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Kallistatin inhibits TGF-β-induced endothelial–mesenchymal transition by differential regulation of microRNA-21 and eNOS expression

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Abstract

Kallistatin, an endogenous protein, consists of two structural elements: active site and heparin-binding domain. Kallistatin exerts beneficial effects on fibrosis by suppressing transforming growth factor (TGF)-β synthesis in animal models. TGF-β is the most potent inducer of endothelial–mesenchymal transition (EndMT), which contributes to fibrosis and cancer. MicroRNA (miR)-21 is an important player in organ fibrosis and tumor invasion. Here we investigated the potential role of kallistatin in EndMT via modulation of miR-21 in endothelial cells. Human kallistatin treatment blocked TGF-β-induced EndMT, as evidenced by morphological changes as well as increased endothelial and reduced mesenchymal marker expression. Kallistatin also inhibited TGF-β-mediated reactive oxygen species (ROS) formation and NADPH oxidase expression and activity. Moreover, kallistatin antagonized TGF-β-induced miR-21 and Snail1 synthesis, Akt phosphorylation, NF-κB activation, and matrix metalloproteinase 2 (MMP2) synthesis and activation. Kallistatin via its heparin-binding site blocked TGF-β-induced miR-21, Snail1 expression, and ROS formation, as wild-type kallistatin, but not heparin-binding site mutant kallistatin, exerted the effect. Conversely, kallistatin through its active site stimulated the synthesis of endothelial nitric oxide synthase (eNOS), sirtuin 1 (Sirt1) and forkhead box O1 (FoxO1); however, these effects were blocked by genistein, a tyrosine kinase inhibitor. This is the first study to demonstrate that kallistatin's heparin-binding site is crucial for preventing TGF-β-induced miR-21 and oxidative stress, while its active site is key for stimulating the expression of antioxidant genes via interaction with an endothelial surface tyrosine kinase. These findings reveal novel mechanisms of kallistatin in protection against fibrosis and cancer by suppressing EndMT.

Graphical Abstract

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Disclosures
The authors declare that they have no competing interests.

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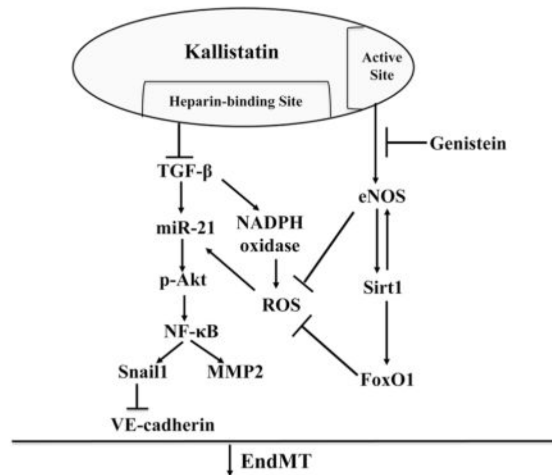

Keywords

Kallistatin; EndMT; miR-21; eNOS; TGF-β; Oxidative stress

1. Introduction

Endothelial–mesenchymal transition (EndMT) plays a pivotal role in organ fibrosis and tumor metastasis [1]. In adults, abnormal differentiation of EndMT-derived fibroblast-like cells results in fibrosis in the heart, kidney and lung [2–4]. Transforming growth factor (TGF)-β signaling is documented to be involved in controlling endothelial and epithelial plasticity by eliciting their transition to a mesenchymal state [5–7]. The TGF-β-Akt-Snail axis is one of the important non-Smad parallel downstream pathways in epithelial–mesenchymal transition (EMT) [8]. TGF-β induces Akt activation and nuclear factor-κB (NF-κB) nuclear translocation [9,10], leading to elevated expression of the Snail transcription factor and reduced epithelial (E)-cadherin expression [11]. Typical morphology changes of EndMT include loss of cell–cell junctions, such as the endothelial markers vascular endothelial (VE)-cadherin and CD31, as well as gain of invasive and migratory properties characterized by mesenchymal markers, including α-smooth muscle actin (SMA) [1,7]. Aberrant expression of microRNAs is also known to be involved in EndMT-related diseases [12]. Expression levels of microRNA (miR)-21 rise to a significant extent during EMT and EndMT [13,14]. TGF-β-induced EndMT is partly regulated by miR-21 via the Akt pathway, as blockade of miR-21 was found to prevent EndMT and inhibit Akt activation [15]. Moreover, reactive oxygen species (ROS) production further stimulates miR-21 synthesis [16], and oxidative stress leads to fibrosis in fibroblasts and other cells. Therefore, miR-21 and ROS are the major mediators of EndMT, and thus EndMT-associated fibrosis and tumor development.

