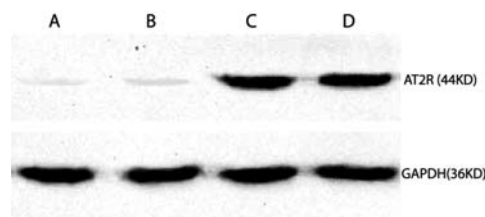
*AT2R protein expression in Dox induced double stable VSMC lines.* Immunoblotting showed no obvious AT2R protein expression in the control group (2.110±1.451), or in the transfected group untreated with Dox. However, induced AT2R protein expression was observed 48 h after treatment with 1 μg/ml Dox in double stable VSMCs (111.876±9.912, P<0.01 versus the control group), and the expression was further enhanced at 72 h (156.477±20.641, P<0.01 versus the group after 48 h) (Fig. 4).

The sustainable induced AT2R expression was also determined in the VSMC lines. The data showed that 1, 2, 4, 6, 8, 10, 12 W after transfection, 72 h treatment with 1 μg/ml Dox significantly induced AT2R protein expression in the VSMCs (P<0.05 versus the control group), and there was no time effect on the expression level, suggesting that the expression of the target gene was tightly regulated by Dox in this cell line, and that this expression inducibility was stable within at least 12 W (data not shown).

Table II. AT2R mRNA expression induced by various concentrations of Dox in double stable VSMCs lines (Intensity value,  $\bar{x} \pm s$ , n=5).

	transfected group transfection+Dox (ng/ml)									
	control group	DOX (-)	1x10 <sup>-3</sup>	1x10 <sup>-2</sup>	0.1	1	10	1x10 <sup>2</sup>	1x10 <sup>3</sup>	1x10 <sup>4</sup>
INT	0.326±0.075	1.515±0.699 <sup>a</sup>	6.192±0.814 <sup>a,b,c</sup>	24.073±5.739 <sup>a,b,c</sup>	51.583±7.479 <sup>a,b,c</sup>	90.315±25.20 <sup>a,b,c</sup>	150.91±20.82 <sup>a,b,c</sup>	238.36±24.59 <sup>a,b,c</sup>	203.52±20.70 <sup>a,b,c</sup>	141.44±17.91 <sup>a,b,c</sup>
Induction multiple		4.09	15.88	34.04	59.61	99.61	157.33	134.33	93.35	

The control group, untransfected VSMCs. AT2R mRNA expression was observed 72 h after treatment with 0, 1x10<sup>-3</sup>, 1x10<sup>-2</sup>, 0.1, 1, 10, 1x10<sup>2</sup>, 1x10<sup>3</sup>, 1x10<sup>4</sup> ng/ml Dox. <sup>a</sup>P<0.01, versus the control group; <sup>b</sup>P<0.01 versus the transfected group Dox(-); <sup>c</sup>P<0.01 between groups (n=5). The results indicated that Dox at 1x10<sup>-3</sup>-10<sup>4</sup> ng/ml dose-dependently increased induced AT2R mRNA expression in the VSMC lines and that induced AT2R mRNA expression and reached a peak at 1x10<sup>-2</sup> ng/ml Dox, and decreased at higher concentrations.

Figure 3. AT2R mRNA expression induced by various concentrations of Dox in the double stable VSMC lines. M, marker; 1, control group; 2, transfected group not treated with Dox; 3-10, 10<sup>-3</sup>-10<sup>4</sup> ng/ml Dox.Figure 4. Immunoblotting of induced AT2R protein expression (GAPDH as protein loading control). A, control group; B, transfected group not treated with Dox; C, transfected group after 48 h treatment with 1  $\mu$ g/ml Dox; D, transfected group after 72 h treatment with 1  $\mu$ g/ml Dox. The results demonstrated no obvious AT2R protein expression in the control group (2.110±1.451), or in the transfected group not treated with Dox (1.894±1.482, P>0.05 versus the control group). However, induced AT2R protein expression was observed after transfection and 48 h treatment with 1  $\mu$ g/ml Dox in double stable VSMCs (111.876±9.912, P<0.01 versus the control group), and the expression was further enhanced after 72 h Dox treatment (156.477±20.641, P<0.01 versus the group after 48 h).

*Effect of AT2R conditional expression on MMP-2 mRNA levels and protein expression in double stable VSMCs.* A very weak MMP-2 baseline expression in the control group (79.263±6.228 INT) was observed. After Ang II stimulation, MMP-2 expression was upregulated significantly with Dox treatment (185.359±8.486 INT) or with out (182.466±9.325 INT). MMP-2 baseline expression did not differ between the double stable cell line and the control group (77.059±6.599 INT, P>0.05). Moreover, after Ang II stimulation, MMP-2 expression increased consistently with the transfected VSMCs (185.660±5.297 INT). Ang II stimulated increase in MMP-2 expression was suppressed (137.594±9.375 INT) in double stable VSMCs which expressed AT2R after Dox treatment. The treatment with AT1R antagonist CV-11974 further reduced MMP-2 expression (92.7983±7.099 INT), which was still higher than that in the control group (P<0.01). The results suggested that Ang II promoted MMP-2 expression increase via AT1R, and that AT1R antagonists exerted a synergistic effect on the action of AT2R. Treatment of double stable cells with AT2R antagonist (PD123319) resulted in MMP-2 protein levels (184.618±7.123 INT), similar to those seen in double stable VSMCs not treated with Dox, but stimulated with Ang II (185.660±5.297 INT, P>0.05). MMP-2

