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Smooth Muscle Enriched Long Non-Coding RNA (SMILR) Regulates Cell Proliferation

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Abstract

**Background**—Phenotypic switching of vascular smooth muscle cells (VSMCs) from a contractile to a synthetic state is implicated in diverse vascular pathologies including atherogenesis, plaque stabilisation, and neointimal hyperplasia. However, very little is known as to the role of long non coding RNA (lncRNA) during this process. Here we investigated a role for long non-coding (lnc)RNAs in VSMC biology and pathology.

**Methods and Results**—Using RNA-sequencing, we identified >300 lncRNAs whose expression was altered in human saphenous vein (HSV) VSMCs following stimulation with IL1α and PDGF. We focused on a novel lncRNA (Ensembl: RP11-94A24.1) which we termed smooth muscle induced lncRNA enhances replication (*SMILR*). Following stimulation, *SMILR* expression was increased in both the nucleus and cytoplasm, and was detected in conditioned media. Furthermore, knockdown of *SMILR* markedly reduced cell proliferation. Mechanistically, we noted that expression of genes proximal to *SMILR* were also altered by IL1α/PDGF treatment, and HAS2 expression was reduced by *SMILR* knockdown. In human samples, we observed increased expression of *SMILR* in unstable atherosclerotic plaques and detected increased levels in plasma from patients with high plasma C-reactive protein.

**Conclusions**—These results identify *SMILR* as a driver of VSMC proliferation and suggest that modulation of *SMILR* may be a novel therapeutic strategy to reduce vascular pathologies.

**Key words:** microRNA; atherosclerosis; non-coding RNA; vascular smooth muscle; proliferation; long non-coding RNA
Introduction

Vessel wall remodelling is an integral pathological process central to cardiovascular diseases including atherogenesis, plaque rupture and neointimal hyperplasia associated vein graft failure and in-stent restenosis. Resident vascular smooth muscle cells (VSMC) are typically quiescent and contractile in the normal physiological state. However, following pathological or iatrogenic vascular injury, the release of cytokines and growth factors from VSMC, aggregated platelets and inflammatory cells on the damaged intimal surface, leads to “phenotypic switching" of VSMC and the adoption of a more synthetic, pro-proliferative and pro-migratory state. In the setting of the pathological injury of atherosclerosis, VSMCs not only contribute to the atherogenic process itself but can also engender plaque stabilisation through the generation of a thick-capped fibroatheroma. For acute iatrogenic vascular injury, over exuberant proliferation of VSMC subpopulations promotes neointimal hyperplasia leading to luminal narrowing such as seen in vein graft failure or in-stent restenosis. Phenotypic switching of VSMCs and release of cytokines and growth factors are therefore critical in vascular disease and understanding the mechanisms involved is critical to gain insights into pathology and identify new opportunities for therapies.

The highly conserved IL1α and PDGF pathways play prominent roles in VSMC-associated pathologies. IL1α is a central mediator in the cytokine cascade and a potent activator of vascular cytokine production. Furthermore, previous studies have demonstrated that ligation injury result in reduced neointimal formation in IL-1 receptor knockout mice. Downstream mediators include the signalling molecules MEKK1, p38 and the transcription factor NF-κB that activate mediators of inflammation and cellular migration. PDGF is a potent mitogen and chemoattractant and expression is increased following vascular injury. Conversely
a reduction in PDGF expression reduces intimal thickening and cellular content of the neointima 9. Activation of both IL1α and PDGF signalling pathways simultaneously can activate common downstream targets leading to additive or synergistic effects. This includes activation of NFκB leading to the up-regulation of MMP 3 and 9 10: genes critical in the development of vasculoproliferative pathologies.

Over the past decade, there has been substantial interest in determining the complex interactions between hierarchical levels of gene regulation. Up to 90% of the human genome is transcribed at different developmental stages and only approximately 2% of RNA molecules are translated into protein 11. The functional complexity of organisms therefore appears to be reliant upon non-coding RNA molecules. Non-coding RNAs are subdivided into several classes, including microRNA (miRNA) and long non-coding RNA (lncRNA). MiRNAs are abundantly expressed in vascular tissues and play an important role in vascular pathology. Interestingly, recent studies have demonstrated that miRNAs are capable of being released into the blood from injured cells. These miRNAs are relatively stable and have been reported as biomarkers for several disease states including myocardial infarction 12 and heart failure13, 14. While the role of miRNAs is reasonably established in the setting of cardiovascular pathology, relatively little is known about the role of lncRNAs. LncRNAs are capable of regulating target DNA, RNA and protein at the pre and post-transcriptional level. It is becoming clear that lncRNAs play a pivotal role in cellular physiology and pathology via localisation in sub populations of cells and through highly controlled temporal expression 15. However, detailed insights into their regulation and biological roles are only beginning to emerge. In the vascular setting, SENCr and MALAT1 have been implicated in the control of vascular cell migration and endothelial cell sprouting, respectively 16, 17. Interestingly, SENCr is implicated in phenotypic switching of VSMCs to a
more pro-migratory phenotype as knockdown of this lncRNA downregulates contractile genes\textsuperscript{17}. A greater understanding of lncRNAs in quiescent and proliferative VSMCs may provide valuable insight into the specific roles of lncRNAs in response to pathological processes.