Kallistatin is a plasma protein that is widely distributed in tissues relevant to cardiovascular function, including the kidney, heart and blood vessel [15,17,18]. Kallistatin contains two structural elements: an active site and a heparin-binding domain [19,20]. Kallistatin's active site is essential for binding to tissue kallikrein, thereby inhibiting kallikrein's enzymatic

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activity [19]. Kallistatin via its heparin-binding site interacts with cell surface heparan sulfate proteoglycans, thus antagonizing signaling pathways mediated by vascular endothelial growth factor (VEGF), tumor necrosis factor (TNF)-α and high mobility group box-1 (HMGB1) [21–23]. Through these two structural elements, kallistatin regulates differential signaling pathways leading to pleiotropic effects, including reduction of blood pressure and inhibition of inflammation, apoptosis, angiogenesis, hypertrophy and fibrosis in many animal models and cultured cells [22,24–28]. Kallistatin gene or protein delivery has been shown to improve cardiac and renal function, and decrease oxidative stress, cardiac and renal fibrosis in conjunction with reduced TGF-β levels and increased endothelial nitric oxide synthase (eNOS) expression and nitric oxide (NO) production in animal models [24,25,28]. Liver fibrosis was also decreased upon kallistatin treatment via inhibition of oxidative stress [29]. Kallistatin antagonizes TGF-β-induced collagen synthesis in cardiac myofibroblasts and TNF-α-induced NF-κB activation in endothelial cells [22,28]. Furthermore, kallistatin through NO formation inhibits superoxide production and NAD(P) H oxidase activity stimulated by TNF-α, H_2O_2, or angiotensin II in cultured renal, cardiac and endothelial cells [25,26,28]. These findings led us to investigate the role and mechanism of kallistatin in modulating TGF-β-induced EndMT in endothelial cells.

2. Materials and methods

2.1. Purification and characterization of recombinant human kallistatins

Recombinant human kallistatin was secreted into the serum-free medium of cultured human embryonic kidney cells (HEK293T). Cultured medium was concentrated by ammonium sulfate precipitation followed by nickel-affinity chromatography as described [23,30]. Recombinant wild-type kallistatin (WT-KS), heparin-binding site mutant (K312A/K313A) kallistatin (HM-KS), and active-site mutant (A377T) kallistatin (AM-KS) were expressed in *E. coli* and purified as described [30]. The purity and identity of human kallistatin were verified by sodium dodecyl sulfate-poly-acrylamide gel electrophoresis (SDS-PAGE) and western blot using a specific monoclonal antibody [23,31].

2.2. Cell culture

Human umbilical vein endothelial cells (HUVECs) were kindly provided by Dr. Robin Muise-Helmericks (Medical University of South Carolina, Charleston, SC, USA). HUVECs were cultured in endothelial cell basal medium (EBM)-2 supplemented with EGM-2 SingleQuots (Lonza, Walkersville, Maryland), and maintained in a humidified atmosphere of 5% CO_2 in air at 37 °C two passages weekly. HUVECs were incubated in the presence or absence of kallistatin (1 μM) for 30 min, followed by the addition of 10 ng/ml TGF-β1 (R&D Systems, Minneapolis, Minnesota) for another 24 or 72 h. Culture medium containing TGF-β1 or kallistatin was replaced every 24 h. For real-time polymerase chain reaction (PCR) experiments, cells were treated with TGF-β1 with or without kallistatin for 24 h. For NADPH oxidase activity, western blot, immunostaining and gelatin zymography, cells were treated with TGF-β1 with or without kallistatin for 72 h. In another set of experiments, cells were pretreated with 5 μM genistein (Sigma, Saint Louis, Missouri) for 30 min, and then further incubated with 2 μM WT-KS, HM-KS or AM-KS in the presence of 10 μg/ml

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polymyxin B sulfate (Sigma, Saint Louis, Missouri) for 30 min followed by stimulation with TGF-β1 for another 24 h.

2.3. Detection of ROS formation and NF-κB activity assay

Intracellular ROS generation was detected using the peroxide-sensitive fluorescent probe 2′, 7′-dichlorofluorescin diacetate (DCFH-DA; Sigma, Saint Louis, Missouri) as described previously [32]. Phosphorylated NF-κB activities in nuclear lysates were determined using ELISA kit (Cell Signaling Technology, Boston, Massachusetts) according to the manufacturer's instructions.

2.4. Real-time polymerase chain reaction

RNA was isolated from cells using Trizol reagent (Invitrogen, Carlsbad, California) per the manufacturer's instructions. Total RNA was reverse-transcribed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster city, California) for messenger RNA and MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster city, California) for microRNA. Real-time PCR was performed with Taqman Gene Expression Assay and was normalized against 18S or U6 using an ABI 7300 Real-time PCR System (Applied Biosystems, foster city, California). Primers were used for the detection of miR-21, eNOS, matrix metalloproteinase 2 (MMP2), Snail1, sirtuin 1 (Sirt1), forkhead box O1 (FoxO1), and NADPH oxidase 4 (Nox4). The following human primers were purchased from Applied Biosystems: 18S (Hs 99999901_sl), U6 snRNA (001973), hsa-miR-21-3p (002438), eNOS (Hs 01574659_ml), MMP2 (Hs 00234422_ml), Snail1 (Hs 00195591_ml), Sirt1 (Hs 01009006_ml), FoxO1 (Hs 01054576_ml), and Nox4 (Hs 00418356_ml). A negative control without cDNA did not produce any amplicons. Data were analyzed with $2^{- \quad Ct}$ value calculation using 18S or U6 for normalization.

2.5. Measurement of NADPH oxidase activity

The enzymatic activity of NADPH oxidase in cell homogenates was assessed by lucigenin enhanced chemiluminescence (ECL) as previously described [33]. The assay solution contained 50 mM HEPES (pH 7.4), 1 mM EDTA, 5 μM lucigenin, and 1 mM NADPH. After preincubation at 37 °C for 10 min, the reaction was started by adding 50 μg of the homogenate. Fluorescence intensity was continuously monitored for 15 min with a TD20/20 luminometer. The chemiluminescent signals observed in the absence of the homogenate were subtracted from the chemiluminescent signals of the samples. The chemiluminescence signal was corrected for by the protein concentration of each cell homogenate.

