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Original Paper

SRY gene transferred by extracellular vesicles accelerates atherosclerosis by promotion of leucocyte adherence to endothelial cells

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Abstract
We set out to investigate whether and how *SRY* (sex-determining region, Y) DNAs in plasma EVs (extracellular vesicles) is involved in the pathogenesis of atherosclerosis. PCR and gene sequencing found the *SRY* gene fragment in plasma EVs from male, but not female, patients; EVs from male patients with CAD (coronary artery disease) had a higher *SRY* GCN (gene copy number) than healthy subjects. Additional studies found that leucocytes, the major source of plasma EVs, had higher *SRY* GCN and mRNA and protein expression in male CAD patients than controls. After incubation with EVs from *SRY*-transfected HEK (human embryonic kidney)-293 cells, monocytes (THP-1) and HUVECs (human umbilical vein endothelial cells), which do not endogenously express SRY protein, were found to express newly synthesized SRY protein. This resulted in an increase in the adherence factors CD11-a in THP-1 cells and ICAM-1 (intercellular adhesion molecule 1) in HUVECs. EMSA showed that SRY protein increased the promoter activity of CD11-a in THP-1 cells and ICAM-1 in HUVECs. There was an increase in THP-1 cells adherent to HUVECs after incubation with SRY-EVs. *SRY* DNAs transferred from EVs have pathophysiological significance *in vivo*; injection of SRY EVs into ApoE$^{-/-}$ (apolipoprotein-knockout) mice accelerated atherosclerosis. The *SRY* gene in plasma EVs transferred to vascular endothelial cells may play an important role in the pathogenesis of atherosclerosis; this mechanism provides a new approach to the understanding of inheritable CAD in men.

Key words: atherosclerosis, CD11-a, extracellular vesicles, gene copy number, ICAM-1, SRY.

INTRODUCTION

EVs (extracellular vesicles) are small membrane vesicles, such as exosomes and shedding vesicles, that are released upon exocytosis of multivesicular bodies and budding from the plasma membrane respectively [1]. EVs may vary in their formation, size, abundance and composition, but they often contain abundant functional transmembrane and cytosolic proteins, mRNAs, miRNAs and DNAs [2–7]. EVs participate in important biological processes, such as surface-membrane trafficking and horizontal transfer of borne molecules among neighbouring cells [8–11].

Our previous study showed the existence of DNAs in EVs that could be transferred from one cell to another by endocytosis or fusion. The transferred EV DNAs have the ability to influence the function of the recipient cells by increasing DNA-coding mRNA and protein levels [12]. However, the role of the EV DNAs in the pathogenesis of disease is not clear. There is increasing evidence that EVs play a pivotal role in tumorigenesis, which can occur in adjacent and remote locations. Indeed, tumour-derived EVs could

Abbreviations: ApoE$^{-/-}$, apolipoprotein E-knockout; AT_1R, angiotensin type 1 receptor; BCECF, 2′,7′-bis-(2-carboxyethyl)-5(6)-carboxyfluorescein; BCECF/AM, BCECF acetoxymethyl ester; CAD, coronary artery disease; CML, chronic myeloid leukaemia; CMV, cytomegalovirus; EV, extracellular vesicle; GCN, gene copy number; HEK, human embryonic kidney; HUVEC, human umbilical vein endothelial cell; ICAM-1, intercellular adhesion molecule 1; RT, reverse transcription; SRY, sex-determining region, Y.
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direct normal cells towards a tumour phenotype [13]. Our previous study provided evidence that *BCR/ABL* DNA transferred by EVs from CML (chronic myeloid leukaemia) cells has pathological significance, for example in the facilitation of metastases [14].

There are several pieces of evidence that EVs, via intercellular communication, play an important pathological role in atherosclerosis by co-ordinating the actions of platelets and leucocytes adherent to endothelial cells [1]. However, the role of EV DNAs in atherogenesis is unclear. The fundamental biological role of the Y chromosome is to impart male characteristics, but it is also linked to cardiovascular disease. For example, several studies have shown that *SRY* (sex-determining region, Y), a gene in the Y chromosome, was responsible for increasing the angiotensin II and noradrenaline levels [15,16]. In addition, polysomy of the Y chromosome (mostly 47, XYY karyotype) was related to increased risk of CAD (coronary artery disease) in men [17]. Importantly, GCN (gene copy number) can regulate gene expression and influence biological function. We hypothesize that *SRY* DNAs in plasma EVs are involved in the pathogenesis of atherosclerosis by their transfer to recipient cells, e.g. leucocytes and endothelial cells, and increased adherence of leucocytes to endothelial cells.

In the present study, we first determined the GCN of *SRY* DNA in plasma EVs from male patients with CAD by quantitative PCR. Then, we quantified the expression of *SRY* GCN, mRNA and protein in the leucocytes from male patients with CAD. We also measured the levels of several intercellular adherent factors to determine the pathophysiological significance of the increased expression of SRY in the leucocytes. To determine the transportability and functionality of the *SRY* DNA in EVs, we used *SRY* gene-containing EVs obtained from *SRY*-transfected HEK (human embryonic kidney)-293 cells that stably expressed SRY, to treat monocytes or endothelial cells. EMSA was used to measure the DNA-binding ability of SRY protein to the consensus promoter-binding sequence of CD11-a and ICAM-1 (intercellular adhesion molecule 1). Finally, we studied the adhesion between the monocytes and endothelial cells caused by *SRY* gene-containing EVs *in vitro* and *in vivo*.

The present study found, by PCR and gene sequencing, *SRY* gene fragment in plasma EVs from male but not from female patients; plasma EVs from male patients with CAD had higher *SRY* GCN than healthy male subjects. In addition, leucocytes, a major source of plasma EVs, had higher *SRY* GCN, mRNA and protein expression in male CAD patients than in healthy male subjects. Incubation of recipient cells with EVs from *SRY*-transfected HEK-293 cells increased SRY expression in these recipient cells, i.e. monocytes (THP-1) and HUVECs (human umbilical vein endothelial cells). Consequently, SRY, acting as a transcript factor, increased the levels of adherence factors, including CD11-a in THP-1 cells and ICAM-1 in HUVECs. There was also an increase in THP-1 cells adherent to HUVECs after incubation with SRY-EVs. Injection of SRY-EVs into ApoE$^{-/-}$ (apolipoprotein E-knockout) mice accelerated atherosclerosis. This study shows the pathological role of the *SRY* gene transferred from plasma EVs to vascular endothelial cells that may play an important part in the pathogenesis of atherosclerosis.

