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The Human- and Smooth Muscle Cell-Enriched lncRNA SMILR Promotes Proliferation by Regulating Mitotic CENPF mRNA and Drives Cell-Cycle Progression Which Can Be Targeted to Limit Vascular Remodeling

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ABSTRACT

Rationale: In response to blood vessel wall injury, aberrant proliferation of vascular smooth muscle cells causes pathologic remodelling. However, the controlling mechanisms are not completely understood.

Objective: We recently showed that the human long non-coding RNA, SMILR, promotes vascular smooth muscle cells (vSMCs) proliferation by a hitherto unknown mechanism. Here, we assess the therapeutic potential of SMILR inhibition and detail the molecular mechanism of action.

Methods and Results: We used deep RNA-sequencing of human saphenous vein smooth muscle cells stimulated with IL1α and PDGF-BB with SMILR-knockdown (siRNA) or -overexpression (lentivirus), to identify SMILR regulated genes. This revealed a SMILR-dependent network essential for cell-cycle progression. In particular, we found using the fluorescent ubiquitination-based cell cycle indicator viral system that SMILR regulates the late mitotic phase of the cell cycle and cytokinesis with SMILR knockdown resulting in ~10% increase in binucleated cells. SMILR-pulldowns further revealed its potential molecular mechanism, which involves an interaction with the mRNA of the late mitotic protein CENPF and the regulatory Staufen1 RNA-binding protein. SMILR and this downstream axis were also found to be activated in the human ex vivo vein graft pathological model and in primary human coronary artery smooth muscle cells and atherosclerotic plaques obtained at carotid endarterectomy. Finally, to assess the therapeutic potential of SMILR, we used a novel siRNA approach in the ex vivo vein graft model (within the 30 min clinical time frame that would occur between harvest and implant) to assess the reduction of proliferation by EdU incorporation. SMILR-knockdown led to a marked decrease in proliferation from ~29% in controls to ~5% with SMILR depletion.

Conclusion: Collectively, we demonstrate that SMILR is a critical mediator of vascular smooth muscle cell proliferation via direct regulation of mitotic progression. Our data further reveals a potential SMILR-targeting intervention to limit atherogenesis and adverse vascular remodelling.

Keywords:
Long non-coding RNA; vascular smooth muscle cells; cell cycle; proliferation; vascular remodelling.
Nonstandard Abbreviations and Acronyms:

vSMCs  Vascular smooth muscle cells
IL1-PDGF  IL1-α and PDGF-ββ-induced
lncRNA  Long non-coding RNA
RNA-seq  RNA-sequencing
PCA  Principal component analysis
HSV  Human saphenous vein
HSVSMCs  Humans saphenous vein derived smooth muscle cells
HCASMCs  Human coronary smooth muscle cells
siRNA  Small interfering RNA
LNT  Lentivirus
GO  Gene ontology
FUCCI  Fluorescence Ubiquitin Cell Cycle Indicator
AurKB  Aurora kinase N
RAP  RNA antisense pulldown
STAU1  Staufen 1 RNA binding protein
FISH  Fluorescence in situ hybridisation

INTRODUCTION

Aberrant proliferation of vascular smooth muscle cells (vSMCs) is a common and functionally important mechanism that impacts on the pathogenesis of many vascular diseases, including intimal thickening associated with remodelling of intra-vascular stents, coronary artery bypass graft failure, atherosclerosis and aortic aneurysm formation. In particular, vSMC proliferation is promoted by the injurious microenvironment, partly through the increased exposure of vSMC to inflammatory cytokines and growth factors such as interleukin-1 (IL-1) and platelet derived growth factor (PDGF), respectively. These often act in synergy to promote a proliferative phenotype with associated activation of critical gene networks, such as metalloproteinases. Clinically, targeting vSMC proliferation is exceptionally effective at reducing adverse vascular remodelling following balloon angioplasty and vessel stenting, evidenced by extensive research and development of anti-proliferative drug eluting stent technologies. For iatrogenic vascular injury, pathogenic SMC proliferation causes intimal hyperplasia and luminal narrowing of blood vessels in the setting of vascular stenting or vein graft failure. In more complex settings, such as atherosclerosis, vSMC proliferation is central to the accumulation of large numbers of plaque-derived vSMC that not only contribute to the atherogenic process itself but can also confer plaque stabilisation.

Despite context-dependent heterogeneity in vSMC pathobiology, the underlying activation of vSMC proliferation is a central phenotype to the progression of vessel wall dysfunction. It is therefore imperative to further understand the molecular mechanisms that govern vSMC proliferation pathways in order to advance innovative therapies.

Recently, robust evidence has revealed that non-coding RNAs may play a vital role in the regulation of tissue homeostasis, including cardiovascular homeostasis, and hence pathophysiological conditions. Mammalian genomes are pervasively transcribed to produce thousands of long non-coding RNAs (lncRNAs). LncRNAs are widely involved in physiological and pathological processes such as cancer, autoimmune diseases and cardiac disease. LncRNA can exert their function via a broad range of activities including, but not limited to, chromatin remodelling, formation of nuclear bodies, and activities as scaffolds and guides. Previous findings have suggested that a substantial proportion of lncRNAs reside within, or are dynamically shuttled to, the cytoplasm in order to regulate mRNA stability, protein translation, microRNA availability and impact upon protein modifications. Such RNA-based regulation generally relies on lncRNA interactions with RNA binding proteins. We previously identified the...
lncRNA SMILR by RNAseq of human vSMCs following activation by IL-1 and PDGF-ββ signalling. SMILR is an intergenic and poorly conserved lncRNA that consists of only a single 3-exon polyadenylated transcript that is vSMC-enriched, and its knockdown by RNA interference blocked IL-1 and PDGF-ββ-induced (IL1-PDGF) vSMC proliferation. Thus, we reasoned that identifying the downstream targets and binding partners of SMILR would reveal the specific mechanism by which it regulates vSMC proliferation and hence provide a novel therapeutic target for preventing adverse vascular remodelling.