2.6. Western blot assay

Cells were lysed with non-denaturing lysis buffer (Cell Signaling Technology, Boston, Massachusetts), separated on a 10% polyacrylamide gel, and transferred on polyvinylidene difluoride (PVDF) membranes (Amersham Biosciences, Amersham place, UK). After being blocked for 1 h in a buffer containing 5% nonfat milk, PVDF membranes were then probed with primary antibodies to phosphorylated and/or total Akt (S473), CD31, VE-cadherin, α-SMA and α-tubulin (Cell Signaling Technology, Boston, Massachusetts) overnight at 4 °C. The respective horseradish peroxidase-labeled secondary antibodies were then added to the

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membrane for 1 h at room temperature. Chemiluminescence was detected by an ECL-plus kit (GE Healthcare, Buckinghamshire, UK).

2.7. Immunofluorescence microscopy

Primary antibodies for VE-cadherin, CD31 and α-SMA were used to detect their respective protein levels with fluorescence microscopy. Briefly, cells were grown and treated in a 12-well plate. At the end of treatment, cells were fixed with 4% formaldehyde diluted in PBS at room temperature for VE-cadherin, or ice-cold 100% methanol for CD31 and α-SMA for 15 min. Cells were permeabilized with 0.2% Triton X-100 and blocked with 3% BSA in PBS for 1 h at room temperature, followed by incubation with primary antibody overnight at 4 °C. At the end of incubation, cells were rinsed three times with PBS and incubated with fluorochrome-conjugated secondary antibody (1:400) for 2 h at room temperature in the dark. Cells were then washed with PBS and counter stained with Hoechst 33342 (Cell Signaling Technology, Boston, Massachusetts) (1 μg/ml) for 5 min. Plates were then observed under a fluorescence microscope (Olympus CK40).

2.8. Gelatin zymography

MMP2 activity was determined by zymography. The serum-free medium of treated HUVECs was centrifuged at 500 rpm for 10 min, and the supernatant was collected and stored at −80 °C. Conditioned HUVEC medium was used for zymography. Briefly, the medium was mixed with non-reducing loading buffer and resolved at 125 V by 10% SDS-PAGE separating gels copolymerized with 1 mg/ml gelatin. The renaturing buffer (2.5% Triton X-100) was used to restore the enzyme activity. The gels were incubated in fresh developing buffer containing 5 mM $CaCl_2$, 0.2 M NaCl and 0.02% Brij-35 in 50 mM Tris (pH 7.6) at 37 °C for 16 h, followed by staining with Coomassie blue to visualize the proteolytic bands.

2.9. Statistical analysis

Data are expressed as means±SE of 3 independent experiments. Statistical significance was determined by analysis of variance with Fisher's probability least-squares difference test or Student *t*-test using GRAPHPAD PRISM software. A value of $P<0.05$ was considered statistically significant.

3. Results

3.1. Kallistatin inhibits TGF-β-induced EndMT and conserves the endothelial phenotype

We first determined the effect of kallistatin on TGF-β1-mediated EndMT in cultured endothelial cells. Exposure of HUVECs to TGF-β1 for 3 days caused an obvious alteration in cellular morphology from a polygonal, cobblestone-like shape to a more spindle, fibroblast-like shape (Fig. 1A). In contrast, kallistatin treatment markedly suppressed the transition by conserving the endothelial phenotype. To examine the effect of kallistatin on TGF-β-mediated EndMT, we assessed the presence of the fibroblast marker α-SMA and the endothelial markers VE-cadherin and CD31 by immunofluorescence. Representative immunostaining showed that TGF-β1-induced EndMT was associated with reduced CD31 and VE-cadherin levels (Fig. 1B and C) and increased α-SMA levels (Fig. 1D). However,

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upon kallistatin treatment, most cells were positive for VE-cadherin and CD31, and negative for α-SMA. These observations were further confirmed by western blot analysis. In the presence of TGF-β1, VE-cadherin and CD31 protein levels were greatly reduced, and α-SMA protein levels were increased (Fig. 1E). However, kallistatin treatment reversed TGF-β1-mediated effects. Quantitative analysis of the western blots verified our results. These findings demonstrate that kallistatin suppresses EndMT induced by TGF-β.

3.2. Kallistatin inhibits TGF-β-induced ROS formation and NADPH oxidase expression and activity

We next determined the effect of kallistatin on ROS formation driven by TGF-β1 in endothelial cells. TGF-β1 significantly stimulated ROS formation compared to the control group, whereas kallistatin reversed TGF-β's effect by restoring ROS production to basal levels (Fig. 2A). We also evaluated the effect of kallistatin on the expression of Nox4, a gene that codes for a protein in the NADPH oxidase family, as NADPH oxidase stimulates the production of ROS [34]. Kallistatin treatment completely antagonized TGF-β1-induced Nox4 mRNA levels and NADPH oxidase activity (Fig. 2B and C). Therefore, kallistatin can block TGF-β-induced oxidative stress in endothelial cells.