**Methods**

**Human tissue samples**

Surplus human saphenous vein tissue was obtained from patients undergoing CABG. Carotid plaques were obtained from patients undergoing endarterectomy following an acute and symptomatic neurovascular event. Human plasma samples were utilised from a previously published study: Carotid Ultrasound and Risk of Vascular disease in Europeans and South Asians (CURVES)\textsuperscript{2}. All patients gave their written, informed consent. All procedures had local ethical approval (06/S0703/110, 12/WS/0227, 09/S0703/118 and 12/NW/0036). All studies were approved by East and West Scotland Research Ethics Committees and all experiments were conducted according to the principles expressed in the Declaration of Helsinki.

**Tissue and Cell culture**

All cells were maintained at 37°C in a humidified atmosphere containing 5\% CO\textsubscript{2}. Primary human saphenous vein derived endothelial cells (HSVECs) were isolated by a modified version of the protocol described by Jaffe and colleagues\textsuperscript{18} and maintained in large vessel endothelial cell culture medium. supplemented with 20\% FCS (Life Technologies, Paisley, UK). Primary human saphenous vein derived smooth muscle cells (HSVSMC) were isolated from medial explants\textsuperscript{19} and maintained in Smooth Muscle Cell Growth Medium 2 (PromoCell, Heidelberg, Germany) with supplements. Human coronary artery VSMC were purchased from Lonza (Basel, Switzerland) and maintained in VSMC media as above.
Sample preparation for RNA-seq library construction and analysis

HSVSMC were plated, quiesced in medium containing 0.2% fetal calf serum for 48 hour before the stimulation with 10 ng/ml IL1α, 20 ng/ml PDGF (R&D Systems) or a combination of both for 72 hours. Total RNA was processed through miRNeasy kit (Qiagen, Hilden, Germany) following the manufacturer’s instructions, treated with DNAse 1 (amplification grade; Sigma, St. Louis, USA) in order to eliminate genomic DNA contamination and quantified using a NanoDrop ND-1000 Spectrophotometer (Nano-Drop Technologies, Wilmington, DE, USA). Following bioanalyzer quality control for RNA integrity number (RIN) values >8, RNA-seq was performed on ribosomal-depleted RNA using an Illumina Hiseq platform by Beckman Coulter Genomics. Paired-end sequencing was carried out with a read depth of 70 million (n=4/group).

RNA-seq reads were processed and trimmed to ensure quality and remove adapter sequences using Flexbar\textsuperscript{20} and mapped to the Ensembl annotation of GRCh37.75 using the TopHat2 version 2.0.9.\textsuperscript{21} The transcriptome was assembled from the aligned reads and quantified using Cufflinks version 2.2.1.\textsuperscript{22} The differential expression levels between the groups was assessed using Cuffdiff version 2.2.1.\textsuperscript{23} The data set are deposited in the GEO repository, study number GSE69637. The biotype of each transcript was annotated according to the Ensembl database. Normalisation and statistical analysis of differentially expressed transcripts were carried out using edgeR and data filtered to find transcripts that were differentially expressed (p<0.01) between 0.2% media and each treatment group. Differentially expressed lncRNAs, between control and both IL1α/PDGF treatment, were explored using more stringent criteria (p<0.01, FDR<0.01, LogFC>2) and filtered according to transcript abundance (FPKM>1 in at least one group). Data outputs such as pie charts and heatmaps were generated using R. IPA analysis was carried out using protein coding genes differentially expressed (FDR<0.01) from Edge R...
analysis.