Table 1 Characteristics of the subjects

Characteristic	Control subjects	CAD patients
Sample size (*n*, male)	14	21
Age (years)	60.3 ± 6.33	66.2 ± 8.08
Height (cm)	170 ± 4.42	166 ± 5.33
Body weight (kg)	66.7 ± 6.91	65.3 ± 9.57
Systolic blood pressure (mmHg)	124.6 ± 11.3	137 ± 18.6
Diastolic blood pressure (mmHg)	75.4 ± 9.76	79.5 ± 14.4
Triacylglycerol (mM)	1.58 ± 1.22	1.31 ± 0.71
Total cholesterol (mM)	4.23 ± 0.93	4.68 ± 1.35
High-density lipoprotein (mM)	1.46 ± 0.33	1.33 ± 0.24
Low-density lipoprotein (mM)	2.15 ± 0.54	2.48 ± 0.94
Blood glucose (mM)	6.08 ± 1.97	5.93 ± 1.95

MATERIALS AND METHODS

Blood collection

Blood was drawn from healthy male donors and male in-patients with CAD (≥50 % stenosis in at least one major coronary artery, newly diagnosed by coronary angiography) at Daping Hospital (Chongqing, China) and stored in test tubes containing 3.8 % trisodium citrate. The baseline characteristics of the male patients with CAD, are listed in Table 1, whereas the healthy male donors (control subjects) were described in our previous study [12]. To minimize the influence of confounding factors, patients with hypertension, diabetes, hypercholesterolaemia and obesity were excluded from the study. Plasma was isolated by centrifugation at 1800 ***g*** for 15 min. All donors or their guardians provided informed consent, and the protocols for handling human blood and tissues were approved by the Ethics Committee of Daping Hospital.

Cell isolation and culture

HUVECs (CRL-1730), monocytes (THP-1), and HEK-293 cells were purchased from the A.T.C.C. (Manassas, VA, U.S.A.). These cells were cultured at 37 °C under 5 % CO_2 in Dulbecco's modified Eagle's medium (Gibco/Life Technologies), supplemented with 10 % FBS (Gibco/Life Technologies).

Isolation of EVs

EVs were isolated from the plasma or cell culture medium by differential centrifugation and then subjected to DNase digestion to remove the DNA exterior of the EVs, as described in our previous study [12]. These EVs were used in the experiments.

DNA extraction from EVs

Total DNA was extracted from EVs with the TIANamp Genomic DNA Kit (Tiangen Biotech Co.), as described in our previous study [12]. The DNA deposit was eluted with 50 μl of sterile deionized water. The quality and quantity of extracted DNA were measured by spectrophotometry (A_{260}) and agarose gel electrophoresis [18].

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Table 2 Sequence of PCR amplification primers and DNA probes for EMSA

Primer/probe name	Sequence (5′→3′)	Product length	Amplified gene
SRY-109FP	5′-TGGCGATTAAGTCAAATTCGC-3′	137 bp	Human *SRY* mRNA/genomic DNA
SRY-245RP	5′-CCCTAGTACCCTGACAATGTATT-3′		
CMV-519FP	5′-CGCAAATGGGCGGTAGGCGTG-3′	329 bp	CMV-*SRY* genomic DNA
SRY-196RP	5′-AGACCACACGATGAATGC-3′		
β-globin-354FP	5′-GTGCACCTGACTCCTGAGGAGA-3′	102 bp	Human β-globin DNA
β-globin-455RP	5′-CCTTGATACCAACCTGCCCAG-3′		
β-actin-822FP	5′-CCACGAAACTACCTTCAACTCC-3′	132 bp	Human β-actin mRNA
β-actin-953RP	5′-GTGATCTCCTTCTGCATCCTGT-3′		
CD11-a FP	5′-Fluorescein-TTCTGGAAACAAATCCCT-3′	–	Binding to SRY protein
CD11-a RP	5′-AGGGATTTGTTTCCAGAA-3′		
ICAM-1 FP	5′-Fluorescein-GACAGAAACAAAGGTCTA-3′	–	Binding to SRY protein
ICAM-1 RP	5′-TAGACCTTTGTTTCTGTC-3′		

Qualitative PCR of DNA

DNAs within EVs were amplified by qualitative PCR, using 2× Taq PCR MasterMix (Tiangen), according to the manufacturer's instructions. A 25 μl volume of the final reaction mixture contained 12.5 μl of 2× Taq PCR MasterMix, 1 μl of sense primer, 1 μl of antisense primer and 10.5 μl of DNA extract. Thermocycling was conducted using a MyCycler™ thermal cycler (Bio-Rad Laboratories), initiated by a 5-min incubation at 94 °C, followed by 40 cycles at 94 °C for 30 s, 58 °C for 45 s and 72 °C for 45 s. All primers used are listed in Table 2.

Quantitative PCR of DNA

DNAs within plasma EVs were quantified by real-time PCR, using SYBR® premix Ex Taq™ (TaKaRa Biotechnology Co.), as described in our previous study [12].

RNA isolation and RT (reverse transcription)–PCR of mRNA

Total RNA of EVs or cells was extracted using TRIzol Reagent (Life Technologies). Residual DNA was removed by DNase I digestion following RNA isolation, and then the purified RNA was reverse-transcribed into complimentary DNA (cDNA), as described in our previous study [12]. Subsequently, qualitative PCR of cDNA was performed using 2× Taq PCR MasterMix (Tiangen) by the MyCycler™ thermal cycler, as described above.

Immunoblotting

The antibodies used in our experiments included: monoclonal mouse anti-human SRY antibody (Santa Cruz Biotechnology); polyclonal goat anti-human CD11-a antibody (Sigma–Aldrich); polyclonal rabbit anti-human ICAM-1 antibody (Cell Signaling Technology); polyclonal mouse anti-human β-actin antibody (Santa Cruz Biotechnology); and polyclonal rabbit anti-human actin antibody (Sigma–Aldrich). Immunoblotting was performed as reported previously except that the transblots were probed with the antibodies against SRY (1:200 dilution) [19], CD11-a (1:400 dilution), ICAM-1 antibody (1:1000 dilution), β-actin (1:600 dilution) or anti-actin antibody (1:400 dilution). Proteins were visualized using the ECL system. Normalization was performed by blotting the same samples with an antibody against β-actin.

Electrophoretic mobility-shift assay

EMSA was used to measure the DNA-binding ability of SRY protein to the consensus promoter-binding sequence of CD11-a and ICAM-1 [20,21]. The *SRY* DNA-containing EVs (SRY-EVs) (10^5/ml) from SRY-HEK-293 cells and control EVs from HEK-293 cells were incubated with HUVECs and THP-1 cells for 24 h. EMSA was performed according to the manufacturer's instructions using a non-radioactive EMSA kit (Thermo Scientific), as previously described [22,23]. All 5′ fluorescein-labelled DNA probes used are listed in Table 2. Protein–DNA complexes were resolved using electrophoresis at 4 °C in a 6.5 % acrylamide gel and subjected to chemiluminescence for visualization.