METHODS

The authors declare that all supporting data and materials are available within the article [and its online supplementary files] and available from the corresponding author upon reasonable request. All RNA sequencing data have been made deposited in the Gene Expression Omnibus (GEO) repository, study number GSE120521 for the atherosclerosis RNAseq and GSE117608 for SMILR RNAseq.

Expanded information about materials and methods are available in Online Supplementary Material.

Declaration of Helsinki.

All studies comply with the Declaration of Helsinki, that the locally appointed ethics committee has approved the research protocol and that informed consent has been obtained from the subjects.

Primary human saphenous vein smooth muscle cells (HSVSMC) were isolated via explant technique from consented patients and cultured as previously described. All procedures had local ethical approval (15/ES/0094). HSVSMC from passage 3 to 5 were used for this study and cells were synchronised in DMEM containing 0.2% FBS for 48 h prior to experimentation. Modulation of SMILR expression was performed through the utilisation of dsiRNA or SMILR lentivirus with appropriate controls and their effect on the genome was assessed via RNA sequencing and confirmed through subsequent qRT-PCR validation. All qRT-PCR data was analysed via the 2-ΔΔCt method. This method utilises a house keeping gene and ubiquitin C (UBC) was selected as a housekeeping gene due to its stability across all groups and conditions studied. Data is graphed as relative quantification normalized to the UBC housekeeping gene.

Assessment of siRNA-SMILR on SMC cell cycle was performed via FUCCI-FACS analysis and confocal imaging for the % of binucleated cells. SiRNA targeting AURKB was used as a positive control in these studies as previously described 19, 20.

To evaluate binding partners of SMILR, antisense SMILR or GFP probes were designed. For each pulldown experiment either 5 GFP or 5 SMILR probes were utilised to capture bound RNA. Prior to RNA extraction, RNA was spiked with 75ng of total C. elegans RNA and AMA1 used as a reference gene as previously described. qRT-PCR was utilised to assess RNA expression.

To assess if SMILR exhibited any venous/arterial differences in expression or function, human coronary artery smooth muscle cells (HCASMC) were utilise and cultured under the same conditions as HSVSMCs. Stimulation of these cells was performed under basal and IL1/PDGF-BB stimulated conditions as described in Ballantyne et al and assessment of the effect of siRNA-SMILR on HCASMC proliferation (Edu-FACS), binucleation (confocal imaging) and downstream target expression (qRT-PCR) was performed.

To address if SMILR exhibited any protein binding partners, SMILR protein pulldowns were performed utilising streptavidin magnetic beads to capture the biotinylated RNA target and any bound proteins from stimulated smooth muscle cells. Mass spectrometric analysis was utilised to identify proteins for subsequent downstream analysis and validation. Anti-Stau1 pulldowns were utilised as validation with appropriate igG control to confirm SMILR and other RNA target binding by qRT-PCR.
Similar to Ballantyne et al., patients with symptomatic carotid artery stenosis scheduled to undergo carotid endarterectomy were recruited from neurovascular clinics at the Royal Infirmary of Edinburgh to undergo separate [18F]-fluoride and [18F]-fluorodeoxyglucose ([18F]-FDG) positron emission tomography combined with computed tomography (CT) scans. Regions of stable and unstable plaque were denoted by low and high tracer uptake respectively and appropriately dissected. RNA sequencing was performed to assess transcriptomic differences between plaque sections and SMILR expression assessed via qRT-PCR. In situ hybridisation was utilised to visualise the localisation of SMILR within the plaque regions.

To assess the potential clinical utilisation of siSMILR, segments of human saphenous vein obtained from consenting patients undergoing bypass surgery were pinned down with minutien pins on a Sylgard coated dissection dish with the luminal surface facing upward for 0, 7 or 14 days with media refreshed every 2 days. At day 0 and after 7 and 14 days of culture, the vein segments were washed in PBS and snap frozen for subsequent RNA extraction or fixed in 4% PFA for histology. Proliferation of segments was assessed through the utilisation of Click-it® EdU Alexa Fluor® 488 In Vivo Imaging Kit. Expression levels of SMILR and target RNA were assessed through qRT-PCR analysis. For siRNA transfections HSV segments cut in equal pieces of approximately 1 cm² were bathed in PBS containing 25 μM siSMILR and scrambled siRNA control for 30 minutes in 24-well plate. After 30 minutes of incubation, the vein segments were washed with PBS and pinned down as described above. To confirm siRNA transfection, cy3 tagged SMILR was transfected and visualised along with DAPI and αSMA co staining via confocal imaging.

Samples of ≥n=5 were subjected to Shapiro-Wilk test to assess normal distribution followed by student t test or ANOVA. Normal distribution cannot be determined on small samples sizes and samples with n<5 were assumed to be not normally distributed and subjected to Iman and Conover non parametric ranking followed by students t test or ANOVA. Statistical significance P<0.05 under all conditions.

RESULTS

Manipulation of SMILR expression identifies a target cell cycle network in HSVSMCs.

SMILR depletion and overexpression was previously shown to decrease and increase, respectively, proliferation induced by stimulation of HSVSMCs with IL1-PDGF. However, no characterisation of the mechanisms of regulation of SMILR by IL1-PDGF was described. Accordingly, we sought to identify the potential transcription factor binding sites within the promoter region of SMILR (Supplemental Figure IA). Within the 2000bp upstream of SMILR’s transcription start site, we identified binding sites for transcription factors that are activated by IL1α and/or PDGF-BB including: NF-KB, CEBPβ, ETS1, AP1, NFAT, IRF8, MYB, and AML (Supplemental Figure IB). Of the commercially available transcription factor inhibitors, we analysed the subsequent effects on SMILR expression following IL1-PDGF stimulation. This identified that the up-regulation of SMILR following IL1-PDGF stimulation may, in part, be due to activation of NF-KB (Supplemental Figure IC and controls in ID). Interestingly, SMILR overexpression does not trigger quiesced HSVSMCs to proliferate in the absence of IL1-PDGF stimulation (Supplemental Figure II). Therefore, to further determine the downstream effects of manipulation of SMILR levels on proliferation, RNA-seq was performed on stimulated HSVSMCs exposed to either SMILR depletion via siRNA (siSMILR) or overexpression via lentivirus (SMILR LNT) treatment to identify a downstream SMILR-dependent transcriptome (Figure 1A).