**Assessment of RNA secretion from HSVSMC**

RNA extraction on conditioned HSVSMC media was performed using a standard volume (2 mL). The conditioned media was first centrifuged (10 min; 2000 g; 4°C) to remove all cells and debris. After addition of 1.4 mL of QIAzol (Qiaagen), 3 μL of c.elegans total RNA at 25 ng/μL was added to each sample. Following 5-min incubation at RT, 140 μL of chloroform was added and samples centrifuged (15 min; 15000 g; 4°C). The clear upper aqueous phase was used to isolate RNA using miRNEasy mini kit (Qiagen) as previously described with alteration of the final wash step (75 % ethanol in DEPC water). Different quantities of total RNA were spiked and a dose response effect was observed (**Suppl. Figure 1A**). The quality of the amplicon was assessed via analysis of melting curves (**Suppl. Figure 1B**) and subsequent visualisation on agarose gel (**Suppl. Figure 1C**). This showed a unique amplification product corresponding to the cDNA fragment of *ama-1*. Due to the correlation observed between quantity of spike-in and *ama-1* expression (**Suppl. Figure 1D**), we utilised 75 ng in all subsequent extractions. This amount allowed reproducibility of our method, with the Ct values of *ama-1* being 29.4±0.3 across 5 separate extractions in non-conditioned media (**Suppl. Figure 1E**).

**Gene expression quantitative RealTime-PCR (qRT-PCR)**

For gene expression analysis, cDNA for mRNA analysis was obtained from total RNA using the Multiscribe Reverse Transcriptase (Life technologies, Paisley, UK). qRT-PCR was performed using Power SYBR green (Life Technologies) with custom PCR primers (Eurofins MWG, Ebersberg, Germany), the specificity of these primers was confirmed by performing a melting curve and running their PCR produce on a gel (**Suppl. Table 1** – primer sequences). Ubiquitin C (UBC) was selected as housekeeping gene due to its stability across all groups studied. Fold-
changes were calculated using the 2-ΔΔCt method 23.

**Statistical Analysis**

Statistical analysis was performed according to figure legends. Data in graphs are shown on relative expression scales as referenced by 24. Data are given as both mean ± standard deviation (StDev) (shown as bars and whiskers) and also as the individual points in order to clearly represent the data. Note that as the relative expression scale is inherently skewed, the bar indicates the geometric mean of the relative expression fold change with the StDev whiskers denoting the relative expression fold change equivalent to an increase of one StDev above the mean on the log transformed scale. All statistical analysis is performed on the dCt scale (a logarithmic transformation of the data shown on the RQ in the plots)24. No evidence of unequal variances across groups was found for any of analyses of the dCt scale data using Levene’s test on minitab version 17 prior to statistical analysis. Comparisons between 2 groups were analysed using 2-tailed unpaired or paired Student’s t test. One-way ANOVA with Tukey’s post hoc or one way ANOVA multiple comparison test for pooled samples, via Graph Pad Prism version 5.0, was used for comparisons among 3 or more groups. Statistical significance is denoted by a P value of less than 0.05.

**Results**

**Induction of inflammatory and cell cycle pathways by IL1α and PDGF**

We sought to identify lncRNAs that are regulated during the induction of proliferative and inflammatory pathways in HSVSMC. RNAs were identified using RNA-seq of HSVSMC treated for 72 h (Figure 1A). Activation of the IL1α and PDGF signalling pathways was confirmed by presence of the inflammatory microRNA miR-146a (Figure 1B) and induction of VSMC
proliferation (Figure 1C). The RNA-sequencing obtained an average of 70 million reads per sample; with 93.5% aligning to the GRCh37 genome reference files. The majority of reads, under all conditions, corresponded to mRNA (49.6±0.48 %; Figure 1D and Suppl. Figure 2A). To identify the biological function, networks, and canonical pathways that were affected by VSMC stimulation, we performed Ingenuity Pathway Analysis (IPA) after RNA-seq analysis. IPA confirmed the mRNAs with altered expression following IL1α treatment were significantly enriched in pathways related to cellular movement and inflammatory disease (Suppl. Table 2), while PDGF stimulation led to the marked enrichment in cell cycle pathways (Suppl. Table 3). Interestingly, co-stimulation led to enrichment in cell cycle and cardiovascular development pathways (Suppl. Table 4). Further analysis of differentially expressed mRNAs with a stringent cut off of FDR<0.01 identified 518 protein coding genes altered following IL1α treatment and 540 following PDGF treatment. Notably, dual stimulation altered 1133 known protein-coding genes with 480 uniquely associated with dual stimulation and not affected by IL1α or PDGF treatment alone (Figure 1E and Suppl. Figure 2B).