3.3. Kallistatin prevents TGF-β-induced miR-21 synthesis, Akt and NF-κB activation, Snail1 expression, and MMP2 synthesis and activation

Since the miR-21-Akt signaling pathway plays an important role in EndMT [14], we tested whether kallistatin regulates TGF-β-induced EndMT through this pathway. Our results showed that TGF-β1 up-regulated miR-21 synthesis, but the effect was prevented by kallistatin (Fig. 3A). In addition, kallistatin prevented TGF-β1-induced Akt phosphorylation (Fig. 3B). These results indicate that kallistatin blocks TGF-β-induced EndMT by inhibiting the miR-21-Akt pathway. Moreover, kallistatin treatment attenuated TGF-β1-mediated NF-κB activation (Fig. 3C). Since NF-κB leads to the activation of the Snail transcription factor [11], we measured Snail1 mRNA levels. TGF-β1 significantly induced Snail1 transcription, whereas kallistatin treatment prevented the effect (Fig. 3D). Furthermore, we analyzed MMP2 synthesis and activation by real-time PCR and zymography, respectively. TGF-β1 stimulated an increase in MMP2 mRNA levels and activity, and these effects were abolished by kallistatin (Fig. 3E). These results indicate that kallistatin blocks TGF-β-mediated EndMT, in part, by inhibiting the miR-21-Akt-NF-κB pathway, Snail1 expression, and MMP2 synthesis and activity.

3.4. Kallistatin's heparin-binding site is essential for preventing TGF-β-induced miR-21 synthesis, oxidative stress and Snail1 expression

We analyzed the role of kallistatin's heparin-binding site in modulating TGF-β1-induced miR-21 synthesis and ROS generation. Wild-type kallistatin significantly reduced TGF-β1-driven miR-21 expression, whereas heparin-binding site mutant kallistatin had no effect (Fig. 4A). Likewise, wild-type kallistatin, but not heparin mutant kallistatin, inhibited TGF-β1-induced ROS formation (Fig. 4B). Moreover, kallistatin's heparin-binding site was found to be essential for abolishing TGF-β1-mediated Snail1 expression (Fig. 4C). Our results demonstrate that kallistatin competes with TGF-β1's ability to bind to heparan sulfate proteoglycans, thus preventing TGF-β1-mediated signaling. These new findings indicate that

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kallistatin's heparin-binding site plays an important role in antagonizing TGF-β-induced miR-21, ROS formation and Snail1 expression.

3.5. Kallistatin via its active site stimulates eNOS, Sirt1, and FoxO1 expression

We next examined the role of kallistatin's structural elements on the expression of antioxidant genes in endothelial cells. Our results showed that wild-type kallistatin and heparin-binding site mutant kallistatin treatment led to a noticeable rise in eNOS, Sirt1 and FoxO1 mRNA levels, whereas active site-mutant kallistatin had no effect (Fig. 5). This finding clearly indicates that the active site of kallistatin is crucial for mediating the expression of eNOS, Sirt1 and FoxO1. Moreover, treatment with genistein, a tyrosine kinase inhibitor, abolished kallistatin-induced eNOS, Sirt1 and FoxO1 expression (Fig. 5). These new results show that kallistatin via its active site interacts with a cell surface tyrosine kinase, thus stimulating expression of the antioxidant genes eNOS, Sirt1 and FoxO1.

4. Discussion

This study provides new insights into the protective role of kallistatin in EndMT-associated pathological processes, such as fibrosis and cancer. We showed that kallistatin prevented EndMT by blocking TGF-β1-induced miR-21 synthesis and oxidative stress, and also by directly stimulating the expression of the antioxidant genes eNOS, Sirt1 and FoxO1. Moreover, this is the first study to demonstrate that kallistatin's structural elements play crucial roles in the differential regulation of TGF-β-mediated effects and eNOS gene expression in endothelial cells. We found that kallistatin via its heparin-binding site inhibited TGF-β1-induced miR-21 expression, ROS formation, and Snail1 synthesis. Conversely, kallistatin's active site was observed to be essential for stimulating the expression of eNOS, Sirt1 and FoxO1 (Fig. 6). Both eNOS and Sirt1 are responsible for increasing NO production, and NO has been shown to be an antioxidant by decreasing ROS levels and NADPH oxidase activity [35]. Kallistatin was previously shown to inhibit ROS production by NO formation in endothelial cells [26]. Kallistatin administration also attenuated vascular injury, and cardiac and renal fibrosis in conjunction with reduced oxidative stress and increased NO production in animal models with hypertension or myocardial infarction [25,28]. In addition, kallistatin reduced liver fibrosis via inhibition of oxidative stress [29]. Thus, our findings reveal a new mechanism by which kallistatin, through its structural elements, inhibits EndMT by down-regulating of miR-21 synthesis and oxidative stress, and up-regulating eNOS, Sirt1 and FoxO1 expression.

miR-21 is an oncogenic microRNA that is overexpressed in most human tumors, and can thus promote tumor growth and progression by acting on multiple target genes [36]. miR-21 elevation has also been documented in cardiac fibrosis during EndMT and EMT [14,37]. Increased miR-21 leads to Akt activation, which in turn up-regulates miR-21 in endothelial cells [12]. These studies are consistent with our findings that TGF-β1 stimulated miR-21 synthesis and Akt phosphorylation/activation; however, kallistatin efficiently blocked TGF-β1-mediated effects. An additional pro-tumorigenic effect of miR-21 is to increase oxidative stress [38]. The present study showed that kallistatin not only antagonized TGF-β1-mediated miR-21-Akt activation, but also blocked TGF-β1-induced oxidative stress. Therefore,

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kallistatin can attenuate TGF-β-induced EndMT by targeting the miR-21-Akt pathway and oxidative stress.