Quantification of miR-146a, -221 and -222 by qRT-PCR confirmed activation of the IL1 and PDGF signalling pathways, respectively (Supplemental Figure III). Alterations in SMILR expression levels were validated by qPCR (Figure 1B). Considering a fold change ≥ 1.5 and an adjusted FDR p <
0.05, 523 (334 downregulated and 189 upregulated) and 183 (126 upregulated and 57 down-regulated) transcripts were significantly differentially expressed following knockdown or overexpression, respectively (Figure 1C). As we observe opposing effects on proliferation with SMILR knockdown and overexpression, we focused on the transcripts that were dysregulated in opposing levels. This revealed 68 transcripts (Figure 1C and D) indicating that such an approach might be powerful in identifying a distinct SMILR-targeted biological interactome. This set of SMILR-regulated genes was enriched for cell division-related and nucleosome assembly Gene Ontology (GO) terms (Figure 1E). Interestingly, analysis by STRING identified that 59% (n=40 of 68 genes) of the overlapping genes were associated with a single network involved in progression through the cell cycle, primarily the mitotic phase (Figure 1F). The top 20 genes identified by RNAseq that were differentially regulated genes by SMILR (Supplemental Figure IV) were selected for further validation in 3 different patient saphenous vein derived smooth muscle cells by qRT-PCR. We observed consistent and robust opposing regulation of the network following SMILR depletion and overexpression in HSVSMCs stimulated with IL1-PDGF (Figure 1G,H). In agreement with the absence of proliferation phenotype after SMILR overexpression in non-stimulated quiescent conditions, SMILR overexpression in non-stimulated quiescent HSVSMCs did not result in transcription changes of the identified network (Supplemental Figure IIB). Collectively, these data suggest that SMILR mechanistically targets the vSMC cell cycle in response to IL1-PDGF stimulation.

**SMILR directly targets CENPF in the cell cycle network.**

With both overexpression and knockdown (Supplemental Figure IV) approaches affecting SMILR expression levels predominantly in the cytoplasmic fraction, we therefore reasoned that SMILR could...
directly regulate the identified affected genes by binding to the mRNA in the cytoplasm. We used a database of predicted lncRNA-RNA interactions by Terai et al \(^\text{27}\) and considered the top100 genes predicted to interact with SMILR based on SumEnergy (Supplemental Table I). These genes were analysed in terms of expression level in stimulated vSMC, differential expression in SMILR depleted or SMILR overexpressed conditions, as well as differential expression upon stimulation with IL1-PDGF (see filtering details in Supplementary methods and summary in Supplemental Table I). This revealed that CENPF, a mitotic centromere protein, was the highest ranked mRNA predicted to interact with SMILR (minimum and sum energy of -35 and -2631 kcal/mol, respectively, Supplemental Table I). The predicted interacting base pair region of the SMILR/CENPF mRNA interaction extends across 51 base pairs (39-90) within the sequence of SMILR and 58 base pairs (3291-3349) within the coding sequence of CENPF transcript (Supplemental Figure VII). We used RNA antisense pulldown (RAP) followed by qRT-PCR to confirm this predicted interaction. Two sets (5 even and 5 odd) of 3'-biotinylated DNA capture oligonucleotides were designed to hybridise specifically to SMILR (see Supplementary Methods) \(^\text{28, 29}\). One set of five GFP-specific 3'-biotinylated DNA capture oligonucleotides were also used as a negative control. A schematic overview of the experimental design is provided in Figure 3A. The relative enrichment of SMILR and the CENPF mRNA present in both the SMILR-even and -odd pools were calculated with respect to the GFP pool, which was used as background reference. We observed a 3-fold and 4-fold enrichment of SMILR with the even and odd probes, respectively. CENPF transcript was also co-enriched by 13-fold and 7-fold in the even and odd SMILR pulldowns, respectively, thereby independently validating the predicted interaction between SMILR and CENPF mRNA. Importantly, MKI67 mRNA another downstream target within the SMILR-dependent cell cycle network was assessed in the even and odd SMILR pulldowns and found to not to be enriched – suggesting specificity for a SMILR: CENPF mRNA interaction within the interactome (Figure 3B). Additionally, in agreement with the RNAseq data, SMILR depletion and overexpression led to a down- and upregulation of CENPF transcript levels, respectively (Figure 3C and D). While having no effect on apoptosis, CENPF depletion (Supplemental Figure VIII) resulted in a significant decrease in EdU incorporation (Figure 3E-F). Additionally, similar to previous findings \(^\text{30, 31}\), CENPF depletion resulted in an increase in the percentage of binucleated cells (12.5 ± 1.2%) comparable to SMILR knockdown (13.7 ± 2.2%), which was significantly greater than that observed under control conditions (6.0 ± 2.0%, \(p < 0.05\), Figure 3G-H). Importantly, knockdown of CENPF also phenotypically mimics the effects of SMILR knockdown on key genes within the cell cycle network (Figure 3I). Thus, these data support the concept that SMILR positively targets CENPF mRNA which is critical for vSMC proliferation.

To further examine the temporal involvement of SMILR and CENPF transcripts in promoting IL1-PDGF induced proliferation, time course experiments were employed and show that SMILR expression is significantly upregulated prior to significant EdU+ incorporation and CENPF mRNA expression is detected (Supplemental Figure IX). Taken together, this suggests that SMILR is required at the early stages of IL1-PDGF stimulation to promote the induction of proliferation and mitotic progression.