Adhesion between the monocytes and endothelial cells

To observe adhesion between the monocytes and endothelial cells that is influenced by SRY-EVs, monocytes (THP-1) and HUVECs were separately incubated with SRY-EVs (10^5/ml) from SRY-HEK-293 cells for 24 h. BCECF/AM [2′,7′-bis-(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester] facilitates the loading of BCECF [2′,7′-bis-(2-carboxyethyl)-5(6)-carboxyfluorescein], a green fluorochrome, into cells. THP-1 was stained with BCECF/AM (5 μM) for 30 min, and then washed with PBS three times to eliminate contamination of unincorporated BCECF. Subsequently, HUVECs were incubated with THP-1 cells stained with BCECF, and then washed with PBS three times to eliminate free THP-1 cells in the culture medium. Fluorescence was measured at 485 nm excitation and 530 nm emission wavelengths, using a fluorescence microscope. All groups were studied under the same conditions. We counted the number of THP-1 cells stained with BCECF and HUVECs in six visual fields, and then calculated the ratio of THP-1 to HUVEC number.

Animal experiments

ApoE$^{-/-}$ male mice were maintained on a 12 h light/12 h dark cycle in a pathogen-free animal facility at Daping Hospital. At 4 weeks of age, the mice were injected, via the tail-vein, every 3 days with PBS (200 μl), EVs from HEK-293 cells (2×10^6 EVs in 200 μl), or EVs from SRY-HEK-293 cells (2×10^6 EVs in 200 μl), After 2 months, the animals were killed by CO_2

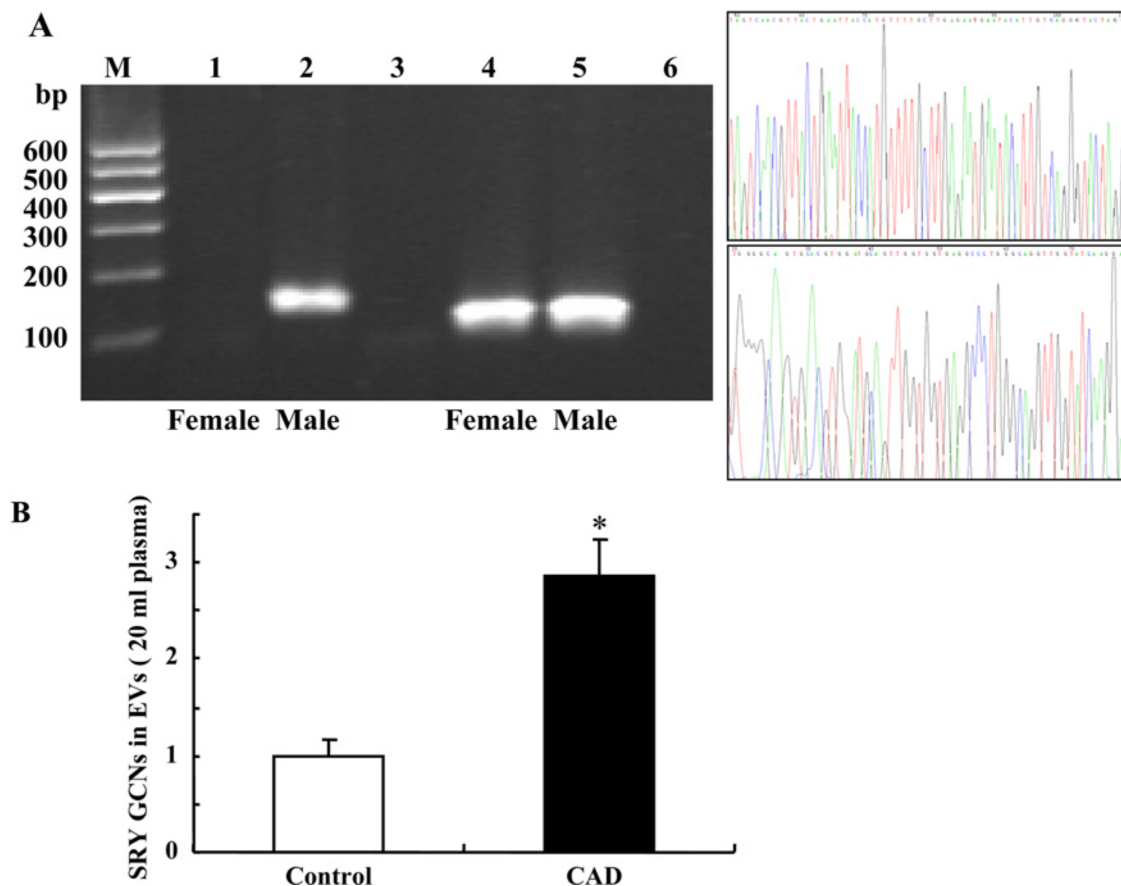

Figure 1 ***SRY* GCNs in plasma EVs from male patients with CAD**
(**A**) Left: the *SRY* gene (lanes 1–3) in EVs from humans was detected by qualitative PCR with the β-globin gene (lanes 4–6) as a control gene. The PCR products were analysed on 2 % agarose gel pre-cast with ethidium bromide. Lane M, DNA marker (sizes indicated in bp); lanes 1 and 4, female EVs; lanes 2 and 5, male EVs; lanes 3 and 6, negative control (double-distilled water). Right: sequencing result of PCR products of the *SRY* gene (top) and the β-globin gene (bottom) in leucocytes from male patients. The sequence identity was more than 99.5 %. (**B**) *SRY* GCNs in EVs, measured by quantitative PCR, from the same amount (20 ml) of plasma from male healthy subjects (control) and patients with CAD ($n = 10$–16, $^{*}P < 0.05$ compared with control).

asphyxiation in accordance with the animal treatment policy of the Third Military Medical University, and the vessels and blood were obtained.

We observed the size of and lipid accumulation in the atherosclerotic plaques in the aorta from the mice with Oil Red O, which stains intracellular triacylglycerol droplets. The detailed methods were described in our previous study [24]. In addition, the levels of CD11-a in monocytes and ICAM-1 in the vessels were measured by ELISA [25]. All assays were performed according to the instructions provided by the manufacturer (Nanjing Jiancheng Bioengineering Institute). All animal experiments were approved by the Third Military Medical University Animal Use and Care Committee.

Statistical analysis

Results are expressed as means $\pm$ S.D. Comparison within groups was made by one-way repeated-measures ANOVA, and comparison between groups was made by one-way factorial ANOVA and Duncan's test (Student's t test when only two groups were compared). A value of $P < 0.05$ was considered significant.

