

Bone Marrow–Derived MicroRNA-223 Works as an Endocrine Genetic Signal in Vascular Endothelial Cells and Participates in Vascular Injury From Kawasaki Disease

Maoping Chu, MD, PhD;* Rongzhou Wu, MD;* Shanshan Qin, MD; Wenfeng Hua, MD, PhD; Zhen Shan, MD, PhD; Xing Rong, MD; Jingjing Zeng, BS; Lanlan Hong, MS; Yeying Sun, PhD; Ying Liu, MD, PhD; Wen Li, MD, PhD; Shenming Wang, MD, PhD; Chunxiang Zhang, MD, PhD, FACC

Background—Kawasaki disease (KD) is now the most common cause of acquired cardiac disease in children due to permanent coronary artery damage with unknown etiology. The study sought to determine the role of blood microRNA miR-223 in KD and KD-induced injuries in vascular endothelial cells (ECs) as well as the mechanisms involved.

Methods and Results—MicroRNA profiles in serum from patients with KD and from healthy controls were assessed by microarray analysis. We noted that multiple serum microRNAs were aberrantly expressed in KD, among them miR-223, which was the most upregulated abundant serum microRNA. We found that bone marrow–derived blood cells (leukocytes and platelets) were able to secrete miR-223 into serum. Vascular ECs had no endogenous miR-223; however, the blood cell–secreted serum miR-223 could enter into the vascular ECs in the vascular walls. The exogenous miR-223 had strong biological effects on EC functions via its target genes such as IGF1R. Interestingly, KD-induced EC injuries were related to increased miR-223 because they were inhibited by miR-223 knockdown. Finally, these observations were verified using miR-223 knockout mice and the chimeric mice generated by transplantation of bone marrow from miR-223 knockout mice into wild-type mice.

Conclusions—In KD patients, the levels of blood cell–derived miR-223 in ECs are significantly increased. The increased miR-223 in ECs could work as a novel endocrine genetic signal and participate in vascular injury of KD. MiR-223 may provide a novel mechanism and a new therapeutic target for vascular complication of KD. (*J Am Heart Assoc.* 2017;6:e004878. DOI: 10.1161/JAHA.116.004878.)

Key Words: blood cells • endothelial cells • Kawasaki disease • microRNA • miR-223 • vascular inflammation

Kawasaki disease (KD) is an acute systemic vasculitis of unknown etiology that occurs predominantly in infants and young children, with vascular injury as the major cause of its morbidity and mortality.^{1,2} If not treated early with high-dose intravenous immunoglobulin, 1 in 5 children could develop coronary artery aneurysms. In KD, a series of inflammatory cellular events occur in blood, including

activation of innate and adaptive immune cells, red and white blood cells, and platelets, although the connections of these cellular events and vascular injury are unclear. Despite current optimal treatment, ≈5% to 15% of children with KD will develop permanent vascular damage. KD has become one of the main pediatric acquired heart diseases and may be an important risk factor for ischemic heart disease in adults

From the Children's Heart Center, The Second Affiliated Hospital & Yuying Children's Hospital, Institute of Cardiovascular Development and Translational Medicine, Wenzhou Medical University, Wenzhou, Zhejiang, China (M.C., R.W., W.H., X.R., J.Z., L.H., C.Z.); Division of Vascular Surgery and the Laboratory of General Surgery, First Affiliated Hospital, Sun Yat-sen University, Guangzhou, China (Z.S., W.L., S.W.); Department of Biomedical Engineering, School of Medicine, The University of Alabama at Birmingham, AL (S.Q., Y.S., Y.L., C.Z.).

*Dr Chu and Dr Wu contributed equally to this work and should be considered joint first authors.

Correspondence to: Chunxiang “Kevin” Zhang, MD, PhD, FACC, Department of Biomedical Engineering, School of Medicine, The University of Alabama at Birmingham, Volker Hall G094, 1670 University Blvd, Birmingham, AL 35294. E-mail: zcx@uab.edu or Maoping Chu, MD, PhD, Children's Heart Center, The Second Affiliated Hospital & Yuying Children's Hospital, Wenzhou Medical University, Wenzhou, Zhejiang 325027, China. E-mail: chmping@hotmail.com

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because of its permanent vascular complications. This situation highlights the importance and urgency of studying novel mechanisms and therapeutic targets of KD-induced vascular injuries.^{1,2}

MicroRNAs (miRNAs), which have strong biological functions in the cardiovascular system, are a class of endogenous small noncoding RNAs that directly regulate >30% of genes in a cell.^{3–5} Once thought to exist only within cells, recent studies by us and others have demonstrated that miRNAs can be exported from cells.^{6–8} Indeed, miRNAs are found in many body fluids including circulating blood and urine. Moreover, the extracellular miRNAs are stable because of binding with microparticles. Interestingly, these extracellular miRNAs may enter into other tissues and cells to serve as novel cell-to-cell communicators.⁹

The miRNA miR-223 is a hematopoietic lineage cell-specific miRNA. Previous studies have shown that miR-223 is expressed exclusively in hematopoietic cells in bone marrow and in bone marrow-derived blood cells, mainly in blood platelets and leukocytes and at a low level in red blood cells.¹⁰ Although vascular cells should not have endogenous miR-223 expression, a significant amount of miR-223 has been identified in normal vascular walls, as shown in our previous study.¹¹ More recently, we noted that miR-223 could be secreted into the circulating blood by bone marrow-derived blood cells such as platelets and leukocytes. The blood cell-secreted miR-223 could then enter into vascular smooth muscle cells (VSMCs) as a novel endocrine genetic signal to regulate their functions and atherogenesis via its target genes.¹² The sources of miR-223 in vascular ECs and its biological functions are still unclear. In addition, the roles of blood miR-223 in KD and KD-induced vascular endothelial cell (EC) injuries are currently completely unknown.

Methods

Cell Isolation and Culture

Vascular ECs from the aortas of C57BL/6 mice, miR-223 knockout mice, and the chimeric mice generated from them were cultured with M199 containing 10% FBS. Human coronary arterial ECs were from the American Type Culture Collection (ATCC). All ECs were verified by the expression of endothelium-specific markers such as vascular endothelial cadherin and CD31, and >90% were ECs, as described in our recent study.¹³ For the platelet study, mouse (C57BL/6) platelets were isolated using platelet-rich plasma and the gel-filtration method.¹⁴ For the leukocyte study, mouse monocytes from blood were used. Mouse monocytes were isolated, as described previously.¹⁵ In addition, cells from the human monocytic cell line THP-1 (from ATCC) were also used.

RNA Isolation and Quantitative Reverse Transcriptase–Polymerase Chain Reaction Assay

RNAs were isolated with TRIzol.^{6,7,11,12} Levels of miRNAs and mRNAs were determined by quantitative reverse transcriptase–polymerase chain reaction (qRT-PCR). For miRNA, cDNA was generated from 100 ng of total RNA using TaqMan miRNA reverse transcription and TaqMan miRNA assays (Life Technologies). For other RNAs, cDNA was generated from 200 ng of total RNA using the High-Capacity RNA-to-cDNA Kit (Life Technologies). The qRT-PCRs for both miRNA and mRNA were performed on cDNAs using TaqMan Fast Universal PCR Master Mix (2X), no AmpErase UNG (Life Technologies), according to the manufacturer's instructions. Amplification and detection of specific products were performed with a Life Technologies 480 ViiA 7 Detection System. As an internal control of cells and tissues, U6 was used for miRNA template normalization and GAPDH was used for other template normalizations. For serum or medium samples, the internal control was either volume or a spiked-in control (cel-miR-39). Fluorescent signals were normalized to an internal reference, and the threshold cycle was set within the exponential phase of the PCR. Relative gene expression was calculated by comparing cycle times for each target PCR.

Expression Profiles of miRNAs

Human miRNA profiling was performed using the miRCURY LNA Universal RT miRNA PCR system and ready-to-use human panel, which included 384 human miRNAs (Exiqon).

