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miR-483 Targeting of CTGF Suppresses Endothelial-to-Mesenchymal Transition: Therapeutic Implications in Kawasaki Disease

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Abstract

Rationale—Endothelial-to-mesenchymal transition (EndoMT) is implicated in myofibroblast-like cell-mediated damage to the coronary arterial wall in acute Kawasaki disease (KD) patients, as evidenced by positive staining for connective tissue growth factor (CTGF) and EndoMT markers in KD autopsy tissues. However, little is known about the molecular basis of EndoMT involved in KD.

Objective—We investigated the microRNA (miRNA) regulation of CTGF and the consequent EndoMT in KD pathogenesis. As well, the modulation of this process by statin therapy was studied.

Methods and Results—Sera from healthy children and KD subjects were incubated with human umbilical vein endothelial cells (HUVECs). Cardiovascular disease-related miRNAs, CTGF, and EndoMT markers were quantified using RT-qPCR, ELISA, and Western blotting. Compared to healthy controls, HUVEC incubated with sera from acute KD patients had decreased miR-483, increased CTGF, and increased EndoMT markers. Bioinformatics analysis followed by functional validation demonstrated that Krüppel-like factor 4 (KLF4) transactivates miR-483, which in turn targets the 3′ untranslated region of CTGF mRNA. Overexpression of KLF4 or pre-miR-483 suppressed, whereas knockdown of KLF4 or anti-miR-483 enhanced, CTGF expression

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DISCLOSURES

None.

in ECs in vitro and in vivo. Furthermore, atorvastatin, currently being tested in a Phase I/IIa clinical trial in KD children, induced KLF4-miR-483, which suppressed CTGF and EndoMT in ECs.

Conclusions—KD sera suppress the KLF4-miR-483 axis in ECs leading to increased expression of CTGF and induction of EndoMT. This detrimental process in the endothelium may contribute to coronary artery abnormalities in KD patients. Statin therapy may benefit acute KD patients, in part through the restoration of KLF4-miR-483 expression.

Clinical Trial Registration—NCT01431105

Keywords

Kawasaki disease; miR-483; CTGF; EndoMT; atorvastatin; microRNA; endothelial dysfunction

Subject Terms

Pediatrics; Translational Studies; Endothelium/Vascular Type/Nitric Oxide; Fibrosis; Vascular Disease

INTRODUCTION

Kawasaki disease (KD) is an acute vasculitis that has emerged as the leading cause of pediatric acquired heart disease in developed countries.[1] Vascular inflammation leads to coronary artery abnormalities (CAA) including aneurysm in 25% of untreated KD children and 5% of those treated with intravenous immunoglobulin (IVIG).[2, 3] While the clinical events of aneurysm formation and remodeling are well-characterized in acute KD,[4] little is understood about the molecular mechanisms underlying aneurysm formation. Spindle-shaped cells in the media express α-smooth muscle actin (αSMA), but not smoothelin, consistent with a myofibroblast-like phenotype.[5] These cells participate in the recruitment of pro-inflammatory cells and are involved in the arterial wall damage by secreting interleukin (IL)-17, matrix metalloproteinases, and connective tissue growth factor (CTGF).[5] These cells are the presumed source for the disordered collagen that reduces the structural integrity of the media and contributes to aneurysm formation. With respect to the origin of these myofibroblast-like cells, several studies suggest that these pro-inflammatory and pro-fibrotic cells may originate from multiple sources including vascular endothelial cells (ECs) through endothelial-mesenchymal transition (EndoMT),[6] perivascular progenitor cells via proliferation,[7] vascular smooth muscle cells by losing differentiation marker smoothelin,[8] and circulating or adventitial fibroblasts via epithelial-mesenchymal transition (EMT).[9, 10]

Damaged coronary artery endothelium has been demonstrated in KD by paradoxical response to intracoronary artery acetylcholine.[11] Other evidence suggesting EC damage associated with KD includes increased arterial stiffness, elevated numbers of circulating endothelial progenitor cells, and EC-derived microparticles (MPs).[12–14] Under a number of conditions including inflammation and transforming growth factor β (TGFβ) signaling, ECs may undergo EndoMT, during which the expression of mesenchymal lineage markers is induced and EC lineage markers decrease.[15, 16] KD autopsy studies with electron

microscopy have confirmed the presence of myofibroblasts in the arterial wall and these cells likely contribute to vascular fibrosis and remodeling.[17]

Genetic association studies in KD patients have established that polymorphisms in the TGFβ pathway influence disease susceptibility and coronary artery aneurysm formation.[18] CTGF is regulated by the TGFβ pathway, which can induce both EMT and EndoMT.[19, 20] At the molecular level, EndoMT is manifested by the induction of mesenchymal markers such as αSMA, vimentin, and fibroblast-specific protein 1 (FSP-1), as well as decreased EC markers such as vascular endothelial cadherin (VE-cadherin) and endothelial nitric oxide synthase (eNOS).[21] Interestingly, EndoMT can be reversed by overexpression of Krüppel-like factor 4 (KLF4),[22] a master regulator of EC homeostasis and preserves EC phenotype.[23, 24]

The mainstay of current therapy for KD is intravenous immunoglobulin (IVIG), which is effective in reducing systemic inflammation and promoting T cell regulation,[25] but has no known direct effect on ECs. To address the need for an acute therapy to modify vascular damage, a dose escalation Phase I/IIa clinical trial of atorvastatin was initiated (NCT01431105).[26] Beside the low density lipoprotein-cholesterol lowering effect, the pleiotropic effects of statins in ECs include improving endothelial function, decreasing oxidative stress, and alleviating inflammation;[27] those benefits may reduce EndoMT in patients with acute KD and early signs of coronary artery damage. Some of these pleotropic effects on vascular protection may be mediated through induction of KLF4 in ECs.[28]

In this study, we developed an in vitro method to assess the molecular basis of EndoMT during acute KD and its modulation by atorvastatin. Our results demonstrate that an impaired KLF4-microRNA-483 (miR-483)-CTGF axis is associated with EndoMT in acute KD and can be mitigated by administration of atorvastatin. These findings have important translational implications that support clinical trials of statins to restore endothelial homeostasis and inhibit EndoMT in acute KD.

METHODS

Human subjects

The demographic and clinical characteristics of study subjects are presented in Supplemental Tables I and II. All patients diagnosed with KD met American Heart Association criteria for KD.[29] Coronary artery dimensions as determined by echocardiography were described by Z-worst, which was defined as the maximal Z score (standard deviation units from the mean) of the internal diameter of the left anterior descending and right coronary arteries normalized for body surface area, within the first year after KD onset. A normal coronary artery dimension was defined as a Z-worst < 2.5. CAA with Z-worst≥2.5 included transiently dilated coronary arteries (Z score>2.5 initially, then <2.5 by 6 weeks after fever onset) and aneurysms (internal diameter≥1.5 times the adjacent segment). All KD patients were initially treated with IVIG (2 g/kg) and aspirin (30–50 mg/kg/day) during the acute phase. IVIG-resistance was defined as persistent or recrudescent fever (T≥38.0°C rectally or orally) at least 36 hr after the completion of the IVIG infusion. A subset of the KD patients who were either IVIG-resistant, had CAA, or were randomized to the infliximab arm in a clinical trial were treated with a single dose of infliximab 5 mg/kg.[30] Statin-treated subjects were

enrolled in a Phase I/IIa dose-escalating clinical trial for KD patients with CAA (NCT01431105).[26] Subjects studied in these experiments received either 0.125 or 0.25 mg/kg/day (equivalent to 8.75 mg/day or 17.5 mg/day, respectively, for a 70 kg adult). Convalescent samples were collected from patients after the resolution of the acute illness and after the erythrocyte sedimentation rate decreased to <40 mm/h and the C-reactive protein level decreased to <1.0 mg/dl. Age-similar healthy subjects were children undergoing minor elective surgery. The Human Research Protection Program of the University of California San Diego approved this research protocol and written informed consent was obtained from the parents of all subjects and adolescent or child assent was obtained as appropriate. Because serum volume from these young patients was limited, not all assays were performed using sera from every subject.