*The SMILR:CENPF RNA interaction is regulated by Staufen1.*

RNAs, including lncRNAs, have been found to occasionally contain structural motifs that can interact with other RNAs to form functional RNA-RNA hybrids, which can then recruit proteins that regulate their function or stability \(^\text{32}\). Accordingly, to understand whether the function of SMILR/CENPF RNA hybrid is dependent on a RNA:protein binding interaction, we performed pulldowns utilising 3'-desthiobiotin-labelled full length SMILR and protein lysates from IL1-PDGF stimulated HSVSMCs (Figure 4A). Mass spectrometry identified 14 potential SMILR-binding proteins (Figure 4B). The RNA binding protein staufen1 (STAU1), known to be involved in mRNA decay and binds lncRNA and mRNA hybrids \(^\text{33}\), was clearly enriched in SMILR pulldowns when compared to beads alone or control 3'-desthiobiotin-labelled full length GFP pulldowns (Supplemental Figure X). Moreover, previous reports have suggested that STAU1 is involved in checkpoint decisions in G2 and/or G2/M transitions, which intersects with the cell cycle defects observed with siSMILR \(^\text{34}\). Hence, STAU1 appears to be a prime
candidate partner for SMILR’s mechanism of action. Immunoprecipitation of STAU1 from HSVSMC lysates stimulated with IL1-PDGF followed by qRT-PCR revealed enrichment of SMILR by 2.8 ± 1 fold (p < 0.05) when compared to IgG controls (Figure 4C), validating the mass spectrometry results. Additionally, we found that STAU1 is likely to bind to SMILR within the first half of its sequence, which as mentioned above, is the predicted interaction site with CENPF (Supplemental Figure VII and XI). To explore the involvement of STAU1 in controlling the proliferative phenotype mediated by SMILR and CENPF, we knocked down STAU1 using dsiRNA, and revealed an increase of SMILR and CENPF mRNA by 3.3 ± 0.9 fold and 3.0 ± 1.1 fold, respectively (Figure 4D). We accordingly sought to further examine the effect of STAU1 knockdown on the SMILR downstream targets. Analysis of the same 20 targets described in Figure 1, which are down- and upregulated following SMILR knockdown and overexpression, revealed that 7 of these genes were significantly upregulated with STAU1 knockdown (Figure 4E). Using RNA FISH, we were also able to examine the co-localisation of SMILR and CENPF with STAU1 knockdown (Figure 4F). This revealed that, when compared to control conditions, SMILR/CENPF transcript co-localisation is not dependent on STAU1 expression, and that there appears to be increased SMILR/CENPF co-localisation events with STAU1 KD (Figure 4F). Collectively, these data suggests that once SMILR expression is upregulated in IL1-PDGF conditions, it is able to bind to CENPF mRNA. This may subsequently counteract STAU1-mediated regulation thereby culminating in a proliferative environment and cell cycle progression in vSMCs.

SMILR and the targeted cell cycle network are activated in atherosclerosis and ex vivo vein model of human saphenous vein.

Despite context-dependent heterogeneity in vSMC pathobiology, defects in SMC cell cycle and hence proliferation, are hallmarks of vascular pathologies including atherosclerosis and neointimal hyperplasia associated with vein graft disease. As SMILR is poorly-conserved, we are limited to human disease and not animal models to study disease association and causality. To interrogate the SMILR:CENPF:STAU1 interaction in human atherosclerosis, we performed an RNAseq on relatively stable and unstable regions dissected from fresh human carotid plaques obtained at carotid endarterectomy in symptomatic patients. Although classified as “stable” these plaques may still contain regions of instability. This is demonstrated by ex vivo 18F- sodium fluoride imaging of explanted plaques which was utilised to confirm the appropriate segregation by regions of relatively more unstable versus stable plaque, where increased uptake of the radiotracer was more apparent in “unstable” dissections and less so in the “stable” regions (Figure 5A). Importantly, Principal Component Analysis (PCA) of the RNAseq showed a clear clustering of the distinct regions separately, and not clustering together within each patient sample (Figure 5B). The differential expression analysis confirmed the changes of protein coding genes linked with plaque instability, including those associated with inflammation, matrix remodelling, and calcification (Supplemental Figure XII). SMILR expression was upregulated in all unstable plaque samples assessed by qRT-PCR (Figure 5C). Additionally, SMILR was detected using in situ hybridisation with varying intensity across all carotid atherosclerotic plaques from symptomatic patients (Figure D, Supplemental Figure XIII). STAU1 pulldowns in whole carotid plaques also further revealed an interaction with SMILR with a 2-fold enrichment compared to IgG controls (p<0.01) (Figure 5E). Remarkably, we also observed that 32 of the 40 SMILR-dependent cell cycle interactome were also upregulated within the unstable plaques compared to stable, including CENPF (Figure 5F and G). Collectively, these data suggest that the SMILR/CENPF-STAU1 axis is activated in unstable atherosclerosis.

With arterial and venous smooth muscle cells differing significantly, we sought to further investigate the role of SMILR in relation to atherosclerosis by validating its mode of action in human coronary artery smooth muscle cells (HCASMCs). Firstly, we confirmed incorporation of EdU in HCASMCs stimulated with IL1-PDGF. This significantly upregulated proliferation, although as previously described (17, Figure 6A and B), the proliferative capacity of HCASMCs are significantly less than that
observed in the HSVSMCs. Nevertheless, with IL1-PDGF induced proliferation in the HCASMCs, we also identified by qRT-PCR significant increases in SMILR, CENPF, MKI67, AURKB, and CDC20 transcripts (Figure 6C and D). Importantly and similar to that observed in the HSVSMCs, knockdown of SMILR and CENPF in HCASMCs (Supplemental Figure XIV) resulted in reduction in proliferation (Figure 6E and F). Taken together, this suggests that although arterial and venous smooth muscle cells differ significantly, SMILR’s mechanism of action remains consistent.