Gene Modulation in Cultured ECs

The expression of miR-223 was downregulated by its inhibitor and upregulated via its mimics or adenovirus-expressing miR-223. In brief, cells were transfected using a transfection reagent (Qiagen) 24 hours after seeding into the wells. Transfection complexes were prepared according to the manufacturer's instructions. The expression of miR-223 was downregulated by its inhibitor (miR-223 Inhibitor [Life Technologies]; 30 nmol/L) and was upregulated via its mimics (mirVana miRNA mimic [Life Technologies]; 10 nmol/L). The transfection medium was replaced 4 hours after transfection by the regular cell-free culture medium for 20 hours before treatments. Vehicle and scramble controls (Integrated DNA Technologies) were applied.

Monitoring the Secretion of miR-223 by Leukocytes and its Ability to Enter Into ECs

Culture medium was collected before and after 12 hours of THP-1 macrophage culture using the method described in our

previous study.⁶ In brief, the medium was centrifuged at 1600g for 15 minutes at 4°C. This technique removed all cells, but the microparticles or exosomes remained; removal of these smaller entities requires centrifuging at a higher speed for a longer time, as we showed previously.^{6,7,12} The levels of miR-223 in collected medium were determined and were increased with the increased THP-1 macrophages.⁶ The medium collected after 12 hours of THP-1 macrophage culture was added into the cultured medium of ECs. After 12 hours, levels of miR-223 in ECs were determined.

Western Blot Analysis

Proteins were isolated from cultured ECs, and vessels were determined by Western blot analysis using antibodies. IGF1R antibody was from Cell Signaling Technology. GAPDH antibody (1:2000 dilution; Cell Signaling Technology) was used as a loading control.

Cell Proliferation and Apoptosis

EC proliferation was induced by platelet-derived growth factor (20 ng/mL) and was determined by MTT assay (Roche) and 5-ethynyl-2'-deoxyuridine assay (EdU kit; RiboBio).¹⁶ EC apoptosis in cultured cells was induced by H₂O₂ (100 μmol/L) for 24 hours and measured by terminal deoxynucleotidyl transferase dUTP nick end labeling assay (Roche).¹⁶

Luciferase Assay

The reporter plasmid, a firefly luciferase reporter construct (psiCHECK-2; Promega), was inserted in a fragment of the 3' untranslated region of IGF1R mRNA containing the putative miR-223 binding sequence. The construct with the mutated fragment of the 3' untranslated region of IGF1R mRNA without the putative miR-223 binding sequences was used as the mutated control. ECs were transfected with the construct or the mutated control construct. These ECs were then treated with vehicle, scramble control, or miR-223 mimics (multiplicity of infection 30). Cell extract was isolated to measure the luciferase expression on a scintillation counter using a dual luciferase reporter system.

Animals

The male C57BL/6 mice and miR-223 knockout mice were from the Jackson Laboratory (Bar Harbor, ME). The chimeric mice were generated in our laboratory by transplantation of bone marrow from miR-223 knockout mice into lethally irradiated wild-type mice, as described previously.¹⁴ The well-established leukocyte depletion model in male wild-type C57BL/6 mice (aged 3 months) was applied in some mice, as

described previously, in which neutrophils in blood were depleted by vinblastine (2.5 mg/kg IP).¹² Platelets in blood were depleted by anti-mouse thrombocyte serum (50 μL IP).¹² All animal protocols were approved by the institutional animal care and use committee and were consistent with the Guide for the Care and Use of Laboratory Animals (2011 version; National Institutes of Health).

Human Serum Samples and Blood Cell Data

This study was approved by the by the institutional review committee of the Second Affiliated Hospital and Yuying Children's Hospital, and the participants gave informed consent, which conformed with the Declaration of Helsinki. Human serum samples were from age- and sex-matched healthy control participants (n=103) and from patients with KD (n=78). Among the KD patients, 12 had coronary artery lesions. In addition, 22 serum samples from patients with KD after treatment with high-dose intravenous immunoglobulin were also collected. In addition, total white blood cell and circulating platelet counts for all participants were obtained from clinical data.

The serum samples were prepared as described in our previous studies.⁶ In brief, peripheral venous blood was collected and was placed for 1 hour at room temperature (26°C). The blood samples were centrifuged at 1600g for 15 minutes at 4°C. All residual blood cells were removed via centrifugation before storage. Serum samples were then carefully transferred into plain propylene tubes and stored at -80°C until miRNA isolation. Serum miRNAs were isolated in 200 μL serum using the solution miRNAs Isolation Kit (RNA Bioscience), according to the kit procedures. In addition to volume control (200 μL serum), we used an exogenous spiked-in control probe (cel-miR-39).

Statistics

The blood cell count data were presented as mean±SD. All other data were presented as mean±SE. For relative gene expression, the mean value of the vehicle control group is defined as 100% or 1. Linear regression analysis was used to determine the relationship between serum miR-223 levels and total white blood cell counts or circulating platelet counts. Two-tailed unpaired Student *t* tests and ANOVAs were used for statistical evaluation of the data. SPSS 17.0 (IBM Corp) was used for data analysis. *P*<0.05 was considered significant.

Results

Serum miRNAs Are Aberrantly Expressed in KD; Among Them, miR-223 Is the Most Upregulated Serum miRNA

Compared with serum from healthy controls, multiple miRNAs were aberrantly expressed in serum from patients with KD.

Table. Top 10 Differentially Expressed Serum microRNAs in Patients With Kawasaki Disease

microRNA	Fold Change	P Value
miR-223	113.9211	2.09E-05
miR-197	50.76238	4.41E-05
miR-27b	97.02541	4.98E-05
miR-125a-5p	10.17754	5.59E-05
hsa-miR-27a	84.45239	5.73E-05
miR-142-5p	126.1471	9.59E-05
miR-186	65.01371	0.000109
miR-23a	64.81441	0.000115
miR-28-3p	33.27843	0.000125
miR-342-3p	16.31398	0.000191

The 10 most deregulated abundant serum miRNAs in KD patients are listed in Table. Among them, miR-223 was the most upregulated serum miRNA in patients with KD. The aberrant expression of miR-223 in KD serum was verified by qRT-PCR, as shown in Figure 1A. The total white blood cell count in KD patients was $(16.82 \pm 6.33) \times 10^9/L$, much higher than that of the healthy control group $([8.07 \pm 2.16] \times 10^9/L; P < 0.001)$. Similarly, the circulating platelet count of KD patients $([416.17 \pm 118.66] \times 10^9/L)$ was higher than that of the healthy control group $([329.58 \pm 61.96] \times 10^9/L; P < 0.01)$. Positive correlations were demonstrated between serum miR-223 and total white blood cell count ($r = 0.85; P < 0.01$) and total platelet count ($r = 0.76; P < 0.05$).

In addition, the high level of miR-223 in KD serum was significantly decreased after treatment with immunoglobulin (Figure 1B). Moreover, the serum miR-223 levels of 12 KD patients with coronary artery lesions were remarkably higher than those of 12 age- and sex-matched KD patients without this critical vascular injury (Figure 1C).