Mouse lung EC isolation

Isolated lung EC RNA from EC-KLF4$^{-/-}$ and EC-KLF4-Tg mice were kindly provided by Dr. Mukesh K. Jain as described.[23] Briefly, lungs were collected from 4–6 week old mice and treated with type 1 collagenase for 30 min, followed by red blood cell lysis buffer treatment. The remaining cells were plated on collagen coated plates. After cells were grown to confluence and selected for using Dynabeads (Invitrogen) bound to anti-CD102 (BD Biosciences), then re-plated on collagen coated plates until further use.

ELISA

Human CTGF was quantified by the use of OmniKine™ ELISA Kit (Assay Biotechnology). Briefly, 2 μL of human serum was added to 98 μL of dilution buffer and incubated with the capture and secondary antibodies according the manufacturer's instructions. The color intensity was measured at 450 nm.

Bioinformatics prediction of transcription factor binding sites and miRNA targeting sites

An upstream region from −1500 to +500 bp from the transcriptional start site was defined as the promoter region of the *insulin-like growth factor 2* (*IGF2*)-*miR-483* gene. Putative KLF4 binding sites were identified using the MATCH algorithm and the KLF4 position weight matrix from TRANSFAC.[31, 32] Putative miR-483 targeting sites in the 3′ untranslated region (3′UTR) of the CTGF gene were predicted using miRanda linux version V3.3a with parameters set as "-sc 55, -scale 2, -en 1", the detailed prediction result showed in Supplemental Fig. I.[33]

Luciferase reporter plasmids, transfection, and luciferase assay

The 1044 bp human CTGF 3′UTR containing three miR-483 putative binding sites was subcloned (Forward primer: CGGACTAGTTGGCATGAAGCCAGAGAGTG, Reverse primer: CGACGCGT ACTTTTGGTCACACTCTCAACA. Restriction enzyme cutting on both ends by Spe I and Mlu I respectively) into the pMIR-REPORT vector (Ambion) to obtain pMIR-Luc-C-3′UTR wild-type reporter [Luc-CTGF(WT)]. Using this construct, we introduced GGA to CCT and GTG to CAC mutations in the miR-483 seed sequence of the CTGF 3′UTR (Fig. 4E) by using QuikChange Lightning Multi Site-Directed Mutagenesis Kit (Agilent Technologies). Renilla luciferase plasmid (pRL-TK) was used as the

transfection control. The constructed plasmids including Luc-CTGF(WT), Luc-CTGF(MT1&2), Luc-CTGF(MT3), and pRL-TK were transfected into bovine aortic endothelial cells (BAECs) using Lipofectamine 2000 (Invitrogen). The luciferase activity was measured using the Dual-Glo Luciferase reporter assay kit (Promega).

Ago1 Immunoprecipitation (Ago1-IP) and Chromatin Immunoprecipitation (ChIP) assays

Ago1-IP was performed as previously described.[34] For ChIP, proteins and DNA were crosslinked with 4% paraformaldehyde and quenched with glycine. HUVECs were then lysed and subjected to sonication. The cell lysates were then incubated with protein A Dynabeads (Life Technologies) conjugated to rabbit anti-KLF4 (Cell Signaling) with rabbit IgG as the isotype control. The immunoprecipitated DNA was analyzed by qPCR. A complete list of primers used for Ago1-IP and ChIP can be found in Supplemental Table III.

Isolation of $CD31^+$ MPs and measurement of miRNA level in $CD31^+$ MPs

$CD31^+$ MPs were isolated using a procedure adapted from Amabile *et al* and Chen *et al*.[34, 35] Briefly, $CD31^+$ MPs were immunoprecipitated from patient sera using anti-CD31 (Santa Cruz Biotechnology). Total RNA from $CD31^+$ MPs was isolated with Trizol (Invitrogen) with Cel-miR-39 added at 1 nM as a spike-in control.

Endothelial-monocyte adhesion assay

Endothelial-monocyte adhesion was determined by using the CytoSelect Adhesion Assay Kit (Cell Biolabs) as previously described.[36] Briefly, THP1 monocytes ($1x10^5$ cells/well) were labeled with LeukoTracker and incubated with monolayer treated HUVECs for 30 min at 37°C. To quantify monocyte adhesion, a Synergy HT multi-detection microplate reader (BioTek) was used to measure fluorescence of lysed cells at 480 nm excitation and 520 nm emission.

Statistical analysis

For normally distributed data, values were expressed as means ± SEM. Student's *t* test or ANOVA followed by the Bonferroni post-hoc test was used to analyze differences among groups. For data that were not distributed normally, values were expressed as medians and interquartile ranges (IQR). Mann-Whitney U test was used to analyze differences between indicated groups. $P<0.05$ was considered statistically significant.

RESULTS

KD sera increases CTGF level in ECs

CTGF is a key factor involved in EMT.[37] Similar to EMT, EndoMT is involved in vascular injury, remodeling, and fibrosis,[15, 21] all of which are features of KD. Given positive staining of CTGF in the coronary arterial wall of KD patients,[5] we first compared the level of circulating CTGF between KD patients (see Supplemental Table I for clinical parameters) and age-matched healthy controls. As shown in Fig. 1A, CTGF levels were elevated in sera from KD subjects when compared with HC. Because damaged endothelium secretes CTGF,[38] we next examined the effect of KD sera on cultured HUVECs. Sera from KD

subjects induced a robust increase in CTGF at both the transcript and protein levels (Fig. 1B and 1C). CTGF and inflammatory cytokines [e.g., tumor necrosis factor α (TNFα) and interleukin (IL)-6] promote EndoMT and indeed levels of TNFα and IL-6 are high in KD sera.[16, 39, 40] Thus, we next investigated whether TNFα and/or IL-6 may contribute to the induction of CTGF by KD sera in ECs. As seen in Fig. 1D and 1E, both TNFα and IL-6 induced CTGF mRNA and protein in ECs. Collectively, these data suggest that KD sera induce CTGF expression in ECs, which may be in part due to elevated levels of TNFα and IL-6.

KD sera attenuate the KLF4-miR-483-CTGF axis in ECs

KLF4 is a master regulator of endothelial homeostasis and the level of KLF4 is often decreased in ECs exposed to inflammatory conditions such as vasculitis.[23, 41] To explore whether altered KLF4 expression participates in the CTGF induction by KD sera, we first examined whether KLF4 expression in ECs is changed by incubation with KD sera. As shown in Fig. 2A and 2B, incubation with KD sera was associated with a decrease in both transcript and protein levels of KLF4 in ECs when compared to HC sera. To decipher whether levels of KLF4 and CTGF were linked, we examined the level of CTGF in ECs following KLF4 knockdown (Fig. 2C and 2D). KLF4 siRNA increased the levels of CTGF transcripts and protein in ECs treated with HC sera. In contrast, KLF4 overexpression via an adenovirus vector attenuated expression of CTGF in KD sera-treated ECs (Fig. 2E and 2F).

