

Deterioration of atherosclerosis in mice lacking angiotensin II type 1A receptor in bone marrow-derived cells

Hideki Kato^{1,2}, Junji Ishida¹, Katsumasa Nagano¹, Kaori Honjo¹, Takeshi Sugaya¹, Norifumi Takeda³, Fumihiro Sugiyama⁴, Ken-ichi Yagami⁴, Toshiro Fujita², Masaomi Nangaku² and Akiyoshi Fukamizu¹

The renin–angiotensin system (RAS) modulates end-organ damages, resulting in cardiovascular and kidney diseases. Experiments both *in vitro* and *in vivo* demonstrate that the angiotensin II (Ang II) type 1 (AT1) receptor pathway also exerts pro-inflammatory and pro-atherogenic effects on bone marrow-derived cells (BMDCs). Here, we investigated how AT1 receptor expression by BMDCs contributes to atherosclerosis and kidney injury *in vivo* by transplanting BM into RAS-activated transgenic mice. There was no difference in the extent of kidney damage between mice receiving BM transplants from mutant mice lacking the angiotensin II type 1a receptor (AT1a) gene and mice receiving transplants from wild-type (WT) mice. However, mice receiving transplants from AT1a ‘knockout’ (KO) mice displayed accelerated lethality and atherosclerotic lesions. These results indicated that the effects of AT1a receptor on BMDCs are organ dependent. Microarray expression profiling of macrophages from AT1a-KO mice revealed significant changes in the mRNA levels for a number of genes implicated in atherosclerosis. In accordance with the *in vivo* atherosclerosis results, AT1a-KO macrophages exhibited greater uptake of modified lipoproteins relative to macrophages from WT mice. We propose that the expression of AT1a receptor by BMDCs limits atherosclerosis *in vivo*.

Laboratory Investigation (2008) 88, 731–739; doi:10.1038/labinvest.2008.42; published online 19 May 2008

KEYWORDS: angiotensin II type 1 receptor; atherosclerosis; bone marrow transplantation; kidney injury; renin–angiotensin system

Angiotensin II (Ang II) is a vasopressor octapeptide in the renin–angiotensin system (RAS), which is a major determinant of blood pressure (BP). The diverse actions of Ang II are mediated by several types of receptors expressed in a variety of target tissues. Two Ang II receptors have been identified in humans, AT1 (type I) and AT2 (type II). In comparison, rodents express two AT1 receptor isoforms, Ang II type 1a (AT1a) and Ang II type 1b (AT1b). In rodents, BP and the pathophysiological effects of RAS are primarily mediated by the AT1a receptor.¹

The role of RAS in the pathogenesis of cardiovascular end-organ damages has been well characterized. Interestingly, clinical studies and animal experiments have demonstrated that inhibition of RAS with pharmacological agents such as angiotensin-converting enzyme inhibitors and AT1 receptor blockers (ARBs), as well as genetic disruption of RAS

signaling components, diminishes end-organ damage, particularly atherosclerosis^{2–4} and kidney injury.^{5,6} The mechanisms underlying these effects have been studied predominately in cells derived from a variety of organs expressing AT1 receptors, such as blood vessel, heart and kidney cells.^{7,8}

AT1 receptors are also expressed by bone marrow-derived cells (BMDCs), such as macrophages and T cells.⁹ Ang II has been proposed to have pro-atherogenic and pro-inflammatory effects on BMDCs that might influence end-organ injury. For example, signaling by AT1 receptors in macrophages and monocytes has been reported to activate pro-inflammatory transcription factors,¹⁰ increase macrophage Ox-LDL (low density lipoprotein) uptake,¹¹ increase macrophage-mediated modification of LDL,^{12,13} generate oxidative stress,¹⁴ produce inflammatory cytokines,¹⁵ contribute to the differentiation of macrophages^{16,17} and

¹Center for Tsukuba Advanced Research Alliance (TARA), University of Tsukuba, Ibaraki, Japan; ²Division of Nephrology and Endocrinology, Department of Internal Medicine, University of Tokyo School of Medicine, Tokyo, Japan; ³Division of Cardiology, Department of Internal Medicine, University of Tokyo School of Medicine, Tokyo, Japan and ⁴Laboratory Animal Resource Center, University of Tsukuba, Ibaraki, Japan

Correspondence: Professor A Fukamizu, PhD, Center for Tsukuba Advanced Research Alliance, Graduate School of Life and Environmental Sciences, Centre for Tsukuba Advanced Research Alliance, University of Tsukuba, Ten-noudai 1-1-1, Tsukuba, Ibaraki 305-8577, Japan.

E-mail: akif@tara.tsukuba.ac.jp

Received 26 September 2007; revised 03 March 2008; accepted 07 March 2008

modulate adhesion molecule expression.¹⁸ However, the lack of an appropriate animal model and the consequential paucity of experimental *in vivo* evidence have hampered efforts to determine the priority of these mechanisms in contributing to end-organ injuries.

Previously, we established a mouse model of persistent hypertension (the Tsukuba hypertensive mouse; THM), by crossing human renin (hREN) and human angiotensinogen (hAGT) transgenic mice. These mice produce higher levels of Ang II¹⁹ and develop atherosclerotic lesions when fed atherogenic diets²⁰ as well as pathognomonic nephrosclerosis.²¹ Therefore, this double transgenic mouse is an ideal model for evaluating RAS-induced end-organ injuries. Here, we investigated the contribution of BMDCs expressing AT1 receptors to atherosclerosis and kidney disease by examining the effects of BMTs in THM and also evaluated its cellular mechanisms using primary cell culture experiments.

MATERIALS AND METHODS

Animal Model

The methods used to generate mice containing both the hREN and hAGT transgenes (THM) were generated on the C57BL/6J background as described previously.¹⁹ Age-matched wild-type (WT) C57BL/6J control mice were obtained from CLEA Japan Company Ltd. (Tokyo, Japan). Angiotensin II type 1a receptor-deficient (AT1-knockout (KO)) mice were described previously²² and backcrossed to the C57BL/6J background. Mice were given free access to food and water. Animal experiments were carried out in a humane manner after receiving approval from the Institutional Animal Experiment Committee of the University of Tsukuba, and in accordance with the Regulation for Animal Experiments in our university and Fundamental Guideline for Proper Conduct of Animal Experiment and Related Activities in Academic Research Institutions under the jurisdiction of the Ministry of Education, Culture, Sports, Science and Technology.