RESULTS

Increased *SRY* GCN in plasma EVs or leucocytes from male patients with CAD

Our previous study showed the existence of DNA in EVs that can influence the function of the recipient cells by increasing DNA-coding mRNA and protein levels [12]. *SRY*, a gene in the Y chromosome responsible for increasing the levels of angiotensin II and noradrenaline, is associated with risk of CAD in men [15–17]. We found, by PCR and gene sequencing, *SRY* DNA in plasma EVs from male, but not female, patients (Figure 1A); the identity of the sequences was more than 99.5 % for each fragment of *SRY* DNA (Figure 1A). Moreover, the GCNs of *SRY* were higher in plasma EVs from male CAD patients than healthy male subjects (Figure 1B).

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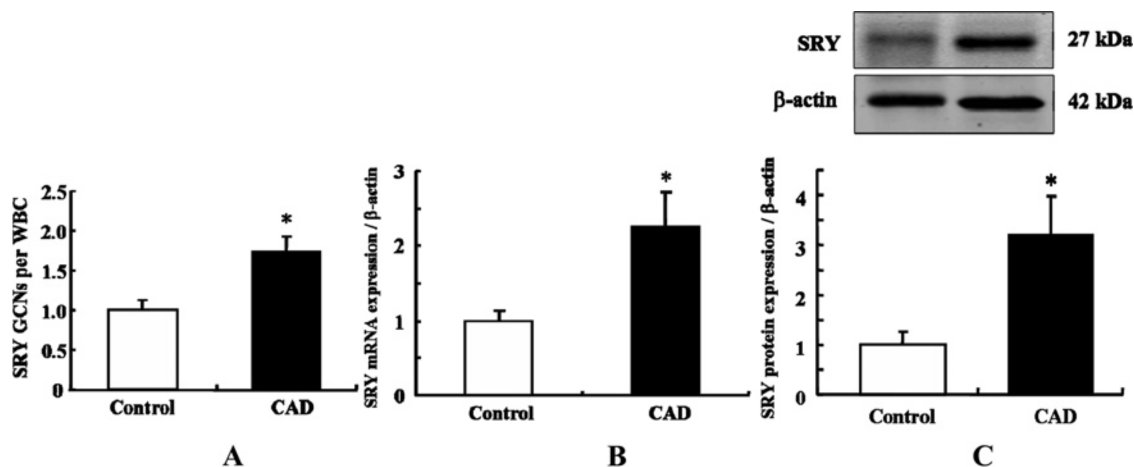

Figure 2 ***SRY* GCNs, mRNA and protein in leucocytes from healthy male subjects and male patients with CAD**
(**A**) *SRY* GCN per leucocyte ('WBC') determined by quantitative PCR ($n = 10$–13, $^*P < 0.05$ compared with control, healthy subjects). (**B**) *SRY* mRNA expression in leucocytes relative to that of β-globin mRNA determined by quantitative RT–PCR ($n = 7$, $^*P < 0.05$ compared with control). (**C**) SRY protein expression in leucocytes relative to that of β-actin (see inset; molecular masses are indicated in kDa) determined by immunoblotting ($n = 9$, $^*P < 0.05$ compared with control).

Although plasma EVs are shed from all blood cells [26], genomic DNA-containing EVs in plasma are potentially secreted from leucocytes, one group of nucleated blood cells. Thus we presumed that the *SRY* GCNs in plasma EVs were from leucocytes. Consistent with this presumption, the *SRY* GCNs in leucocytes from males were higher in CAD patients than healthy controls (Figure 2A). Moreover, the mRNA and protein expression of SRY in leucocytes was also higher in CAD patients than in healthy controls (Figures 2B and 2C).

Because leucocyte adherence to endothelial cells plays an important role in atherosclerosis, we measured the levels of several intercellular adherent factors to elucidate the pathophysiological significance of the increased expression of SRY in the leucocytes of CAD patients. Immunoblotting showed that CD11-a protein expression was significantly increased in leucocytes from male patients with CAD compared with healthy male subjects (Figure 3), whereas CD11-b or CD11-c expression was not different between the two groups (results not shown). CD11-a, which is a receptor for ICAM-1 typically expressed on endothelial cells, is expressed on the cell surface of all leucocytes,. As these two intercellular adherent factors are involved in leucocyte–endothelial cell interactions, we investigated the relationship between SRY and CD11-a or ICAM-1.

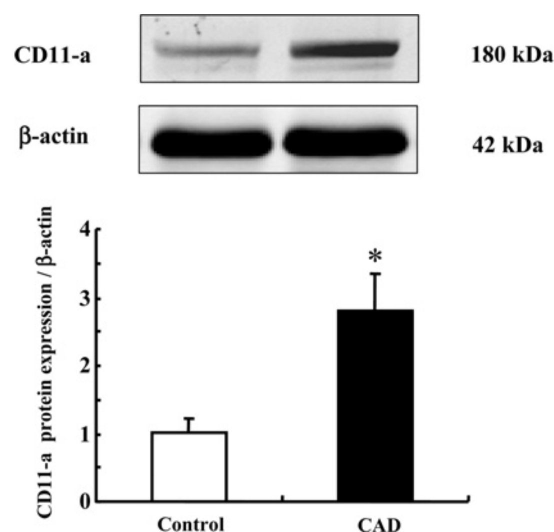

Figure 3 CD11-a protein expression in leucocytes from healthy male subjects and male patients with CAD
CD11-a protein expression in leucocytes relative to that of β-actin was determined by immunoblotting ($n = 4$, $^*P < 0.05$ compared with control, healthy subjects). Molecular masses are indicated in kDa.

Transportable and functional *SRY* DNA in EVs from *SRY*-transfected HEK-293 cells to recipient cells

To determine whether or not the *SRY* DNA fragments in EVs are transportable and functional, we used *SRY*-transfected HEK-293 (SRY-HEK-293) cells, as described in our previous study [12]. Analysis by PCR showed CMV (cytomegalovirus)-*SRY* DNA in SRY-HEK-293 cells, as well as in their EVs, but not in vector-transfected HEK-293 cells or their EVs (Figure 4A). The presence of CMV-*SRY* DNA in SRY-EVs was confirmed by sequencing, and the sequence identity was more than 99.5 % (Figure 4B). We also found by immunoblotting that SRY protein was expressed in SRY-HEK-293 cells but not in vector-transfected HEK-293 cells (Figure 4C), monocytes (THP-1) or HUVECs (results not shown).

