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The myristoylated alanine-rich C kinase substrate (MARCKS) differentially regulates kinase interacting with stathmin (KIS) in vascular smooth muscle and endothelial cells and potentiates intimal hyperplasia formation

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Abstract

Objective: Restenosis limits the durability of all cardiovascular reconstructions. Vascular smooth muscle cell (VSMC) proliferation drives this process, but an intact, functional endothelium is necessary for vessel patency. Current strategies to prevent restenosis employ anti-proliferative agents that affect both VSMCs and endothelial cells (ECs). Knockdown of the myristoylated alanine-rich C kinase substrate (MARCKS) arrests VSMC proliferation and paradoxically potentiates EC proliferation. MARCKS knockdown decreases expression of the kinase interacting with stathmin (KIS), increasing $p27^{kip1}$ expression, arresting VSMC proliferation. Here, we seek to determine how MARCKS influences KIS protein expression in these two cell types.

Methods: Primary human coronary artery VSMCs and ECs were used for *in vitro* experiments. MARCKS was depleted by transection with small interfering RNA (siRNA). Messenger RNA (mRNA) was quantitated with the real-time reverse transcription polymerase chain reaction (qPCR). Protein expression was determined by Western blot analysis. Ubiquitination was determined with immunoprecipitation. MARCKS and KIS binding was assessed with co-immunoprecipitation. Intimal hyperplasia was induced in CL57/B6 mice with a femoral artery wire injury. MARCKS was knocked down *in vivo* by application of 10 μM siRNA targeting MARCKS suspended in 30% pluronic F-127 gel. Intimal hyperplasia formation was assessed by measurement of the intimal thickness on cross sections of the injured artery. Re-endothelialization was determined by quantitating the binding of Evans blue dye to the injured artery.

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Results: MARCKS knockdown did not affect KIS mRNA expression in either cell type. In the presence of cyclohexamide, MARCKS knockdown in VSMCs decreased KIS protein stability, but had no effect in ECs. The effect of MARCKS knockdown on KIS stability was abrogated by the 26s proteasome inhibitor MG-132. MARCKS binds to KIS in VSMCs but not ECs. MARCKS knockdown significantly increased the level of ubiquitinated KIS in VSMCs, but not ECs. MARCKS knockdown *in vivo* resulted in decreased KIS expression. Furthermore, MARCKS knockdown *in vivo* resulted in decreased 5-ethynyl-2'-deoxyuridine integration and significantly reduced intimal thickening. MARCKS knockdown enhanced endothelial barrier function recovery four days after injury.

Conclusions: MARCKS differentially regulates the KIS protein stability in VSMCs and ECs. The difference in stability is due to differential ubiquitination of KIS in these two cell types. The differential interaction of MARCKS and KIS provides a possible explanation for the observed difference in ubiquitination. The effect of MARCKS knockdown on KIS expression persists *in vivo*, potentiates recovery of the endothelium, and abrogates intimal hyperplasia formation.

Abstract

This basic research study demonstrates that MARCKS depletion decreases KIS expression through increased ubiquitination in smooth muscle cells but not endothelial cells in vitro, and results in decreased KIS expression, decreased intimal hyperplasia formation, and more rapid reendothelialization in vivo. This study suggests that both MARCKS and KIS are potential targets for therapy to prevent intimal hyperplasia formation.

Keywords

cell proliferation; cell migration; MARCKS; KIS

Introduction

Restenosis plagues all arterial reconstructions and significantly limits the durability of both open surgical and endovascular procedures. Current clinical strategies to prevent restenosis employ anti-proliferative agents that nonspecifically target both vascular smooth muscle cell (VSMC) and vascular endothelial cell (EC) proliferation (1-3). Because such therapies prevent the recovery of endothelium, patients must remain on dual anti-platelet therapy for an extended duration to prevent potentially life-threatening *in situ* thrombosis (4, 5). An ideal strategy would specifically inhibit the pathologic VSMC proliferative response while promoting restoration of the endothelium.

The myristolated alanine-rich C kinase substrate (MARCKS) differentially regulates VSMC and EC proliferation (6). *In vitro* and *in vivo* siRNA-mediated MARCKS knockdown inhibits VSMC proliferation but enhances EC proliferation (7). MARCKS, therefore, is a promising target for novel therapies to prevent restenosis. However, the underlying molecular mechanisms for MARCKS-mediated differential regulation of VSMC and EC proliferation are incompletely understood. The rationale for the proposed work is to further delineate the downstream effects of MARCKS signaling to identify other, potentially better or synergistic targets to prevent intimal hyperplasia formation.

MARCKS is a major substrate of protein kinase C (PKC) and is also implicated in the mitogen-activated protein kinase (MAP kinase) signaling pathway (8-10). In VSMCs and ECs MARCKS regulates $p27^{kip1}$ expression through the kinase interacting with stathmin (KIS) (7). KIS is a serine/threonine kinase transiting between the nucleus and cytoplasm during cell cycle (11, 12). On entry into phase G_1, $p27^{kip1}$ bound to the cyclin-dependent kinases (CDKs) in the nucleus, is phosphorylated by KIS at serine 10, which facilitates $p27^{kip1}$ transfer to the cytoplasm for proteasome-mediated degradation (13). KIS is associated with cell cycle progression in 3T3 cells (13) corneal ECs (14), and multiple human cancers including osteosarcoma (15) and breast cancer (16). In VSMCs, depleting KIS protein inhibits cell growth (17), and MARCKS knockdown in vascular ECs results in increased KIS expression and increased proliferation (7). What we do not know is how KIS is paradoxically regulated in these two cell types.

We seek to identify the point of regulation in both VSMCs and ECs where MARCKS exerts its differential effects on KIS expression. We will also provide *in vivo* evidence that MARCKS knockdown inhibits intimal hyperplasia formation and enhances re-endothelialization through regulation of KIS protein abundance.

Methods

Animals

The animal research in the present investigation was approved by the University of Maryland Institutional Animal Care Committee. CL57/B6 wild-type mice 8-12 weeks-old of both genders were purchased from the Jackson Lab (Bar Harbor, ME). Mice were maintained following the guidelines and protocols of the Animal Care and Use Committee of the University of Maryland School of Medicine.

Cell culture

Human coronary artery VSMCs and ECs (Lonza, Walkersville, MD) were cultured per the supplier's instructions. VSMCs were maintained in Smgm-2 Bulletkit (Lonza, cat#CC4147 culture media; ECs were maintained in Ebm-2 Bulletkit (Lonza, cat#CC3156). Cells from passage 3 to 7 were used in experiments.

siRNA transfection

MARCKS siRNA (siMARCKS) (5'-GGU GCC CAG UUC UCC AAG AUU-3'), and non-targeting, control siRNA (siControl) (5'-CGC ACC AGA ACA AAC ACA UU -3') were purchased from Dharmacon (Lafayette, CO). Cells were transfected using the DharmaFECT-Duo transfection reagent (Thermo Fisher Scientific, Waltham, MA). Unless otherwise stated, cells were maintained in culture for 72 hours after transfection before they were used in experiments.

Western blot analysis

Protein expression in VSMCs and ECs was determined by western blot analysis. The antibodies used were as the following: Anti-KIS antibody (Cat#SAB1300125), was purchased from Sigma Aldrich (St. Louis, MO). Anti-MARCKS antibody (Cat#5607S) and

anti-GAPDH antibody (Cat#2118L) were from Cell Signaling Tech. (Danvers, MA). Protein expression was quantitated through densitometry using the Image Station 4000 MMPro (Carestream Health, Rochester, NY). Signal intensity of the protein of interest was normalized to GAPDH signal intensity.

KIS protein stability assay

To determine KIS protein stability in VSMCs and ECs, cells were cultured in normal growth media and treated with the translation inhibitor cycloheximide (CHX) (10 μg/ml). Total protein samples were harvested at 0, 4 and 8 hours post CHX treatment. Data are expressed as folds of the relative KIS (KIS/GAPDH ratios) as compared with the relative KIS level at time 0 (before CHX treatment).

The role of the 26s proteasome on KIS degradation in VSMCs was determined with the proteasome inhibitor MG-132. Sub-confluent VSMCs were pre-treated with MG-132 (10 nM) for 16 hours then incubated with cycloheximide as described above.