Isolation of RNA and RT-PCR

Total RNA was isolated from kidney, testis, brain, spleen and BM of WT female mice aged 12 weeks using Isogen (Nippon Gene, Tokyo, Japan). Isolated total RNA samples were treated with deoxyribonuclease I (Promega, Madison, WI, USA) according to the manufacturer's instructions to avoid possible contamination with genomic DNA. The synthesis of cDNA was performed using ReverTra Ace M-MuLV Reverse Transcriptase (Toyobo, Osaka, Japan) according to the manufacturer's instructions using 1 µg of total RNA. PCR was performed for 35 cycles with the following amplification conditions: 94°C for 30 s, 55°C for 45 s and 72°C for 1 min. The following PCR primers were employed to amplify mouse Ang II receptor subtypes and control GAPDH:

AT1a receptor: 5'-AGTGCATTTTGAACAGTGTCTG-3' and 5'-ATCCTAAAGATGTCATCATTTTC-3'²³

AT1b receptor: 5'-TCACCTGCATCATCATCTGG-3' and 5'-AGCTGGTGAGAATAATAACG-3'

AT2 receptor: 5'-CTGACTCTGAACATGTTTGCA-3' and 5'-GGTGTCCATTTCTCTAAGAG-3'

GAPDH: 5'-ACCACAGTCCATGCCATCACT-3' and 5'-TCCACCACCCTGTTGCTGTAG-3'.

Diets

The standard chow (MF; Oriental Yeast Company Ltd., Tokyo, Japan) contained 5.6% fat and 0.09% cholesterol. The atherogenic diet, prepared according to a method described previously,²⁰ contained 15% fat and 1% cholesterol. Mice were initially fed with the standard chow. After the BMT at 8–9 weeks of age, mice were then switched to the atherogenic diet at 14 weeks of age until the end of the study, as noted.

BMT

Recipient female THM and WT mice aged 8–9 weeks were lethally irradiated with 940 cGy and THM were divided into two groups. Recipient mice received 5×10^6 BM cells by tail vein injection 6 h after irradiation. BM cells were harvested from male donor WT or AT1-KO mice.²⁴ One THM group received BM cells from male donor WT mice (designated as THM ← WT), while the second THM group received cells from male donor AT1-KO mice (designated as THM ← AT1-KO). As a control, irradiated female WT mice also received BM cells from male donor WT mice (designated as WT ← WT). At 14 weeks of age, animals were switched to an atherogenic diet.

BMT for three groups, THM ← WT, THM ← AT1-KO and WT ← WT, was performed on the same day in one set of experiments. At 14 weeks of age, genotype exchange was confirmed as described previously.²⁴

Measurement of BP

Systolic BP was measured using a non-invasive computerized tail cuff BP system on awake animals (BP-98A; Softron, Japan) as described previously.¹⁹

Serum Blood Urea Nitrogen Analysis and Urinary Albumin Analysis

Prior to killing, mice were deprived of food for 8 h. Mice were anesthetized with diethylether and blood samples were collected by cardiac puncture. Serum blood urea nitrogen (BUN) was determined with an automated analyzer, DRICHEM 3500 (Fuji Film Inc., Tokyo, Japan). Urinary albumin concentrations were measured using a Mouse Albumin ELISA Kit (AKRAL-121; Shibayagi, Gunma, Japan). The detection range was from 50 to 1000 ng/ml. Urinary albumin excretion was expressed as the total amount excreted in 24 h.

Renal Histology

Kidney tissues were fixed in 10% buffered formalin and embedded in paraffin. Sections were stained with Masson's trichrome and periodic acid-Schiff. The extent of renal injury was estimated by morphometric assessment of the tubulointerstitial injury and glomerular damage. Random cortical

fields were viewed at a magnification of $\times 200$. The percent interstitial fibrosis volume was calculated from the area occupied by Masson's trichrome-stained interstitium divided by the total area with the computer-assisted MacSCOPE image analysis system (Mitani Corp., Tokyo, Japan). The mean value of five cortical fields was determined for each section. To avoid selection bias, the areas to be viewed for morphometric analysis were anatomically identical for each section. Glomerulosclerosis was quantified using a score of 0 (normal glomerulus) to 4 (global sclerosis or global extracapillary fibrosis or proliferation, or complete collapse of the glomerular tuft) as described previously.²⁵ The number of glomeruli per renal cross section was 100 and the sum of 100 glomerular indices was used as glomerular sclerosis score.

Quantification of Aortic Atherosclerotic Lesions

Mice surviving at 11 weeks after BMT were analyzed. The mice were killed and perfused transcardially with 30 ml of PBS. Aortic tissue was removed from the ascending aorta to the ileal bifurcation and placed in 10% buffered formalin. After tissue fixation, the entire aorta was cleaned of peripheral fat, opened longitudinally and stained with Oil red O. Photographs of the stained aortas were digitized with a dissecting microscope equipped with a high-resolution camera (DS6041; Canon, Tokyo, Japan). Digitized images were then evaluated with the computer-assisted MacSCOPE image analysis system. Lesion area measurements were performed and the proportion of aortic intimal surface area occupied by red stain was calculated as described previously.⁴

Microarray Experiment of Macrophages

Mouse peritoneal macrophages were collected as described previously.²⁶ In brief, WT and AT1a-KO mice were injected i.p. with 2.5 ml of 4% thioglycollate and peritoneal exudates were collected by lavage after 84 h. The collected peritoneal cells were suspended in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 0.1 mg/ml streptomycin and 100 U/ml penicillin G. For the microarray experiment, 2.0×10^7 cells from WT and AT1-KO mice were seeded onto 10-cm dishes. After 90 min incubation, non-adherent cells were removed by washing. After 48 h, the cells were incubated with Ang II (Peptide Institute, Osaka, Japan) at a concentration of 100 nM. After 24 h, acetylated LDL was added at a final concentration of 25 $\mu\text{g}/\text{ml}$. Cells were then maintained for 36 h. Total RNA was prepared from cells using an RNeasy Mini Kit (Qiagen, Valencia, CA, USA) from WT or AT1-KO mice macrophages. Total RNA quantity and quality were assessed with an Agilent 2100 Bioanalyzer. Universal mouse reference RNA (Stratagene, La Jolla, CA, USA) was used as a control for all hybridizations and was labeled with Cy3-CTP dye using a Low RNA Input Fluorescent Linear Amplification Kit (Agilent Technologies, Palo Alto, CA, USA). Targets from WT or AT1-KO macrophages were prepared from 500-ng aliquots of total RNA samples and labeled with Cy5-CTP dye. Targets were purified using RNeasy mini-

spin columns (Qiagen) in accordance with Agilent's clean-up protocol. A total of six microarray slides were hybridized for two groups using independent macrophage samples (WT; $n = 3$, AT1-KO; $n = 3$). All hybridizations compared a Cy5-CTP-labeled experimental target to the single Cy3-CTP-labeled reference target. Samples were hybridized on Whole Mouse Genome Oligo Microarrays (G4122A) (Agilent Technologies) and washed according to the manufacturer's protocol. Slides were scanned on an Agilent Technologies Microarray Scanner using standard settings.