One possible mechanism underlying the inverse relationship between the expression levels of KLF4 and CTGF is that KLF4 may induce miRNAs that suppress CTGF expression. Such a mechanism would be attenuated in KD sera-treated ECs due to the lower level of KLF4. Using bioinformatics analysis, we predicted that KLF4 can induce miR-483 through binding to the promoter region of the host gene *insulin-like growth factor 2* (*IGF2)* to induce the intronic miR-483. We also predicted that miR-483 can potentially target the 3′UTR of human CTGF mRNA at 3 putative sites (Fig. 3A, and Supplemental Fig. I). Indeed, ECs treated with sera from KD patients showed lower levels of miR-483, when compared with cells treated with HC sera (Fig. 3B). This is consistent with the lower levels of miR-483 in $CD31^+$ MPs, predominantly derived from the endothelium,[42] in KD patient sera in comparison with HC individuals, suggesting the lower production and/or secretion of miR-483 from ECs (Fig. 3C). We then tested whether KLF4 directly binds to the putative promoter region of the human *IGF2-miR-483* gene. The ChIP assay shown in Fig. 3D demonstrated that KLF4 was enriched in two of the three predicted binding sites in ECs overexpressing KLF4. As a control, minimal enrichment was detected in IgG isotype control experiments (Supplemental Fig. II). In terms of gene expression, KLF4 overexpression was associated with increased levels of transcripts encoding IGF2 and miR-483, but decreased that of CTGF (Fig. 3E). The same trend was found in lung ECs isolated from EC-specific KLF4 overexpressing (EC-KLF4-Tg) mice when compared to the EC-specific KLF4 ablation (EC-$KLF4^{-/-}$) mice (Fig. 3F). Taken together, KLF4 induces the expression of miR-483, which is negatively associated with CTGF levels in ECs.

Next, we used gain- and loss-of-function approaches to validate miR-483 targeting of CTGF mRNA. Although the three putative miR-483 targeting sites are non-canonical

(Supplemental Fig. I), overexpression of miR-483 using pre-miR substantially suppressed the levels of CTGF mRNA and protein in ECs (Fig. 4A, upper panel and 4B). In contrast, anti-miR-483 increased the expression of CTGF either in ECs treated with HC sera or ECs infected with Ad-KLF4 (Fig. 4C, lower panel and 4B). To investigate whether miR-483 directly targets CTGF mRNA, we performed Ago1-IP and found that in pre-miR-483-transfected ECs, there was an increase in the Ago1-associated miR-483 and CTGF mRNA. However, such increased association of CTGF mRNA with Ago-1 was not seen in ECs transfected with anti-miR-483 (Fig. 4D), indicating that miR-483 targeting of CTGF mRNA occurred within the RNA-induced silencing complex (RISC). As the isotype control, mouse IgG did not result in detectable association of Ago1 with miR-483 or CTGF mRNA (Supplemental Fig. II).

We then measured the activity of a luciferase reporter fused to the wild-type CTGF-3′UTR [Luc-CTGF (WT)] or mutant CTGF 3′UTR [Luc-CTGF (MT)] in which the miR-483 targeting sites were mutated. Whereas pre-miR-483 significantly decreased the luciferase activity in ECs co-transfected with Luc-CTGF (WT), pre-miR-483 had little effect on the activity in cells co-transfected with mutated Luc-CTGF (MT) (Fig. 4E). However, luciferase activity was increased in ECs transfected with anti-miR-483 and incubated with HC sera. Such increase in luciferase activity was not seen in ECs transfected with Luc-CTGF (MT) (Fig. 4F). To corroborate the involvement of miR-483-CTGF targeting in ECs subjected to KD sera, we found that KD sera (which reduced levels of KLF4-miR-483) increased luciferase activity in ECs transfected with Luc-CTGF (WT), but not Luc-CTGF (MT), when compared with HC (Fig. 4G). In additional control experiments, ECs treated with pre-miR-483, anti-miR-483, or KD sera had no effect when transfected with the empty vector (pMIR-REPORT™) (data not shown). As a rescue experiment, miR-483 supplementation reduced the KD sera-induced CTGF at both the mRNA and protein levels (Fig. 4H, 4I). Together, these data suggest that KLF4-induced miR-483 directly targets CTGF mRNA to suppress its expression. This protective mechanism appears to be impaired in KD patients, because lower levels of KLF4 led to decreased levels of miR-483 and de-repression of CTGF.

Atorvastatin activation of KLF4-miR-483 axis decreases CTGF

In light of the ongoing clinical trial testing the safety and pharmacokinetics of atorvastatin in children with acute KD,[26] we next investigated whether atorvastatin acts on the KLF4-miR-483-CTGF axis and thereby reduces KD-associated EndoMT. As seen in Fig. 5A and 5B, atorvastatin induced KLF4 and miR-483 and suppressed CTGF in ECs treated with KD sera in a dose-dependent manner. Furthermore, atorvastatin decreased luciferase activity of Luc-CTGF (WT), but not that of Luc-CTGF (MT) (Fig. 5C), which reinforces the concept that atorvastatin induces KLF4-miR-483 to target CTGF.

Next, we examined the miR-483-CTGF axis in KD patients treated with or without atorvastatin (patient characteristics listed in Supplemental Table II). Six weeks after treatment with IVIG, infliximab, and aspirin, subjects had moderately decreased levels of serum CTGF (P=0.135). Importantly, patients receiving the same therapy with the addition of atorvastatin (0.125–0.25 mg/kg/day) had a significant reduction in serum CTGF levels

(P=0.044) accompanied by an increase in miR-483 levels (P=0.040, Fig. 5D). Furthermore, while ECs treated with sera from subjects receiving standard therapy or standard therapy plus atorvastatin showed increased miR-483 and decreased CTGF level; the changes were more robust with sera from the atorvastatin-treated subjects (Fig. 5E and 5F). Taken together, these data indicate that the pharmacodynamics of atorvastatin involves the induction of the KLF4-miR-483 axis in ECs, which inhibits CTGF expression.

Atorvastatin and miR-483 attenuate EndoMT in KD

Because of the involvement of CTGF in EndoMT, we next examined whether sera from KD patients can promote EndoMT, and whether atorvastatin therapy can attenuate this process. Indeed, incubating ECs with KD sera increased the expression of mesenchymal markers, including αSMA, vimentin, and fibroblast specific protein 1 (FSP-1). Conversely, expression of vascular endothelial (VE)-cadherin, an endothelial-specific marker, and eNOS, the hallmark for endothelial function, was decreased (Fig. 6A). Accordingly, KLF4 knockdown promoted EndoMT (Fig. 6B). Furthermore, lung ECs isolated from EC-KLF4$^{-/-}$ mice had increased expression of mesenchymal markers (e.g., αSMA and vimentin) when compared to EC-KLF4-Tg mice (Supplemental Fig. III). In addition to EndoMT, KD sera also impaired EC function as manifested by increased monocyte adhesion (Supplemental Fig. IV) and EC apoptosis (Supplemental Fig. V). Significantly, atorvastatin co-incubation with KD sera decreased the expression of mesenchymal markers while increasing expression of healthy EC markers, suggesting attenuation of EndoMT but enhancement of EC function (Fig. 6C, Supplemental Fig. IV). A similar attenuation of the EndoMT phenotype was achieved by overexpressing miR-483, which was associated with a moderate increase in eNOS expression (Fig. 6D). Taken together, these data suggest that the therapeutic effect of atorvastatin is in part due to suppression of CTGF and EndoMT by inducing miR-483.