The transportable *SRY* DNA was functional because THP-1 cells or HUVECs incubated with SRY-EVs (10^5/ml), but not EVs from control cells, for 24 h, had newly synthesized SRY protein (Figures 5A and 5B). Consequently, CD11-a protein expression was significantly increased in SRY-EV-treated THP-1 cells (Figure 5A), and ICAM-1 protein expression was higher in SRY-EV-treated HUVECs than EV-treated control cells (Figure 5B).

EMSA was used to detect the DNA-binding ability of SRY protein to the consensus binding sequences of ICAM-1 and

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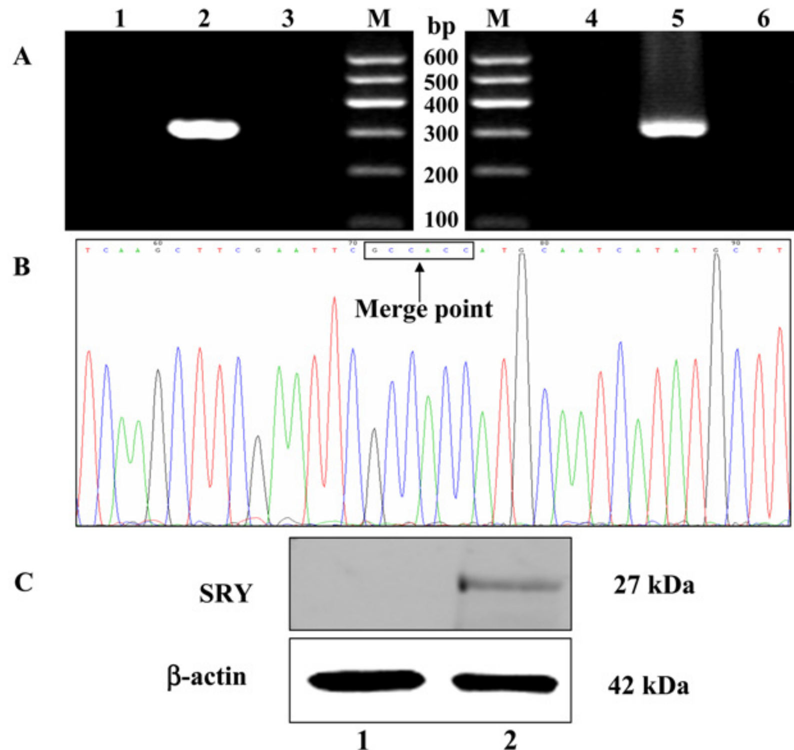

Figure 4 Secreted *SRY* gene-containing EVs (SRY-EVs) from SRY-HEK-293 cells
(**A**) CMV-*SRY* gene in SRY-HEK-293 cells and SRY-EVs was detected by qualitative PCR. The PCR products were analysed on a 2 % agarose gel pre-cast with ethidium bromide. Lane 1, HEK-293 cells; lane 2, SRY-HEK-293 cells; lane M, DNA marker (sizes in bp), lanes 3 and 6, negative controls (double-distilled water; lane 4, HEK-293 EVs; lane 5, SRY-HEK-293 EVs. (**B**) Sequencing result of the PCR product of *SRY* DNA in the SRY-HEK-293 cells and SRY-EVs. The sequence identity was more than 99.5 %. The black arrow indicates the merge point of the CMV DNA and *SRY* DNA. (**C**) SRY protein expression in SRY-HEK-293 cells was determined by immunoblotting. Molecular masses are indicated in kDa.

CD11-a. We found increased DNA-binding ability of SRY protein to the promoters of ICAM-1 and CD11-a in SRY-EV-treated cells compared with vehicle and control EV groups (Figure 5C). The results indicated that SRY-EVs, via the synthesis of *SRY* DNA-coding protein, enhanced the promoter activity of ICAM-1 in HUVECs and CD11-a in THP-1 cells.

To determine the pathophysiological significance of *SRY* DNA in EVs transferred between cells, we counted the number of monocytes (THP-1) adherent to HUVECs, incubated in the presence or absence of SRY-EVs. We found that the presence of SRY-EVs increased the number of THP-1 cells that adhered to HUVECs (Figure 6).

Effect of *SRY* DNA in EVs on atherosclerosis in ApoE$^{-/-}$ mice

We found that the size of atherosclerotic plaques in the aorta was increased in ApoE$^{-/-}$ mice injected with SRY-EVs relative to the control mice (Figure 7). Moreover, the area of lipid vacuoles (Oil Red O-staining) was also increased in the vascular wall of ApoE$^{-/-}$ mice treated with SRY-EVs (Figure 7). To elucidate the functional role of SRY on ICAM-1 and CD11-a *in vivo*, we measured, using ELISA, the levels of CD11-a in monocytes and ICAM-1 in the vessels of the mice. We found increased levels of CD11-a in monocytes (Figure 8A) and ICAM-1 in the vessels (Figure 8B) from ApoE$^{-/-}$ mice injected with SRY-EVs. These results indicated that *SRY* gene transferred by EVs can increase the formation of atherosclerotic plaques in ApoE$^{-/-}$ mice.

DISCUSSION

EVs can carry DNA, mRNA, miRNA and protein, providing a horizontal genetic information transfer between cells [12,27,28]. The packaged mRNAs or miRNAs in EVs can be delivered into target cells and modulate the biological functions of the recipient cells by regulating the expression of target genes [1,4,29]. As with mRNA, miRNA and protein, the packaged DNAs in EVs, secreted from maternal cells, are transportable and functional.

Our previous study showed that genomic DNA in EVs increased the coding mRNA and protein in the recipient cells [12]. We found the presence of AT_1R (angiotensin type 1 receptor) gene-coding region, 5′ promoter region and 3′ UTR in EVs from vascular smooth muscle cells. Actinomycin D, an inhibitor of DNA transcription, blocked the stimulatory effect of EVs on AT_1R mRNA expression in the recipient cells, indicating that the transcription of EV DNA occurred in the recipient cells. The DNA in EVs could be transferred into the recipient cells, localize