Data Analysis

Intensity of 41 232 gene features per array was extracted from scanned microarray images, and the data were analyzed using GeneSpring version GX software (Agilent Technologies). The ratios of intensity for Cy3- to Cy5-labeled probes were determined for each DNA spot. The Lowess normalization method was applied to the log of background-corrected raw signal intensities to remove signal intensity-dependent dye effects on each slide. Spot intensities were quantified and corrected for background signals. Of the 41 232 genes, unqualified genes and genes whose signal intensities were below background levels were eliminated, leaving 17 914 genes for evaluation. Genes showing both a Welch's *t*-test *P*-value of < 0.05 and an expression ratio of > 1.5 -fold in both directions were considered differentially expressed for pairwise comparisons. Heatmaps were generated using Genepilot software (TG Services, El Sobrante, CA, USA).²⁷ Average difference values were normalized to the median over the array. Gene lists represent a select group of genes (expressed sequence tags excluded) with higher or lower expression in AT1-KO macrophages whose enriched expression in macrophages were confirmed with the publicly available Digital Differential Display (http://www.ncbi.nlm.nih.gov/UniGene/info_ddd.shtml) databases (ID12268 and ID18091). Several important genes related to atherosclerosis were added to the heatmap data.

Gene Ontology Analysis

Each list of differentially expressed genes was analyzed in the context of gene ontology (GO) describing the Biological Process to identify groups of genes with similar functions or processes. GO annotations for genes represented on the Agilent oligonucleotide microarray were downloaded from GO (www.geneontology.org/index.shtml). We applied Fisher's exact test to evaluate the statistical significance of the association between the gene lists of the GO category and the up- or downregulated genes from microarray data, as described previously.²⁸ GO categories that contained more than five significantly changed genes were selected and redundant categories were eliminated.

Macrophage Cell Culture and Acetylated LDL Uptake Assays

For the LDL uptake experiment, 3.0×10^5 macrophage cells from WT and AT1-KO mice were seeded onto 96-well plates.

After a 90-min incubation, non-adherent cells were removed by washing. After a 48-h incubation, the medium was changed to RPMI-1640 supplemented with 5% bovine serum albumin (Sigma-Aldrich, St Louis, MO, USA) and incubated for 48 h. The cells were then incubated with 2.5 µg/ml of acetylated LDL-labeled with 1,1'-dioctadecyl-3,3,3',3'-tetramethyl-indocarbocyanine perchlorate (DiI-Ac-LDL) (Biomedical Technologies Inc., MA, USA) for 8 h. The cells were washed with PBS, and counterstained with 10 µg/ml Hoechst 3569 nuclear dye (Invitrogen, Madison, WI, USA) for 15 min. Cells were then fixed with 4% paraformaldehyde and washed in PBS. Images were immediately captured at $\times 20$ magnification with an IN Cell Analyzer 1000 (GE Healthcare Biosciences). DiI was detected with a S555/25 nm excitation filter and a S620/60 nm emission filter. Similarly, Hoechst staining was determined with D360/40 nm and HQ460/40 nm excitation/emission filters, respectively. The area of fluorescence was measured and normalized to the total number of nuclei. The images were analyzed with the Developer Toolbox 1.5 software (GE Healthcare Biosciences). Experiments were performed in sextuplicate. Ten random images were acquired per well and two independent sets of experiments were performed.

Statistical Analysis

Data are presented as means \pm s.e.m. Statistical significance between groups was evaluated with the unpaired Student's *t* test. The log-rank test was used for survival studies. A *P*-value of <0.05 was considered statistically significant.

RESULTS

Ang II Receptor Expression in Spleen and BM Cells

First, we examined which Ang II receptor subtypes are expressed by spleen and BM cells of WT mice. AT1a receptor expression was detected in both cells by RT-PCR.^{9,29} In contrast, AT1b receptor expression was slightly detectable in spleen cells, but not in BM cells, and AT2 receptor expression was not detected in both cell types (Figure 1).

BMT and Survival Rate on an Atherogenic Diet

To evaluate how atherosclerosis and kidney injury are affected by AT1 activity in BMDCs, we performed BMT experiments.²⁴ In the first set of BMT experiments, animals were challenged with an atherogenic diet for 14 weeks. Survival rates of both THM groups were significantly lower than that of the WT \leftarrow WT group (THM \leftarrow WT ($n=31$), 59.4%; THM \leftarrow AT1-KO ($n=21$), 33.3% and WT \leftarrow WT ($n=13$), 92.3% at age 28 weeks). Unexpectedly, the THM \leftarrow AT1-KO group exhibited significantly accelerated lethality as compared to the THM \leftarrow WT group (THM \leftarrow WT vs THM \leftarrow AT1-KO, $P=0.009$; THM \leftarrow AT1-KO vs WT \leftarrow WT, $P=0.002$ and THM \leftarrow WT vs WT \leftarrow WT, $P=0.075$) (Figure 2a). Given this high lethality, we shortened the duration of the atherogenic diet period in a second set of BMT experiments to 11 weeks. However, the survival rates were essentially

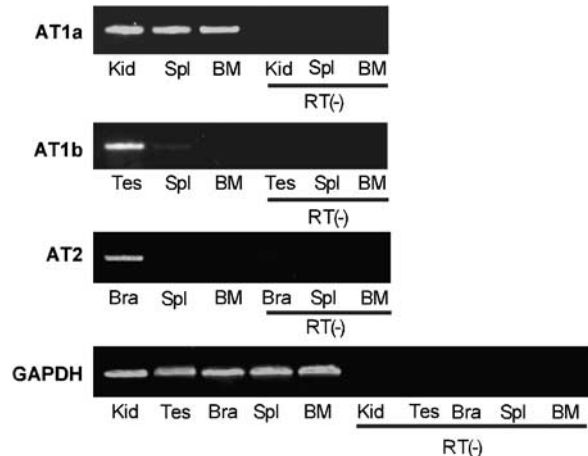


Figure 1 RT-PCR analysis of AT1a receptor, AT1b receptor and AT2 receptor mRNA in spleen and bone marrow (BM) cells of WT mice. Total RNA was isolated from spleen (Spl), BM, kidney (Kid), testis (Tes) and brain (Bra) of WT C57BL/6J mice. cDNA was synthesized and amplified for 35 cycles with each Ang II-specific primer. Expression of AT1a receptor, AT1b receptor, AT2 receptor and GAPDH are shown. RT(-) indicates that the PCR products from the RNA templates were treated with deoxyribonuclease I, but not with reverse transcriptase. A positive control reaction of an appropriate organ sample for each Ang II receptor subtype is provided at the left of each panel.

the same as those in the first experiment (survival rate of THM \leftarrow WT ($n=14$), 85.7%; THM \leftarrow AT1-KO ($n=15$), 46.7% and WT \leftarrow WT ($n=13$), 92.3% at age 25 weeks). Surviving mice in the second experiment were evaluated for atherosclerosis and kidney injury (THM \leftarrow WT, $n=12$; THM \leftarrow AT1-KO, $n=7$ and WT \leftarrow WT, $n=12$).