KIS ubiquitination assay

KIS ubiquitination was assessed in the presence and absence of MARCKS knockdown in VSMCs and ECs. After siRNA transfection, cells were incubated with 10 nM of the 26s proteasome inhibitor MG-132 (18) for 16 hours. Polyubiquitin chains were immunoprecipitated from 150 μg protein sample using Polyubiquitin Affinity Resin (Pierce Biotechnology, Rockford, IL; Cat#89899). The resins were centrifuged to pull down the ubiquitinated proteins and then washed and eluted. The polyubiquitinated proteins were analyzed for KIS protein levels.

KIS and MARCKS immunoprecipitation assay

VSMCs and ECs cultured subconfluently were starved for 48 hours and then stimulated with 20% FBS for 10 min. Total protein lysates were collected with lysis buffer (10 mM TrisHCl, 100 mM NaCl, 1 mM EDTA, 1% Triton-X100, 0.5% NP-40, and Halt protease inhibitors (Thermo Scientific, Rockford, IL)) and then immunoprecipitated with anti-MARCKS antibody (EMD Millipore, Cat#AB9298, Darmstadt, Germany) and protein A-plus-agarose beads (Pierce Biotechnology, Rockford, IL). Immunoprecipitants were washed with cold lysis buffer, and heat denatured with 2x SDS Sample buffer (Invitrogen, Cat#NP0007, Carlsbad, CA) containing 0.1 M DTT. Blots were probed with the following antibodies: anti-KIS antibody (Santa Cruz, Cat#SC-393605, Santa Cruz, CA) and anti-MARCKS antibody. Reverse immunoprecipitation was performed to confirm the interaction of MARCKS and KIS,

Qualitative and quantitative assessment of mRNA expression

Messenger RNA (mRNA) expression was qualitatively assessed using the reverse transcription polymerase chain reaction (RT-PCR). Total RNA was purified from VSMCs and ECs with the Total RNA Purification Kit (Qiagen, Gaithersburg, MD).

mRNA expression was quantified using real-time reverse transcription quantitative polymerase chain reaction (qPCR). Total RNA (1 μg) was reverse transcribed with

SuperScript IV Master Mix (Thermo Fisher, Waltham, MA). The reactions were performed with Taqman probes and primers, Taqman Fast Advanced Master Mix and 50 ng of total cDNA equivalent per reaction. Real-time PCR reaction and signal detection was quantified with the Thermo Fisher ABI QuantStudio 3 qPCR machine. The Taqman probes used were: KIS, ABI gene assay ID Hs00332674_m1; housekeeping gene 18S rRNA, ABI gene assay ID Hs03928990_g1. The PCR was run with denaturation at 95°C for 30 s, annealing at 51°C for 30 s, and extension at 72°C for 45 s, total cycler number was 40. To quantify relative fold of mRNA levels, the delta Ct method was used.

Mouse femoral artery wire injury

Mice were anesthetized with inhaled 2% isoflurane. An incision was made over the upper left hind limb and the medial/caudal femoral artery branch was exposed. The branch was isolated with 6-0 silk suture over the proximal and distal segments of this vessel. A partial-thickness incision was made on the artery. A 0.014-inch diameter Spartacore wire (Abbott Vascular, Cat#1005201, Santa Clara, CA) was introduced into the proximal end of the vessel through the arteriotomy and passed proximally into the superficial femoral artery 5 mm proximal to the arterial bifurcation. This injury was repeated five times over the full distance of the wire injury zone to ensure complete stripping of the intimal layer. The proximal suture was tied as the wire was removed to achieve hemostasis.

in vivo siRNA transfection

The femoral arteries were transfected with either siControl or siMARCKS (10 μM) suspended in 30% Pluronic F-127 gel (Sigma Aldrich, St. Louis, MO) in PBS. This solution was prepared under sterile conditions and stirred for 48 hours at 4°C. The siRNA pluronic gel mixture was applied topically to the femoral artery inclusive of the entire zone of injury and allowed to solidify for 10 minutes.

Immunohistochemistry

At the predetermined time of euthanasia, the anesthetized mice were perfused through the left ventricle with 4% paraformaldehyde via a sternotomy. The femoral arteries were excised and snap-frozen in OCT compound (Sakura, Torrance, CA). The arteries were sectioned at 5 μm intervals beginning 2.5 mm proximal to the suture. For assessment of intimal hyperplasia formation, the sections were stained with hematoxylin and eosin, then counterstained with Verhoeff-Van Gieson (VVG) stain (Sigma Aldrich, Cat#HT25A; St. Louis, MO) to allow visualization of the internal elastic lamina (19). To assess MARCKS expression and KIS expression in the femoral arteries, sections were immunostained with anti-MARCKS antibody (EMD Millipore, Cat# AB9298; Billerica, MA), anti-KIS antibody (Sigma Aldrich, Cat#SAB1300125; St. Louis, MO). These sections were counterstained with α-smooth muscle actin (Sigma Aldrich, Cat#2547; St. Louis, MO) and 4’,6-diamidino-2-phenylindole (DAPI) (Sigma Aldrich, St. Louis, MO). Confocal microscopic images of the stained sections were acquired using a Zeiss 510 laser confocal microscope (Carl Zeiss, Germany). Zeiss Zen imaging software was used to collect and analyze the image data.

in vivo cell proliferation assay with 5-ethynyl-2'-deoxyuridine (EdU) labeling

Mice were injected with 5-ethynyl-2'-deoxyuridine (EdU) (50 μg/g body weight) intraperitoneally at 24 hours and again at 4 hours prior to euthanasia. At the time of euthanasia, the animal was perfused, and the femoral artery was dissected and fixed as described above. Frozen sections were permeabilized and treated with Click-it cocktail solution for 30 minutes to label EdU before the antibodies were applied (Invitrogen Cat# C10337, Carlsbad, CA). EdU labeled sections were analyzed using Zeiss Zen software (Carl Zeiss, Germany).

Evans blue staining and quantification

Evans blue dye was used to determine endothelial permeability after femoral artery wire injury. At the time of euthanasia, the animals were perfused with 5 ml of 0.3% Evans blue dye (Sigma Aldrich, Cat#E2129) through the left ventricle followed by 5 ml PBS. The injured femoral arteries were then incised longitudinally to expose the intima and examined for Evans Blue staining. A quantification method was used based on Dr. Aoki's protocol with minor modification (20). Femoral arteries were weighed and homogenized in 50% trichloroacetic acid solution (500 μl/mg tissue). Lysates were then centrifuged at 15,000 relative centrifuge force for 30 minutes at 4°C. The fluorescence (emission 610 nm/ excitation 680 nm) of supernatant was measured in a microplate fluorescence reader (BioTek, Winooski, VT).

Statistical analysis

All experiments were performed in three independent replicates unless otherwise noted. Data are presented as means ± standard deviation. Statistical analysis was performed on STATA software (STATA, College Station, TX). Significance of association of two means was assessed using the two-tail Student's *t*-test. A p-value < 0.05 was considered statistically significant.

Results

MARCKS knockdown does not affect KIS mRNA expression in VSMCs or ECs

MARCKS knockdown resulted in decreased MARCKS protein expression in both cell types three days after transfection. siMARCKS resulted in decreased KIS expression in VSMCs, but increased KIS expression in ECs (Figure 1A and 1B). However, siMARCKS did not alter KIS mRNA expression in either cell type (Figure 1C). This result was confirmed with qPCR. (Figure 1D).

MARCKS knockdown decreases KIS protein stability in VSMCs but not in ECs

To confirm that regulation of KIS protein expression by MARCKS knockdown occurs at the protein level (protein stability), we analyzed the KIS protein expression over time after treatment with the translation inhibitor cyclohexamide (CHX) (21). In VSMCs, MARCKS knockdown resulted in significantly decreased KIS protein expression from time 0 evident as early as 4 hours after CHX treatment. This decrease in KIS expression subsequently became

greater at 8 hours (Figure 2A, $p<0.05$ by two-tailed Student's t-test). In contrast, MARCKS knockdown did not affect KIS protein expression in ECs (Figure 2B).