Vascular EC miR-223 Comes From Bone Marrow-Derived, Blood Cell-Released Serum miR-223

As shown in Figure 2A, it was difficult to find miR-223 in passaged ECs cultured with serum-free medium by qRT-PCR (FBS has a high level of miR-223). The threshold cycle level was very close to water (H₂O; the negative control, which still had a peak and a threshold cycle level when we measured the miR-223 level by qRT-PCR). To further confirm that there was no endogenous miR-223 in passaged ECs, we added 3 positive controls: miR-34a and miR-222, which are known miRNAs expressed in ECs, and human monocyte line THP-1 cells, which have a high level of endogenous miR-223. As shown in Figure 1A, compared with miR-34a in ECs, miR-223

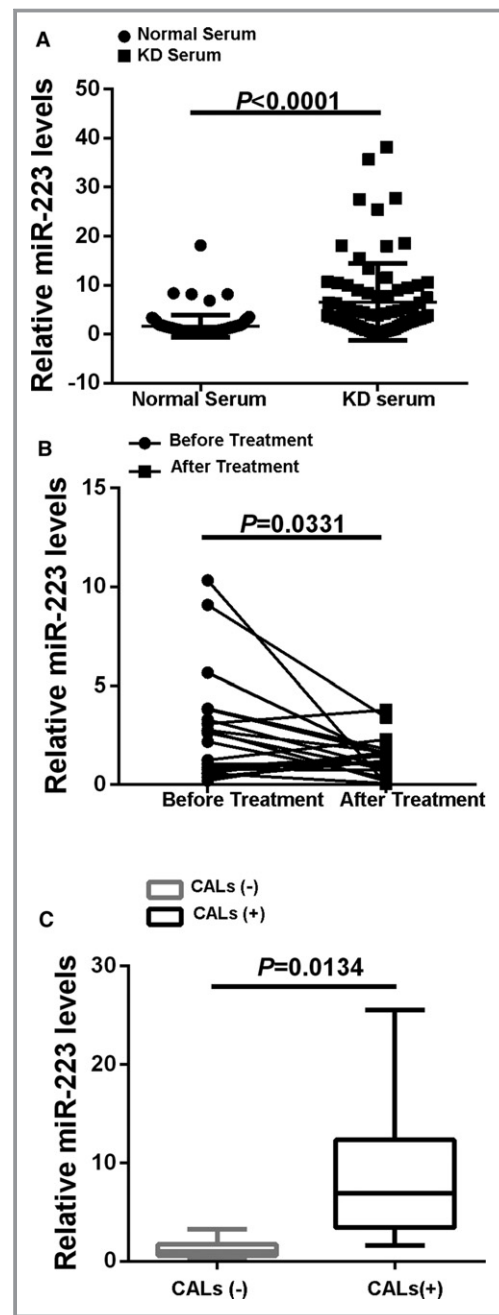


Figure 1. Serum microRNA miR-223 levels are increased in patients with Kawasaki disease (KD). A, Serum levels of miR-223 in 78 KD patients and 103 healthy control participants. B, Changes in serum miR-223 levels in 22 KD patients before and after treatment with high-dose intravenous immunoglobulin. C, Serum levels of miR-223 in 12 KD patients with coronary artery lesions (CALs) and in 12 sex- and age-matched KD patients without CALs.

was almost undetectable, although the expression level of miR-34a in ECs was much lower than miR-223 in THP-1 cells after normalization by their individual U6 expression. The

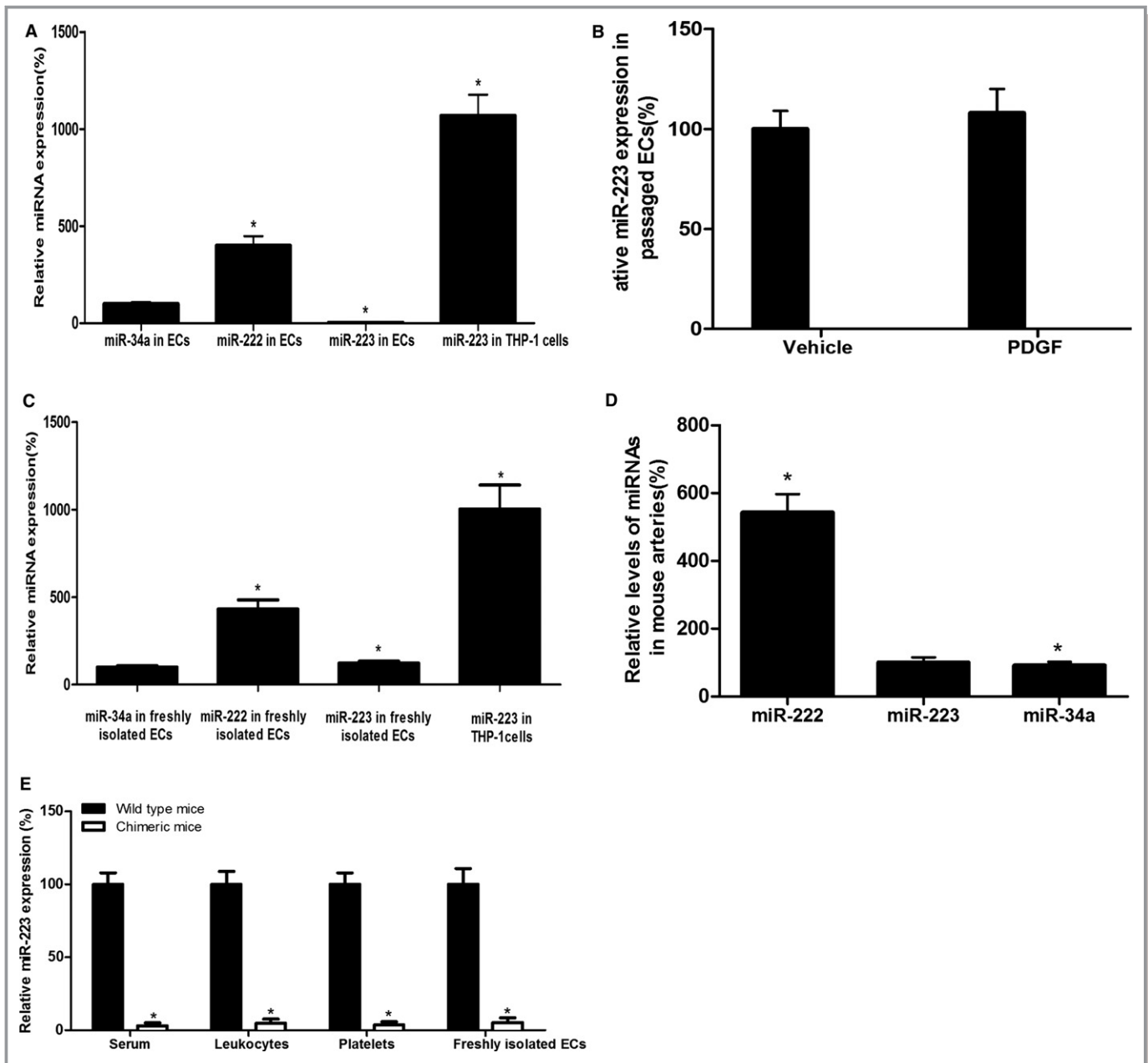


Figure 2. Vascular endothelial cell (EC) microRNA miR-223 is from bone marrow–derived, blood cell–released serum miR-223. A, No expression was found in passaged vascular ECs cultured in serum-free medium. In this experiment, 2 known EC-expressed microRNAs, miR-34a and miR-222, were used as positive controls. Another positive control was the human monocyte line THP-1 cells, which have high levels of endogenous miR-223. Water (H₂O) was used a negative control (its mean threshold cycle level was similar to miR-223). The mean level of miR-34a in ECs (normalized by its U6) was expressed as 100%. The relative levels of miR-222, miR-223 in ECs, and miR-223 in THP-1 cells (normalized by their U6) are shown. Note: n=6; *P<0.05 compared with miR-34a levels. B, Platelet-derived growth factor (PDGF; 20 ng/mL) stimulation does not induce any miR-223 expression in passaged ECs (normalized by its U6) compared with vehicle-treated control, for which the level of miR-223 was expressed as 100% (P>0.05). C, A significant amount of miR-223 was found in freshly isolated ECs (n=6). *P<0.05 compared with the miR-34a levels. D, A significant amount of miR-223 was found in healthy vascular walls. Two well-known miRNAs in vascular walls, miR-222, and miR-34a, were used as positive controls. The mean level of miR-223 in vascular walls was expressed as 100%. Note: n=6; *P<0.05 compared with the level of miR-223. E, miR-223 levels in serum (normalized by spiked-in cel-miR-39), blood cells (platelets, leukocytes; normalized by U6), and freshly isolated aortic ECs (normalized by U6) were almost undetectable in chimeric mice. The mean level of miR-223 in serum, blood cells (platelets, leukocytes), and freshly isolated aortic ECs from wild-type mice were expressed as 100%. Note: n=6; *P<0.05 compared with wild-type control mice.

negative result of miR-223 in passaged ECs was not related to their growth status, as platelet-derived growth factor stimulation did not change the levels of miR-223 in serum-free cultured ECs (Figure 2B). However, a significant amount of miR-223 was identified in freshly isolated ECs and in normal vascular walls (Figure 2C and 2D). To confirm the discovery, we compared the levels of miR-223, miR-222 (an abundant miRNA in ECs and vessels), and miR-34a (another well-studied miRNA in ECs and vessels) in mouse artery vessels. We found that in normal vascular walls, levels of miR-223 were lower than the abundant miR-222 but were similar to levels of miR-34a (Figure 2D).