AT1 Receptor in BM Cells does not Affect Blood Pressure

Systolic BP was measured in each group at three time points: at 6 weeks (before BMT), 13 weeks (after BMT but before initiation of the atherogenic diet) and 20 weeks (after BMT and during the atherogenic diet) of age. BP was significantly higher in the two THM groups than in the WT \leftarrow WT group but did not significantly differ between the two THM groups. BP decreased after BMT by approximately 10 mm Hg in the two THM groups, but showed no change in the WT \leftarrow WT group (Figure 2b).

Severity of Kidney Injury

To explore the relationship between cause of death and end-organ injury, we evaluated mice from each group for kidney injury as this is one of the most clinically significant consequences of RAS activation and subsequent hypertension. In both the THM \leftarrow WT and THM \leftarrow AT1-KO groups, serum BUN levels were significantly elevated (48.32 ± 1.94 and 43.33 ± 1.32 mg/100 ml, respectively) as compared to those of the WT \leftarrow WT group (29.01 ± 1.24 mg/100 ml) ($P < 0.0001$), but did not differ significantly between the two THM groups ($P=0.079$, NS) (Figure 3a). Representative kidney sections stained with Masson's trichrome are shown in Figure 3b.

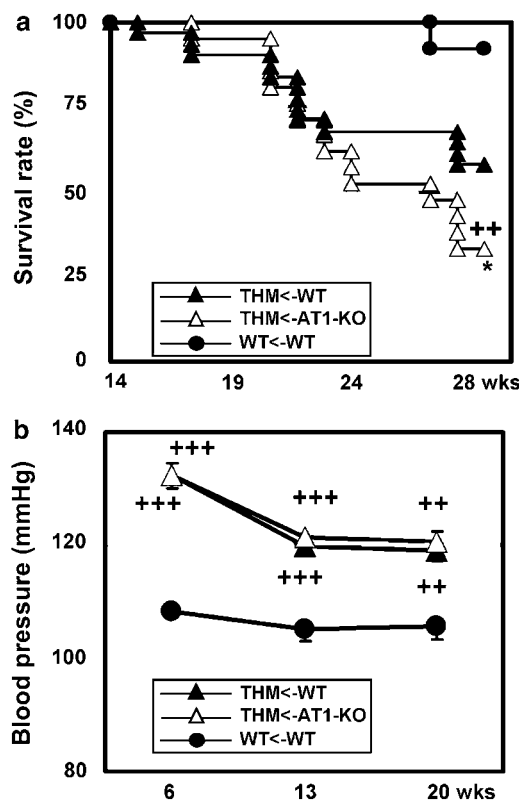


Figure 2 Survival rate and changes in blood pressure after bone marrow transplantation in mice. (a) Lethally irradiated female THM recipients aged 8 or 9 weeks received transplanted bone marrow from WT (designated as THM<-WT, closed triangle, $n = 31$) or AT1-KO (THM<-AT1-KO, open triangle, $n = 21$) mice. An atherogenic diet was initiated at 14 weeks of age and survival was carefully monitored for 14 weeks. As a control, WT mice were transplanted with bone marrow from WT mice (WT<-WT, closed circle, $n = 13$). (b) Systolic blood pressure was measured at three points: 6 weeks (before BMT), 13 weeks (after BMT but before the atherogenic diet) and 20 weeks (after BMT and during the atherogenic diet) of age. At least eight animals were tested from each group. $++P < 0.01$, $+++P < 0.0001$ vs WT<-WT; $*P < 0.05$ vs THM<-WT; NS, not significant.

Interstitial fibrosis area was significantly higher in the THM<-WT and THM<-AT1-KO groups (27.67 ± 2.75 and $33.93 \pm 9.50\%$, respectively) than in the WT<-WT group ($3.44 \pm 0.65\%$) ($P < 0.0001$), but did not differ significantly between the two THM groups ($P = 0.49$, NS) (Figure 3c). Distinct glomerular hypertrophy and glomerulosclerosis were evident in both THM groups. Semiquantitative scores of the extent of glomerulosclerosis were significantly greater in the THM<-WT and THM<-AT1-KO groups (42.56 ± 4.41 and 40.05 ± 5.71 , respectively) than in the WT<-WT group (10.82 ± 1.04) ($P < 0.0001$), but did not differ significantly between the two THM groups ($P = 0.73$, NS) (Figure 3d). Urinary albumin excretion was significantly higher in the THM<-WT and THM<-AT1-KO groups (623.1 ± 90.8 and $425.5 \pm 86.6 \mu\text{g/day}$, respectively) than in the WT<-WT group ($13.7 \pm 1.9 \mu\text{g/day}$) ($P < 0.001$), but did not differ significantly between the two THM groups ($P = 0.18$, NS) (Figure 3e).

Atherosclerotic Lesion Formation

In addition, we evaluated mice from each group for the development of atherosclerotic lesions. After 11 weeks on an atherogenic diet, the severity of aortic lesions was quantified by Oil red O staining followed by macroscopic *en face* analysis. Atherosclerotic formations containing fatty streak lesions were conspicuously accelerated in both THM groups, particularly in the region from the ascending to the thoracic aorta. In contrast, atherosclerotic lesions were barely detected in the control WT<-WT group. Subsequently, we evaluated and compared atherosclerotic lesions of the aortic wall from the ascending to thoracic aortic region area, the region reported to be the most sensitive to atherosclerosis.³⁰

Figure 4a provides representative preparations of Oil red O-stained aorta from the ascending to thoracic regions for all three groups. Figure 4b is the quantification of the percentage of mean lipid-containing atherosclerotic lesion area per total surface area. In both the THM<-WT and THM<-AT1-KO groups, the formation of atherosclerotic lesions was significantly accelerated (1.97 ± 0.67 and $11.40 \pm 5.46\%$, respectively) compared to the WT<-WT group ($0.11 \pm 0.06\%$, $P < 0.05$). Atherosclerotic lesions were markedly more severe in the THM<-AT1-KO group than in the THM<-WT group ($P < 0.05$).

Differential Gene Expression Profiles Between WT and AT1-KO Macrophages

To investigate the molecular basis of atherogenic effects of AT1-KO macrophages, we performed microarray expression profiling. Expression profiles of primary peritoneal macrophages from three independent WT and three independent AT1-KO mice were examined. We considered expression changes greater than 1.5-fold with P -values less than < 0.05 to be significant. This filter identified 321 genes as upregulated (Supplementary Table 1) and 647 genes as downregulated (Supplementary Table 2). These transcripts represent approximately 5% of all genes on the microarrays.