The EC-derived miR-483 inversely correlates with CAA in KD

To explore the relationship between coronary artery damage and EC dysfunction, we measured miR-483 levels in ECs cultured with sera from patients with different coronary artery outcomes. Incubation of ECs with sera from KD subjects with CAA^{+} resulted in lower miR-483 level (Fig. 7A). This pattern was in line with the circulating miR-483 levels measured in patient sera, which follows the order of HC > KD CAA^{-} > KD CAA^{+} (Fig. 7B). Furthermore, we identified an inverse correlation between CD31^{+}-MPs containing miR-483 with coronary artery Z-score (r=−0.786; p=0.001, in Fig. 7C), the clinical index measured by echocardiography to quantify the severity of CAA.[29]

These data suggest that the circulating miR-483 was originated at least in part from endothelium and its level may serve as an additional marker to monitor the prognosis of CAA.

DISCUSSION

The main finding of this study is that the higher levels of CTGF in KD sera are the consequence of lower levels of KLF4 in ECs leading to reduced induction of miR-483, which targets CTGF and prevents EndoMT (depicted in Fig. 7D). This newly defined

mechanism likely influences vascular wall injury and aneurysm formation in acute KD. Furthermore, the translational implication of this finding builds on the known function of statins to induce KLF4,[28] a transcription factor that induces miR-483. Thus, statins may improve EC function and preserve the EC phenotype in KD patients by restoring the KLF4-miR-483 axis and hence reduce levels of CTGF. Given that miR-483 levels in ECs and EC-derived MPs that are secreted into the circulation are closely related, circulating miR-483 may serve as a surrogate marker for EC impairment in KD (Fig. 7D).

KLF4 is a master regulator maintaining the EC phenotype and homeostasis. Vaso-protective stimuli (e.g., statins, metformin, or shear stress) result in an optimal level of KLF4.[28, 43, 44] In contrast, pro-inflammatory and low shear stress conditions, such as those encountered in KD aneurysms, decrease KLF4 expression,[45, 46] which in turn promotes an EndoMT phenotypic switch.[22] Our study demonstrates a novel mechanism by which KLF4 regulates EC homeostasis, namely through the induction of miR-483, which in turn attenuates CTGF expression. In the endothelium of KD patients, this protective machinery is impaired, as evidenced by the increased CTGF levels both in KD sera and in ECs treated with KD sera. Of therapeutic importance, atorvastatin restored KLF4-induced miR-483 levels and lowered the level of CTGF both in the sera from acute KD subjects and in ECs treated with these sera (Fig. 5). Collectively, these results suggest the pivotal role of KLF4-miR-483-CTGF pathway in physiological, pathological, and pharmacological contexts.

In addition to CTGF, miR-483-3p and -5p are likely to target the 3′UTR of several other mRNA transcripts involved in the EndoMT phenotype, including αSMA, vimentin, and FSP-1 (Supplemental Fig. VI). miR-483 also targets B-cell lymphoma 2 binding component 3 and SMAD family member 4, both of which are involved in EMT.[47, 48] Other miR-483 targets include mitogen-activated protein kinase-activated protein kinase 2, Yes-associated Protein 1, proliferation marker Ki-67, extracellular signal-regulated kinase 1, and IGF1, the suppression of which inhibits proliferation of keratinocytes, gliomas, pancreatic cancer cells, and natural killer cells.[49–51] Therefore, miR-483 is predicted to not only negatively regulate growth and proliferation but also fibrosis and metastasis via targeting multiple genes.

miR-483 is encoded by a conserved sequence in intron 2 of the *IGF2* gene, an oncogene implicated in EMT.[52] As the host gene of miR-483, IGF2 expression varied in concert with that of miR-483, i.e., decreased in ECs treated with KD sera, but increased in ECs treated with atorvastatin (Supplemental Fig. VII). Although IGF2 is a pro-mesenchymal determinant, co-transcribed miR-483 may provide a check-and-balance, which negatively regulates EndoMT by targeting CTGF (Fig. 4 and 6). Hence, KLF4 induction of IGF2/miR-483 represents a fine-tuning mechanism by which an intronic miRNA (i.e., miR-483) plays an antagonistic role to negatively regulate the host gene's (i.e., IGF2) downstream pathways. This mode of IGF2/miR-483 regulation may be important in the context of endothelial homeostasis, which warrants further investigation. Of note, KLF4 is expressed at high levels in both healthy endothelium as well as in a synthetic phenotype of vascular smooth muscle cells, in which KLF4 plays a pro-inflammatory role.[53] Thus, the induction and regulation of the KLF4-IGF2/miR-483 axis in various cell types in the vessel wall may be crucial to maintaining vascular homeostasis.

We show that KD sera had elevated CTGF (Fig. 1A) and that CTGF was increased in the KD sera-treated ECs (Fig. 1B); one possible mechanism is that CTGF could be transferred directly from the circulation into the ECs and/or vice versa. To exclude this possibility, we found that ECs treated with KD sera following depletion of CTGF still showed increased CTGF expression (Supplemental Fig. VIIIA). In a complementary experiment, supplementation of CTGF in culture did not increase CTGF expression in ECs (Supplemental Fig. VIIIB). Thus, it appears more likely that EC-produced CTGF is responsible, at least in part, for the elevated levels of circulating CTGF in KD sera. This is supported by the findings that while *de novo* synthesized CTGF in KD sera-treated ECs were continuously secreted into freshly replaced culture media, the concentrations of CTGF in HC-conditioned media remained low (Supplemental Fig. VIIIC). Interestingly, activating any part of the KLF4-miR-483 axis (i.e., using statins) decreased CTGF production by ECs as well as its secretion into the culture media (Fig. 2E, 2F, 3E, 4A, 4B, 4G, 4H, 5 and Supplemental Fig. IX). The pro-inflammatory cytokines TNFα, IL-6, IL-1β, and interferon γ have been shown to trigger EndoMT in human ECs.[15, 16] High circulating levels of these cytokines are associated with the acute phase of KD,[39, 54] so these pro-inflammatory molecules either alone or in concert may be responsible for the induction of EndoMT observed following exposure to KD serum. Indeed, TNFα and IL-6, two pro-inflammatory cytokines that are increased in KD suppressed the KLF4-miR-483 axis and thereby induced CTGF (Fig. 1D, 1E and Supplemental Fig. X). Consistent with this finding, heat-inactivation of KD sera (to denature the protein component) attenuated the effect of KD sera in increasing CTGF (Supplemental Fig. XI). Significantly, organic extractions also increased CTGF with a concomitant decrease in KLF4 in ECs (Supplemental Fig. XI). This result suggests the additional engagement of lipid molecules in mediating the EC damage associated with KD. Thus, both pro-inflammatory cytokines (e.g., TNFα and IL-6) and lipid factors in KD circulation may contribute to EC damage.

Of translational significance, EC-derived miR-483 may serve as an additional marker to assess endothelial damage and recovery in the KD patients. This is supported by several findings. First, consistent with lower miR-483 levels in ECs treated with acute KD sera, $CD31^+$-MPs containing miR-483 were also lower in KD sera (Fig. 3C). Currently, there is uncertainty as to the best clinical practice for these patients with different centers advocating for steroids, TNFα blockers, IL-1β antagonists, or calcium channel inhibitors in a vacuum of evidence-based guidelines.[55] Thus, EC-derived miR-483 may serve as a molecular marker to assist in the assessment of the EC effects and dose selection for different proposed adjunctive therapies for acute KD patients with early CAA.