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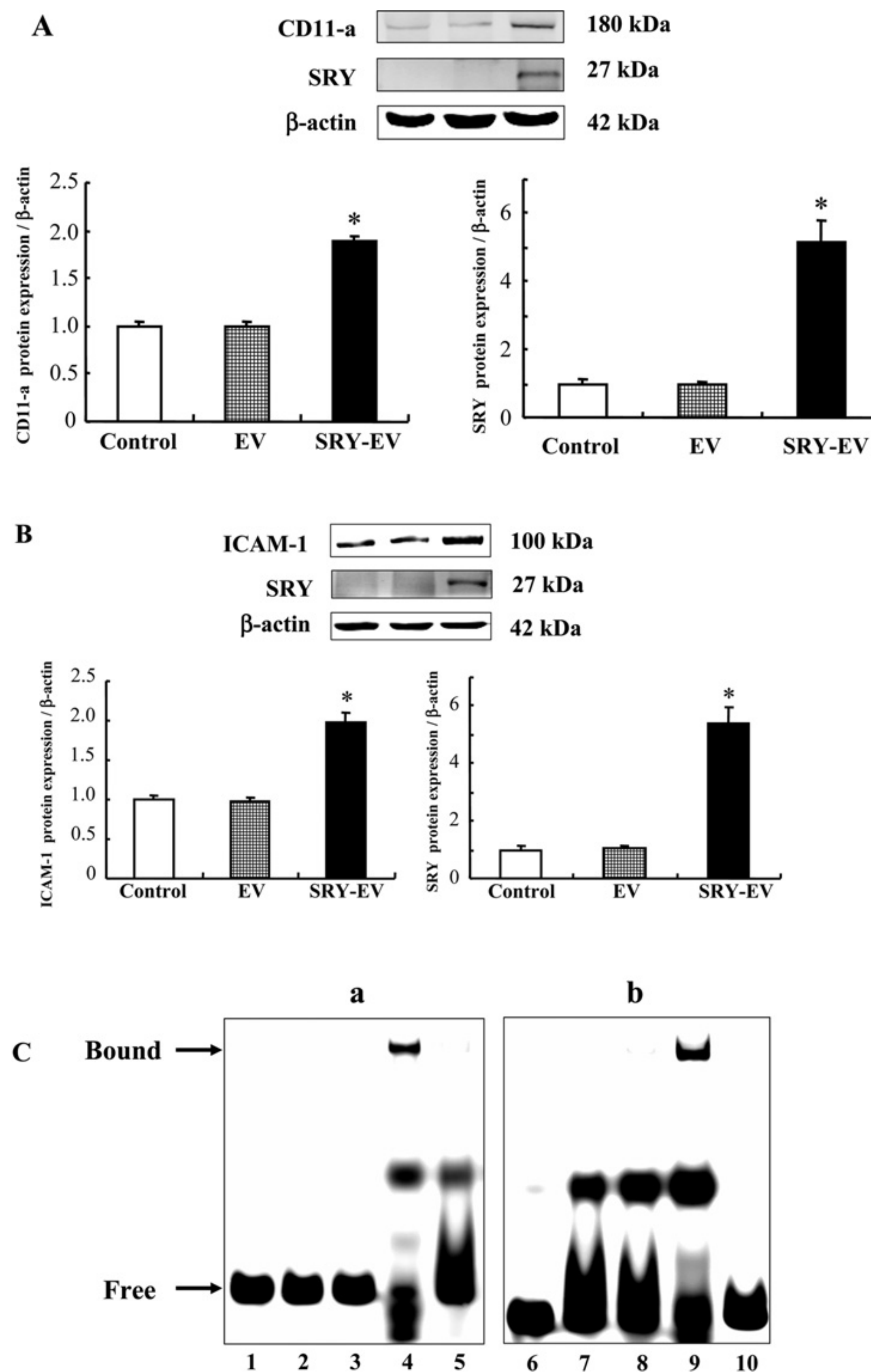

Figure 5 The effect of SRY-EVs on CD11-a in monocytes and ICAM-1 in endothelial cells
(**A** and **B**) Protein expression of CD11-a in THP-1 cells (**A**) and ICAM-1 in HUVECs (**B**) co-cultured with PBS (control), EVs from control HEK-293 cells without *SRY* DNA (EV) or *SRY* gene-containing EVs (SRY-EV). CD11-a and ICAM-1 protein expression were determined by immunoblotting ($n = 3$, $^{*}P < 0.05$ compared with others). Molecular masses are indicated in kDa. (**C**) DNA-binding ability of SRY protein to the promoters of CD11-a in THP-1 cells (a; lanes 1–5) and ICAM-1 in HUVECs (b; lanes 6–10) by EMSA. The DNA probe for the promoters of CD11-a (lane 1) or ICAM-1 (lane 6) without nuclear proteins incubation was set as a negative control. Nuclear proteins (4 μg) from the control group (lanes 2 and 7), EVs (lanes 3 and 8) and SRY-EVs (lanes 4 and 9) were incubated with the labelled DNA probes binding to SRY protein. The probes were competed with their respective unlabelled identical oligonucleotides (lanes 5 and 10). Arrows indicate the positions of bound protein–DNA complexes and free probe. The EMSA was performed in triplicate and the gel shown is representative of the three assays.

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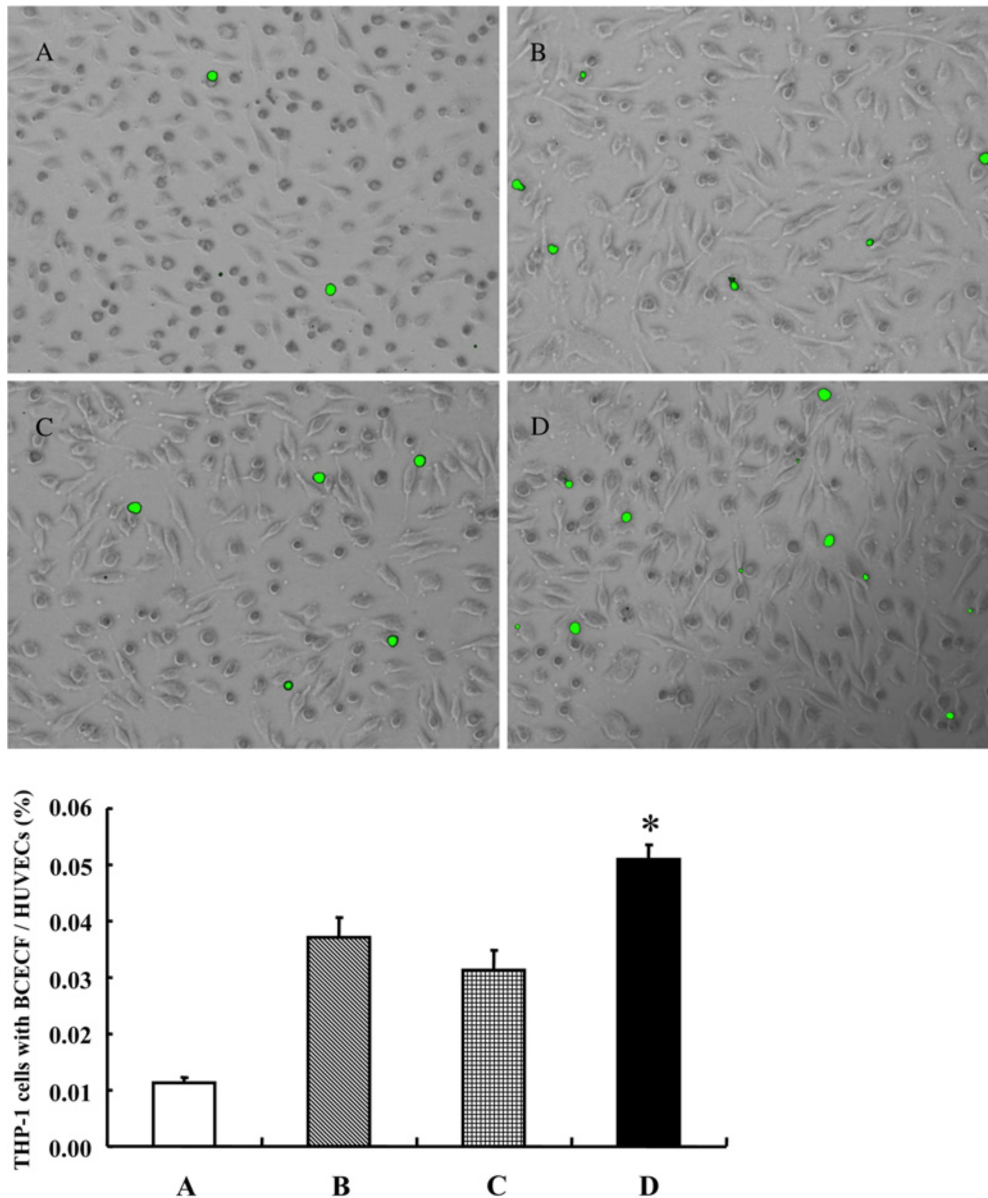