We found significantly upregulated expression of a number of atherosclerosis-related genes, including sterol *O*-acyltransferase 1 (*Soat1*: an intracellular protein that forms cholesterol esters from cholesterol), macrophage receptor with collagenous structure (*Marco*: scavenger receptor class A), collectin subfamily member 12 (*Colecl12*: scavenger receptor with c-type lectin domain), and chemokine (C-C motif) ligand 5 (*Ccl5*: one of ligand for the chemokine receptor CCR5). Other atherosclerosis-related genes were significantly downregulated, including sphingosine kinase 1 (*Sphk1*: blocker of the formation of sphingosine-1-phosphate), tissue inhibitor of metalloproteinase 3 (*Timp3*: zinc-binding endopeptidases involved in the degradation of the extracellular matrix), chemokine (C-X-C motif) ligand 4 (*Cxcl4*: a strong chemoattractant for neutrophils), caveolin 1 (*Cav1*: a scaffolding protein for organizing caveolin-interacting molecules), matrix metalloproteinase 13 (*Mmp13*: a collagenase against type II collagen), thrombospondin 1 (*Thbs1*:

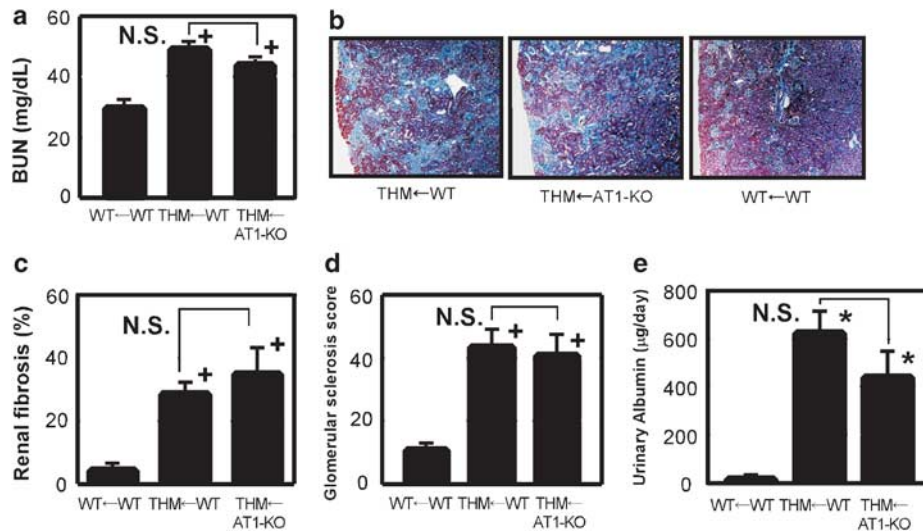


Figure 3 Assessment of kidney injury. (a) Serum blood urea nitrogen levels. (b) Light microscope photographs of representative cortical areas of Masson's trichrome-stained renal sections. (c) Morphometric analysis of relative interstitial fibrosis by measurement of the area occupied by Masson's trichrome-stained interstitium divided by total area. (d) Estimation of glomerulosclerosis by semiquantitative score. (e) Urinary albumin levels. * $P < 0.05$, + $P < 0.0001$ vs WT←WT; NS, not significant.

a secreted protein that associates with the extracellular matrix and with antiangiogenic activity), procollagen, type XVIII alpha 1 (*Col18a1*: extracellular matrix proteins with multiple triple-helical domains) and platelet-derived growth factor B (*PDGFb*: a growth factor that promotes cellular proliferation) (Figure 5a).

GO Analysis

To interpret the differential expression of gene categories, we classified genes that were differentially expressed between WT and AT1-KO macrophages according to GO categories. We found significant functional differences between the two gene populations. The categories most highly represented among upregulated genes included 'phagocytosis' and 'endocytosis', while those among downregulated genes included 'morphogenesis', 'development', 'cell adhesion' and 'migration' among others. Other examples of these are shown in Supplementary Tables 3 and 4. We conclude from these results that AT1-KO macrophages are pro-atherogenic, exhibit greater phagocytic activity and have downregulated gene clusters that are essential to the maintenance of macrophage function.

Upregulation of Acetylated LDL Uptake in AT1-KO Macrophages

To determine whether the increased atherosclerosis in THM←AT1-KO was a consequence of differences in macrophage function, and to validate the phenotype changes between WT and AT1-KO macrophages, we tested the uptake of DiI-ac-LDL by mouse primary peritoneal macrophages derived from WT and AT1-KO mice. As shown in Figure 5b, macrophages from AT1-KO accumulated more DiI-ac-LDL than WT mice. The amount of DiI-ac-LDL uptake was measured as the fluorescent area normalized to total number

of nuclei. Macrophages from AT1-KO mice had significantly greater uptake of ac-LDL than macrophages from WT mice (WT, $5.51 \mu\text{m}^2$ per cell, $n = 4$; AT1-KO, $18.8 \mu\text{m}^2$ per cell, $n = 4$; $P = 0.018$) (Figure 5c).

DISCUSSION

In this study, we demonstrate that AT1a receptor function in BMDCs attenuates atherosclerotic lesion formation and increases survival in a chronically RAS-induced atherosclerotic and nephrosclerotic mouse model, but does not influence kidney injury. Furthermore, we determined that phagocytic activity is enhanced in primary cultures of macrophages from AT1-KO mice.

Ang II receptors are expressed in spleen and BM cells. Although RT-PCR studies detected AT1a receptor transcripts in both cells as reported previously,^{9,29} AT1b receptor transcripts were only slightly detectable in spleen cells, but not in BM cells (Figure 1). Therefore, we conclude that the AT1a receptor is the predominant AT1 receptor subtype expressed in BMDCs. Thus, in the THM←AT1-KO group, transplanted BMDCs lacked AT1 receptor function and associated AT1 signaling.

After BMT, the survival rates of both THM groups were significantly lower than that of the WT←WT group, and the THM←AT1-KO group showed significantly higher lethality (Figure 2a). We previously reported that THM developed aortic aneurysm and accelerated mortality with a high-salt diet³¹ and Apolipoprotein E (Apo-E) KO mice with Ang II infusion were reported to promote the abdominal aortic aneurysms.³² Thus, we attempted to determine the cause of death in both THM groups by necropsy. In addition to atherosclerosis, we observed abdominal aortic aneurysm, elevated serum BUN levels, severe lipid deposition in the