We recognize several strengths and limitations to our work. We have delineated a novel molecular pathway through which KD sera promote EndoMT, which is amenable to modulation through atorvastatin administration. While a small number of subjects were involved in this initial study, a larger sample size with a range of clinical outcomes will help to clarify the potential beneficial role of statins in acute KD. In addition, the maximum tolerated dose has not yet been defined in the on-going clinical trial and there may be important differences in effects on the KLF4-miR-483 pathway as a function of drug level in the serum. Further investigation is also required to determine the origin of myofibroblast-like cells in the vascular wall of KD patients. Lineage tracing studies using the *Lactobacillus*

casei cell wall extract mouse model of KD would be one approach to clarify if modulation of the KLF4-miR-483 axis in EC is the major pathway to generate myofibroblasts in the vascular wall in vivo.[56]

In summary, the assays and the assessment of the KLF4-miR-483-CTGF pathway reported here may be useful in the evaluation of the pro-inflammatory and pro-EndoMT status of KD patients and in monitoring therapeutic interventions with statins and other agents targeting vascular inflammation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Nonstandard Abbreviations and Acronyms

Ago1	argonaute 1
αSMA	α-smooth muscle actin
BAECs	bovine aortic endothelial cells
CAA	coronary artery abnormalities
CTGF	connective tissue growth factor
ECs	endothelial cells
EMT	epithelial-mesenchymal transition
EndoMT	endothelial-mesenchymal transition
eNOS	endothelial nitric oxide synthase
FBS	fetal bovine serum
FSP-1	fibroblast-specific protein 1
HC	healthy controls
HUVECs	human umbilical vein endothelial cells
IGF2	insulin-like growth factor 2

IL	interleukin
IVIG	intravenous immunoglobulin
KD	Kawasaki disease
KLF4	krüppel-like factor 4
miR	miRNA, microRNA
MPs	microparticles
RISC	RNA induced silencing complex
TGFβ	transforming growth factor β
TNFα	tumor necrosis factor α
VE-cadherin	vascular endothelial cadherin

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NOVELTY AND SIGNIFICANCE

What Is Known?

- Endothelial cell (EC) damage is associated with coronary artery inflammation in patients with acute Kawasaki disease (KD).
- The vascular wall of KD patients shows myofibroblast-like cells together with increased expression of connective tissue growth factor (CTGF).
- Krüppel-like factor 4 (KLF4) is a key transcription factor for EC homeostasis.

What New Information Does This Article Contribute?

- KD sera induces endothelial-to-mesenchymal transition (EndoMT), in part by suppressing KLF4-induced miR-483 that targets CTGF.
- Statin therapy may benefit acute KD patients through the induction of the KLF4-miR-483 pathway in ECs.
- Incubation of cultured human ECs with sera from KD patients treated with escalating doses of atorvastatin provides an ideal model with which to choose the optimal dose of atorvastatin to block EndoMT.

Kawasaki disease (KD), the most common cause of acquired heart disease in children, is a systemic vasculitis of unknown etiology. Although KD is characterized by an upregulation of the innate immune system, the molecular mechanisms by which KD causes endothelial damage are largely unknown. Our study indicates that sera from acute KD patients decrease the KLF4-miR-483 axis, critical for EC homeostasis. Given that miR-483 targets CTGF, suppression of KLF4 and miR-483 by KD sera may potentiate EndoMT due to increased levels of CTGF. Atorvastatin, which is known to induce KLF4, reduces KD-induced CTGF and EndoMT in cultured ECs. Similarly, KD patients treated with atorvastatin, in addition to standard treatment, show further reduced serum levels of CTGF. In acute KD patients, circulating levels of miR-483 are inversely correlated with coronary artery dimensions. Collectively, these results delineate the molecular pathway that leads to EndoMT in acute KD and its blockade by atorvastatin. Significantly, this novel therapy supports repurposing of statins for the treatment of vasculitis seen in acute KD in an ongoing clinical trial.

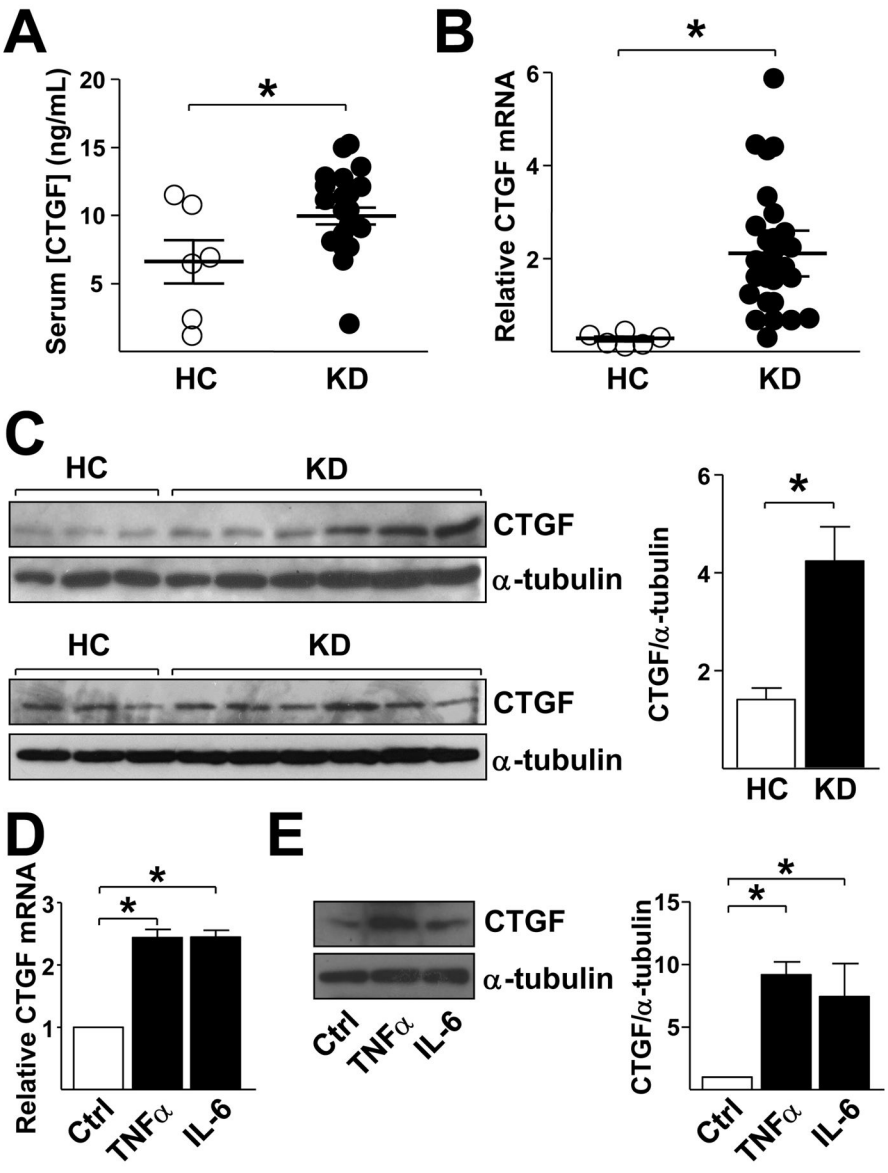

Figure 1. Elevated CTGF in KD sera and sera-treated ECs

(**A**) Circulating levels of CTGF were quantified by ELISA in age-similar healthy controls (HC, n=6), KD subjects (n=20). (**B, C**) HUVECs were treated with medium containing 15% sera from HC (n=6) and KD (n=28) subjects. After 96 hr, CTGF mRNA levels were measured by qPCR (B, all samples were normalized to HUVECs without patient sera treatment) and protein levels in ECs were measured by Western blot (C, HC, n=6 and KD, n=12). The right panel of (C) is the statistical analysis of immunoblots. **(D, E)** HUVECs were treated with TNFα (100 ng/mL), IL-6 (100 ng/mL), or left untreated (Ctrl) for 72 hr. CTGF mRNA and protein levels were measured by qPCR and Western blotting, respectively. The bar graphs are mean±SEM. **P*<0.05 compared with respective control group.