Our recent study demonstrated that miR-223 is the most abundant miRNA in serum from blood cells (platelets, leukocytes).¹⁰ To further define the sources of miR-223 in blood, we produced the chimeric mice by transplantation of bone marrow from miR-223 knockout mice into wild-type mice. As shown in Figure 2E, miR-223 in serum, platelets, leukocytes, and freshly isolated aortic ECs from these chimeric mice was almost undetectable. The result suggested that serum miR-223 was mainly from bone marrow-derived blood cells.

Blood Cells Are Able to Secrete miR-223 Into the Extracellular Space and Thus Into ECs

To test whether the blood cells could secrete miR-223 into the extracellular space, THP-1 macrophages were seeded into cell culture wells with different cell numbers but with a fixed amount of serum-free medium. At 12 hours after culture, the culture medium was collected and miR-223 levels in medium were determined. No miR-223 was found in the medium without THP-1 macrophages; however, a significant amount of miR-223 was identified in THP-1 macrophage-cultured medium, as described previously.⁶ We then applied the culture medium from THP-1 macrophages as the culture medium of ECs. At 12 hours after culture, the ECs were collected to determine the levels of miR-223 inside ECs. As shown in Figure 3A, the blood cell-secreted miR-223 could enter into ECs, as demonstrated by the increased levels of miR-223 in these ECs cultured with miR-223-containing medium.

Bone Marrow–Derived, Blood Cell–Released Serum miR-223 Could Enter ECs and Vascular Walls Both In Vitro and In Vivo

To test whether serum miR-223 could enter ECs, fresh serum isolated from mice was added into culture medium (at 10%) of ECs for 24 hours. Then the cells were washed with serum-free medium and kept in serum-free medium for another 24 hours.

The result revealed that the serum miR-223 could enter ECs (Figure 3B).

To verify the blood cells as the major sources of miR-223 in serum and the ability of miR-223 to move into the vascular walls, we applied the mouse models of leukocyte (neutrophil) depletion. As expected, the leukocytes in blood were successfully depleted by their special antibodies (vinblastine), shown by the decreased numbers of these blood cells in mice.¹² At 7 days after depletion, the serum, ECs from aortas, and the aortas of these mice were collected to measure the levels of miR-223. As shown in Figure 3C, the levels of miR-223 in serum, vascular ECs, and aortas were significantly decreased in mice with the depletion of leukocytes. Similarly, platelets in blood were depleted by antimouse thrombocyte serum.¹² The levels of miR-223 in serum vascular ECs and aortas were also decreased in mice with depletion of platelets (Figure 3D). The results suggested that blood cells were indeed the major sources of miR-223 in serum, vascular ECs, and vascular walls.

Finally, the levels of miR-223 in aortas from the chimeric mice were determined. As shown in Figure 3E, only a very low level of miR-223 was identified in these vessels from the chimeric mice, consistent with the results determined in serum and vascular ECs from these special mice (Figure 2E).

Exogenous miR-223 Has Strong Biological Effects on ECs

The effects of miR-223 on proliferation and apoptosis of ECs were determined using our well-established cell models. Because ECs have no or negligible endogenous miR-223, we could use only the gain-of-function approach to test them. To increase the levels of miR-223, miR-223 mimic (10 nmol/L) was used (the miR-223 level after miR-223 mimics in ECs was slightly higher than that in KD serum-treated ECs). We found that miR-223 had a strong negative effect on EC proliferation, as determined by cell counting (Figure 4A), MTT (Figure 4B), and EdU assay (Figure 4C). In contrast, apoptosis of ECs was significantly increased by miR-223 overexpression (Figure 4D).

Levels of miR-223 in KD Serum-Cultured Human Vascular ECs Are Significantly Increased and Have a Role in KD-Induced EC Injury

In this experiment, human coronary arterial ECs were cultured with 20% serum either from KD patients or healthy controls. As shown in Figure 5A, the levels of miR-223 in KD serum-cultured human vascular ECs were significantly increased, which could be inhibited by miR-223 inhibitor (30 nmol/L). The increased miR-223 was from serum and thus exogenous