Figure 6 Adhesion of monocytes (THP-1) to HUVECs
THP-1 and/or HUVECs were incubated with SRY-EVs (10^5/ml) for 24 h. EVs from control HEK-293 cells without the *SRY* gene were used as control EVs. THP-1 was stained with BCECF, a green fluorochrome, and then used to determine adherence to HUVECs. Free THP-1 cells in the culture medium were washed three times with PBS. Fluorescence was measured at 485 nm excitation and 530 nm emission wavelengths, using a fluorescence microscope. (**A**) Both THP-1 and HUVECs were incubated with control EVs. (**B**) THP-1 was incubated with SRY-EVs; HUVECs were incubated with control EVs. (**C**) THP-1 was incubated with control EVs; HUVECs were incubated with SRY-EVs. (**D**) Both THP-1 and HUVECs were incubated with SRY-EVs. All groups were under the same conditions. We counted the number of THP-1 cells stained with BCECF in HUVECs in six visual fields, and then calculated the ratio of the number of THP-1 cells adhered to HUVECs and total HUVECs ($n = 6$, $*P < 0.05$ compared with others).

to and enter the nuclear membrane, and combine with transcription factor, providing direct evidence that the transferred gene can be transcribed in the recipient cells. However, it is possible that the endogenous DNA in recipient cells can be activated by some other substances in EVs. To overcome this limitation, we treated HEK-293 cells with EVs bearing AT_1R–EGFP DNA derived from AT_1R-HEK-293 cells, so that the endogenous AT_1R can be distinguished from the exogenous (i.e. transferred) AT_1R–EGFP DNA-transcribed receptor. Moreover, we chose the *BCR/ABL* hybrid gene, which is uniquely present in the CML cell line K562, but not endogenously present in the HEK-293 cells or neutrophils. With these studies, we provided direct evidence that EV genomic DNA can be transferred to and expressed in recipient cells [12].

We also reported that the transferred DNA from EVs has pathophysiological significance. Transferred *BCR/ABL* gene

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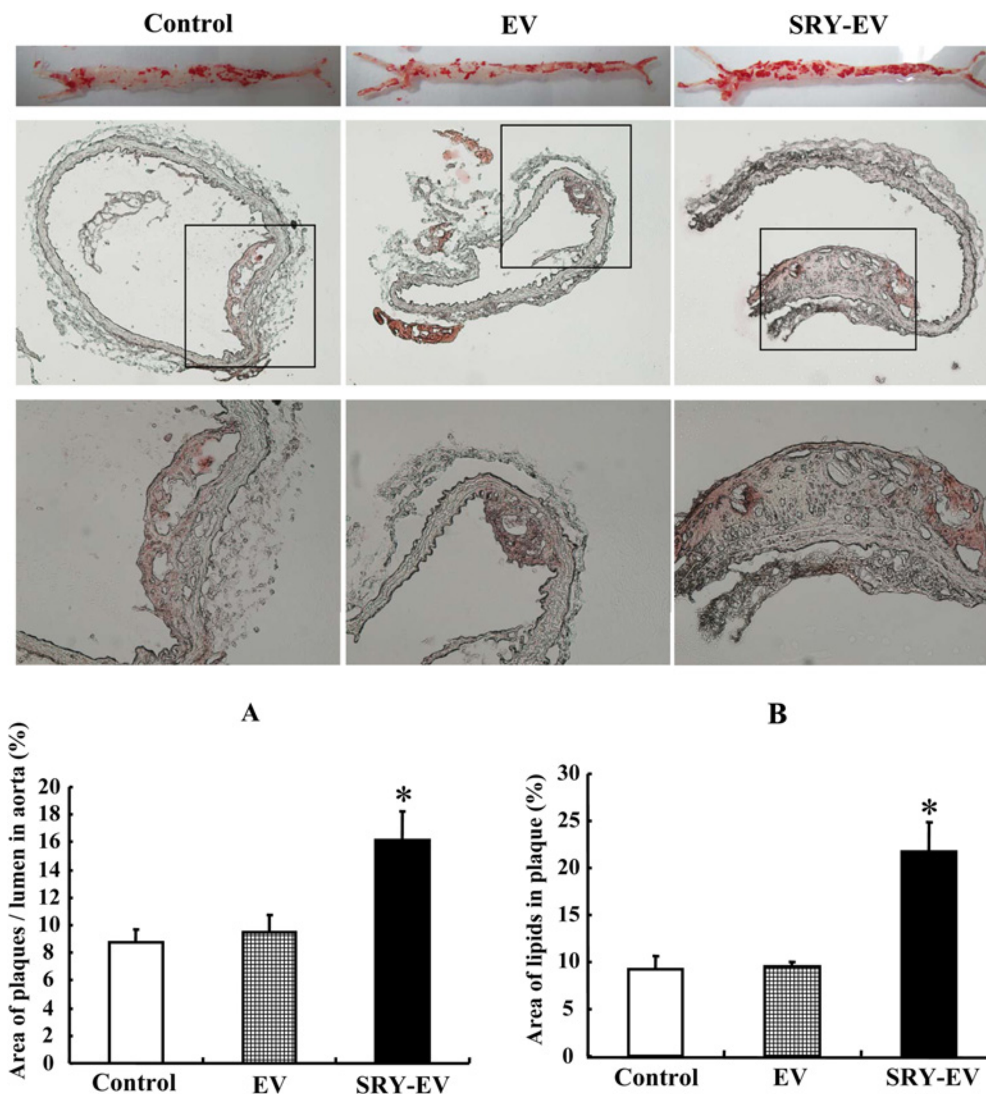