Identification of differentially expressed LncRNAs in HSVSMC treated with IL1α and PDGF

We next assessed whether lncRNAs were dynamically regulated by growth factor and cytokine stimulation. Approximately 33% of reads in each condition aligned to known or predicted lncRNAs (Suppl. Figure 3A). Differential expression analysis confirmed substantial differences in lncRNA expression between control and stimulated cells. Using the stringent criteria FDR ≤0.01 and log2 fold change (FC) ≥2, to declare significance and fragments per kilobase of exon per million fragments mapped (FPKM) >1, to confirm quantifiable expression we identified 224, 215 and 369 differentially expressed lncRNAs following IL1α, PDGF or dual stimulation respectively (Suppl. Figure 3A). Since lncRNAs can typically contain multiple splice variants,
the numbers quoted refer to a single consensus gene model and therefore do not reflect the
multiple transcripts of each lncRNA. To determine if specific biotypes of lncRNA were enriched
following HSVSMC stimulation, those differentially expressed were further subdivided
according to biotype in the Ensembl database. These are based upon their relative orientation to
protein coding genes; intervening lncRNA (lincRNA), antisense, overlapping and processed
transcripts. Utilising control and dual stimulation as an example, the distribution of different
lncRNA biotypes was: intervening (45.5%), antisense (45.3%), overlapping (1.4%) and
processed transcripts (7.9%) (Suppl. Figure 3B). Focusing on lincRNA, the candidates (control
vs IL1α and PDGF, FDR<0.01, LogFC<2, FPKM>1) were ranked according to their FPKM and
level of up/down-regulation (Figure 2A, suppl Figure 4 for heat map of all conditions). A
subset of the most differentially expressed transcripts was identified and validated by qRT–PCR
(RP11-91k9.1, RP11-94a24.1, RP11-709B3.2, RP11-760H22.2 and AC018647.3; Figure 2B,
chromosomal locations in Suppl. Table. 1). This was consistent with the RNA-seq results,
showing RP11-94a24.1 and RP11-91k9.1upregulated 20.2±30 and 45±26.4 fold, respectively
following co-stimulation and lncRNAs RP11-709B3.2, RP11-760H22.2 and AC018647.3 being
down regulated 16, 28 and 1209 fold, respectively (Figure 3A) (RQ = 0.06±0.04, 0.035±0.01
and 0.0008±0.001 respectively). The dissociation curves and gel products of each primer set are
shown in Supplemental Figure 5.

Vascular enriched expression of RP11-94a24.1

The expression of each lncRNA was quantified in a range of 10 normal human tissues including
specimens derived from brain, gastrointestinal, reproductive, and endocrine systems. In general,
lncRNAs were expressed at relatively low levels across the tissue panel. However, we observed
that RP11-91k9.1 was expressed highest in the heart, while RP11-91K9.1 and AC018647.3
showed preferential expression within the liver and brain respectively. RP11-709B3.2 and RP11-760H22.2 displayed highest expression in spleen and thyroid respectively (Suppl. Figure 6A). We next examined the expression of each lncRNA in primary HSVEC, HSVSMC and human coronary artery SMC (HCASM). All lncRNAs had higher expression in VSMCs of either venous or arterial lineage compared to endothelial cells, suggesting VSMC enrichment (Suppl. Figure 6B). We also assessed whether the expression of these lncRNAs could be modulated by IL1α and PDGF in HSVEC as had been found in the HSVSMCs. Notably, subsequent down regulation of RP11-709B3.2, RP11-760H22.2 and AC018647.3 was not observed in HSVECs as was the case in HSVSMC (data not shown). Stimulation of HSVECs produced a significant 3.8±0.7 and 8.7±2.1 fold up regulation of RP11-91K9.1 following IL1α and IL1α/PDGF treatment respectively (Figure 3B). However, stimulation had no effect upon RP11-94a24.1 expression (Figure 3B), indicating selective regulation in HSVSMC. Due to the expression of RP11-94a24.1 in HSVSMC and its cell specific induction in response to pathological mediators of vascular injury, we focused further studies on RP11-94a24.1. We termed this lncRNA, smooth muscle induced lncRNA enhances replication (SMILR). SMILR expression was assessed through the utilisation of 3 independent primer sets targeting differential exons of the lncRNA. qRT-PCR revealed similar Ct and fold changes amongst the 3 sets, further confirming our previous data (Suppl. Figure 7). The longest open reading frame within SMILR is 57 amino acids. Analysis of this open reading frame using the Coding Potential Calculator (http://cpc.cbi.pku.edu.cn) did not reveal any similarity to known protein coding sequences suggesting that this RNA has no protein coding potential (data not shown).

**IL1α/PDGF treatment induces the expression of SMILR in a time dependent manner**

To investigate the longitudinal regulation of SMILR, we stimulated HSVSMC with PDGF, IL1α
or a combination of both (1.5 h, 4 h, 24 h, 48 h and 72 h). We found significant up regulation of SMILR in response to PDGF as early as 4 h post stimulation. By 24 h SMILR expression was increased by treatment with PDGF or IL1α as well as both together (Suppl. Figure 8). The combination of PDGF and IL1α induced a synergistic increase in SMILR expression at 72 h.