MARCKS knockdown affects KIS protein ubiquitination

Computer modeling demonstrates that KIS possess several lysine residues which are candidates for ubiquitination. These residues are lysine 190, 282, and 410 (low confidence of binding) and lysine 387 (high confidence of binding) (22) suggesting that KIS expression is regulated through degradation (data not shown). To test this hypothesis, we repeated the KIS protein stability assay in the presence of the 26s proteasome inhibitor MG-132. Blocking the 26s proteasome with MG-132 in VSMCs abrogated the effect of MARCKS knockdown on KIS in VSMCs (Figure 3A and 3B). MARCKS knockdown resulted in significantly increased KIS ubiquitination in VSMCs but reduced KIS ubiquitination in ECs (Figure 3C and 3D).

MARCKS binds to KIS in VSMCs but not in ECs

To determine the involvement of MARCKS in the regulation of KIS stability during cell proliferation, we tested our hypothesis that MARCKS directly interacts with KIS and this association protects KIS protein from degradation. Total cell lysates were sampled for the expression of MARCKS and KIS with Western blot (Figure 4A). Total cell lysate was immunoprecipitated with anti-MARCKS. The precipitate was then blotted for KIS. Equal loading of precipitate was ensured by blotting for MARCKS (Figure 4B). MARCKS and KIS coprecipitated in VSMCs, but not ECs. Coprecipitation was potentiated with the addition of serum (Figure 4C). To confirm this finding, we immunoprecipitated with KIS and then blotted for MARCKS in VSMCs. As in Figure 4B, we demonstrated that MARCKS and KIS coprecipitate in VSMCs and this relationship is potentiated with serum stimulation (Supplemental Figure 1).

MARCKS knockdown prevents intima hyperplasia formation *in vivo*

The mouse femoral artery wire injury model of intimal hyperplasia and re-endothelialization was used to evaluate in vivo effects of MARCKS knockdown on VSMC proliferation, intimal hyperplasia formation, and re-endothelialization. Both femoral arteries were injured at the time of surgery. One side was treated with siControl and the contralateral side was treated with siMARCKS. At 6 weeks after injury, vessels treated with siControl had formed more intimal hyperplasia (Figure 5A) than those treated with siMARCKS (Figure 5B). The intima-to-media ratio (I/M ratio) was 1.16 ± 0.57 for vessels treated with siControl and 0.09 ± 0.02 for vessels treated with siMARCKS (Figure 5C).

MARCKS knockdown decreases time to return of endothelial integrity after wire injury

The mouse femoral artery wire injury model allows quantification of the re-endothelialization after vascular injury. Normally, the intact endothelium prevents penetration of Evans blue into the vessel wall and prevents staining of the artery (Figure 6A). At the time of injury, the femoral artery was treated with either siControl or siMARCKS. Four days after injury, the animals were euthanized and perfused with Evans blue. The injured femoral arteries treated with siControl were stained dark blue, indicating

that the injured endothelium had not recovered barrier function (Figure 6B). Whereas, arteries treated with siMARCKS resulted in less Evans blue staining (Figure 6C) compared with the siControl treated group. Treatment with siControl resulted in significantly more dye bound to the artery (297±15 ng/mg) compared to treatment with treatment with siMARCKS (170 ± 37 ng/mg, $p<0.05$) (Figure 6D).

KIS protein expression and VSMC proliferation are attenuated by MARCKS knockdown *in vivo*

Animals were euthanized 7 days after injury. Treatment with siMARCKS resulted in significantly less MARCKS expression (teal) throughout the vessel wall than treatment with siControl (Figure 7A and B). MARCKS knockdown resulted in decreased KIS expression (orange) (Figure 7C and D). Vessels treated with siMARCKS had less medial proliferation as assessed by EdU incorporation (green) (Figure 7E and F). The vessels were also counterstained with α-smc actin (red) and DAPI (blue) (Figure 7G-J). Images for KIS, EdU, α-smc actin, and DAPI are presented to demonstrate the co-localization of KIS and DAPI stained nuclei in vessels treated with siControl, but not siMARCKS (Figure 7K and L). Vessels treated with siControl had greater expression of KIS in the vessel wall compared with vessels treated with siMARCKS (4.13 ± 1.17 /1,000 μm^2, compared to 1.47` ± 0.15 / 1,000 μm^2, $p<0.05$) (Figure 7M). Vessels treated with siControl incorporated significantly more EdU than vessels treated with siMARCKS (2.90 ± 0.92 /1,000 μm^2 compared to 0.33 ± 0.11 /1,000 μm^2, $p<0.05$) (Figure 7N).

Discussion

MARCKS over expression is correlated with intimal hyperplasia formation in large animal bypass models (23, 24). Depletion of MARCKS through genetic manipulation attenuates intimal hyperplasia formation in the murine carotid ligation model (25). siRNA-mediated MARCKS knockdown attenuates vascular smooth muscle cell proliferation and accelerates re-endothelialization in the murine aortic injury model (7). Interestingly, MARCKS knockdown attenuates VSMC proliferation, but potentiates EC proliferation. This paradoxical effect of MARCKS on VSMC and EC proliferation makes MARCKS an attractive therapeutic target to prevent intimal hyperplasia formation. The effect of MARCKS on proliferation in these two cell types occurs through a $p27^{kip1}$, KIS-dependent mechanism (7). In this investigation, we sought to determine how MARCKS exerts opposite effects on KIS expression and proliferation in these two cell types.

The present investigation brings some clarity to the understanding of KIS regulation in these two cell types. KIS mRNA is not affected by MARCKS knockdown in either VSMCs and ECs. KIS possesses four lysine residues which are putative sites for ubiquitination as determined by computer modeling (22). MARCKS knockdown results in increased KIS ubiquitination and decreased KIS protein stability in VSMCs but not ECs. The effect of MARCKS knockdown on KIS stability was abrogated by the 26s proteasome inhibitor MG-132. It is plausible that MARCKS prevents KIS ubiquitination and thus KIS degradation in VSMCs. This hypothesis is supported by the finding that MARCKS and KIS coprecipitate in VSMCs, suggesting that these two proteins have a binding relationship that

prevents KIS ubiquitination. While there is evidence to support this mechanism of regulation in VSMCs, we failed to see increased ubiquitination of KIS or coprecipitation of KIS and MARCKS in ECs.

A mechanism for the observed difference in these two cell types is that different splice variants of KIS might be expressed in these two cell types and one variant possesses the MARCKS binding site (VSMCs) and the other does not (ECs). The 46 kDa protein KIS contains 7 introns and alternative promoters (different exon 1 and amino terminus), an alternately spliced exon 7, and differential usage of polyadenylation signal in exon 8. Thus, KIS has many options for alternative splicing and some splice variants have been associated with schizophrenia (26). Differential splicing is responsible for changes in protein ubiquitination in other systems (27). Alternative splicing of KIS in VSMCs and ECs is plausible if MARCKS knockdown had no effect on KIS expression in ECs.

In this investigation, we confirmed our *in vitro* findings that MARCKS knockdown attenuates VSMC proliferation by decreasing KIS expression. Interestingly, Dr. Nabel and colleagues report that absence of KIS results in increased intimal hyperplasia after femoral wire injury in KIS −/− mice (28), due to another effect of KIS on phosphorylation of stathmin, which results in microtubule instability and increased VSMC motility. This is in contrast to both our *in vitro* results demonstrating the relationship between KIS and MARCKS on proliferation through regulation of $p27^{kip1}$ protein expression and *in vivo* data generated in the same model of intimal hyperplasia. Furthermore, we assert in this work and in previous publications, that the effect of KIS on VSMC and EC phenotype is driven through its effect on $p27^{kip1}$ as opposed to its effects on microtubule stability. The difference between these results is possibly due to the fact that Dr. Nabel and colleagues employed a pan KIS knockout mouse where as our intervention was to knockdown MARCKS. There might be a systemic effect of KIS knockdown not seen with local administration of siRNA. Alternatively, the effect of MARCKS knockdown might have additional, yet identified effects that modulate proliferation in these two cell types.

The present work establishes a mechanism for differential regulation of KIS protein expression in VSMCs and ECs. One significant limitation of this work is that while we have proved a binding relationship between MARCKS and KIS in VSMCs, but not ECs, the immunoprecipitation assay used in this investigation only proves that the two proteins are bound in the cell lysate. In an intact cell these proteins might be segregated to separate subcellular compartments. Foster resonance energy transfer (FRET) imaging would complement the coprecipitation data presented herein. FRET imaging would allow observation of the interactions of these two proteins in intact, live cells. This technique would also allow us to study the binding of these two proteins at different stages of the cell cycle and in response to mitogens.