We also assessed the SMILR: CENPF axis in the context of vSMC proliferation associated with vein graft disease. Hereeto, we used an ex vivo human saphenous vein (HSV) model, which is associated with time-dependent SMC proliferation, migration and formation of neointima over 14 days in culture (Figure 7A). We first validated this approach by monitoring EdU incorporation at 0, 7, and 14 days and found significant increases (Figure 7B and C). Thus, we hypothesised that SMILR expression may be regulated during the culture period. Accordingly, saphenous veins were cultured for 0, 7 or 14 days and the expression of SMILR, CENPF and the downstream cell cycle associated targets assessed by qRT-PCR. When compared to day 0 control, SMILR expression was increased 28 ± 13 (p < 0.05) and 53 ± 19 fold (p < 0.01), respectively, at day 7 and 14 (Figure 7D). We also identified a time-dependent increase in CENPF expression to 8 ± 1 fold (p < 0.05) at day 7 and 19 ± 7 fold (p < 0.05) at day 14 (Figure 7E). Similar to the qRT-PCR data obtained in Figure 6E, we are able to detect using immunohistochemistry increases in CENPF positive cells in the medial layer from 30% at day 0 to 51% at day 7 (Supplemental Figure XV). Concordantly, expression of other SMILR downstream targets within the cell cycle network, namely AurKB, BUB1B, MKI67 and CDC20, were upregulated at day 7 (22 ± 8, 11 ± 3, 22 ± 8 and 18 ± 7 fold change, respectively) and day 14 (41 ± 14, 34 ± 14, 75 ± 29 and 50 ± 31 fold change, respectively) (Supplemental Figure XVI). Overall, these data suggests that SMILR expression and its downstream network has a strong association with pathological remodelling in human ex vivo vein grafts.

We then sought to manipulate SMILR expression in the ex vivo saphenous vein graft to assess the clinical relevance and therapeutic potential. We therefore used a novel siRNA approach within the clinically-relevant time window of an initial 30 minutes (clinical window from harvesting of the saphenous vein to grafting) prior to culture in which to attempt to knockdown SMILR. Cy3-tagged siSMILR was first used to visualise the successful infiltration of the siRNA into the vein (Figure 7F). Due to the limitations of the longevity of siRNA chemistry, by day 14 the siSMILR effects were found to be diminished (Supplemental Figure XVII). In veins with siRNA intervention leading to a significant decrease in SMILR levels assessed at Day 7 (Figure 7G), we also observed significant decreases in CENPF and MKI67 mRNA expression (Figure 7H and I). Finally, quantified proliferation by EdU incorporation in the cultured vein revealed a strong reduction from 28.7 ± 5.3 % EdU +ve/DAPI +ve nuclei in control conditions to 5.2 ± 2.6 % with SMILR knockdown (Figure 7J and K, p < 0.01).

DISCUSSION

Aberrant growth of vSMCs is a common and functionally important mechanism, which may ultimately contribute to the etiology of numerous cardiovascular diseases. Although the general mechanism of cell-cycle regulation are well established, cell-enriched regulators such as IncRNA are not at all well defined in terms of expression, association and mechanism, which is crucial for the successful development of targeted therapeutics and improved knowledge of how the human transcriptome can impact physiological and pathological pathways. Here, we identify the mechanism and downstream network of the vSMC-enriched human IncRNA, SMILR, and demonstrate its therapeutic potential in the ex vivo HSV model (Figure 7). This has the potential to not only enhance our understanding of atherogenesis, neointimal hyperplasia and plaque formation, but also provides a clear therapeutic target for future investigation in a broad range of cardiovascular diseases.

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The human genome contains a wide range of lncRNAs that are dynamically expressed in a temporal and cell specific manner. These lncRNAs can influence the level and spatial distribution of many proteins and mRNAs in order to control key aspects of cellular function. LncRNAs have previously been shown to modulate cell-cycle, primarily in cancer cell lines \(^{40,41}\). Additionally, lncRNAs, such as smooth muscle and endothelial cell enriched migration/differentiation-associated lncRNA (SENCR) and myocardin-induced smooth muscle lncRNA, inducer of differentiation (MYOSLID), have also been previously shown to be influential in cardiovascular diseases and essential in controlling the phenotypic switching of VSMCs to maintain their contractile phenotype \(^{42,43}\). More recently, the role of MEG3 in patients with pulmonary arterial hypertension was examined which revealed significantly reduced MEG3 expression levels in patients compared to healthy controls \(^{44}\). In vitro siRNA silencing of MEG3 resulted in increased SMC proliferation and migration while mechanistic investigation revealed that MEG3 regulates the p53 pathway in PASMCs \(^{45}\). Although several lncRNAs have been identified that control key aspects of SMC and EC function, very little is known about their role in atherosclerosis. A key atherosclerotic lncRNA is ANRIL, which was identified via GWAS studies, in which several SNPs located within this lncRNA were associated with atherosclerosis. It was later identified that ANRIL regulates gene expression epigenetically through recruiting repressive components of the polycomb complexes 1 and 2 to ANRIL-target gene promoters via Alu-repeats \(^{46}\).