Figure 7 Effect of *SRY* DNA in EVs on the atherosclerosis in ApoE$^{-/-}$ mice
Male ApoE$^{-/-}$ mice were injected with PBS (200 μl), EVs from HEK-293 cells (2×10^6 EVs in 200 μl) or EVs from SRY-HEK-293 cells (2×10^6 EVs in 200 μl), via the tail-vein every 3 days for 2 months. The atherosclerotic plaques of the aorta from the mice were stained with Oil Red O. The top images are representative photographs of the aorta from the mice; the middle and bottom images were observed under a microscope using a 10×10 and a 10×20 lens respectively. We quantified the size (**A**) and lipid accumulation (**B**) of the atherosclerotic plaques in the aorta ($n = 3$, $^*P < 0.05$ compared with others).

from CML-derived EVs to normal neutrophils is functional not only *in vitro*, but also *in vivo*, causing CML in the recipient mice [12,14]. In this study, we found that EVs from male CAD patients had higher *SRY* GCNs than healthy male subjects. The *SRY* DNAs in EVs were expressed in the recipient cells, increased adherence factor levels in monocytes and endothelial cells, and enhanced the adherence between the two cells. Moreover, injection of SRY-EVs accelerated atherosclerosis in ApoE$^{-/-}$ mice.

Of all of the human chromosomes, the haploid Y chromosome contains the smallest number of genes. The main part of Y chromosome is transmitted intact from father to son and contains single or multicopy genes that encode approximately 27 distinct proteins [30,31]. The *SRY* gene in the Y chromosome imparts its role in testicular development and sexual differentiation. However, there are also data showing a relationship between SRY and cardiovascular diseases [17]. For example, SRY is responsible for the elevated blood pressure caused by activation of the renal sympathetic nervous and renin–angiotensin systems [15,16]. SRY has also been reported to be associated with the increase in blood pressure in peripubertal males, and circulating concentrations of total and LDL (low-density lipoprotein)- and HDL (high-density lipoprotein)-cholesterol, and paternal history of CAD in the general population [32–34]. There are studies indicating that SRY may be involved in the pathogenesis of atherosclerosis. Because SRY is a transcription factor, we studied the effect of SRY protein on adherent factor expression. EMSA showed that SRY

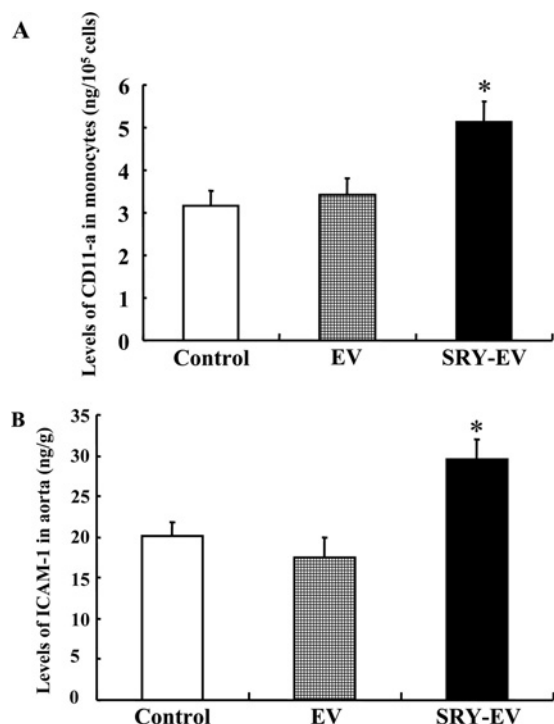

Figure 8 Levels of CD11-a in monocytes and ICAM-1 in the vessels from ApoE$^{-/-}$ mice

Male ApoE$^{-/-}$ mice were injected with PBS (200 μl) (control), EVs from HEK-293 cells (2×10^6 EVs in 200 μl) (EVs) or EVs from SRY-HEK-293 cells (2×10^6 EVs in 200 μl) (SRY-EVs), via the tail-vein every 3 days for 2 months. The levels of CD11-a in monocytes (**A**) and ICAM-1 in the vessels (**B**) from the mice were determined by ELISA ($n = 8$, $^*P < 0.05$ compared with others).

protein increased the promoter activities of CD11-a in monocytes and ICAM-1 in HUVECs. The SRY-induced increase in CD11-a and ICAM-1 expression has pathophysiological significance because the adherence between monocytes and vascular endothelial cells was also increased. Moreover, the plasma levels of CD11-a and ICAM-1 expression were also higher in SRY-EV-treated ApoE$^{-/-}$ mice, indicating involvement in the pathogenesis of atherosclerosis.

In conclusion, we have shown that EVs had higher *SRY* GCNs in male CAD patients than in healthy male subjects. The *SRY* DNA in EVs could be transferred to recipient cells and influence the function of the recipient cells by the newly synthesized SRY protein. The DNA-coding mRNA and protein levels increased adherence factor levels in monocytes and endothelial cells and their adherence, which are involved in the pathogenesis of CAD. Thus the present study provides a new approach to the understanding of inheritable CAD in men.

CLINICAL PERSPECTIVES

- Our previous studies showed the existence of DNAs in EVs that could be transferred from one cell to another by endocytosis or fusion. The transferred EV DNAs have the ability to influence the function of the recipient cells by increasing DNA-coding mRNA and protein levels. However, the role of EVs DNAs in the pathogenesis of atherosclerosis is not known.
- We have shown that the *SRY* DNA in EVs could be transferred to recipient cells and influence the adherence function of the recipient cells by the newly synthesized SRY protein.
- The *SRY* gene in plasma EVs transferred to vascular endothelial cells may play an important role in atherogenesis; this mechanism provides a new approach to the understanding of inheritable CAD in men.

AUTHOR CONTRIBUTION

The study was designed and conducted by Jin Cai, Weiwei Guan, Xiaorong Tan, Caiyu Chen, Liangpeng Li and Chunyu Zeng. Data analysis was performed by Jin Cai, Na Wang, Xue Zou, Faying Zhou, Jialiang Wang, Fang Pei, Xinjian Chen, Hao Luo, Xinquan Wang, Duofen He, Lin Zhou and Chunyu Zeng. The paper was written by Jin Cai, Lin Zhou, Pedro Jose and Chunyu Zeng.

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