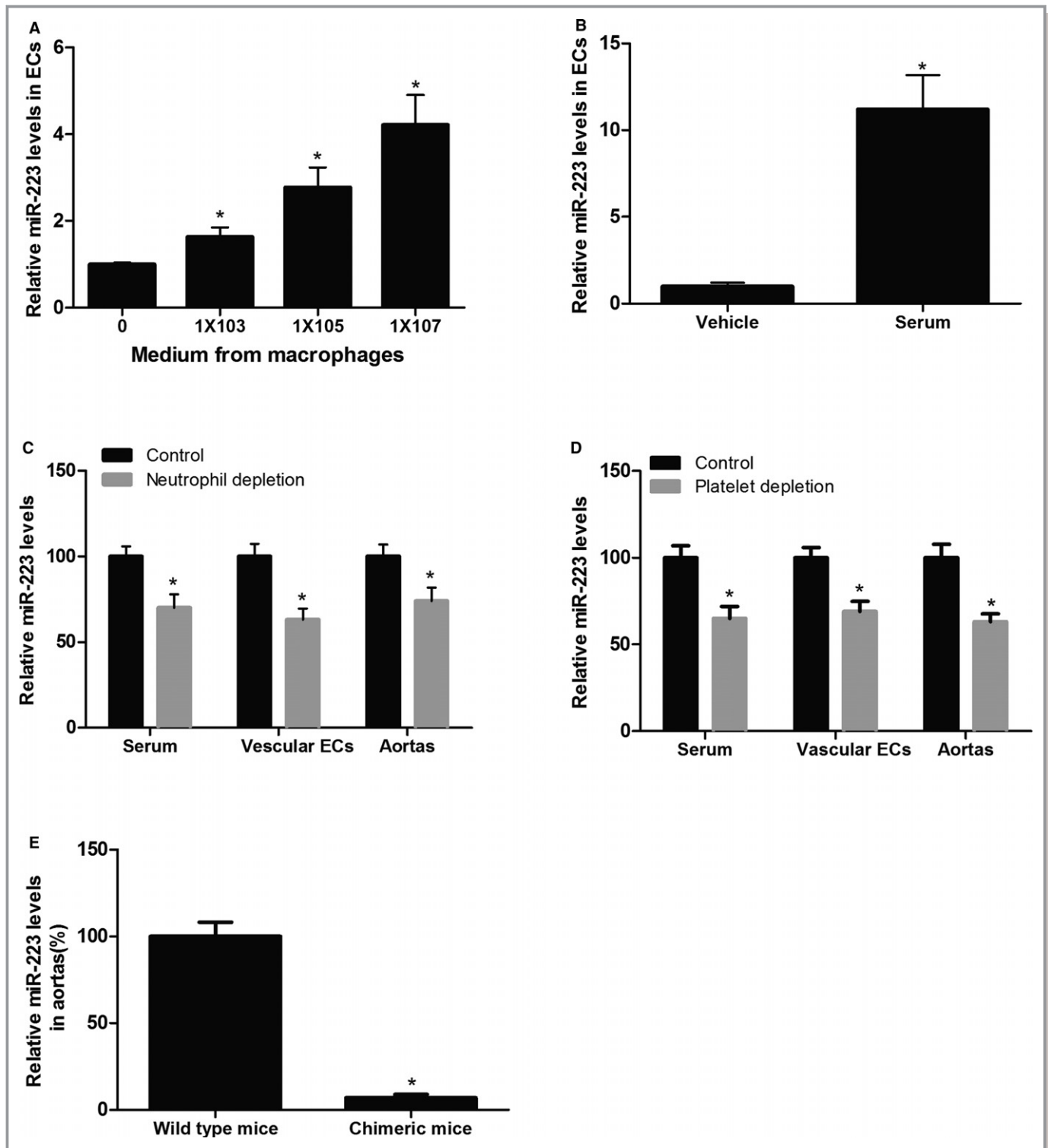


Figure 3. Blood cell–secreted microRNA miR-223 in extracellular space, vascular endothelial cells (ECs), and vascular walls. A, Culture medium of THP-1 macrophages was added into culture medium of ECs. The miR-223 levels in ECs were increased with miR-223–containing THP-1 macrophage medium. B, MiR-223 levels were higher in the group in which fresh serum isolated from mice was added into a culture medium (at 10%) of ECs for 24 hours followed by washing and then kept in serum-free medium for another 24 hours. C, The levels of miR-223 in serum, vascular ECs, and aortas were decreased in mice with neutrophil depletion. D, The levels of miR-223 in serum (normalized by spiked-in cel-miR-39), vascular ECs, and aortas (normalized by U6) were decreased in mice with platelet depletion. E, Only a very low level of miR-223 was identified in aortas from the chimeric mice compared with wild-type control mice (normalized by U6). Note: n=6 to 8; *P<0.05 compared with control groups.

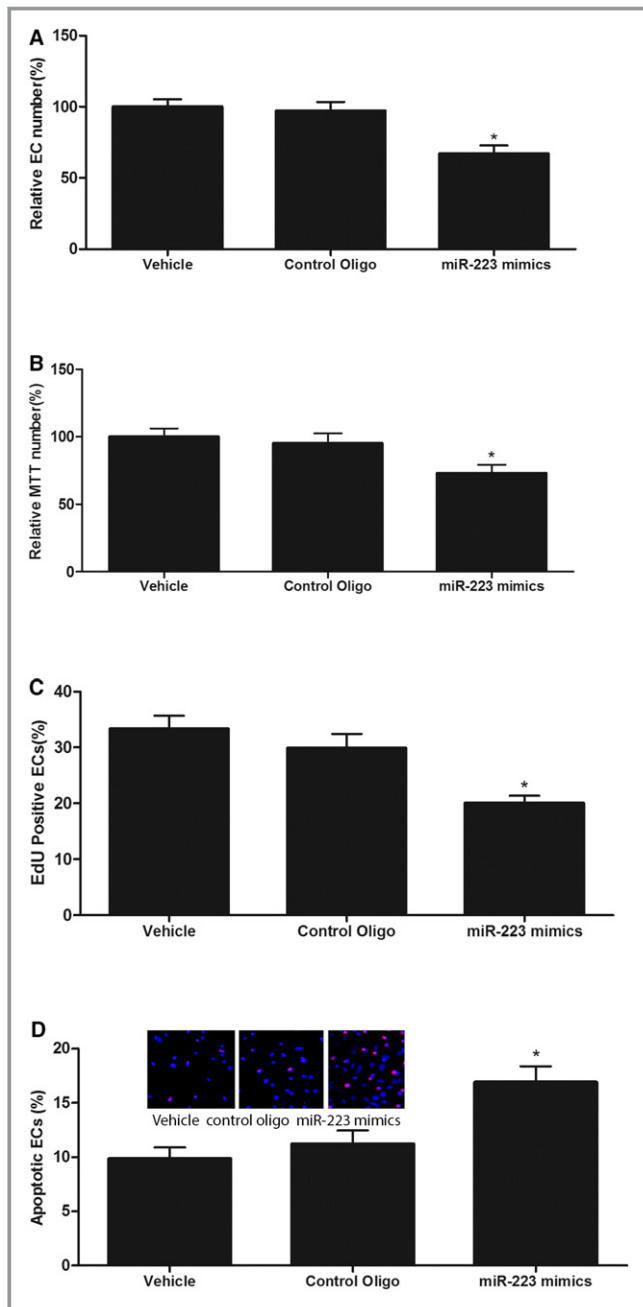


Figure 4. Cellular functions of microRNA miR-223 in endothelial cells (ECs). The effect of miR-223 on platelet-derived growth factor–induced EC proliferation determined by (A) cell counting, (B) MTT assay, and (C) 5-ethynyl-2'-deoxyuridine (EdU) assay. D, The effect of miR-223 on vascular smooth muscle cell apoptosis induced by H_2O_2 (100 μ mol/L) and representative images determined by terminal deoxynucleotidyl transferase dUTP nick end labeling analysis. Note: $n=6$; $*P<0.05$ compared with control groups. Oligo indicates Oligonucleotides.

because it was mature miR-223, and no endogenous primary miR-223 could be detected in these ECs. The increased exogenous miR-223 participated in KD-induced EC injury. Indeed, as shown in Figure 5B and 5C, KD serum could induce EC injury, as shown by increased EC apoptosis. KD-induced

EC apoptosis, however, was significantly inhibited by miR-223 inhibitor.

IGF1R Is a Signaling Pathway Involved in miR-223–Mediated Effects on ECs

Computational analysis suggested that human IGF1R, a key antiapoptosis gene in ECs, has a miR-223 binding site in its 3' untranslated region that is conserved among mouse, rat, and human.^{12,17} IGF1R is thus a potential direct target gene of miR-223. To test whether miR-223 is able to bind directly to IGF1R and inhibit its expression, a construct in which a fragment of the 3' untranslated region of IGF1R mRNA containing the conserved miR-223 binding sequence was cloned into a firefly luciferase reporter construct and transfected into HEK 293 cells. We identified in our recent study that plasmid-expressing miR-223, but not the empty plasmid pDNR-CMV, inhibited luciferase activity.¹² In the mutated control groups, the inhibitory effect of plasmid-expressing miR-223 disappeared.¹² We then performed the luciferase assay in ECs by using 223 mimics (30 nmol/L), as described in our previous study.¹⁶ The results revealed that 223 mimics, but not scramble control (control oligo) or vehicle, inhibited luciferase activity.¹² In the mutated control groups, the inhibitory effect of miR-223 mimics disappeared (Figure 6A). The results suggested that miR-223 was able to bind to IGF1R directly and inhibit its expression in ECs. The target gene of a miRNA may be cell specific. To test whether the endogenous IGF1R is a true target gene of miR-223 in vascular ECs, miR-223 mimic was transfected into ECs, and the expression of IGF1R was determined at both protein and mRNA levels. Indeed, the expression of IGF1R was downregulated in miR-223–overexpressed ECs (Figure 6B–6D). In addition, in KD serum–treated ECs, the IGF1R level was also decreased at both protein and mRNA levels, which could be blocked by miR-223 inhibitor (30 nmol/L) (Figure 6E and 6F). To further confirm that the exogenous miR-223 in KD serum could enter ECs and affect its target genes, we determined another miR-223 known target gene, F-box/WD repeat-containing protein 7 (FBXW7), by qRT-PCR.¹⁸ As shown in Figure 6G, we found that KD serum could also decrease the expression of FBXW7 in ECs. To confirm that miRNAs in KD serum could enter into ECs, we determined the expression of semaphorin 6A (SEMA6A), which is a known target of miR-27a.¹⁹ miR-27a was also increased in KD serum (Table). As shown in Figure 6H, the expression of SEMA6A was decreased after treatment with KD serum. Finally, IGF1R as a target gene of miR-223 in vascular ECs was verified using knockout mice. As shown in Figure 6I through 6K, the expression of IGF1R in freshly isolated vascular ECs from miR-223 knockout mice was higher than in those from wild-type control mice.