The role of TGF-β-induced Akt-NF-κB activation in the context of EndMT is not well understood. In the EMT process, Akt stimulates the nuclear translocation of the NF-κB complex. NF-κB acts as a transcriptional factor for Snail1 and MMP2 expression [11,39]. Snail factors are known to repress both VE-cadherin and E-cadherin transcription. Consequently, elevated Snail expression can stimulate the loss of endothelial cell–cell junctions. Moreover, during EndMT the basal lamina is degraded by matrix metalloproteinases, such as MMP2 [40]. In the present study, our results showed that NF-κB was induced by TGF-β1 in endothelial cells undergoing EndMT, resulting in elevated Snail1 and MMP2 expression and loss of the endothelial marker VE-cadherin. In contrast, kallistatin treatment blocked TGF-β1-mediated miR-21-Akt activation, leading to suppression of TGF-β-induced NF-κB-Snail1/MMP2 signaling. Thus, these results indicate that kallistatin inhibits EndMT through blockade of the TGF-β downstream signaling pathway.

Kallistatin is a serine proteinase inhibitor and heparin-binding protein [20,41]. Kallistatin has been localized in vascular smooth muscle cells and endothelial cells of human blood vessels [18]. Heparan sulfate proteoglycans are present on the surface of vascular smooth muscle cells and endothelial cells [41,42]. TGF-β1 is a heparin-binding growth factor, and the ability of TGF-β1 to bind to heparin or related proteoglycans is the first step to initiate metabolic signals [43]. Our previous studies showed that the heparin-binding site of kallistatin is essential for antagonizing signaling pathways mediated by other heparin-binding proteins, such as VEGF, TNF-α, and HMGB1 [21–23]. Since TGF-β1 signaling is dependent on the binding to heparin, it is likely that kallistatin competes with TGF-β1 from binding to proteoglycans or heparin on the endothelial cell surface. Indeed, we showed that kallistatin's heparin-binding site is essential for preventing TGF-β1-induced miR-21-Akt signaling and ROS formation in endothelial cells. Therefore, these results provide new information regarding the role of kallistatin's heparin-binding site in antagonizing TGF-β-induced miR-21 expression and oxidative stress in endothelial cells.

Kallistatin's active site is necessary for covalent complex formation with tissue kallikrein, thereby inhibiting kallikrein's enzymatic activity [19]. Kallistatin administration has been shown to reduce organ damage in conjunction with reduced oxidative stress and increased eNOS expression and NO levels in animal models [24–26,28]. Moreover, kallistatin inhibited superoxide production and NAD(P)H oxidase activity stimulated by TNF-α or H_2O_2 via increased NO formation in cultured renal, cardiac and endothelial cells [25,26,28]. NOS-derived NO is an antioxidant by inhibiting NADPH oxidase activity [35,44]. In addition to eNOS/NO, both Sirt1 and FoxO1 are capable of suppressing ROS formation. Sirt1 can function to deacetylate and activate FoxO1, which is known to promote activation of the antioxidant enzyme manganese super-oxide dismutase [45]. Our present finding indicates that kallistatin's active site is essential for stimulating the expression of the antioxidant genes eNOS, Sirt1 and FoxO1, and the effect was blocked by genistein, a tyrosine kinase inhibitor. Therefore, kallistatin's active site is crucial for interacting with a tyrosine kinase, leading to increased eNOS expression in endothelial cells. The identity of

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the putative kallistatin-binding protein or kallistatin receptor on the endothelial cell surface awaits further investigation.

Taken together, the present study indicates that kallistatin has a novel role in inhibiting EndMT, and thus protection against fibrosis and cancer development. Mechanistically, kallistatin through its two structural elements differentially regulates miR-21, oxidative stress and eNOS expression. Kallistatin's heparin-binding site plays an important role in suppressing miR-21-Akt signaling and ROS formation, whereas its active site is key for stimulating the expression of eNOS, Sirt1 and FoxO1. Moreover, kallistatin via its active site interacts with a tyrosine kinase to stimulate eNOS synthesis, which can lead to NO formation and reduced oxidative stress. Thus, kallistatin exerts potent antioxidant activity by dual mechanisms: (1) blocking TGF-β-induced oxidative stress, and (2) increasing eNOS/Sirt1/FoxO1 levels. These new findings shed light on the pleiotropic functions and mechanisms of kallistatin in the potential treatment of fibrosis and tumorigenesis.