We were able to use siRNA to silence MARCKS *in vivo*. This work is the proof of principle that will serve as the basis for developing a method of delivery in a clinical setting. In the case of bypass surgery, the conduit could be transfected through immersion in the siRNA solution as was done in the PREVENT trial (29) or topically as was done in the PATENCY-1 trial (30). Transfection in the setting of an endovascular procedure could be accomplished

with an siRNA eluting stent. We are currently developing a methodology to create a stainless-steel stent with a polymer-based coating to provide a controlled release of siRNA to the treated artery.

Conclusion

In summary, MARCKS is an important mediator of proliferation in VSMCs and ECs. MARCKS binds to KIS in VSMCs, but not ECs. This binding interaction protects KIS from degradation. Disruption of this binding relationship allows KIS ubiquitination and subsequent degradation. This work more clearly delineates the mechanism through which MARCKS impacts VSMC and EC proliferation and has identified additional, and possibly synergistic targets to prevent intimal hyperplasia formation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

References

1. Duda SH, Bosiers M, Lammer J, Scheinert D, Zeller T, Tielbeek A, et al. Sirolimus-eluting versus bare nitinol stent for obstructive superficial femoral artery disease: the SIROCCO II trial. J Vasc Interv Radiol. 2005;16(3):331–8. [PubMed: 15758128]
2. Tepe G, Zeller T, Albrecht T, Heller S, Schwarzwalder U, Beregi JP, et al. Local delivery of paclitaxel to inhibit restenosis during angioplasty of the leg. N Engl J Med. 2008;358(7):689–99. [PubMed: 18272892]
3. Holmes DR Jr., Leon MB, Moses JW, Popma JJ, Cutlip D, Fitzgerald PJ, et al. Analysis of 1-year clinical outcomes in the SIRIUS trial: a randomized trial of a sirolimus-eluting stent versus a standard stent in patients at high risk for coronary restenosis. Circulation. 2004;109(5):634–40. [PubMed: 14769686]
4. Finn AV, Joner M, Nakazawa G, Kolodgie F, Newell J, John MC, et al. Pathological correlates of late drug-eluting stent thrombosis: strut coverage as a marker of endothelialization. Circulation. 2007; 115(18):2435–41. [PubMed: 17438147]
5. Hayashi S, Yamamoto A, You F, Yamashita K, Ikegame Y, Tawada M, et al. The stenteluting drugs sirolimus and paclitaxel suppress healing of the endothelium by induction of autophagy. Am J Pathol. 2009;175(5):2226–34. [PubMed: 19815708]
6. Monahan TS, Andersen ND, Martin MC, Malek JY, Shrikhande GV, Pradhan L, et al. MARCKS silencing differentially affects human vascular smooth muscle and endothelial cell phenotypes to inhibit neointimal hyperplasia in saphenous vein. FASEB J. 2009;23(2):557–64. [PubMed: 18940893]
7. Yu D, Makkar G, Dong T, Strickland DK, Sarkar R, Monahan TS. MARCKS Signaling Differentially Regulates Vascular Smooth Muscle and Endothelial Cell Proliferation through a KIS-, p27kip1- Dependent Mechanism. PLoS One. 2015;10(11):e0141397. [PubMed: 26528715]
8. Schonwasser DC, Palmer RH, Herget T, Parker PJ. p42 MAPK phosphorylates 80 kDa MARCKS at Ser-113. Febs Letters. 1996;395(1):1–5. [PubMed: 8849678]
9. Ohmitsu M, Fukunaga K, Yamamoto H, Miyamoto E. Phosphorylation of myristoylated alanine-rich protein kinase C substrate by mitogen-activated protein kinase in cultured rat hippocampal neurons following stimulation of glutamate receptors. Journal of Biological Chemistry. 1999;274(1):408–17. [PubMed: 9867858]
10. Monahan TS, Andersen ND, Martin MC, Malek JY, Shrikhande GV, Pradhan L, et al. MARCKS silencing differentially affects human vascular smooth muscle and endothelial cell phenotypes to inhibit neointimal hyperplasia in saphenous vein. Faseb Journal. 2009;23(2):557–64. [PubMed: 18940893]

11. Maucuer A, Le Caer JP, Manceau V, Sobel A. Specific Ser-Pro phosphorylation by the RNA-recognition motif containing kinase KIS. European Journal of Biochemistry. 2000;267(14):4456–64. [PubMed: 10880969]
12. Pedraza N, Ortiz R, Cornado A, Llobet A, Aldea M, Gallego C. KIS, a Kinase Associated with Microtubule Regulators, Enhances Translation of AMPA Receptors and Stimulates Dendritic Spine Remodeling. Journal of Neuroscience. 2014;34(42):13988–97. [PubMed: 25319695]
13. Boehm M, Yoshimoto T, Crook MF, Nallamshetty S, True A, Nabel GJ, et al. A growth factor-dependent nuclear kinase phosphorylates p27(Kip1) and regulates cell cycle progression. Embo Journal. 2002;21(13):3390–401. [PubMed: 12093740]
14. Lee JG, Song JS, Smith RE, Kay EP. Human Corneal Endothelial Cells Employ Phosphorylation of p27(Kip1) at Both Ser10 and Thr187 Sites for FGF-2-Mediated Cell Proliferation via PI 3-Kinase. Investigative Ophthalmology & Visual Science. 2011;52(11):8216–23. [PubMed: 21948550]
15. Archangelo LF, Greif PA, Maucuer A, Manceau V, Koneru N, Bigarella CL, et al. The CATS (FAM64A) protein is a substrate of the Kinase Interacting Stathmin (KIS). Biochimica Et Biophysica Acta-Molecular Cell Research. 2013;1833(5):1269–79.
16. Zhang DW, Tari AM, Akar U, Arun BK, LaFortune TA, Nieves-Alicea R, et al. Silencing Kinase-Interacting Stathmin Gene Enhances Erlotinib Sensitivity by Inhibiting Ser(10) p27 Phosphorylation in Epidermal Growth Factor Receptor-Expressing Breast Cancer. Molecular Cancer Therapeutics. 2010;9(11):3090–9. [PubMed: 21045138]
17. Langenickel TH, Olive M, Boehm M, San H, Crook MF, Nabel EG. KIS protects against adverse vascular remodeling by opposing stathmin-mediated VSMC migration in mice. Journal of Clinical Investigation. 2008;118(12):3848–59. [PubMed: 19033656]
18. Lee DH, Goldberg AL. Selective inhibitors of the proteasome-dependent and vacuolar pathways of protein degradation in Saccharomyces cerevisiae. J Biol Chem. 1996;271(44):27280–4. [PubMed: 8910302]
19. Puchtler H, Waldrop FS. On the mechanism of Verhoeff's elastica stain: a convenient stain for myelin sheaths. Histochemistry. 1979;62(3):233–47. [PubMed: 90671]
20. Aoki T, Sumii T, Mori T, Wang XY, Lo EH. Blood-brain barrier disruption and matrix metalloproteinase-9 expression during, reperfusion injury - Mechanical versus embolic focal ischemia in spontaneously hypertensive rats. Stroke. 2002;33(11):2711–7. [PubMed: 12411666]
21. Obrig TG, Culp WJ, McKeehan WL, Hardesty B. The mechanism by which cycloheximide and related glutarimide antibiotics inhibit peptide synthesis on reticulocyte ribosomes. J Biol Chem. 1971;246(1):174–81. [PubMed: 5541758]
22. Chen Z, Chen YZ, Wang XF, Wang C, Yan RX, Zhang Z. Prediction of ubiquitination sites by using the composition of k-spaced amino acid pairs. PLoS One. 2011;6(7):e22930. [PubMed: 21829559]
23. Kalish JA, Willis DJ, Li C, Link JJ, Deutsch ER, Contreras MA, et al. Temporal genomics of vein bypass grafting through oligonucleotide microarray analysis. Journal of Vascular Surgery. 2004;39(3):645–54. [PubMed: 14981462]
24. Willis DJ, Kalish JA, Li C, Deutsch ER, Contreras MA, LoGerfo FW, et al. Temporal gene expression following prosthetic arterial grafting. Journal of Surgical Research. 2004;120(1):27–36. [PubMed: 15172187]
25. Yu D, Makkar G, Strickland DK, Blanpied TA, Stumpo DJ, Blackshear PJ, et al. Myristoylated Alanine-Rich Protein Kinase Substrate (MARCKS) Regulates Small GTPase Rac1 and Cdc42 Activity and Is a Critical Mediator of Vascular Smooth Muscle Cell Migration in Intimal Hyperplasia Formation. J Am Heart Assoc. 2015;4(10).
26. Bristow GC, Lane TA, Walker M, Chen L, Sei Y, Hyde TM, et al. Expression of kinase interacting with stathmin (KIS, UHMK1) in human brain and lymphoblasts: Effects of schizophrenia and genotype. Brain Res. 2009;1301:197–206. [PubMed: 19747464]
27. Roy A, Al-Qusairi L, Donnelly BF, Ronzaud C, Marciszyn AL, Gong F, et al. Alternatively spliced proline-rich cassettes link WNK1 to aldosterone action. J Clin Invest. 2015;125(9):3433–48. [PubMed: 26241057]