**Cellular localisation of SMILR in HSVSM cells**

Rapid amplification of cDNA ends, was utilised to design specific RNA FISH probes. RNA-FISH highlighted a SMILR isoform, consisting of an additional 6bp at the 5’ end and 316bp at the 3’ end (Suppl. Figure 9A and B). RACE data is supported by the raw RNA-seq files (Suppl. Figure 10 A-C).

We performed RNA-FISH to provide visuospatial information as to the location of SMILR within HSVMSC. Negative control samples showed no fluorescent signal while SNORD3 fluorescent activity confirmed the nuclear permeabilisation of cells (Figure 4A). In the absence of growth factor and cytokine stimulation, HSVSMC typically exhibited between 0 and 3 positive fluorescent signals corresponding to SMILR localisation (Figure 4A). IL1α/PDGF treatment induced a marked increase in fluorescent signal within the nucleus and cytoplasm of HSVSMC. Further specificity of the FISH probes was confirmed through the utilisation of cells treated with either lentivirus containing SMILR or siRNA targeting SMILR. In each case an increase and decrease in SMILR transcripts was observed (Figure 4A). Quantification of FISH samples is provided in Figure 4B. In the absence of stimulation 2±3.6 SMILR molecules were observed. Following stimulation, 25±5 individual SMILR molecules were observed within the nucleus and cytoplasm (Figure 4B).

**Identifying upstream mediators of SMILR expression in HSVSMC**

It is well established that IL1α and PDGF work through distinct pathways leading to vascular
cell activation. To assess the functional consequences of inhibition of these pathways on SMILR expression, selective pharmacological inhibitors AZD6244 (MEKK1) and SB 203580 (p38) were utilised (Figure 5A). Following 60 min pre-treatment with inhibitors, VSMC were stimulated with IL1α/PDGF and the expression of SMILR was determined at 24 h. Pre-treatment with AZD6244 (5, 10 or 15 μM) prevented the induction of SMILR in response to PDGF and IL1α (Figure 5B), while inhibition of p38 with SB203580 induced a dose-dependent reduction in SMILR expression in response to PDGF and IL1α (Figure 5C).

IL1α/PDGF treatment induces the release of SMILR into conditioned media

MicroRNAs have been reported to be secreted from cells as a means of cell to cell communication. To investigate whether HSVSMCs release SMILR as an indication of expression, we modified a method commonly utilised to evaluate miRNA expression. As no endogenous control was stably expressed across all conditions in this study, an exogenous control was added in order to monitor extraction efficiency and to normalise data. Consequently, total RNA from C. elegans was used as a spike-in and ama-1 encoding polymerase II was chosen as a control for its high constitutive expression (see methods). Interestingly, SMILR was detected at low levels in media from quiesced VSMCs and those stimulated by either PDGF or IL1α, while conditioned media obtained from VSMC stimulated by combination contained significantly higher levels of SMILR (4.8±4.5 fold) (Figure 5D), consistent with the increased intracellular expression of SMILR following co-stimulation of VSMC. Thus, treatment with PDGF and IL1α increased intracellular and released levels of SMILR.

Additionally we sought to identify if SMILR was encapsulated within exosomes or microvesicles (MV). We utilised both ultracentrifugation, to remove cell debris, and an exosome isolation kit. Supplemental Figure 11A and B confirms the presence of microvesicles and
exosomes using Nanosight technology and the expression of the previously described miR-143 within the exosomes/MV. Our data highlights the expression of SMILR restricted to exosome free media (Suppl. Figure 11C) and inability to detect SMILR expression in the exosomes/MV compartment using both techniques of isolation. This observation has been confirmed by agarose gel electrophoresis (Suppl. Figure 11D). Primer melting curves are also shown in Supplemental Figure 11E. Our data confirm that SMILR is secreted into the media and located in a non-exosome/MV fraction. This could possibly be through interaction with specific membrane channels but requires additional experimentation.

Additionally, we examined the release of SMILR following lentiviral overexpression in IL1 and PDGF treated cells. Lentiviral overexpression resulted in a 10-fold increase in SMILR RNA intracellularly. However, only a marginal (not significant) increase was observed within conditioned media analysed from infected cells (Suppl. Figure 11F). When this media was transferred onto additional quiesced cells, no change in proliferation was detected (Suppl. Figure 11G). This may suggest that the release of SMILR is under stringent control mechanism and simply increasing SMILR expression via lentiviral approach is not sufficient to induce the additional release of this lncRNA from the cells. In addition, these cells were stimulated with IL1 and PDGF, which strongly enhances SMILR expression in VSMC. The secretory machinery may have been saturated with the high levels of LncRNA within the cytoplasm. This has previously been demonstrated with microRNA where high levels of miR, via overexpression with microRNA mimics, saturated the exportin-5 pathway of endogenous miRNAs with fatal consequences.