Here, we showed that SMILR specifically targets the late mitotic pathway in proliferating HSVSMCs and interacts with CENPF mRNA and STAU1. Two recent studies have demonstrated that the lncRNAs, SNHG5 and TINCR, counteract STAU1-mediated decay to promote the stabilisation of specific mRNAs to control tumour cell survival in colorectal cancer and somatic tissue differentiation, respectively \(^{29,47}\). Similar to TINCR and its target mRNA PGLYRP3, SMILR’s interaction with CENPF mRNA appears to occur independent of STAU1 protein interaction as revealed by RNA-FISH. Although we see up-regulation of both CENPF mRNA and SMILR with STAU1 knockdown we cannot exclusively conclude whether STAU1’s interaction with SMILR: CENPF mRNA is regulating CENPF at a post-transcriptional and/or post-translational stage. Additionally, STAU1 may not only affect the levels of CENPF at an RNA and/or protein levels but also regulate its sub-cellular localisation since STAU1 has been found to be involved in mRNA transport and localisation to mediate further translation \(^{48}\).

Whether the SMILR/CENPF interaction is dependent on base complementarity and/or secondary structure is a key future scientific question as the secondary structure of SMILR may be crucial for its localisation, downstream interactions, and hence function \(^{49,51}\). Also, other mRNAs might be regulated by SMILR and STAU1 and sequencing of associated mRNAs may further provide a comprehensive network of interactions in proliferating vSMCs.

Consistent with our findings, previous studies have indicated that STAU1 primarily binds to protein coding mRNAs of key mediators of cell cycle and that STAU1 expression and function necessarily fluctuates throughout the cell cycle, being highest during the S-phase and rapidly decreasing during mitotic progression \(^{34}\). Additionally, STAU1 overexpression affects mitotic entry and impairs proliferation of transformed cells, therefore highlighting STAU1-function must be inhibited in a temporally dependent manner during the cell cycle for proper mitotic progression \(^{34}\). With STAU1 being a ubiquitously expressed and a multi-functional protein, lncRNAs may be crucial for providing its cell-specific function and accordingly mediate cell-specific phenotypes. This may also be the case for CENPF, which is also ubiquitously expressed and shown to be multi-functional to control mitotic control, transcriptional regulation, and muscle cell differentiation \(^{52}\). Intriguingly, increased levels of CENPF have also been previously associated with increased proliferation in malignant conditions \(^{31}\) and associated with a poor prognosis in human cancers \(^{53,54}\). However, the mechanism by which increased CENPF results in increased proliferation is not entirely understood. One possibility is that the role of CENPF in assembling kinetochore structures required for correct chromosome alignment and separation during mitosis is a rate-limiting step.
for mitotic progression. Taken together, our study therefore suggests that SMILR may provide such a critical cell-specific regulation of STAU1 and CENPF function in human vSMCS to trigger cell cycle progression and proliferation. Further studies are required to dissect mechanistically the consequence of CENPF mRNA regulation by SMILR. Particularly CENPF’s mRNA stability, transport, and translation as well as the intersection of this with the mitotic phenotype that we observe when SMILR levels are reduced.

SMILR was previously suggested to function, at least in part, by regulating its neighbouring gene, HAS2\(^1\), although HAS2 is located \(\approx 750\)kb from SMILR. However, we showed using RNAseq that HAS2 is downregulated with SMILR knockdown but was not affected by SMILR overexpression, confirming previous findings \(^1\). We also demonstrated the proliferative effects of SMILR occur in the cytoplasmic fraction since the siRNA approach used selectively blocked cytoplasmic SMILR expression and would therefore unlikely involve a direct targeting of the HAS2 gene in the nucleus. Here, we focused on the direct regulation by SMILR in the cytoplasm and find effects mediated by a distinct proliferative network, but we cannot rule out a downstream effect in the nucleus due to SMILR manipulation, or indeed a further proliferative effect mediated selectively in the nucleus by SMILR by an independent mechanism. In particular, we noticed the presence of histone mRNAs among the dysregulated genes from the RNAseq data. Although histone mRNAs are not within the list of SMILR predicted targets, the observed level change at the RNA level could lead to protein level changes and subsequent transcriptional changes.

The up- or downregulation of the SMILR-axis and its consequential effects on vSMC proliferation could influence numerous cardiovascular diseases. This was apparent in the \textit{ex vivo} vein graft model in this study and suggests that this can influence neointimal hyperplasia and hence the long term success of revascularisation of vein graft after coronary artery bypass surgery. Interestingly we found a similar role for SMILR in HCASMCs and may therefore also be involved in atherosclerosis. However targeting of SMILR may not be beneficial due to the potential reduction in stability and formation of a fibrous cap. Further studies are however required to fully understand the influence of the SMILR-axis with respect to SMC proliferation in the atherosclerotic environment and hence the susceptibility to plaque rupture and ultimately myocardial infarction and stroke.

Significantly, within a clinically-amenable timeframe, siRNA-based gene therapy targeting SMILR is sufficient to markedly reduce proliferation in the \textit{ex vivo} vein model. This excitingly provides a vSMC-specific target, which reduces the possibility of off-target effects in the remainder of the vessel wall, i.e. inhibited re-endothelialisation. This strongly suggests that such an intervention may reduce vein graft failure rates. Although our studies only show successful knockdown with siRNA for a limited time frame, whether this is sufficient to maintain a long term anti-proliferative effect is something that requires further studies. Nonetheless, other routes of SMILR-targeting gene therapy may be required for maximum longevity such as LNA-GapmeR antisense oligonucleotides (ASOs) \(^{13}\). However, ASOs target both nuclear and cytoplasmic fractions of a cell whereas siSMILR only has a cytoplasmic effect (Supplemental figure 6). Accordingly, the subsequent effects of ASOs knockdown of SMILR in the nuclear fraction must be studied to ensure no detrimental effects.