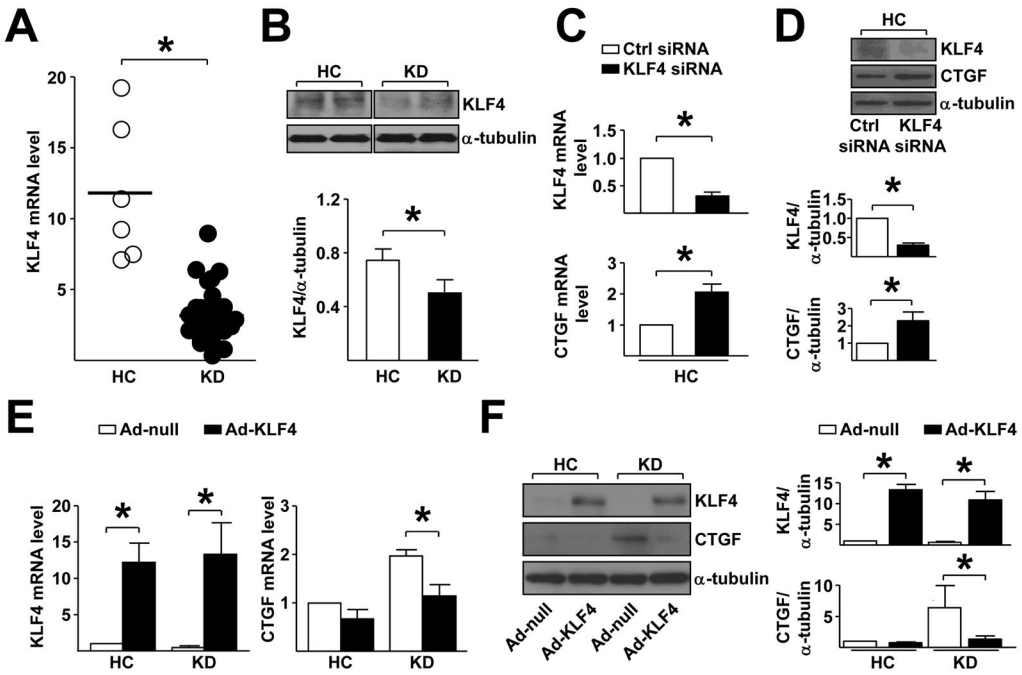

Figure 2. CTGF induction by KD sera is inversely associated with KLF4

(**A, B**) HUVECs were treated with individual KD sera (n=28) for 96 hr as described in Fig. 1B. KLF4 mRNA and protein were measured by qPCR and Western blotting, respectively. (**C, D**) HUVECs were incubated with HC sera pooled from 5 individuals, and transfected with control siRNA (Ctrl siRNA) or KLF4 siRNA for 48 hr. (**E, F**) HUVECs were treated with HC or KD sera (pooled from 8 patients) for 24 hr, followed by infection with Ad-null or Ad-KLF4 for an additional 48 hr. Levels of CTGF and KLF4 mRNA were measured using qPCR (in C, E) and protein using Western blotting (in D, F). Data in C-F are mean ±SEM from 3 independent experiments involving different batches of cells but the same pooled HC or KD sera. **P*<0.05 when compared with respective controls or between indicated groups.

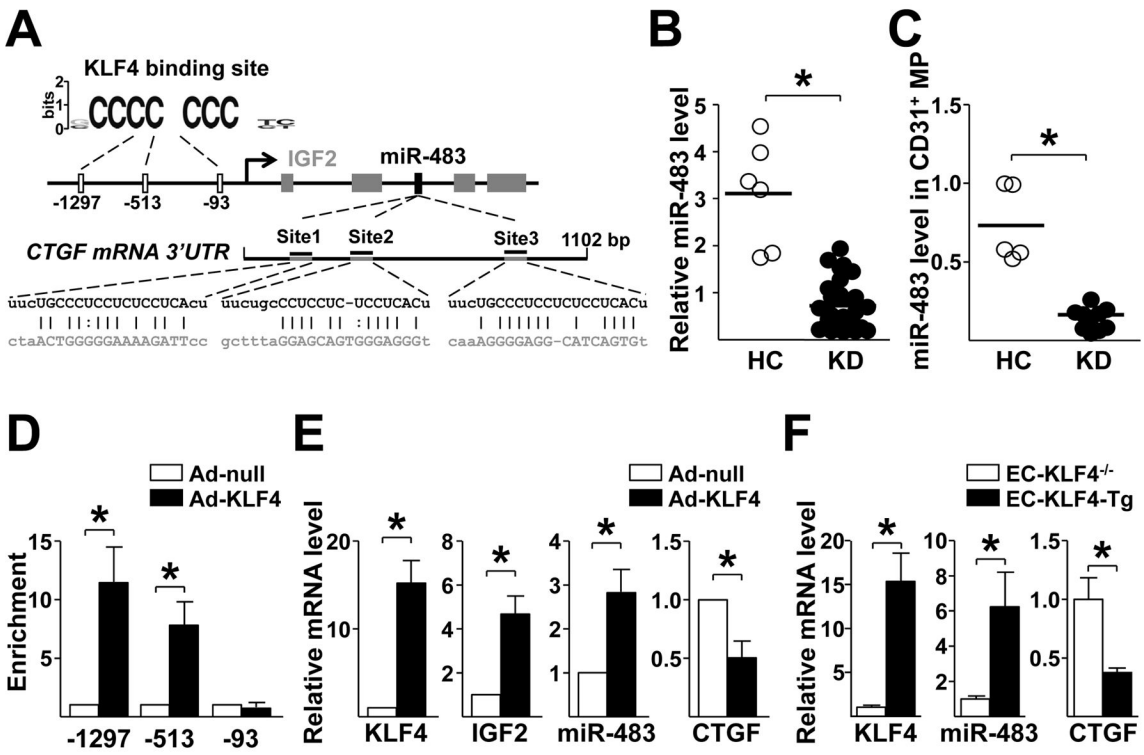

Figure 3. KLF4 transactivates miR-483

(**A**) Bioinformatics prediction of KLF4 binding sites in the promoter region of the *IGF2-miR-483* and putative miR-483 targeting sites in the 3′UTR of human CTGF mRNA. (**B**) HUVECs were treated with patients' sera as described in Fig. 1B, and **(C)** $CD31^+$ MPs were isolated from sera from KD patients (n=10) and age-matched HC (n=5). All samples were normalized to the subject with the highest miR-483 expression. (B, C) The miR-483 level was detected by qPCR. (**D, E**) HUVECs were infected with Ad-null or Ad-KLF4 for 72 hr. In (D), ChIP assay was performed using anti-KLF4 and HUVEC extracts. The enrichment of KLF4 binding to the putative binding sites in the promoter region of *IGF2-miR-483* was quantified by qPCR. In (E), the mRNA levels of KLF4, IGF2, miR-483, and CTGF were detected by qPCR. (**F**) Lung ECs were isolated from EC-$KLF4^{-/-}$ or EC-KLF4-Tg mice (n=3 per group), mRNA and miR-483 levels were quantified by qPCR. Data in (D, E) are mean±SEM from 3 independent experiments, and comparisons were made with control groups arbitrarily set as 1. *P<0.05 when compared between indicated groups.