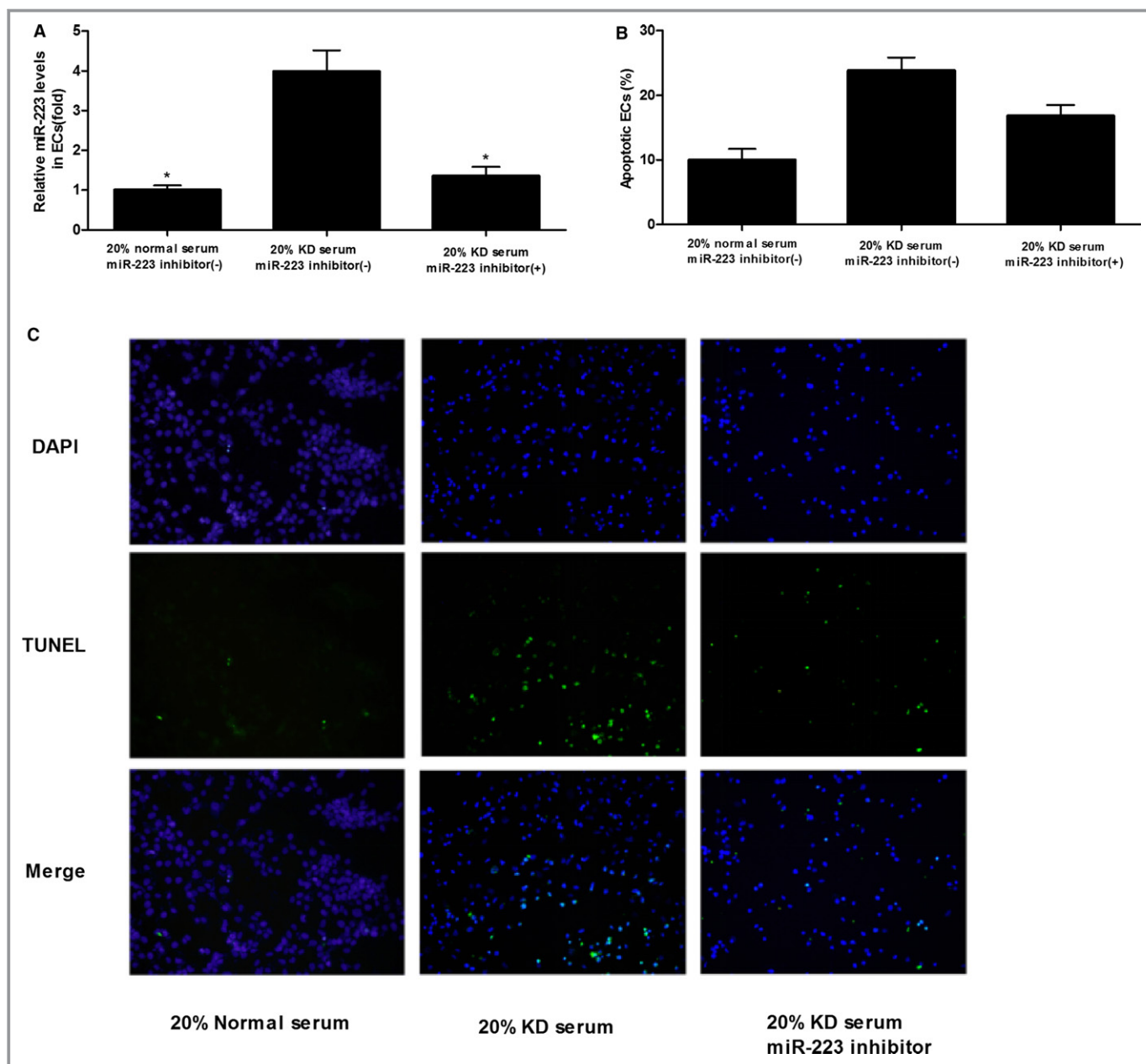


Figure 5. The effect of microRNA miR-223 on endothelial cell (EC) injury induced by Kawasaki disease (KD). A, The levels of miR-223 in KD serum–cultured human vascular ECs are significantly increased in cultured human arterial ECs and could be inhibited by miR-223 inhibitor (30 nmol/L). B, EC apoptosis was induced by KD serum and inhibited by miR-223 inhibitor (30 nmol/L). C, Representative terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) images of different groups. Note: n=6; *P<0.05 compared with 20% KD serum without an miR-223 inhibitor group.

Discussion

KD has become one of the main pediatric acquired heart diseases and may be an important risk factor for ischemic heart disease in adults, given its vascular injury complication. Because of our very limited knowledge about the real cause of vascular injury in KD, there is still a lack of effective therapy. Studying the novel mechanisms and new therapeutic targets of KD-induced vascular injuries is now an important task for both vascular biologists and clinical cardiologists.

It is well established that KD accompanies a series of inflammatory cellular responses in blood, including blood cell activation and release of their contents into circulating blood. The biological roles of these released cell-free contents in KD and KD-induced vascular injuries are still unclear.

The miRNAs, which are a class of noncoding RNAs, have strong biological functions in the cardiovascular system. Interestingly, blood has a large number of cell-free miRNAs that not only could be used as novel biomarkers for human