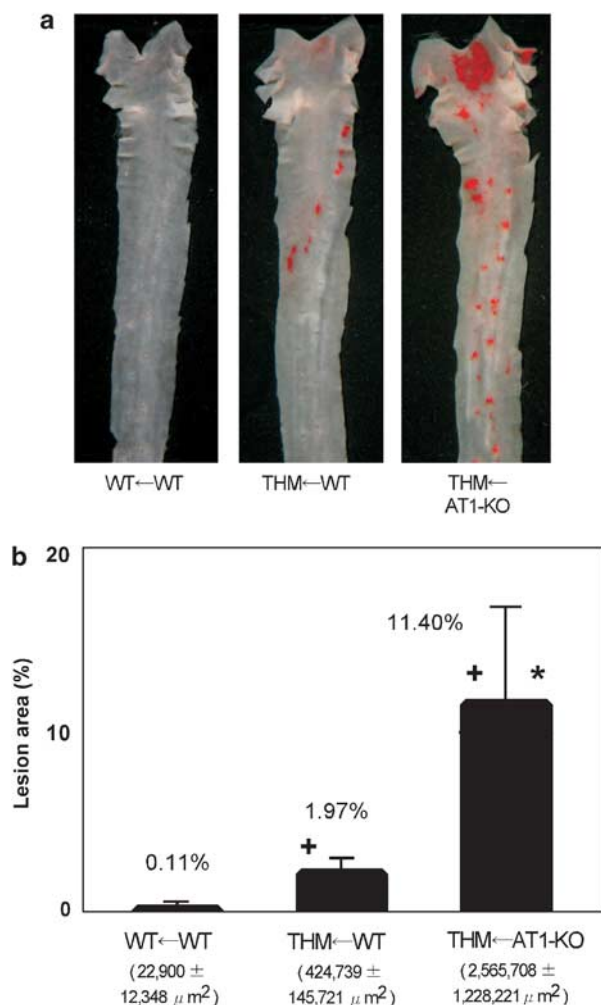


Figure 4 Atherosclerotic lesions in aortas. (a) Oil red O-stained and longitudinally opened aortas 11 weeks after initiating an atherogenic diet. Photographs show representative lesions from the ascending to thoracic aorta for each group. (b) Quantitative analysis of atherosclerotic lesion size. Lesion size in each mouse was measured by morphometry and is shown at bottom. The percentage of atherosclerotic lesion area per total surface area from an ascending to thoracic *en face* aortic lesion of each group is provided. $^+P < 0.05$ vs WT-WT, $^*P < 0.05$ vs THM-WT.

renal artery lesions and almost complete occlusion of the renal artery in several mice in THM groups (data not shown). Although further analysis is necessary to explain the exact proportion of the causes of death in both THM groups, one might speculate that the vascular injuries and subsequent renal failure contributed to the lethality.

BP unexpectedly decreased by approximately 10 mm Hg in both THM groups after BMT (Figure 2b). Although the underlying mechanism remains unclear, several important determinants of BP are aberrant in these mice, including the attenuation of hREN and hAGT transgene expression following irradiation. Irrespective of the actual changes in BP, the levels were similar in the two THM groups and elevated relative to the WT-WT group. Thus, AT1a receptors on BMDCs play only a minor role in BP regulation and the

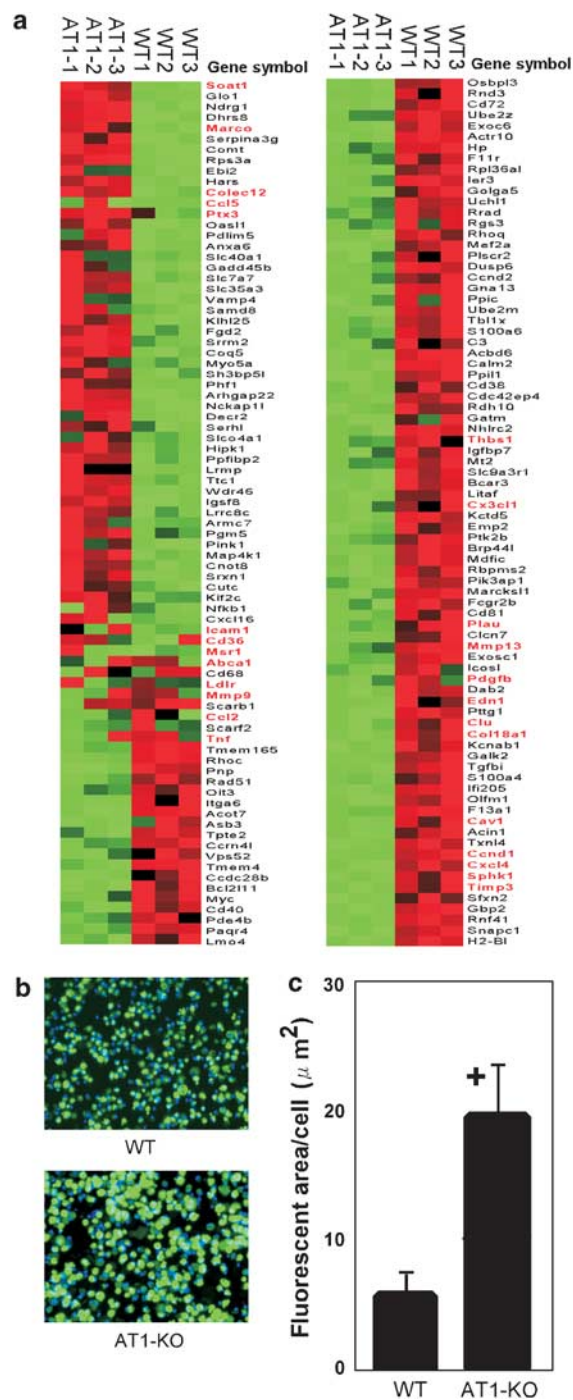


Figure 5 Differential gene expression and macrophage LDL uptake. (a) The heatmap reflects normalized gene expression ratios for each of the three replicates for each mouse. A substantial number of genes were differentially expressed between WT and AT1-KO primary macrophages. Highest expression corresponds to bright red, and the lowest expression corresponds to bright green. Genes reported to relate to atherosclerosis are presented in red. (b) Primary mouse peritoneal macrophages were incubated with 2.5 $\mu\text{g}/\text{ml}$ of Dil-Ac-LDL. Upper panel: WT macrophages. Lower panel: AT1-KO macrophages. (c) The area of Dil fluorescence was measured and normalized to the total number of nuclei. Average fluorescent area per cell is shown. $^+P < 0.05$ vs WT.

pathogenic differences between the two THM groups cannot be attributed to disparities in BP.

To date, the role of AT1a receptors on BMDCs in mediating the end-organ injuries is controversial. Regarding the kidney injury, there are at least two conflicting studies. In a unilateral ureteral obstruction mouse model, Nishida *et al*³³ reported that WT mice receiving marrow from AT1-KO mice developed more severe interstitial fibrosis than those receiving marrow from WT mice in the kidney in response to an obstructive insult. In contrast, we reported previously that AT1a receptor activity in BMDCs played only a minor role in an immune-mediated renal injury model of anti-glomerular basement membrane (anti-GBM) nephritis.²³ Similar to our results with the anti-GBM nephritis model, in the present study we demonstrate that kidney injury in the THM ← AT1-KO group did not differ from that of the THM ← WT group (Figure 3).