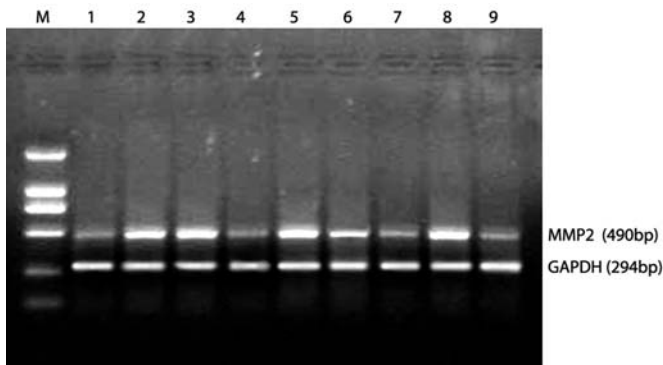


Figure 5. Effect of AT2R conditional expression on MMP2 mRNA expression in double stable VSMCs. M, marker; 1, control group; 2, untransfected VSMCs + Ang II  $10^{-7}$  Mol/l; 3, untransfected VSMCs + Ang II  $10^{-7}$  Mol/l + Dox 1 g/l; 4, transfected group; 5, transfected group + Ang II  $10^{-7}$  Mol/l; 6, transfected group + Ang II  $10^{-7}$  Mol/l + Dox 1 g/l; 7: transfected group + Ang II  $10^{-7}$  Mol/l + Dox 1 g/l + CV-11974  $10^{-6}$  Mol/l; 8: transfected group + Ang II  $10^{-7}$  Mol/l + Dox 1 g/l + PD123319  $10^{-6}$  Mol/l; 9, transfected group + Ang II  $10^{-7}$  Mol/l + Dox 1 g/l + CV-11974  $10^{-6}$  Mol/l + PD123319  $10^{-6}$  Mol/l.

was expressed at high levels in these cells, suggesting that AT2R mediated the negative regulation of the extracellular protease. Treatment of double stable VSMCs with both AT1R and AT2R antagonists abolished the AT1R and AT2R mediated Ang II effect on MMP-2 expression ( $78.7461 \pm 6.143$  INT). The results demonstrate that AT2R expression suppresses the Ang II stimulated enhancement in MMP-2 expression, and Ang II exerted its effect via AT1R. AT2R antagonized AT1R in mediating the biological effect of Ang II. Given the expression of both AT1R and AT2R on VSMCs, AT1R antagonists blocked the biological effect of AT1R, possibly through more Ang II binding to AT2R (Fig. 5). The same tendency of MMP-2 protein expression in double stable VSMCs was observed using immunoblotting (Fig. 6).

## Discussion

Neointima formation after vascular injury is a process of matrix reconstruction, which involves multiple growth factors and cytokines (24,25). Local renin-angiotensin system (RAS) participates in the process of restenosis, and plays a crucial role in its promotion (26,27). It was shown that after percutaneous coronary intervention (PCI), angiotensin II (Ang II) gene expression and protein synthesis increase in the injured blood vessel walls, and that the resulting Ang II cooperates with multiple cytokines and growth factors to promote cell proliferation, migration, and hypertrophy of VSMCs and suppress VSMC apoptosis. Ang II overexpression also promotes secretion of extracellular matrix and thus participates in vascular remodeling (28). Evidence suggests that most of the biological effects of Ang II are primarily mediated by AT1R. After transfection and expression of AT2R in VSMCs resulted in suppression of cell migration and proliferation were with an increased apoptotic rate, consistent with our previous studies (29,30).

Tetracycline regulatable system (TRS) (31) involves two chimeric structures, involving placement of the target gene under the control of Tet promoter. For the Tet-On regulatable