We demonstrate that SMILR is a vSMC-enriched lncRNA, essential in the control of cell-cycle through binding of CENPF mRNA and STAU1. Our studies provide early but compelling evidence that SMILR is an exciting and novel target in the treatment of aberrant growth of vascular smooth muscle cells, with the potential to significantly reduce the rate of vein graft failure.
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AUTHOR CONTRIBUTIONS
A.H.B conceived and supervised the study. A.H.B, A.D.M, M.D.B and J.R designed experiments, interpreted data, and wrote the manuscript. A.D.M, M.D.B, V.M, K.P, J.H, and J.P.S. performed experiments. K.P, J.H, J.K and A.S.T sampled and conducted imaging on patient atherosclerotic plaques. J.R. did the RNA-seq analysis. I.U provided pipeline for RNA-seq analysis. G.W.G provided guidance for the cell cycle studies. S.J.G provided guidance on ex vivo studies. N.L.M, D.E.N. and J.C.S provided guidance on atherosclerosis and clinical studies. A.C.B and J.C.S provided samples for studies. All the authors discussed the data and edited the manuscript.

DECLARATION OF INTERESTS
The authors declare no competing interests.

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REFERENCES


FIGURE LEGENDS

Figure 1. Transcriptomics identifies a cell cycle-associated network targeted by SMILR in proliferative vSMCs.

(A) Schematic of experimental design for SMILR knockdown and overexpression using dsiRNA and lentivirus (LNT), respectively. MOI = multiplicity of infection. (B) Validation of SMILR knockdown and overexpression via qRT-PCR of 3 technical replicates used for RNAseq from one patient sample. (C) Venn diagrams illustrating the number of genes dysregulated by SMILR knockdown and overexpression. (D) Heatmap of all overlapping 68 significant changes observed upon SMILR depletion and overexpression. Fold change calculated compared to the average FPKM in the control (CONT) samples. (E) Gene Ontology (GO) terms for the SMILR-regulated gene cohort. (F) Protein network of 40 proliferative and cell-cycle associated genes found to be dysregulated with SMILR depletion and overexpression. (G,H) Further validation by qRT-PCR of the top 20 identified dysregulated proliferative and cell-cycle associated genes with siRNA Control (siCtrl) versus siSMILR and control LNT (Null LNT) versus SMILR LNT. * = p < 0.05, by Iman and Conover non parametric ranked paired student’s t-test of deltaCT values between gene of interest and the housekeeper gene UBC, n = 3 biological replicates.

Figure 2. SMILR manipulation regulates the cell cycle of vSMC.

(A) Schematic of FUCCI viral analysis. (B) Flow cytometric analysis tracking cell cycle changes indicated by colour changes of the FUCCI viruses. (C) Bar chart representing average changes in the % of cells in each stage of the cell cycle. Repeated measures ANOVA for * p<0.05, Iman and Conover ranked non parametric analysis followed by one way ANOVA, n = 3 biological replicates. (D) Fluorescent Microscopy of HSVSMCs infected with the FUCCI viral system. Scale bars at 50 µm. Red arrows indicate binucleated cells. (E) Schematic of characterisation method of binucleated cells. (F) Fluorescent images of HSVSMCs stained with DAPI and phalloidin. Scale bars at 50 µm. Red arrows indicate binucleated cells. (G) Bar chart representing the % of total cells that were binucleated. * = p < 0.05, Iman and Conover ranked non parametric analysis followed by one way ANOVA vs IL1α/PDGF-ββ treatment alone (I+P); + = p < 0.05 vs siControls, n = 3 biological replicates. (H) Bar chart representing caspase-3 activity in HSVSMCs cultured with siRNA or hydrogen peroxide as a positive control. Caspase activity measured by OD405. n = 3 biological replicates, vs I+P treatment. ns = not significant.

Figure 3. RNA: RNA analysis reveal a SMILR: CENPF interaction.

(A) Schematic of DNA antisense biotinylated probes site for SMILR and GFP and the experimental design of RNA: RNA pulldowns. (B) Bar charts representing relative enrichments of SMILR, CENPF, and MKI67 in SMILR even and odd pulldowns versus the GFP control pulldown. Each even and odd SMILR probe pulldown was performed across one two independent biological replicates. Effects of (C) SMILR knockdown and (D) SMILR overexpression on CENPF mRNA. ** = p < 0.05 Iman and Conover non parametric ranked analysis followed by students t-test, n = 3 biological replicates. (E) Representative FACs histogram plots depicting EdU uptake in siCtrl and siCENPF treated HSVSMCs. Gate represents EdU+ cells. (F) Bar chart showing mean changes of EdU incorporation in siCtrl and siCENPF treated HSVSMCs. Iman and Conover ranked non parametric analysis followed by t-test, n=3 biological replicates. * = p < 0.05. (G) Fluorescent images of HSVSMCs stained with DAPI and phalloidin. Scale bar represents 50 µm. Red arrows indicate binucleated cells. (H) Bar chart representing the % of total cells that were binucleated. Iman and Conover ranked non parametric analysis followed by paired t-test * = p < 0.05 vs siCtrl, n = 3 biological replicates. (I) The effects of CENPF knockdown on mitotic associated genes compared to effects observed with knockdown of SMILR, * = p < 0.05, by Iman and Conover ranked non parametric analysis followed by paired t-test, n = 4 biological replicates.

Figure 4. STAU1 degrades the SMILR: CENPF interaction to mediate vSMC proliferation.

(A) Schematic showing methodology of biotinylated SMILR-pulldowns. (B) Mass spectrometry identified 14-enriched SMILR-binding proteins. Staufen1 (STAU1) was specifically enriched in the SMILR pulldown.
with 11-unique peptides detected. (C) Anti-STAU1 pulldowns confirming interaction with SMILR and CENPF; * = p < 0.05, by Iman and Conover ranked non parametric analysis followed by t-test, n = 3 pulldowns from 3 independent patient samples. (D) Relative quantification of STAU1, SMILR, and CENPF expression with SiSTAU1 at 10nM. * = p < 0.05, by Iman and Conover ranked non parametric analysis followed by t-test, n = 3 biological replicates. (E) The effects of STAU1 knockdown on the top 20 downregulated cell-cycle associated genes regulated by SMILR. * = p< 0.05, by Iman and Conover ranked non parametric analysis followed by t-test, n = 3 biological replicates. (F) RNA fluorescence in situ hybridization (FISH) for SMILR (red) and CENPF (green) in stimulated vSMCs under control, siSMILR, siCENPF, and siSTAU1 conditions. Scale bar represents 20 µm. Yellow arrows show some co-localisation events.