28. Langenickel TH, Olive M, Boehm M, San H, Crook MF, Nabel EG. KIS protects against adverse vascular remodeling by opposing stathmin-mediated VSMC migration in mice. J Clin Invest. 2008;118(12):3848–59. [PubMed: 19033656]

29. Conte MS, Bandyk DF, Clowes AW, Moneta GL, Seely L, Lorenz TJ, et al. Results of PREVENT III: a multicenter, randomized trial of edifoligide for the prevention of vein graft failure in lower extremity bypass surgery. J Vasc Surg. 2006;43(4):742–51; discussion 51. [PubMed: 16616230]

30. Peden EK, O'Connor TP, Browne BJ, Dixon BS, Schanzer AS, Jensik SC, et al. Arteriovenous fistula patency in the 3 years following vonapanitase and placebo treatment. J Vasc Surg. 2017;65(4):1113–20. [PubMed: 27986480]

ARTICLE HIGHLIGHTS

Type of Research: Basic Science Research

Key Findings: MARCKS differentially regulates smooth muscle cell and endothelial cell proliferation. MARCKS knockdown increases KIS ubiquitination and decreases KIS expression in smooth muscle cells but not endothelial cells. In vivo, MARCKS knockdown decreases KIS expression, decreases intimal hyperplasia and increases the rate of reendothelialization.

Take home Message: MARCKS and KIS are both potential targets for therapy to prevent intimal hyperplasia formation.

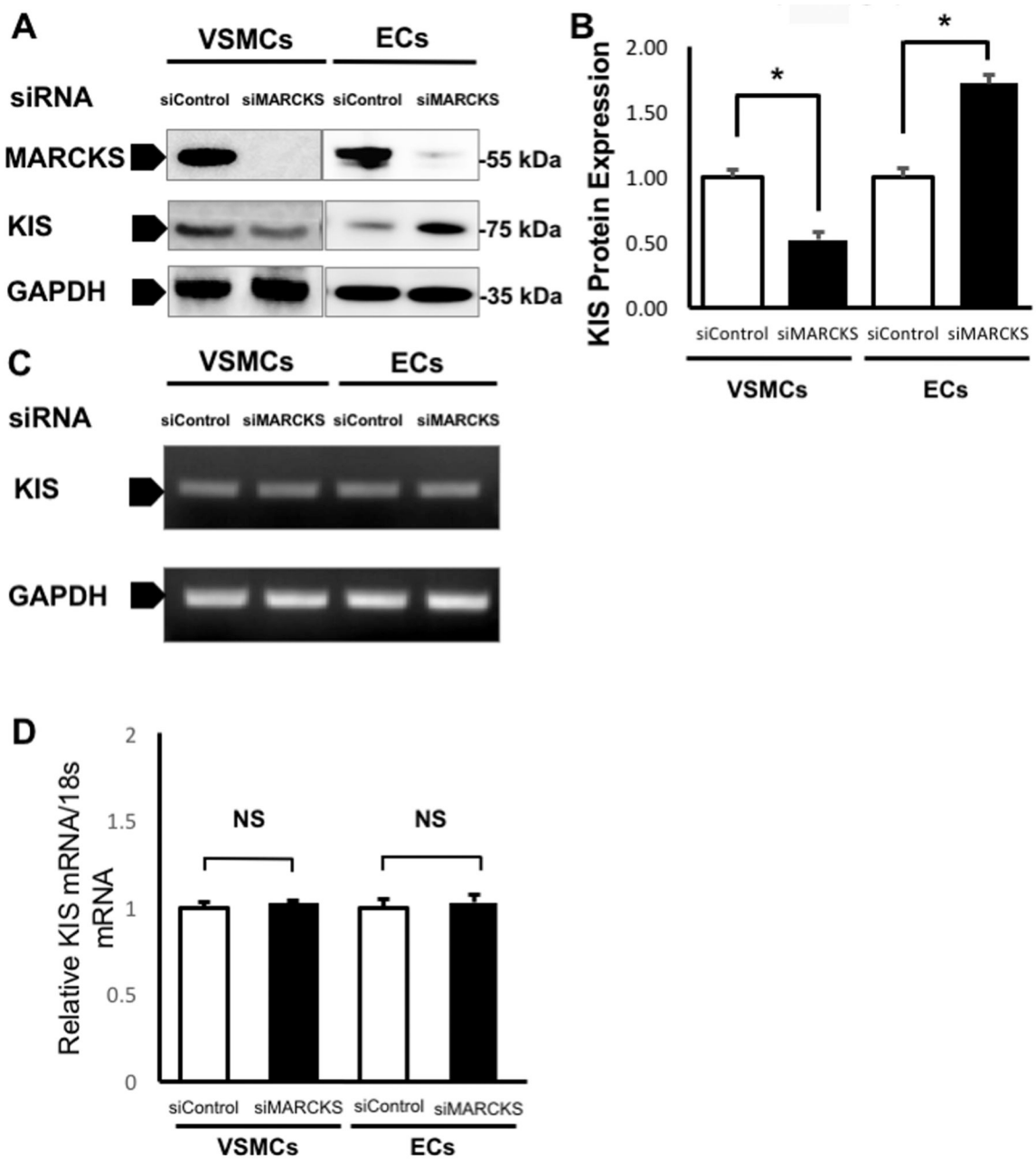

Figure 1. MARCKS knockdown does not affect KIS mRNA expression in either VSMCs or ECs.

A. Representative Western blots showing successful inhibition of MARCKS protein expression at 72 hours after treatment with siMARCKS. Total cell lysates from human coronary artery VSMCs and human coronary artery ECs were prepared and analyzed with Western blot. GAPDH was used as loading control. **B.** Data from three independent experiments demonstrate that MARCKS knockdown decreases KIS expression in VSMCs but increases KIS expression in ECs. * denotes $p<0.05$. **C.** RT-PCR analysis of KIS mRNA expression in siRNA-treated cells as described in Figure 1A. MARCKS knockdown does not affect KIS mRNA expression. **D.** Quantitative real-time RT-PCR (qPCR) was used to quantify mRNA expression in VSMCs and ECs after treatment with siMARCKS or

siControl. Values are presented as means ± standard deviation from three separate experiments. NS indicates no significant difference ($p>0.05$) between cells treated with siMARCKS and siControl.