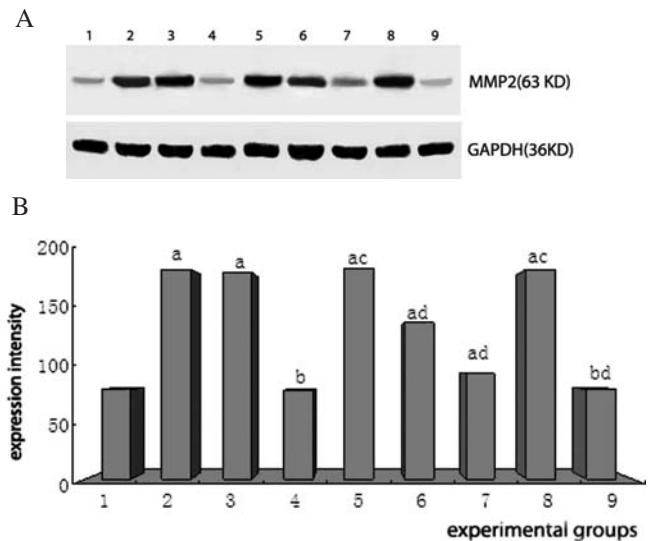


Figure 6. Effect of AT2R conditional expression on MMP2 protein expression in double stable VSMCs. 1, control group; 2, untransfected VSMCs + Ang II  $10^{-7}$  Mol/l; 3, untransfected VSMCs + Ang II  $10^{-7}$  Mol/l + Dox 1 g/l; 4, transfected group; 5, transfected group + Ang II  $10^{-7}$  Mol/l; 6, transfected group + Ang II  $10^{-7}$  Mol/l + Dox 1 g/l; 7, transfected group + Ang II  $10^{-7}$  Mol/l + Dox 1 g/l + CV-11974  $10^{-6}$  Mol/l; 8, transfected group + Ang II  $10^{-7}$  Mol/l + Dox 1 g/l + PD123319  $10^{-6}$  Mol/l; 9, transfected group + Ang II  $10^{-7}$  Mol/l + Dox 1 g/l + CV-11974  $10^{-6}$  Mol/l + PD123319  $10^{-6}$  Mol/l. <sup>a</sup> $P < 0.01$  versus the control group; <sup>b</sup> $P > 0.05$  versus the control group; <sup>c</sup> $P > 0.05$  versus the untransfected Ang II group; <sup>d</sup> $P < 0.01$  versus the transfected Ang II group (n=10).

system, in the presence of tetracycline, tetracycline repressor protein (TetR) binds to the operator sequence (TetO), resulting in activation of transcription of the quiescent target gene. This feature of the conditional gene expression system is suitable for clinical applications, particularly with restenosis which occurs in 30-50% patients after vascular injury.

Matrix metalloproteinases (MMPs) contribute to the matrix remodeling and play an essential role in SMC migration. Collagen is a major component of ECM. In vessel walls, intracellular collagen is dominated by types I/III, and type IV collagen contributes to the formation of basal lamina (32,33). MMP-2 is a major type IV collagenase (gelatinase), which mainly degrades types IV, V, and VIII collagen. In restenosis lesions, a number of smooth muscle cells lose their contractile function, and secrete large amounts of MMPs by autocrine or paracrine mechanisms to degrade the extracellular matrix. MMP-2 and -9 secreted by smooth muscle cells effectively degrade basal lamina and facilitate cell migration (34,35). A positive staining of MMPs in neointima one week after balloon injury, particularly at the luminal surface of neointima and its persistence throughout VSMC migration and proliferation was demonstrated (36). MMP inhibitors significantly reduce VSMC migration into intima, suggesting a functional role for these proteases in the vascular pathology. Uzui *et al* (37) showed that MMP-2 is required for VSMCs to penetrate the basal lamina barrier.

Accordingly, in the present study, the Tet-On regulatable system was used to control AT2R expression in VSMCs using Dox (a tetracycline analog). Regulated AT2R expression exerts the favorable antagonistic effect on AT1R-mediated





events and it reduces the potential adverse effects of AT2R associated with conventional gene delivery systems. In the present study, therefore, we successfully established a double stable VSMC line by *in vitro* transfection of VSMCs with AT2R gene construct followed by clone screening, and alterations in the MMP-2 gene expression, with and without induction of the ectopic gene.

Our studies demonstrated that MMP-2 expression at mRNA and protein levels was increased significantly after Ang II stimulation. After the expression of the AT2R protein (by Dox treatment), the Ang II promoted MMP-2 levels were significantly suppressed, suggesting that AT2R reduces extracellular matrix degradation, and consequently, curtail the VSMC migration. We also showed that the AT1R antagonist CV-11974 further downregulated MMP-2 expression in AT2R induced cells, and that the AT2R antagonist PD123319 abolished the suppressing effect of AT2R on MMP-2 expression. These findings suggest that Ang II enhances MMP-2 expression via AT1R, and AT2R antagonizes the AT1R actions in this biological effect. Therefore, it may be possible to control the fate and dynamics of extracellular matrix in damaged vasculature through gene therapy approaches. Our findings provide a sound experimental basis for introducing VSMCs that stably express AT2R into injured blood vessels, and investigate the role of these cells in the prevention and treatment of restenosis. We believe that the Dox-On regulatable mammalian expression system used artificially controls AT2R gene expression and modulates MMP activity or alters the MMP:TIMP (tissue inhibitors of metalloproteinases) ratios, which promise to be useful for clinical management of vascular diseases. The positive effect of AT2R gene expression can be exploited for therapeutic purposes, and such an approach merits further investigation.

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