Regarding the atherosclerosis, the role of AT1a receptors on BMDCs has been more controversial. Recent studies demonstrated that atherosclerosis in Ang II-infused Apo-E KO mice transplanted with BM from AT1a receptor KO mice showed reduction in atherosclerosis.³⁴ In addition, Yamada *et al*³⁵ reported that Ang II accelerated BMDC differentiation into smooth muscle-like cells via AT1 receptors. These results suggested that blockade of AT1 receptor in BM cells might have inhibitory effects on atherosclerosis. In contrast, little or no changes of atherosclerosis in LDL receptor KO mice by transplantation with BM from AT1a receptor KO mice are also reported^{36,37} and these reports suggest the ameliorative role of AT1 receptor blockade in vascular cells for the ARB-mediated atherosclerosis inhibition. In accordance with the latter reports, our results strongly suggest that the beneficial effects of ARB in end-organ injuries are attributed to the blockade of AT1 receptor expressed in the end organs, but not in BMDCs (Figures 2 and 4). One possible explanation for the distinct results observed in the kidney injury and atherosclerosis is differences in the pathogenesis of mouse models. Therefore, we speculate that AT1 receptor function in BMDCs may have differential action points, depending upon the tissues and model systems examined.

To evaluate the characteristics of AT1-KO macrophages, we examined the expression profiles of macrophages from WT and AT1-KO mutant mice. Macrophages from AT1-KO mice exhibited an upregulation of phagocytosis- and endocytosis-related genes and a downregulation of a number of gene clusters essential for maintaining the cellular functions of macrophages (Figure 5a and Supplementary Tables 1–4). Interestingly, our microarray results revealed a marked increase in class A scavenger receptor, *Colec12/SRCL/CL-P1* and *MARCO* in AT1-KO macrophages (Figure 5a). *Colec12* has been reported to react with oxidized LDL³⁸ and *MARCO* was recently shown to have profound effects on binding of acetylated LDL.³⁹

Little is known about the role of Ang II on phagocytosis by macrophages. Keidar *et al*¹¹ reported that although systemic

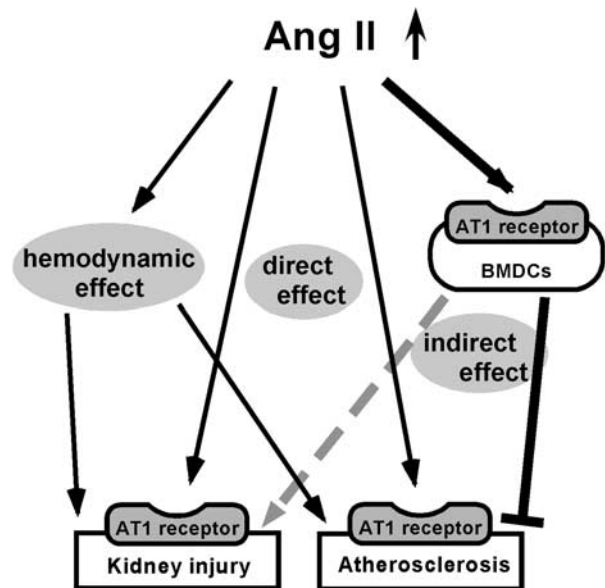


Figure 6 Schematic of Ang II pathways in end-organ injuries. Ang II affects end organs through at least three separate pathways: the hemodynamic, direct and BMDC-mediated indirect effects. AT1 receptor function in BMDCs contributes to the prevention of atherosclerosis but has a negligible effect on kidney injury.

Ang II administration in mice resulted in an increase in modified LDL uptake by macrophages *in vivo*, no change of phagocytic activity was observed in peritoneal macrophage cell culture *in vitro* following addition of Ang II or ARB. However, we demonstrated that cultured AT1-KO macrophages had enhanced phagocytic activity. These differences may result from differences between the systemic effects of Ang II and local direct effects of Ang II on cultured macrophages. Alternatively, there may be differences associated with the short-term pharmacological effects of AT1 receptor antagonists and the genetic deletion of AT1 receptors. Moreover, in contrast to our results, Nishida *et al*³³ reported that cultured AT1-KO primary macrophages had decreased phagocytic activity of fluorescent beads. Considering these results, we interpret that the influence of AT1 receptors on phagocytosis may differ with the substrate tested.

Figure 6 shows a schematic of Ang II pathways that contribute to end-organ injuries consistent with our results. Given that the systemic inhibition of AT1 receptors reduces the development of atherosclerosis as well as high BP, these beneficial effects have been proposed to be mediated by the blockade of end-organ AT1 receptors expressed by specific end organs such as the vasculature and kidney. Thus, in addition to AT1 receptor blockade in end organs, a sustained AT1 receptor activation in BMDCs might play additive protective roles from end-organ injuries. Our results provide new insights into the pathogenic role of the AT1 receptor in BMDCs for end-organ injuries.

Supplementary Information accompanies the paper on the Laboratory Investigation website (<http://www.laboratoryinvestigation.org>)

ACKNOWLEDGEMENT

We thank Dr T Yokoo, Dr J Fan, Dr T Koike and Dr S Matsumoto for their technical advice. This study was supported by the following grants: The 21st Century COE Program, Grant-in-Aid for Scientific Research (S), and Grant-in-Aid for Young Scientists (B) from the Ministry of Education, Culture, Sports, Science and Technology of Japan, the University of Tsukuba Special Research Program, Japan, and the Heart Foundation Research Grant.