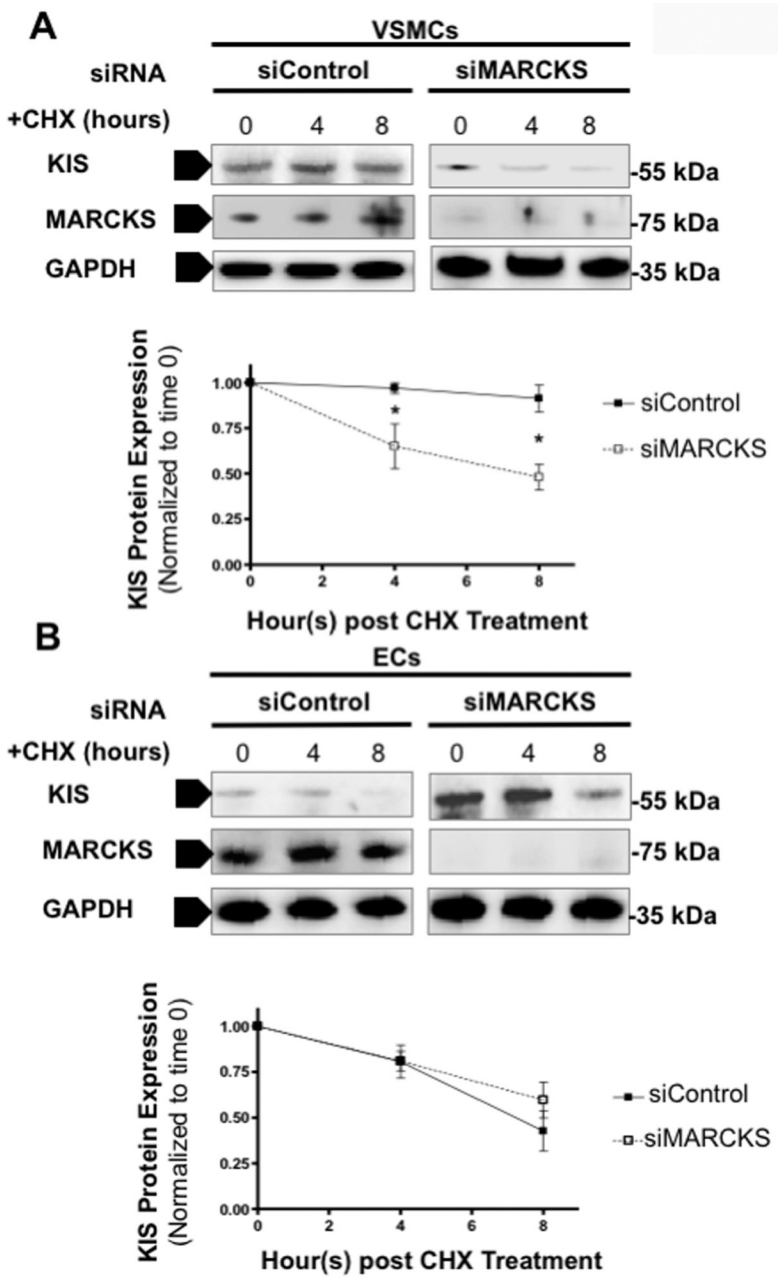

Figure 2. MARCKS knockdown decreases KIS protein stability in VSMCs but increases KIS stability in ECs.

Human coronary artery VSMCs and ECs were grown in culture and transfected with either siMARCKS or siControl. The cells were then treated with 10 μg/ml cyclohexamide (CHX) to inhibit protein synthesis. Total cell lysates were prepared at time points indicated. KIS expression was determined with Western blot and GAPDH was used as loading control. **A.** In VSMCs, MARCKS KD decreased KIS expression at 4- and 8-hours after CHX treatment compared to time 0 (two-tailed Student's t-test, $p<0.05$). **B.** In contrast, MARCKS knockdown did not affect KIS protein expression in ECs. Data are expressed as relative KIS/

GAPDH ratios as compared to time 0. Values are presented as means ± standard deviation from three separate experiments.

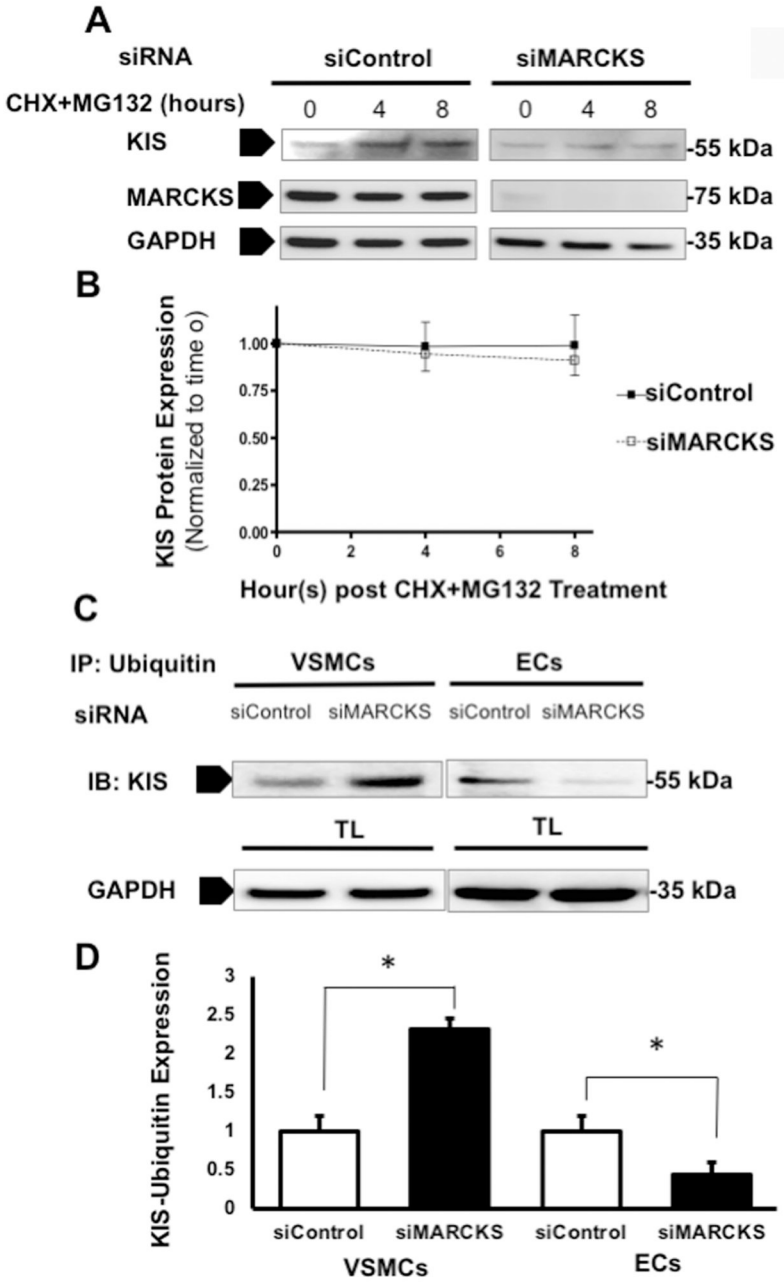

Figure 3. MARCKS KD affects KIS ubiquitination.

A. Human coronary artery VSMCs were grown in culture and transfected with either siMARCKS or siControl. The cells were pretreated with MG-132 (10 nM) for 16 hours before incubated with cyclohexamide (CHX) (10 μg/ml) for protein degradation assay. Pre-treating cells with MG-132 (10 nM) abrogated the decreased KIS protein stability previously observed with MARCKS knockdown. **B.** Data are expressed as relative KIS/GAPDH level as compared with time 0. Data are presented as means ± standard deviations of three separate experiments. Statistical significance was determined by the two-tail Student's *t*-test. No difference in KIS expression was detected in VSMCs with MARCKS knockdown in the presence of MG-132. **C.** KIS ubiquitination was analyzed in human coronary artery VSMCs

and ECs. Cells were grown in culture and transfected with either siMARCKS or non-targeting, siControl. The cells were then treated with MG-132 to block the 26s proteasome function and prevent the degradation of ubiquitinated protein. Total cell lysates were prepared immunoprecipitated with an Ubiquitin Enrichment Kit (Pierce). The precipitate was blotted with anti-KIS (IP). To confirm equal target loading, the total cell lysate (TL) was blotted for GAPDH. **D**. MARCKS knockdown resulted in increased KIS ubiquitination in VSMCs, but paradoxically decreased KIS ubiquitination in ECs. Data are presented as means ± standard deviations of three separate experiments. Statistical significance was determined by the two-tail Student's *t*-test. * denotes $p<0.05$).