Acknowledgments

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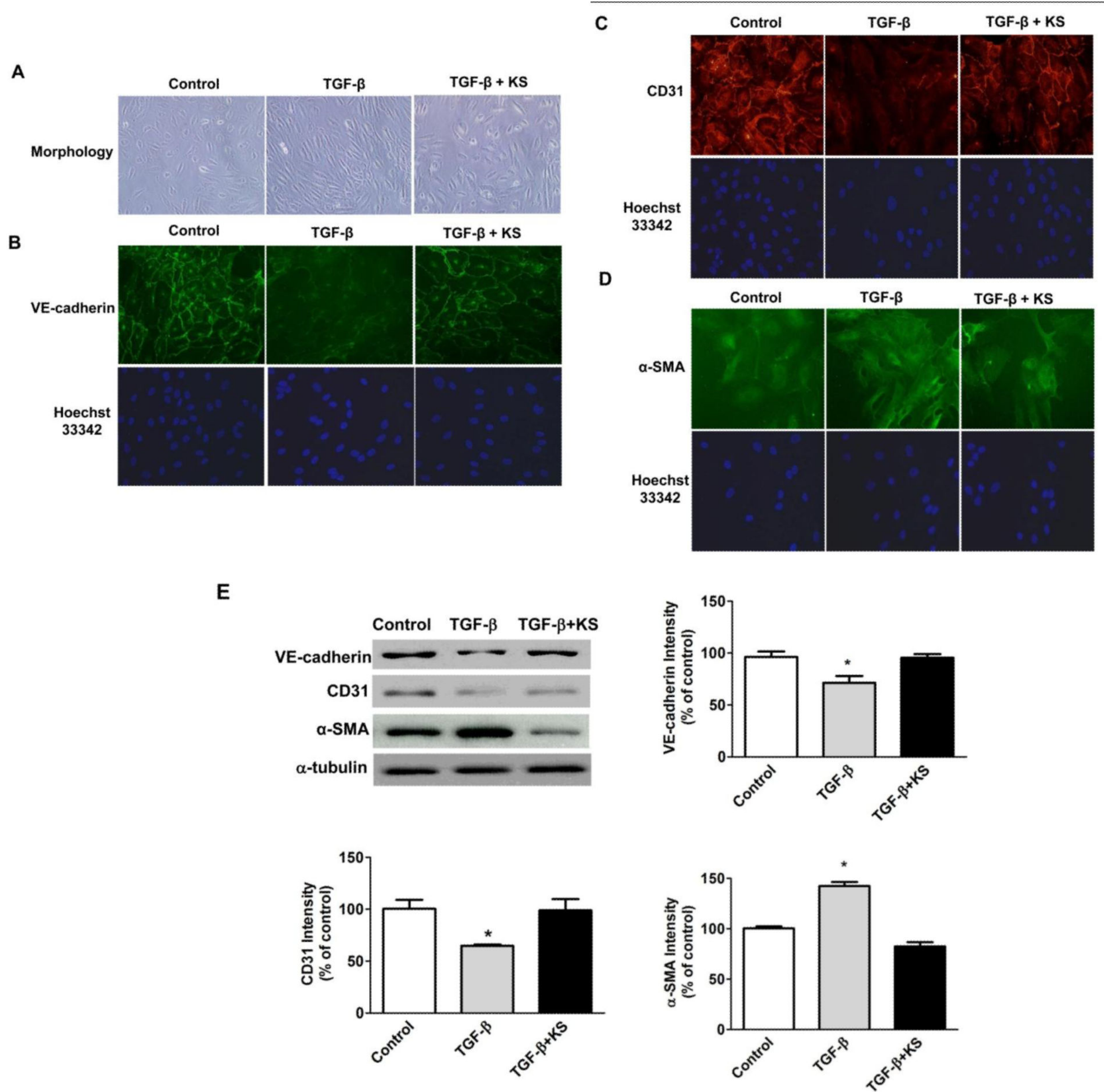

Fig. 1.

Kallistatin (KS) inhibits TGF-β1-induced EndMT. Kallistatin suppresses (A) TGF-β1-promoted morphology changes as indicated by light microscopy. Kallistatin also prevented TGF-β1-induced reduction of expression of the endothelial markers (B) VE-cadherin and (C) CD31, and elevation of expression of the mesenchymal marker (D) α-SMA in HUVECs, as indicated by representative immunofluorescence. Hoechst 33342 stains the nucleus. (E) Protein levels were determined by western blot and quantified. *P<0.05 vs. other groups. Values are expressed as mean±SE; n=3 experiments.

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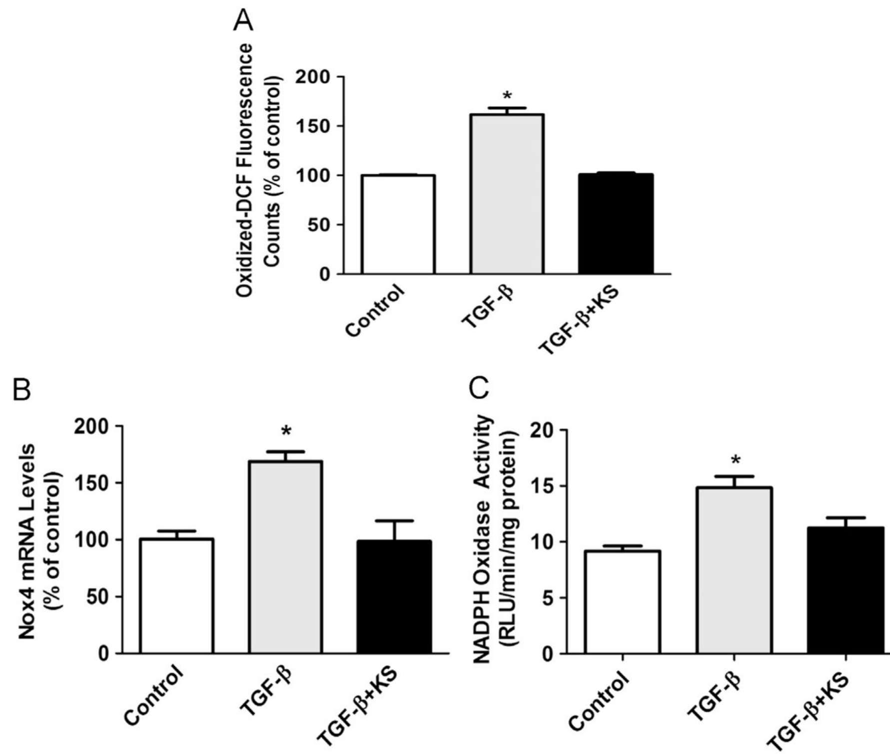

Fig. 2.