- Crowley SD, Tharaux PL, Audoly LP, *et al*. Exploring type I angiotensin (AT1) receptor functions through gene targeting. *Acta Physiol Scand* 2004;181:561–570.
- Yusuf S, Sleight P, Pogue J, *et al*. Effects of an angiotensin-converting-enzyme inhibitor, ramipril, on cardiovascular events in high-risk patients. The Heart Outcomes Prevention Evaluation Study Investigators. *N Engl J Med* 2000;342:145–153.
- Ferrario CM. Use of angiotensin II receptor blockers in animal models of atherosclerosis. *Am J Hypertens* 2002;15(1 Part 2):95–135.
- Wassmann S, Czech T, van Eickels M, *et al*. Inhibition of diet-induced atherosclerosis and endothelial dysfunction in apolipoprotein E/angiotensin II type 1A receptor double-knockout mice. *Circulation* 2004;110:3062–3067.
- Taal MW, Brenner BM. Renoprotective benefits of RAS inhibition: from ACEI to angiotensin II antagonists. *Kidney Int* 2000;57:1803–1817.
- Wolf G, Butzmann U, Wenzel UO. The renin–angiotensin system and progression of renal disease: from hemodynamics to cell biology. *Nephron Physiol* 2003;93:P3–P13.
- Ruiz-Ortega M, Lorenzo O, Ruperez M, *et al*. Role of the renin–angiotensin system in vascular diseases: expanding the field. *Hypertension* 2001;38:1382–1387.
- Touyz RM. Molecular and cellular mechanisms in vascular injury in hypertension: role of angiotensin II. *Curr Opin Nephrol Hypertens* 2005;14:125–131.
- Nataraj C, Oliverio MI, Mannon RB, *et al*. Angiotensin II regulates cellular immune responses through a calcineurin-dependent pathway. *J Clin Invest* 1999;104:1693–1701.
- Kranzhofer R, Browatzki M, Schmidt J, *et al*. Angiotensin II activates the proinflammatory transcription factor nuclear factor-kappaB in human monocytes. *Biochem Biophys Res Commun* 1999;257:826–828.
- Keidar S, Heinrich R, Kaplan M, *et al*. Angiotensin II administration to atherosclerotic mice increases macrophage uptake of oxidized LDL: a possible role for interleukin-6. *Arterioscler Thromb Vasc Biol* 2001;21:1464–1469.
- Keidar S, Kaplan M, Hoffman A, *et al*. Angiotensin II stimulates macrophage-mediated oxidation of low density lipoproteins. *Atherosclerosis* 1995;115:201–215.
- Scheidegger KJ, Butler S, Witztum JL. Angiotensin II increases macrophage-mediated modification of low density lipoprotein via a lipoxygenase-dependent pathway. *J Biol Chem* 1997;272:21609–21615.
- Yanagitani Y, Rakugi H, Okamura A, *et al*. Angiotensin II type 1 receptor-mediated peroxide production in human macrophages. *Hypertension* 1999;33(1 Part 2):335–339.
- Hahn AW, Jonas U, Buhler FR, *et al*. Activation of human peripheral monocytes by angiotensin II. *FEBS Lett* 1994;347:178–180.
- Muller DN, Shagdarsuren E, Park JK, *et al*. Immunosuppressive treatment protects against angiotensin II-induced renal damage. *Am J Pathol* 2002;161:1679–1693.
- Nahmod KA, Vermeulen ME, Raiden S, *et al*. Control of dendritic cell differentiation by angiotensin II. *FASEB J* 2003;17:491–493.
- Hernandez-Presa M, Bustos C, Ortego M, *et al*. Angiotensin-converting enzyme inhibition prevents arterial nuclear factor-kappa B activation, monocyte chemoattractant protein-1 expression, and macrophage infiltration in a rabbit model of early accelerated atherosclerosis. *Circulation* 1997;95:1532–1541.
- Fukamizu A, Sugimura K, Takimoto E, *et al*. Chimeric renin–angiotensin system demonstrates sustained increase in blood pressure of transgenic mice carrying both human renin and human angiotensinogen genes. *J Biol Chem* 1993;268:11617–11621.
- Sugiyama F, Haraoka S, Watanabe T, *et al*. Acceleration of atherosclerotic lesions in transgenic mice with hypertension by the activated renin–angiotensin system. *Lab Invest* 1997;76:835–842.
- Shimokama T, Haraoka S, Horiguchi H, *et al*. The Tsukuba hypertensive mouse (transgenic mouse carrying human genes for both renin and angiotensinogen) as a model of human malignant hypertension: development of lesions and morphometric analysis. *Virchows Arch* 1998;432:169–175.
- Sugaya T, Nishimatsu S, Tanimoto K, *et al*. Angiotensin II type 1a receptor-deficient mice with hypotension and hyperreninemia. *J Biol Chem* 1995;270:18719–18722.
- Hisada Y, Sugaya T, Tanaka S, *et al*. An essential role of angiotensin II receptor type 1a in recipient kidney, not in transplanted peripheral blood leukocytes, in progressive immune-mediated renal injury. *Lab Invest* 2001;81:1243–1251.
- Kato H, Ishida J, Imagawa S, *et al*. Enhanced erythropoiesis mediated by activation of the renin–angiotensin system via angiotensin II type 1a receptor. *FASEB J* 2005;19:2023–2025.
- Hartner A, Cordasic N, Klanke B, *et al*. The alpha8 integrin chain affords mechanical stability to the glomerular capillary tuft in hypertensive glomerular disease. *Am J Pathol* 2002;160:861–867.
- Kim JG, Keshava C, Murphy AA, *et al*. Fresh mouse peritoneal macrophages have low scavenger receptor activity. *J Lipid Res* 1997;38:2207–2215.
- Kaushik N, Fear D, Richards SC, *et al*. Gene expression in peripheral blood mononuclear cells from patients with chronic fatigue syndrome. *J Clin Pathol* 2005;58:826–832.
- Korostynski M, Kaminska-Chowaniec D, Piechota M, *et al*. Gene expression profiling in the striatum of inbred mouse strains with distinct opioid-related phenotypes. *BMC Genomics* 2006;7:146.
- Rodgers KE, Xiong S, Steer R, *et al*. Effect of angiotensin II on hematopoietic progenitor cell proliferation. *Stem Cells* 2000;18:287–294.
- Zhou Y, Chen R, Catanzaro SE, *et al*. Differential effects of angiotensin II on atherogenesis at the aortic sinus and descending aorta of apolipoprotein E-deficient mice. *Am J Hypertens* 2005;18(4 Part 1):486–492.
- Nishijo N, Sugiyama F, Kimoto K, *et al*. Salt-sensitive aortic aneurysm and rupture in hypertensive transgenic mice that overproduce angiotensin II. *Lab Invest* 1998;78:1059–1066.
- Daugherty A, Manning MW, Cassis LA. Angiotensin II promotes atherosclerotic lesions and aneurysms in apolipoprotein E-deficient mice. *J Clin Invest* 2000;105:1605–1612.
- Nishida M, Fujinaka H, Matsusaka T, *et al*. Absence of angiotensin II type 1 receptor in bone marrow-derived cells is detrimental in the evolution of renal fibrosis. *J Clin Invest* 2002;110:1859–1868.
- Fukuda D, Sata M, Ishizaka N, *et al*. Critical role of bone marrow angiotensin II type 1 receptor in the pathogenesis of atherosclerosis in apolipoprotein E deficient mice. *Arterioscler Thromb Vasc Biol* 2008;28:90–96.
- Yamada T, Kondo T, Numaguchi Y, *et al*. Angiotensin II receptor blocker inhibits neointimal hyperplasia through regulation of smooth muscle-like progenitor cells. *Arterioscler Thromb Vasc Biol* 2007;27:2363–2369.
- Cassis LA, Rateri DL, Lu H, *et al*. Bone marrow transplantation reveals that recipient AT1a receptors are required to initiate angiotensin II-induced atherosclerosis and aneurysms. *Arterioscler Thromb Vasc Biol* 2007;27:380–386.
- Lu H, Rateri DL, Feldman DL Jr RJ, *et al*. Renin inhibition reduces hypercholesterolemia-induced atherosclerosis in mice. *J Clin Invest* 2008;118:984–993.
- Ohtani K, Suzuki Y, Eda S, *et al*. The membrane-type collectin CL-P1 is a scavenger receptor on vascular endothelial cells. *J Biol Chem* 2001;276:44222–44228.
- Chen Y, Sankala M, Ojala JR, *et al*. A phage display screen and binding studies with acetylated low density lipoprotein provide evidence for the importance of the scavenger receptor cysteine-rich (SRCR) domain in the ligand-binding function of MARCO. *J Biol Chem* 2006;281:12767–12775.