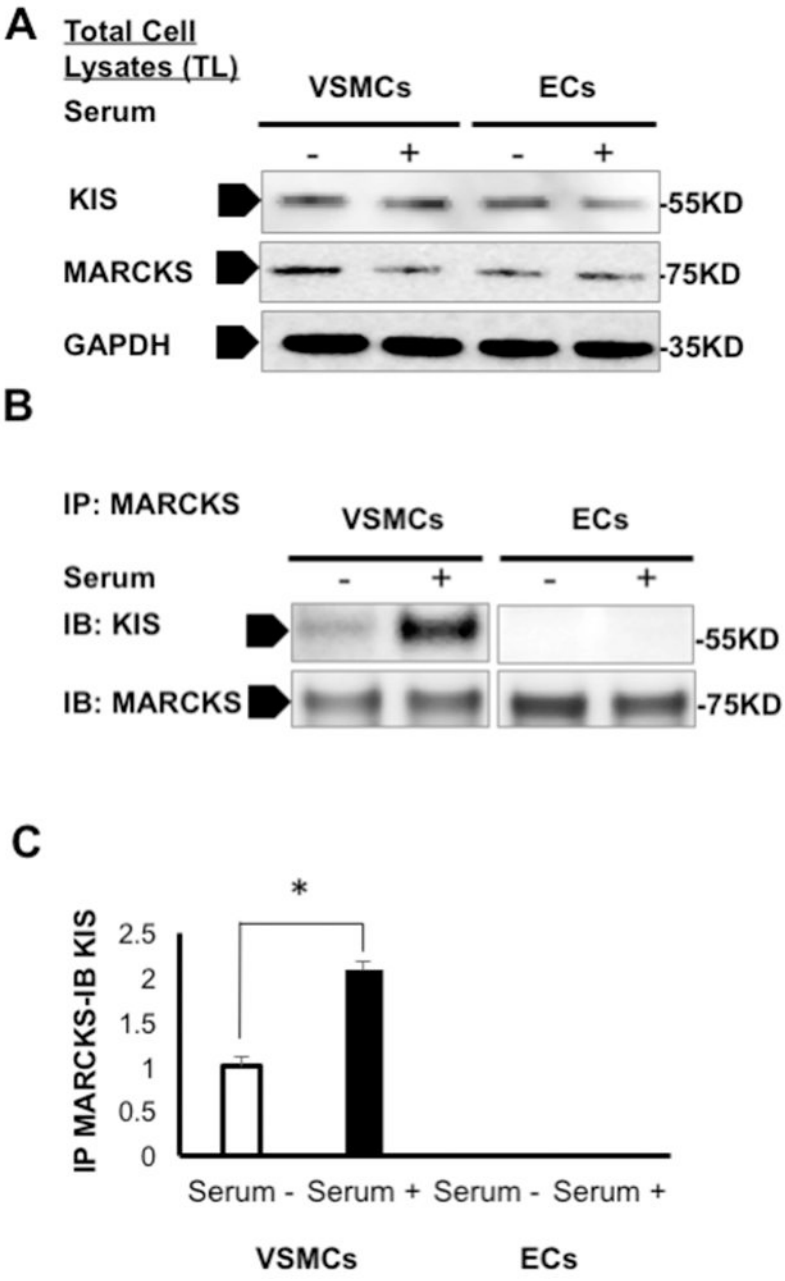

Figure 4. MARCKS binds KIS in VSMCs but not in ECs.

A. Human coronary artery VSMCs and ECs were cultured sub-confluently and starved for 48 hours. Cells were then stimulated with 20% fetal bovine serum (FBS) for 10 min. Total cell lysates were analyzed with Western blot to determine the expression of MARKCS and KIS. GAPDH was used as loading control. **B.** MARCKS protein was immunoprecipitated (IP) with anti-MARCKS antibody from the total cell lysate then immunoblotted (IB) with anti-KIS antibody. The precipitate was also blotted with anti-MARCKS antibody to demonstrate equal input loading. **C.** MARCKS coprecipitates with KIS in VSMCs, but not ECs. This relationship was potentiated with mitogen (serum) exposure in VSMCs.

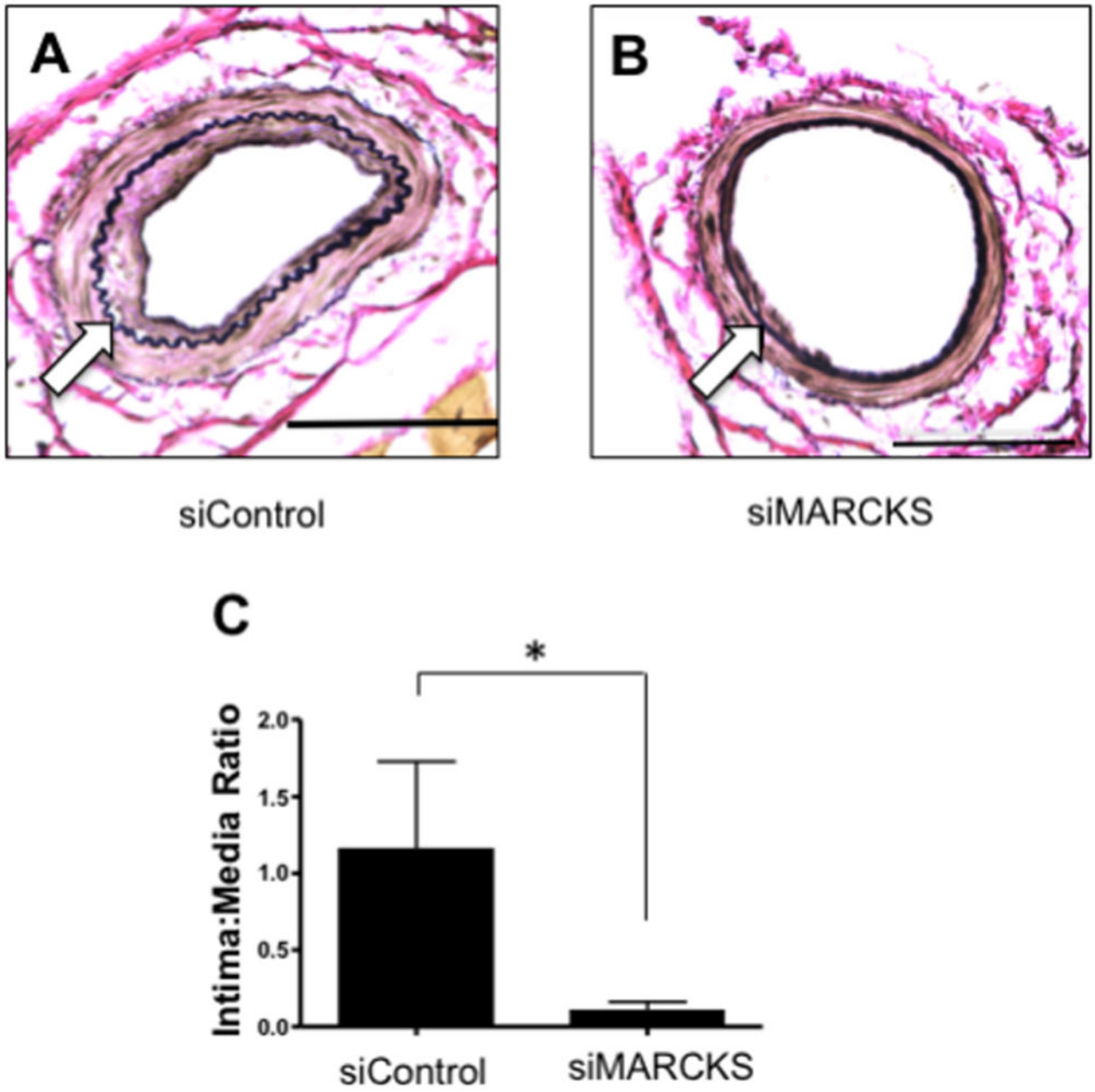

Figure 5. MARCKS knockdown prevents intima hyperplasia formation *in vivo*.

The mouse femoral artery wire injury model was used to generate intimal hyperplasia formation. The animals were subjected to bilateral femoral artery wire injury. At the time of surgery, the external surface of the artery was coated with either siControl or siMARCKS (10 μM) in a 30% pluronic gel solution. Animals were euthanized, and the femoral arteries were processed for histology at 6- and 12-weeks after injury. The sectioned femoral arteries were stained with hematoxylin and eosin and counter stained with Verhoeff-Van Gieson stain (VVG) to demonstrate the border between the intima and the media (arrows). **A and B.** At 6 weeks after injury, vessels treated with siControl had formed more intimal hyperplasia (Fig 5A) than those treated with siMARCKS (Fig 5B). **C.** The intima-to-media ratio (I/M

ratio) was 1.16 ± 0.57 for vessels treated with siControl and 0.09 ± 0.02 for vessels treated with siMARCKS. Data are presented as means ± standard deviations for three independent experiments. * denotes $p<0.05$ as determined by the two-tailed Student-t test. Scale bars = 100 μm.