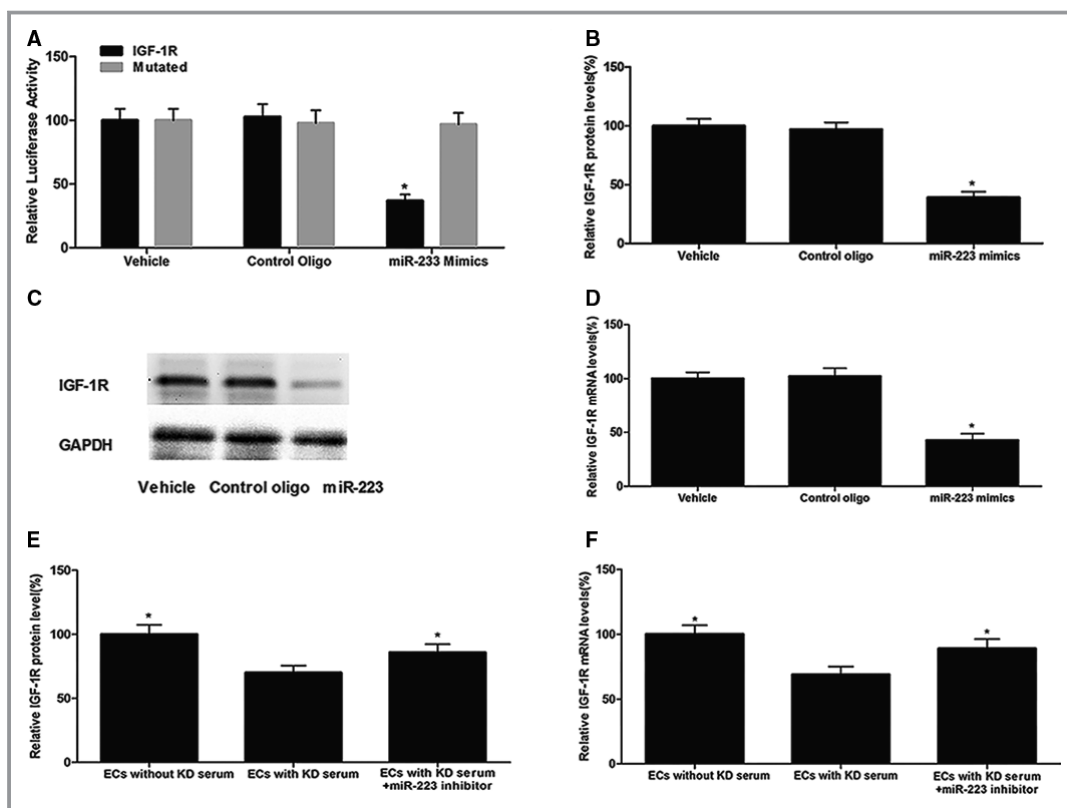


Figure 6. IGF1R is a direct target gene of microRNA miR-223 in endothelial cells (ECs), and Kawasaki disease (KD) serum could affect its target gene expression in ECs. A, miR-223, but not scramble control (control oligo) or vehicle, inhibited luciferase activity in ECs. In the mutated control groups, the inhibitory effect of miR-223 mimics disappeared. B, Overexpression of miR-223 decreased the expression of IGF1R at the protein level in ECs. C, Representative Western blots showing the IGF1R protein levels from different groups. D, Overexpression of miR-223 decreased the expression of IGF1R at the mRNA level in ECs. E, In KD serum–treated ECs, the IGF1R protein level was decreased; that effect could be partially blocked by miR-223 inhibitor (50 nmol/L). F, In KD serum–treated ECs, the IGF1R mRNA level was decreased; that effect could be partially blocked by miR-223 inhibitor (50 nmol/L). G, KD serum decreased the expression of FBXW7 in ECs. H, KD serum decreased the expression of SEMA6A in ECs. I, IGF1R protein levels in freshly isolated vascular ECs from miR-223 knockout mice and wild-type control mice. J, Representative Western blots of IGF1R protein in freshly isolated vascular ECs from miR-223 knockout mice and wild-type control mice. K, IGF1R mRNA levels in freshly isolated vascular ECs from miR-223 knockout mice and wild-type control mice. Note: $n=6$; $*P<0.05$ compared with KD groups in (D and F) and with control groups in others.

diseases but also may be critical participants in many diseases. Understanding of the roles of blood miRNAs in KD and KD-induced vascular injury research is still at an early stage. In this respect, Yun et al found some miRNAs in the serum of patients with KD, such as miR-200c and miR-371-5p, which are involved in the inflammatory response.²⁰ In contrast, Rowley et al measured serum miRNAs in KD but failed to identify any diagnostic miRNAs for KD.²¹ Shimizu et al showed that miR-145 was expressed at high levels in blood and plasma samples from patients with acute KD but not from adenovirus-infected control patients.²²

In this study, we identified that multiple miRNAs were aberrantly expressed in serum from patients with KD. Among them, miR-223 was the most upregulated abundant serum miRNA in KD. The high level of miR-223 in KD serum was

significantly decreased after treatment with immunoglobulin. Interestingly, the serum miR-223 levels of 12 KD patients with vascular damage were remarkably higher than those of KD patients without vascular complication. It should be noted that the 10 most changed miRNAs were upregulated miRNAs, although some miRNAs were also downregulated. We think that leukocytes and platelets, 2 important sources of circulating cell-free miRNAs, are increased in KD patients. The global mean threshold cycle per 200 μ L serum of miRNAs in KD patients was lower than that in healthy controls. This suggested that more miRNAs may be released into serum in KD. Although we focused on miR-223 in this study, other miRNAs listed in Table may also play roles in KD and should be studied in the future. We found in this study that serum miR-223 had a positive correlation with blood cell (platelet

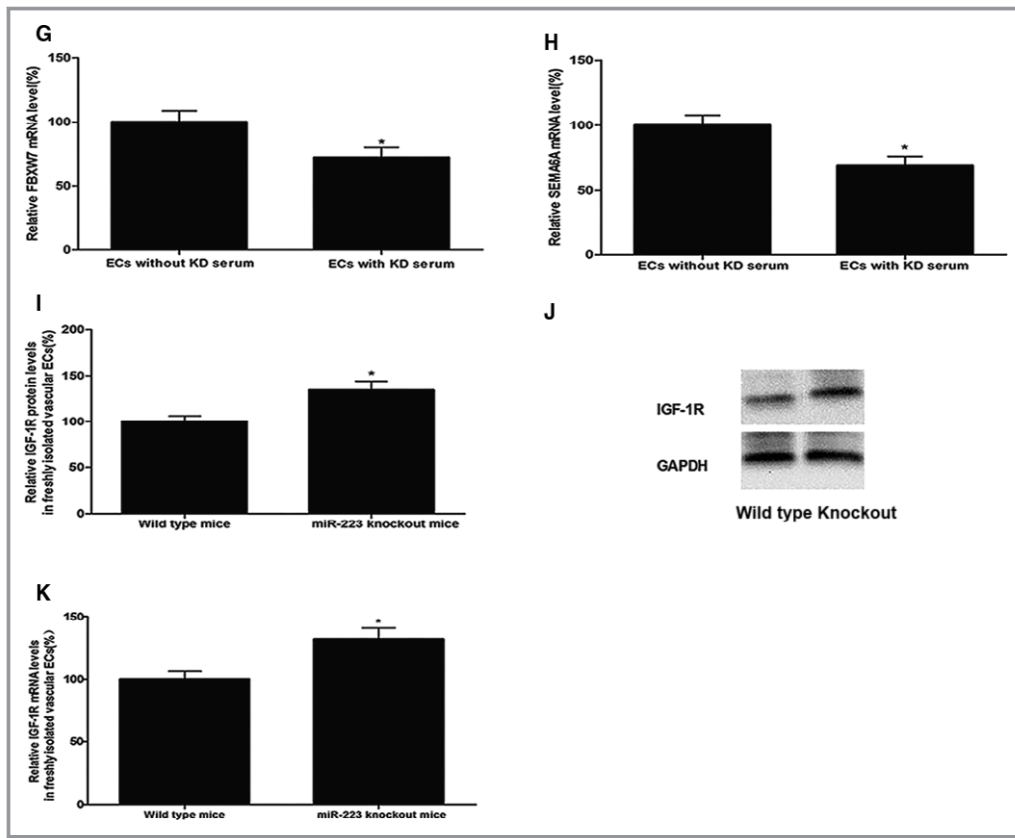


Figure 6. Continued

and leukocyte) counts. Interestingly, miR-92, another serum miRNA that was also increased in KD, had no correlation with platelet counts and total leukocyte counts in KD patients, as shown previously.²³ We think the reason is that miR-223 is a blood cell-specific miRNA, as we showed in this study and previously.¹² However, miR-92 is not a blood cell-specific miRNA. Although the changes of miR-223 are consistent with blood cell changes, it is a novel molecular biomarker, in addition to the blood cell markers.

miR-223 is a hematopoietic lineage cell-specific miRNA. Theoretically, vascular cells such as ECs should not produce or should generate only negligible amounts of endogenous miR-223. Indeed, it is difficult to find miR-223 in passaged ECs. The negative result was not induced by growth stopping under the serum-free condition, because in platelet-derived growth factor-stimulated, proliferative ECs, miR-223 could not be detected; however, a significant amount of miR-223 could be found in both freshly isolated ECs and normal vascular walls. Thus, the miR-223 in ECs and in vascular walls should mainly be from other sources. In this study, we identified that blood cell-secreted miR-223 in serum could enter ECs and that the bone marrow-derived blood cells are the major sources of miR-223 in ECs and vascular walls. It should be noted that free miRNAs have difficulty entering ECs. In addition, free miR-223 is not stable at all.^{6,7} However, blood cell-released miRNAs

bind easily with microparticles, and other proteins like high-density lipoprotein are within microparticles when they are released from the cells.^{6,7,24} The microparticles and proteins binding miRNAs are stable and could easily enter into ECs. Finally, we further verified our discovery using the chimeric mice via transplantation of bone marrow from miR-223 knockout mice into wild-type mice. The results are consistent with our recent study showing that miR-223 in another type of vascular cell, VSMCs, is also from circulating blood.¹¹

More important, we identified that the extracellular serum miR-223 not only could enter into ECs but also has strong biological functions in ECs via its target gene. We identified that miR-223 had antiproliferative and proapoptotic effects on ECs, as shown by decreased proliferation but increased apoptosis in miR-223-overexpressed ECs, which is also consistent with its cellular functions in VSMCs.¹² To test the direct involvement of this bone marrow-derived, blood cell-released serum miR-223 in KD-induced vascular injury, human ECs were cultured with either KD serum or healthy serum. We found that KD serum could increase EC apoptosis, accompanied by increases in exogenous miR-223 levels within ECs. Interestingly, KD serum-induced vascular EC apoptosis could be partially inhibited by blocking miR-223 via its inhibitor. The results suggest that miR-223 is not only a biomarker but also an active participant in KD-induced vascular cell injuries.