Kallistatin (KS) inhibits TGF-β1-induced ROS generation and NADPH oxidase (Nox) expression and activity. (A) 2′,7′-dichlorofluorescein was used to quantify ROS formation. (B) Nox4 mRNA levels were measured by real-time PCR. (C) NADPH oxidase activity was determined by lucigenin–chemiluminescence. *P<0.05 vs. other groups. Values are expressed as mean±SE; n=3 experiments.

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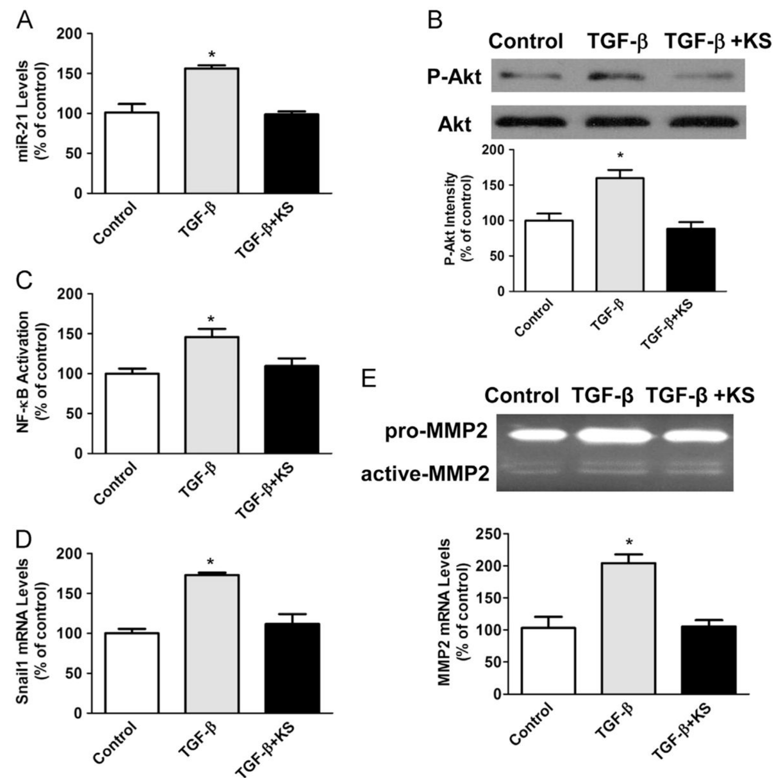

Fig. 3.

Kallistatin (KS) inhibits TGF-β1-induced miR-21 synthesis, Akt and NF-κB activation, Snail1 expression, and MMP2 synthesis and activity. (A) miR-21 expression was detected by real-time PCR, (B) Akt phosphorylation was detected by western blot, (C) NF-κB activation was detected by enzyme-linked immunosorbent assay kit, (D) Snail1 mRNA level was determined by real-time PCR, and (E) MMP2 activity was determined by zymography, and MMP2 mRNA expression was determined by real-time PCR. *P<0.05 vs. other groups. Values are expressed as mean±SE; n=3 experiments.

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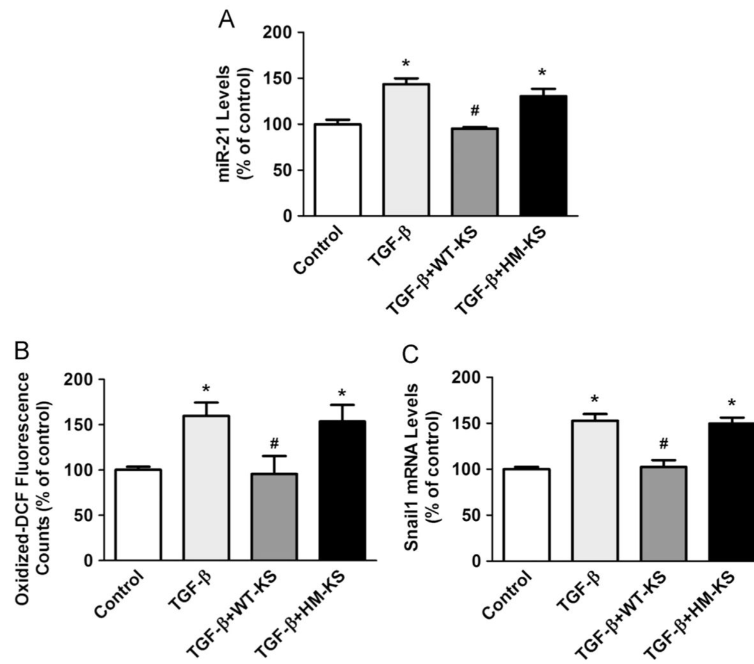

Fig. 4.