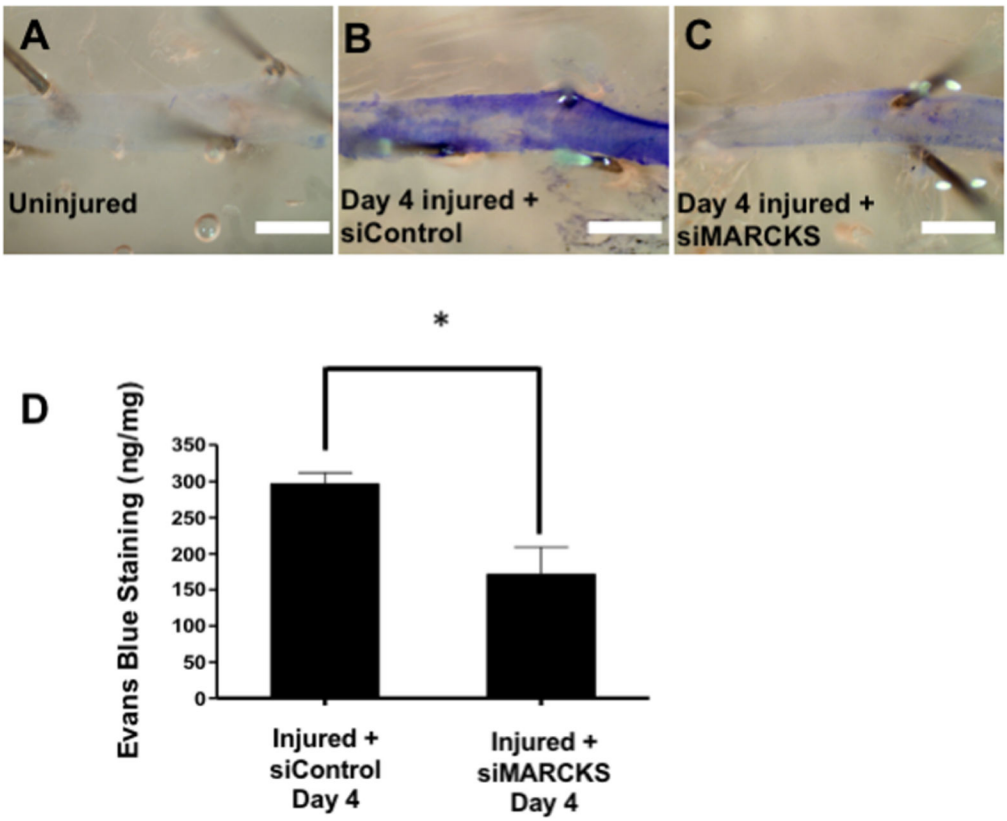

Figure 6. MARCKS knockdown decreases time to return of endothelial integrity after wire injury.

The mouse femoral artery wire injury model was used to generate an endothelial injury. The animals were subjected to bilateral femoral artery wire injury. At the time of surgery, the external surface of the artery was coated with either siControl or siMARCKS (10 μM in a 30% pluronic gel solution. At the time of euthanasia, the animals were perfused with 0.3% Evans Blue dye. **A.** Evans Blue dye is excluded from vessels with an intact endothelium and thus there is the minimal staining in the uninjured femoral artery. Wire injury disrupted the barrier function of the endothelium allowing the dye to penetrate the arterial wall and stain it blue. **B and C**. Four days after injury, vessels treated with siControl exhibited greater Evans Blue staining than vessels treated with siMARCKS. **D.** Evans Blue staining was further quantitated by measuring the amount of Evans Blue (ng) bound to the vessel and normalized to total vessel weight. Vessels treated with siControl bound significantly more Evans Blue (297 ±15.0 ng/mg) than vessels treated with siMARCKS (171 ± 37.5 ng/mg, $p<0.05$). Data are presented as means ± standard deviations for three independent experiments. * denotes $p<0.05$ as determined by the two-tailed Student-t test. Scale bars = 500 μm.

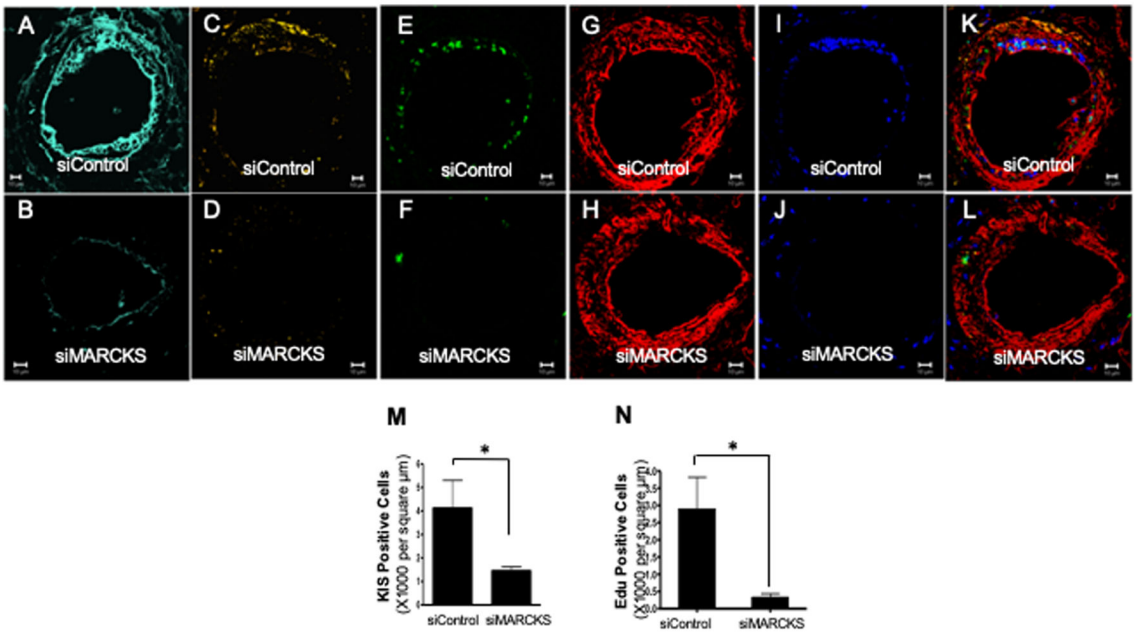

Figure 7. KIS protein expression and VSMC proliferation are attenuated by MARCKS knockdown.

The mouse femoral artery wire injury model of intimal hyperplasia was used to evaluate in vivo effects of MARCKS knockdown on VSMC proliferation and KIS expression. The animals were subjected to bilateral femoral artery wire injury. At the time of surgery, the external surface of the artery was coated with either siControl or siMARCKS (10 μM) in a 30% pluronic gel solution. Animals were euthanized 7 days after injury. **A and B**. Treatment with siMARCKS greatly reduced the amount of MARCKS protein expression throughout the vessel wall. **C and D.** MARCKS knockdown resulted in decreased KIS expression throughout the arterial wall. **E and F.** MARCKS knockdown reduced the vascular proliferative response to injury as measured by EdU incorporation. **G-J.** The vessels were counterstained for α-smc actin, and DAPI. **K and L.** Images for KIS, EdU, α-smc actin, and DAPI demonstrate colocalization of KIS and DAPI in vessels treated with siControl, but not siMARCKS. **M.** Treatment with siMARCKS resulted in significantly less KIS expressed in the vessel wall compared to treatment with siControl (1.47 ± 0.15 /1,000 μm^2 compared to 1.47 ± 0.15 /1,000 μm^2, $p<0.05$). **N.** Treatment with siMARCKS significantly reduced the vascular proliferative response to injury as determined by EdU incorporation (0.33 ± 0.11 / 1,000μm^2 compared to 2.90 ± 0.92 /1,000 μm^2, $p<0.05$). Data are presented as means ± standard deviations for three independent experiments. * denotes $p<0.05$ as determined by the two-tailed Student's-t test. Scale bars = 10 μm.