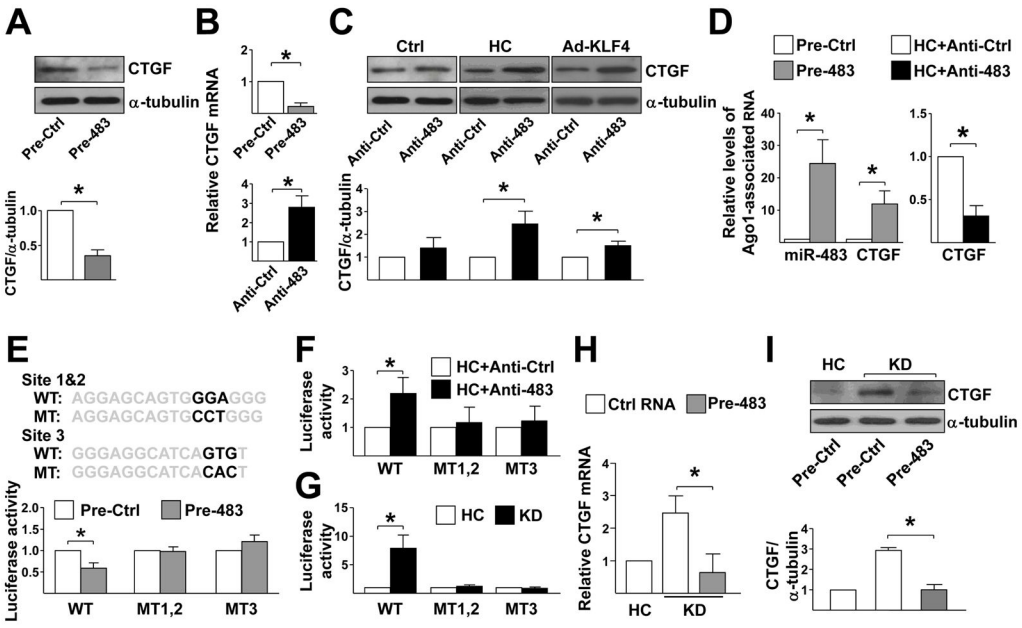

Figure 4. miR-483 targets CTGF in ECs

(**A–C**) HUVECs were transfected with miR-483 mimic (pre-483) or inhibitor (anti-483) for 48 hr. In (C), HUVECs were pre-treated with or without pooled HC sera or infected with or without Ad-KLF4 for 48 hr. CTGF mRNA and protein were detected and quantified. (**D**) HUVECs were transfected with pre-483 or pooled HC sera with anti-483 (HC+Anti-483) for 48 hr followed by Ago1 immunoprecipitation. Ago1-associated miR-483 and CTGF mRNA were quantified by qPCR. (**EG**) BAECs transfected with Luc-CTGF (WT) or Luc-CTGF (MT1&2, 3) were co-transfected with pre-483 in (E), pooled HC sera with anti-483, or treated with pooled KD sera as indicated in (G). Luciferase activity was measured with that of Renilla as transfection control. (**H, I**) HUVECs were treated with pooled sera from HC or KD patients for 48 hr before transfection with pre-control or pre-483. CTGF expression was determined by qPCR and Western blot. Controls for the miRNA mimic (Pre-Ctrl) or miRNA inhibitor (Anti-Ctrl) were used. Data are mean±SEM from 3 independent experiments, and comparisons were made with control groups arbitrarily set as 1. **P*<0.05 when compared between indicated groups.

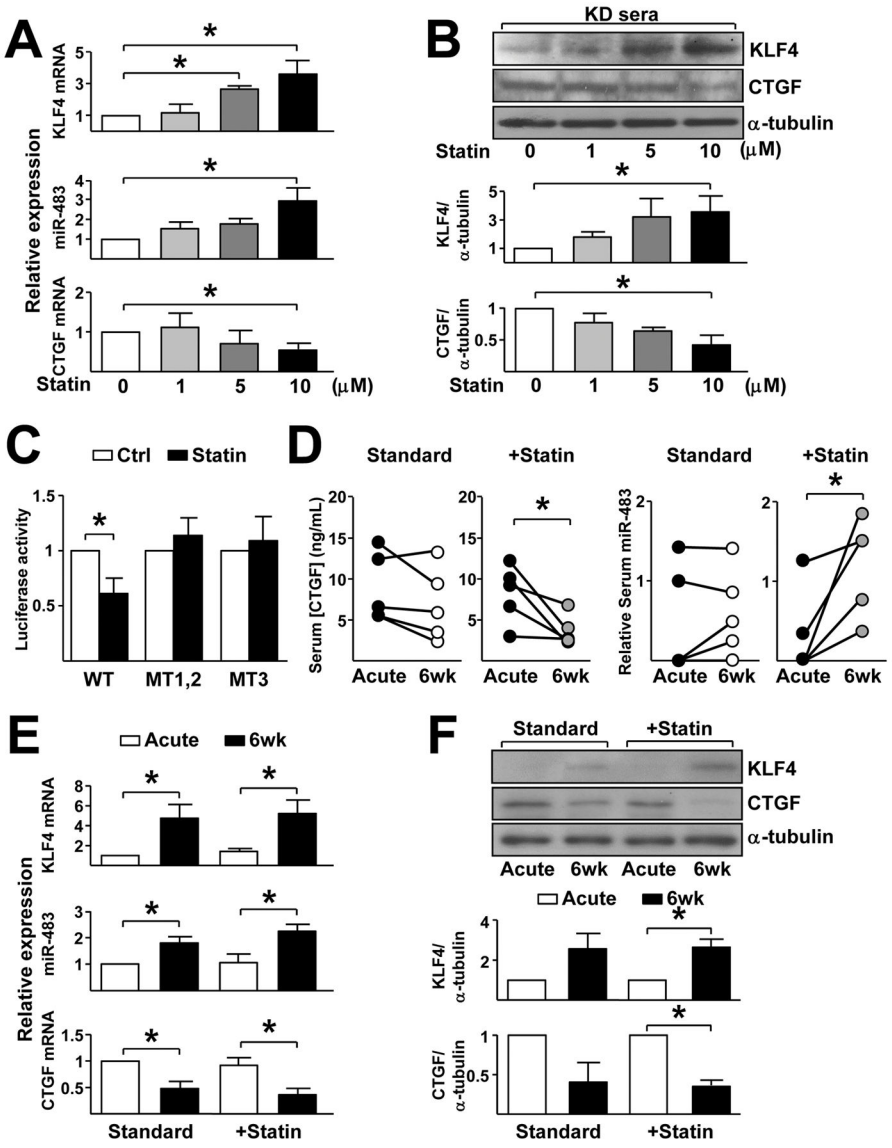

Figure 5. Atorvastatin decreases CTGF expression in ECs by activating the KLF4-miR-483 axis

(**A, B**) HUVECs were treated with pooled KD sera for 48 hr followed by atorvastatin treatment with indicated dosage for an additional 48 hr. The changes in miR-483, CTGF, and KLF4 were determined by qPCR and Western blotting. (**C**) BAECs were transfected with Luc-CTGF (WT) or Luc-CTGF (MT1,2, and MT3) before atorvastatin treatment (5 μM) for 48 hr. Luciferase activity was measured. (**D**) Comparison of circulating levels of CTGF and miR-483 in KD patients between acute phase (pre-treatment) and 6 weeks post-treatment with standard treatment (IVIG+ASA+infliximab) with or without atorvastatin (0.125–0.25 mg/kg/day, n=5/group). Control subjects were matched for age, illness day and Z-worst (Supplemental Table II). (**E, F**) HUVECs were treated with pooled patients' sera from acute phase (pre-treatment) and 6 weeks post-treatment. The changes in miR-483, CTGF, and KLF4 levels were determined by qPCR and Western blot. Data are mean±SEM from 3

independent experiments (A-C) or from 5 subjects per group (D–F). *P<0.05 compared between the indicated group.