Effect of dicer substrate siRNA mediated knockdown of SMILR on HSVSMC proliferation

We investigated the function of SMILR using dicer substrate siRNA (dsiRNA)-mediated
knockdown and EdU incorporation. Forty-eight hours post stimulation, IL1 and PDGF treatment induced a 34±15% increase in VSMC proliferation compared to control (Suppl. Figure 12). DsiRNA SMILR caused 75±24% decrease in SMILR expression when compared to dsiControl (Figure 5E). Following SMILR knock down with dsiRNA, VSMC proliferation was reduced by 56±15% (Figure 5F). Results were confirmed through the use of a second dsiRNA targeting an alternative region of SMILR (Suppl. Figure 13A and B). No effect on the interferon pathway was observed upon assessment of the response genes OAS1 and IRF7, which have previously been linked to disRNA off target effects31 (Suppl. Figure 13C and D).

Additionally the effect of SMILR overexpression on SMC proliferation was investigated. SMC were infected with SMILR lentivirus or empty control for 24 hours prior to stimulation. Infection at a multiplicity of infection of 25 and 50 produced a 5.5 ± 3.5 and 11.4 ± 4.7 fold increase in SMILR expression compared to the empty control, with no apparent toxicity effects (Figure 5G). Overexpression produced a dose dependent increase of 1.3 ± 0.3 fold and 1.66 ± 0.5 fold in SMC proliferation respectively (Figure 5H), confirming the knock down data.

SMILR expression correlates with other proximal genes

The expression of lincRNAs can correlate with the expression of adjacent genes and other RNAs within the genomic locale32. We therefore assessed the expression of genes and non-coding RNAs within 5 million base pairs of SMILR, from COL14A1 on the forward strand to FERIL6-AS1 on the reverse strand (Figure 6A), using the RNA-seq data set (Figure 6B). Up-regulation of SMILR was not associated with a widespread increase in transcriptional activity within the region (Figure 6B). However, similar changes in expression in response to VSMC stimulation were observed in two proximal transcripts (HAS2 and HAS2-AS1). SMILR is located ~750 kbp downstream of HAS2 on the same (reverse) strand and ~350 kbp from ZHX2 and ~ 750 kbp from
HAS2-AS1 on the opposite strand of chromosome 8 (Figure 6C). The upregulation of HAS2 was accompanied by an increase in HAS1 but not HAS3 following dual stimulation (Figure 6 D-F). Interestingly, IL1 and PDGF in combination had no effect on HAS3 expression as IL1 and PDGF have opposing effects on HAS3 expression (Full graph with single stimulation Suppl. Figure 13 E and F). In addition to SMILR, up-regulation of HAS2-AS1 was evident following IL1α and PDGF treatment, but not ZHX1 in the RNA-seq data (Data not shown). This observation was validated by qRT-PCR (Figure 6 G,H and I).

It has been previously shown that lncRNA can modulate the expression of nearby protein coding genes. Thus, the expression of proximal genes HAS2, ZHX2 and HAS2-AS1 were determined following SMILR knockdown. RNAi-mediated knockdown of SMILR significantly altered levels of HAS2 mRNA. However, no change in the HAS2-AS1 lncRNA or the ZHX2 gene was observed via qRT-PCR (Figure 6 J-L). Results were confirmed using a second siRNA targeting SMILR (Suppl. Figure 13 G - I). Additionally, no effect on HAS1 or HAS3 expression was observed while utilising SMILR siRNA indicating that the effect of SMILR knockdown is specific to HAS2 and not all isoforms of HAS (Figure 6 M and N).

Additionally, knockdown of HAS2-AS1 greatly reduced HAS2 expression with no effect on SMILR expression (Suppl. Figure 14 A and 14B). However, the reverse experiment utilising HAS2 knockdown, did not affect the expression of HAS2-AS1 nor SMILR (Suppl. Figure 14C). Finally, lentiviral mediated overexpression did not affect HAS1, 2,3 or HAS2-AS1 expression (Suppl. Figure 14 D-G).