The heparin-binding site of kallistatin (KS) is essential to inhibiting TGF-β1-induced miR-21 synthesis, ROS production and Snail1 expression. (A) miR-21 synthesis was detected by real-time PCR, (B) ROS production was quantified with 2′,7′-dichlorofluorescein, and (C) Snail1 mRNA levels was determined by real-time PCR. WT-KS indicates wild-type kallistatin; HM-KS indicates heparin-binding site mutant kallistatin. *P<0.05 vs. control; #P<0.05 vs. TGF-β. Values are expressed as mean±SE; n=3 experiments.

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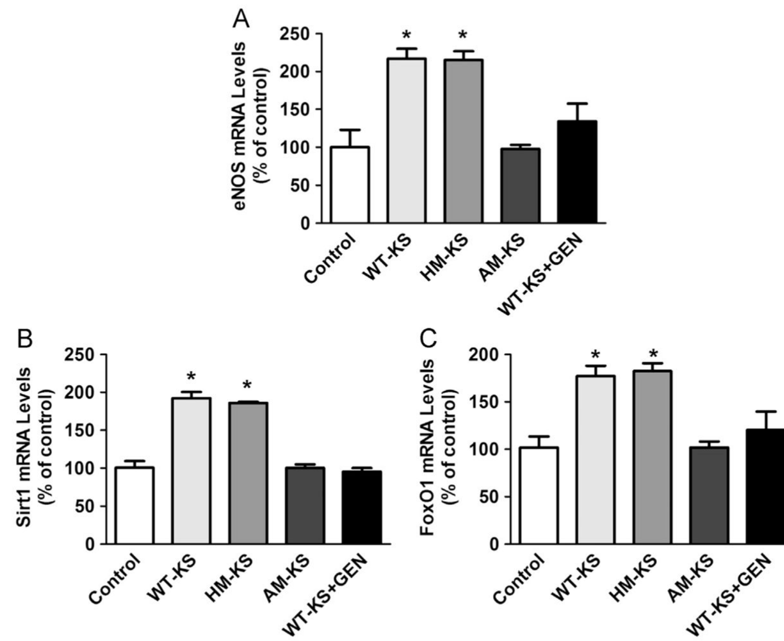

Fig. 5.

The active site of kallistatin is essential for increasing the expression of eNOS, Sirt1 and FoxO1 via a tyrosine kinase, as genistein blocked kallistatin's effect. (A) eNOS, (B) Sirt1 and (C) FoxO1 mRNA levels were determined by real-time PCR. WT-KS indicates wild-type kallistatin; HM-KS indicates heparin-binding site mutant kallistatin; AM-KS indicates active site mutant kallistatin; GEN indicates genistein. *$P<0.05$ vs. control. Values are expressed as mean±SE; $n=3$ experiments.

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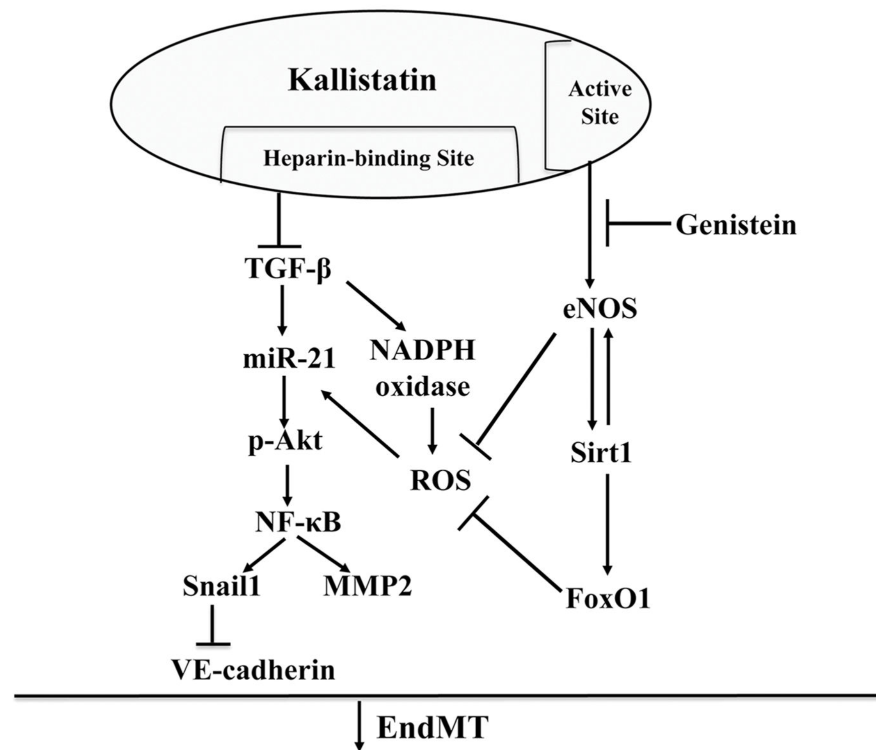

Fig. 6.

Hypothetic scheme: Kallistatin's heparin-binding site is responsible for suppressing TGF-β-induced miR-21-Akt signaling and ROS formation, while its active site is key for stimulating the expression of eNOS, Sirt1 and FoxO1. Kallistatin's effect on the expression of antioxidant genes is blocked by genistein, a tyrosine kinase inhibitor, suggesting that kallistatin's active site interacts with a tyrosine kinase. Our current data, together with previous studies, suggest that kallistatin's structural elements play an important role in attenuating endothelial-mesenchymal transition (EndMT).