Figure 5. SMILR and the targeted cell cycle network are activated in atherosclerosis
(A) Ex vivo 18F imaging of unstable versus stable plaques. (B) Principal Component Analysis (PCA) of the RNAseq of the stable and unstable samples. (C) Relative FPKMs of protein coding genes linked with plaque instability, including those associated with inflammation and calcification. (D) Representative images of in situ detection of SMILR in plaques obtained from the carotid artery derived from symptomatic patients at carotid endarterectomy (n = 5 biological replicates per plaque type, replicates in Supplemental Figure 12). SMILR is visualised using NBT/BCIP (purple) at varying intensities across plaques exhibiting either intraplaque haemorrhage or thick fibrous cap. Nuclei are stained with fast red. L, indicates arterial lumen; NC, lipid core; scale bar represents 200 µm (E) SMILR enrichment in STAU1 pulldowns in whole carotid plaques, n = 6, ** = p < 0.01, by paired student t-test with paired experiments matched by colour. Protein network (F) and heatmap (G) of the 34 proliferative and cell-cycle associated genes found to be dysregulated with SMILR manipulation and unregulated in unstable plaques.

Figure 6. Role of SMILR in human coronary artery smooth muscle cells.
(A) Representative FACs histogram plots depicting EdU uptake in 0.2% and I+P treated HCASMCs. Gate represents EdU+ cells. (B) Bar chart showing mean changes of EdU incorporation in 0.2% and I+P conditions. Iman and Conover ranked non parametric analysis followed by t-test, n=3 biological replicates. * = p < 0.05. Bar charts showing relative expression of (C) SMILR and (D) CENPF, MKI67, AURKB, and CDC20 by qRT-PCR in 0.2% and I+P stimulated HCASMCs. Iman and Conover ranked non parametric analysis followed by t-test, n=3 biological replicates. ** = p < 0.05. (E) Representative FACs histogram plots depicting EdU uptake in siCtrl, siSMILR, and siCENPF treated HCASMCs. Gate represents EdU+ cells. (F) Bar chart showing mean changes of EdU incorporation in siCtrl, siSMILR, and siCENPF treated cells. Iman and Conover ranked non parametric analysis followed by t-test, n=3 biological replicates, * = p < 0.05.

Figure 7. SMILR modulates the proliferation of the ex vivo human saphenous vein organ culture.
(A) Graphical representation of ex vivo HSV proliferation model. (B) Quantification of EdU +ve nuclei in the media of HSV in culture expressed as % of EdU +ve/DAPI +ve nuclei. # = p < 0.05, * = p < 0.0,. * versus Day 0, # versus Day 7 using Iman and Conover ranked non parametric analysis followed by one-way ANOVA (n=3 biological replicates per time point). (C) Representative images of HSV in culture stained for EdU (green) and with DAPI (blue) at day 0, 7, and 14 (n = 3 biological replicates per time point). SMILR (D) and CENPF (E) expression determined by qRT-PCR analysis at day 0, 7 and 14. * P<0.05 and *** P<0.001 vs day 0 analysed by one way ANOVA (n=7). (F) Left: Graphical representation of the model of HSV siSMILR intervention; Right: Representative image of Cy3-labeled SMILR siRNA localization in HSV at 3 days post-siSMILR intervention; the section is co-stained with DAPI (blue) and for α-smooth muscle actin (αSMA, green) at 60x magnification. Relative quantification of (G) SMILR, (H) CENPF, and (I) MKI67 expression in HSV after siSMILR intervention at day 7 normalized to UBC. * = p < 0.05, ** = p < 0.01 by paired two-tailed Student’s t-test, n = 5 biological replicates. (J) Representative images of HSV post-siSMILR intervention stained for EdU (green) and with DAPI (blue). (K) Mean ± SEM of EdU +ve nuclei in the media of HSV after siSMILR intervention expressed as % of EdU +ve/DAPI +ve nuclei. (n
= 3 biological replicates). Values are * = p < 0.05; vs siCtrl using Iman and Conover ranked non parametric analysis followed by student’s t-test.
NOVELTY AND SIGNIFICANCE

What Is Known?

- Like other non-coding RNA, long non-coding RNA (lncRNA) can regulate key aspects of smooth muscle cell function.
- LncRNA SMILR regulates SMC proliferation via an unknown mechanism.

What New Information Does This Article Contribute?

- LncRNA SMILR regulates a network of cell cycle-associated mRNAs in vascular smooth muscle cells.
- LncRNA SMILR binds directly to the mitotic cell regulator centromere protein F (CENPF) mRNA.
- Inhibition of SMILR by RNA interference blocks vascular smooth muscle cell proliferation in intact saphenous vein.

VSMC proliferation has been recognised as central to the pathology of many major forms of vascular disease including intimal hyperplasia associated with vein graft failure. Previously, RNA-sequencing identified SMILR as a novel intergenic lncRNA activated by vSMC proliferation. Understanding the molecular mechanisms governing the mode of action of SMILR is an important next step. Specific modulation of SMILR levels revealed its role in regulating a mitotic mRNA network and more specifically a central role for binding to the cell cycle associated CENPF mRNA. Specific knockdown of SMILR resulted in the accumulation of binucleated cells and reduced proliferation and was phenotypically copied by silencing of CENPF. Further, we show that SMILR binds the RNA-binding protein Stau1 which may aid in the regulation of cell cycle. Finally, SMILR inhibition in whole vein segments resulted in the reduction of SMC proliferation through modulation of the key cell cycle network. Our findings provide compelling evidence that SMILR is a novel target in the treatment of aberrant growth of vascular smooth muscle cells.
FIGURE 2

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