SMILR expression is dysregulated in unstable human carotid plaques

To investigate the importance of SMILR in human vascular pathologies, we assessed levels of SMILR in unstable atherosclerotic plaques. We used two established inflammatory ([18F]-
fluorodeoxyglucose (FDG) and calcification ([18F]-fluoride) PET radiotracers to define prospectively portions of high-risk plaque for RNA extraction. Plaque and relatively ‘healthy’ adjacent sections of vessel were assessed from within individual patients (Suppl. Table 5 for patient characteristics). This is of key importance as it permits the assessment of non-coding RNA expression from within each micro environment (plaque vs. non plaque) from within the one vessel. Compared to quiescent adjacent tissue, portions of high-risk plaque showed higher uptake of both [18F]-FDG (maximum tissue-to-background ratio (TBR_max) 1.81±0.21 versus 1.31±1.6) and [18F]-fluoride (TBR_max 2.32±0.52 versus 1.31±0.43) indicating that plaques subjected to RNA analysis had enhanced rates of inflammation (Figure 7A-G for image examples and Figure 7 H-K for graphs of tracer uptake). Since non-coding RNAs have not been assessed in a similar sample set before, we first confirmed whether expression of a panel of miRNAs associated with atherosclerosis processes were dysregulated As expected, inflammation-associated miRNAs 146a-and 146b were significantly upregulated in unstable plaques compared to adjacent quiescent tissue, while miR-29 and miR-204, which are inversely associated with osteoblastogenesis and arterial calcification, were down regulated in mineralised regions of the atherosclerotic plaque. In addition we also found a downregulation of the miR-143/145 cluster, which is associated with SMC differentiation and aortic aneurysm formation, an event which has previously been linked to osteogenic differentiation of SMC (Figure 7L). Thus expression of small non-coding RNAs (miRs) was associated with PET/CT defined high-risk plaques. Therefore, we utilised the same cohort of samples to assess SMILR, HAS2 and HAS2-ASI levels. A 3.9±2.3 fold increase in SMILR expression was observed in high-risk plaques compared to adjacent stable regions of the carotid artery (Figure 7M). Intriguingly, we also observed an increased in HAS2 (Figure 7N) but not HAS2-ASI (Figure 7O).
SMILR is detectable in human plasma and correlates with inflammatory CRR

Due to the release of SMILR into conditioned media from VSMC following stimulation with inflammatory mediators, we evaluated whether SMILR was detectable in stored samples from a cohort of men with varying metabolic dysfunction. These samples were ranked in order of the serologic parameter CRP levels into 3 groups: CRP <2, CRP 2-5 and CRP >5 mg/L representing broad tertiles of CRP. SMILR showed no difference in patients with CRP levels below 2 mg versus 2-5 mg/L. However, a 3.3±5.7 fold increase in SMILR was observed when CRP concentrations were greater than 5 mg/L (Figure 8A). Furthermore, a significant positive correlation was seen between SMILR and CRP (R²=0.33, P<0.0001) (Figure 8B). There was no correlation between SMILR and additional risk factors including age (P=0.66), blood pressure (P=0.12), BMI (P=0.14) or social deprivation status (P=0.11) (Suppl. Table 6). Melting curves and gel products of SMILR primers in plasma are shown in Supplemental Figure 15. Further information regarding the statistical analysis of SMILR CRP correlation can be found in Supplemental Figure 16.

Discussion

We have shown that stimulation of HSVSMCs with PDGF and IL1α increases expression of SMILR. This novel lincRNA increases cell proliferation which may be linked to its ability to regulate the proximal gene HAS2. In a clinical setting, we found increased expression of SMILR in unstable atherosclerotic plaques suggesting an association with fundamentally important vascular pathologies linked to inflammation and VSMC proliferation. We also discovered that SMILR can be released from cells and is detectable in plasma from patients with enhanced inflammation and thus susceptibility to atherosclerosis. These findings support the growing body
of evidence that non-coding RNAs can act as mediators to modulate disease pathways.

Recent advances in RNA-sequencing have demonstrated that previously thought "genome deserts" are in fact pervasively transcribed and are populated by IncRNAs. Utilising paired end-sequencing allowed accurate alignment of reads to the human genome (GRCh37), the 93% alignment rate met quality standards for the RNA-seq technique and ensured that our RNA-seq provided a high quality profile of the HSVSMC transcriptome during quiescent and stimulated conditions. Notably, compared to control cells, protein-coding genes accounted for 3-4 fold greater abundance than IncRNAs. Our RNA-seq depth of 70 million reads was sufficient to identify IncRNAs within VSMC, however, it should be noted that greater read depths and use of refined capture-seq technique would be beneficial in order to offer greater annotation of specific areas within the genome.