Our recent report identified that IGF1R is a direct target gene in VSMCs¹² as well as a key regulator of apoptosis in both VSMCs and ECs.^{20,25} In this study, we identified that IGF1R was also a direct target gene in vascular ECs. It is well established that a miRNA has multiple targets. We selected IGF1R for study for several reasons. First, we found that miR-223 had a proapoptotic effect on ECs. Consequently, we thought that an antiapoptosis gene might be a direct functional target gene of miR-223. Second, by computational analysis, we found that several antiapoptosis genes including IGF1R had the miR-223 binding sites in their 3' untranslated region. Third, IGF1R is conserved among mouse, rat, and human. Fourth, expression of IGF1R is decreased in KD serum-treated ECs. Finally, experimental and miR-223 knockout approaches have confirmed that IGF1R was indeed a direct target gene. Currently, it remains unclear that whether Bcl2, a key apoptosis-related gene, is involved in the miR-223-mediated effect on EC apoptosis. Although our unpublished data identified that Bcl2 is decreased in miR-223-treated ECs, we think Bcl2 is not a direct target gene of miR-223, based on 2 results: (1) Bioinformatics analysis did not find any potential binding site of miR-223 in BCL2, and (2) in 2007, we performed a series luciferase assay on miRNAs and their target genes in VSMCs and ECs, including miR-223 and BCL2. We did not find any binding between miR-223 and BCL2 (data not shown); however, IGF1R is a well-known upstream signal molecule of Bcl2, whereas IGF1R is a direct target of miR-223. Consequently, we think the miR-223-mediated effect on apoptosis might be related to the miR-223/IGF1R/Bcl2 pathway. Other target genes of miR-223 might be involved in KD-induced EC injuries and should be studied in the future. It should be noted that multiple miRNAs could regulate a protein-coding gene. We found in this study that multiple miRNAs were aberrantly expressed in KD serum and KD serum-treated ECs; therefore, other miRNAs might also be involved in KD-induced IGF1R expression changes and EC injuries, and this needs to be studied.

Although the cause and molecular mechanism of KD-induced vascular injuries are unknown, the major cellular events and histological and pathological changes in KD-injured vascular walls are now clear. In brief, KD could induce vascular EC injury. The injured ECs are able to elicit vascular thrombosis and increase the entry of numerous blood inflammatory mediators into VSMCs. Apoptosis of both ECs and VSMCs and decreased production of extracellular matrix occur and could finally induce the classical vascular complication: artery aneurysms in KD. The results from the current study and our recent report¹² strongly suggest that serum miR-223 may play an important role in the pathogenesis of vascular complications in KD: (1) Blood cell-released serum miR-223 is significantly increased in KD; (2) increased serum miR-223 in KD could enter both vascular ECs and VSMCs in the vascular walls; (3) increased miR-223 within ECs and VSMCs could

increase apoptosis and impair proliferation of these vascular cells, which are key cellular events in KD-injured vascular walls; and (4) inhibition of miR-223 is able to inhibit, at least in part, KD serum-induced apoptosis of vascular cells.

A traditional concept of the endocrine system includes 4 key components: (1) endocrine cells and glands, (2) endocrine ducts, (3) endocrine target cells and organs, and (4) endocrine substances that have biological functions in endocrine target cells. The current study identified that bone marrow-derived blood cells, miR-223, vessels, and vascular cells might be considered a novel endocrine system. In this system, bone marrow and blood cells are endocrine cells and glands; miR-223 is the endocrine substance; the vasculature is the endocrine duct; and vascular cells (ECs and VSMCs) and walls

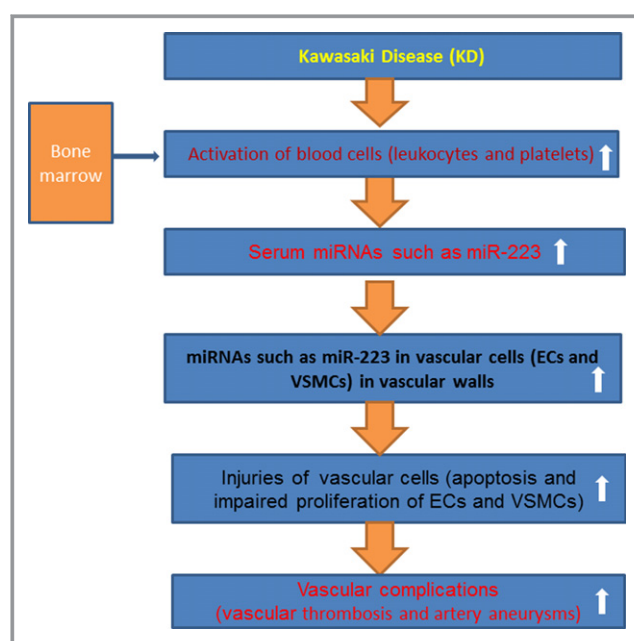


Figure 7. Bone marrow-derived, blood cell-released microRNAs (miRNAs) such as miR-223 work as endocrine genetic signals in vascular cells and have a role in vascular injury from Kawasaki disease (KD). The inflammatory blood cells such as leukocytes and platelets, which originate from hematopoietic cells in bone marrow, could secrete the hematopoietic lineage cell-specific miRNAs such as miR-223 into circulating serum. The blood cell-secreted serum miRNAs such as miR-223 could then enter vascular cells such as endothelial cells (ECs) and vascular smooth muscle cells (VSMCs) in vascular walls and work as a novel endocrine genetic signal (like a hormone does) in ECs and VSMCs to regulate their biological functions such as proliferation and apoptosis. With the pathological condition of KD, blood cells are activated to release more miRNAs such as miR-223 into serum and result in increased levels of miRNAs such as miR-223 in ECs, VSMCs, and vascular walls. The increased miRNAs such as miR-223 in vascular cells in KD could increase vascular cell injuries such as enhanced apoptosis and impaired proliferation, which could finally induce the vascular damage in KD such as vascular thrombosis and artery aneurysms.

are the endocrine target cells and organs. A key difference from the traditional endocrine system is that the endocrine substance miR-223 is genetic molecule, unlike the peptides or proteins such as hormones.

We are not able to determine the therapeutic effect of miR-223 inhibition on KD-induced vascular injury in vivo in animals, because of the lack of a KD animal model of this disease of unknown etiology disease, or in KD patients in vivo, because an miR-223 inhibitor has not yet been approved for clinical applications. Nevertheless, the current study indicates that miR-223 could have an effect in vivo on vascular injury, as shown by decreased serum miR-223 levels in patients with effective immunoglobulin treatment.

Conclusions

In this study, combined with another of our reports,¹² we have identified a novel biomarker; a new molecular mechanism; and a therapeutic target for KD, which is of unknown cause, and KD-related vascular injury (Figure 7). The inflammatory blood cells such as leukocytes and platelets, which originate from hematopoietic cells in bone marrow, could secrete the hematopoietic lineage cell-specific miRNAs such as miR-223 into circulating serum. The blood cell-secreted serum miRNAs such as miR-223 could then enter vascular cells such as ECs and VSMCs in vascular walls and work as novel endocrine genetic signals (as hormones do) in ECs and VSMCs to regulate their biological functions such as proliferation and apoptosis. With the pathological condition of KD, blood cells are activated to release more miRNAs such as miR-223 into serum and cause increased levels of miRNAs such as miR-223 in ECs and VSMCs in vascular walls. The increased miRNAs such as miR-223 in vascular cells in KD could increase vascular cell injuries, such as enhanced apoptosis and impaired proliferation, that could finally induce vascular damage in KD such as vascular thrombosis and artery aneurysms.

Author Contributions

Chu and Zhang designed the research and wrote the paper. All others performed research and analyzed data. Chu and Wu contributed equally to this work and should be considered joint first authors.

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Disclosures

None.

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