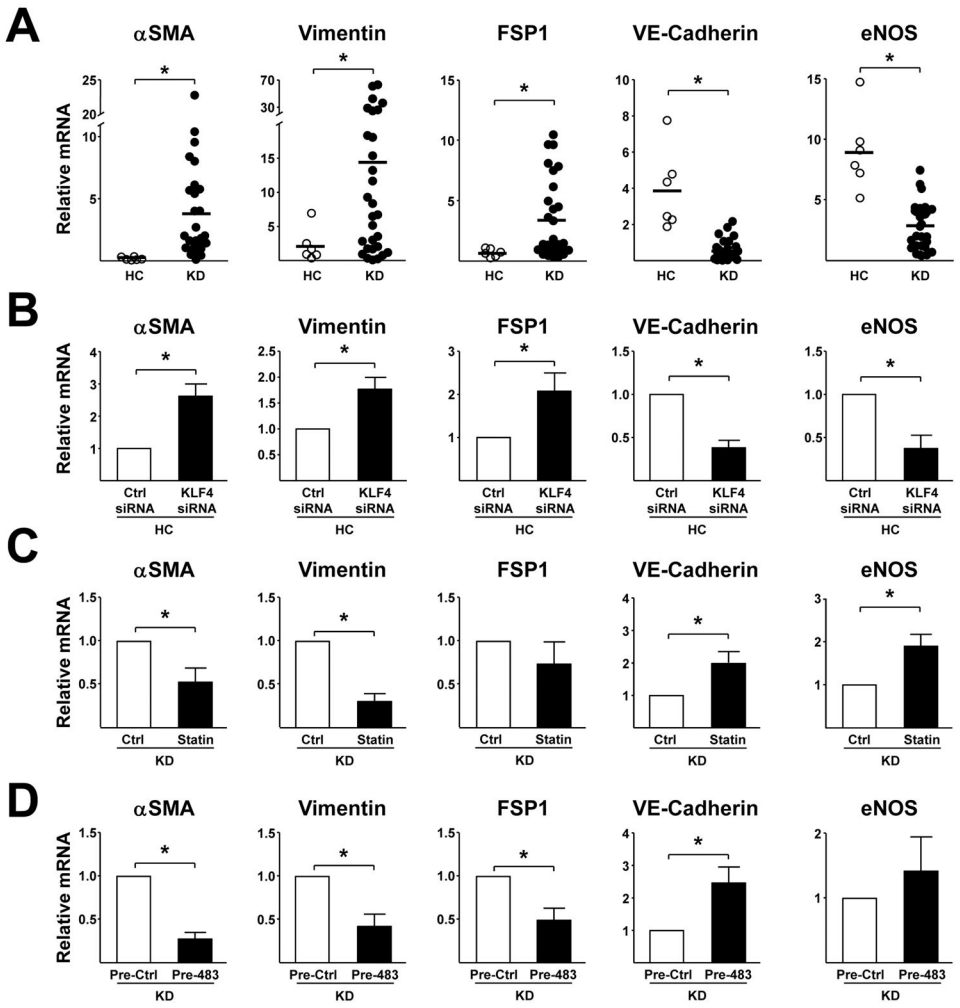

Figure 6. Atorvastatin and miR-483 alleviate KD sera-induced EndoMT

Expression levels of EndoMT markers were quantified by qPCR. In (**A**) HUVECs were treated with individual sera from HC (n=6) or KD (n=29) patients as described in Fig. 1B. (**B**) HUVECs were treated with HC sera (pool from 5 subject) for 48 hr and transfected with Ctrl siRNA or KLF4 siRNA for 48 hr. In (**C, D**) HUVECs were treated with KD sera (pooled from 8 patients) for 48 hr before transfection with pre-Ctrl, pre-483, or atorvastatin for an additional 48 hr. Data are mean±SEM from 3 independent experiments. **P*<0.05 compared with the control or indicated group.

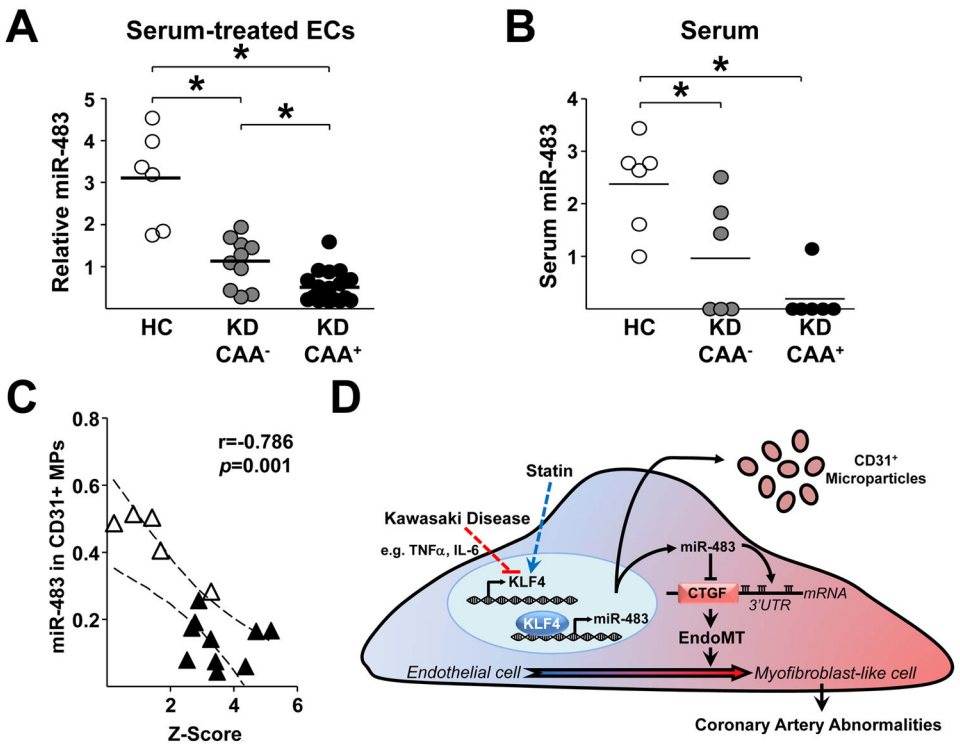

Figure 7. The EC-derived miR-483 inversely correlates with CAA in KD subjects and a schematic illustration of KD- and statin-modulated KLF4-miR-483-CTGF pathway in EndoMT

(**A, B**) miR-483 expression was determined by qPCR in (A) HUVECs treated with sera from HC (n=6), KD CAA^{-} (n=10), and KD CAA^{+} (n=18) patients as described in Fig. 1B and in (B) respective patient sera (all samples were normalized to the HC subject with the lowest miR-483 expression). *P<0.05 when compared between indicated groups. (**C**) Spearman's correlation between miR-483 level in CD31^{+} MPs and Z-score. Data were from 10 acute patients (closed triangles) and 5 convalescent patients (open triangles). (**D**) Schematic illustration of KD- and statin-modulated KLF4-miR-483-CTGF pathway in EndoMT. In healthy endothelial cells, KLF4 binds to the promoter region of miR-483 and transcriptionally induces miR-483. miR-483 in turns binds to the 3′UTR of CTGF mRNA to inhibit CTGF expression. In KD, the KLF4-miR-483 axis is suppressed in ECs and CD31^{+}-MPs have decreased miR-483 enrichment. As a result, CTGF is de-suppressed, thereby inducing EndoMT (the transition of endothelial cells into myofibroblast-like cells) and contributing to coronary artery abnormalities. Such pathological modulation may be reversed by statin therapy, which restores the KLF4-miR-483 axis.