Analysis of the RNA-seq data pinpointed SMILR as an IL1α/PDGF responsive lincRNA located on chromosome 8, 750 kbp from the closest protein-coding gene, on the same strand. This gene, HAS2, encodes an enzyme which synthesises hyaluronic acid (HA), a critical component of the extracellular matrix that accumulates in human restenotic and atherosclerotic lesions. Our results show knockdown of SMILR reduces HAS2 expression and VSMC proliferation. This mechanism of action is supported by a number of studies demonstrating HA can enhance VSMC proliferation and migration. Recent studies using transgenic mice with VSMC specific over-expression of HA have found increased susceptibility to atherosclerosis and enhanced neointima formation in response to cuff injury. The ability of SMILR to specifically target HAS2 with no effect on HAS1 or HAS3 allows a means of specifically altering HAS2 expression, the main HAS isoform expressed and functioning in SMC pathology.
LncRNAs can regulate other RNAs via a number of mechanisms\textsuperscript{47}, including changes in chromatin signatures within their locus. For example, the *HOTAIR* lncRNA is capable of repressing transcription in trans across 40 kbp of the HOXD locus\textsuperscript{48}. Thus *SMILR* may act as an enhancer or scaffold via interaction with the promoter region, and potentially other transcription factors of *HAS2*, to regulate expression following inflammatory cytokine stimulation. However, further detailed co-immunoprecipitation or site directed mutagenesis studies would be required to demonstrate whether *SMILR* participates in transcription factor complexes with NF-κβ or other transcription factors. Previous work has found *HAS2* is regulated by an additional lncRNA, *HAS2-AS1*\textsuperscript{49}. Interestingly, our RNA-seq data show *HAS2-AS1* expression was also upregulated by PDGF treatment alone and in combination with IL1α. However, knockdown of *SMILR* did not alter *HAS2-AS1* expression. LncRNA *HAS2-AS1* modulates chromatin structures around the gene in order to allow more efficient binding of the RNA polymerase 2, and enhance *HAS2* gene expression\textsuperscript{49}. This suggests both *SMILR* and *HAS2-AS1* can regulate *HAS2* by independent mechanisms. Interestingly, knockdown of *HAS2* did not affect either *SMILR* nor *HAS2-AS1* expression indicating that the expression of these lncRNA are not directly linked to *HAS2* expression.

The composition of ECM assists in the determination of the stability of the atherosclerotic plaques, the phenotype of cells within it and the volume of neointima. During the progression of atherosclerosis, VSMC are exposed to a plethora of signalling molecules, including inflammatory cytokines. Using the clinical gold-standard methods of 18F-FDG and 18F-fluoride PET/CT imaging to identify inflamed, necrotic and mineralising atherosclerotic plaque\textsuperscript{33,34}, our results indicate miRs 29, 143, 145, 146 and 204 are differentially expressed in unstable regions of atherosclerotic plaques. These miRs have previously been linked to VSMC
differentiation, inflammatory cell signalling\(^5^0\) and VSMC calcification\(^5^1\). The strong association and co-localisation of \(SMILR\) with this classical miRNA profile and focal 18F-FDG and 18F-fluoride uptake within atherosclerotic plaque suggests that \(SMILR\) may play a role in atherosclerosis through inflammatory and proliferative pathways. In keeping with our results showing \(HAS2\) regulation by \(SMILR\), HA content has been shown to reflect the progression of atherosclerotic disease and promotes vessel wall thickening\(^5^2\). Indeed, HA has been implicated in the recruitment of inflammatory cells, known to play a prominent role in the initiation and progression of atherosclerotic lesion to an unstable plaque phenotype.

Our results demonstrate \(SMILR\) is up-regulated by a combination of PDGF and IL1α in VSMCs but not ECs, suggesting modulation of \(SMILR\) could specifically alter VSMC proliferation without detrimental effects on vessel re-endothelialisation. If this is the case, it would provide a suitable candidate to improve current anti-proliferative therapies since current pharmacological agents target cell proliferation in a non-cell specific manner, events which can lead to late stent thrombosis\(^5^3\).

The ability to identify confidently a plaque, or patient, at particular risk of a major adverse cardiovascular event (i.e. plaque rupture) remains an important goal of Cardiovase Res. Long RNA, both mRNA and non-coding RNA, have been previously shown to be stable in vivo for up to 3 weeks\(^5^4\). As such the search for prognostic biomarkers has greatly increased in recent years. \(SMILR\) was expressed in both the nucleus and cytoplasm of cells following stimulation and was released into the media. It will be important to determine whether the cytoplasmic copies induce functional effects, such as regulation of gene expression through post-translational mechanisms or if they are simply being processed for release. Dual transcriptional functions of lncRNAs have been shown previously\(^5^5\), but to date no reports of a single lncRNA affecting both
transcription and translation have been published. The release of SMILR could affect function in neighbouring cells, particularly in a vascular injury setting where phenotypic switching of VSMCs occurs in distinct areas of the vessel wall. In support of this theory, it has been shown that miR 143/145 can be transferred from VSMC into EC\(^56\). This transfer produced physiological effects within EC including modulation of angiogenesis. We also found that SMILR could be detected in the plasma of patients with higher CRP levels indicative of chronic low grade inflammation. In light of our studies, we propose this release could be due to the increased levels of SMILR in the diseased vasculature, although delineating whether plasma SMILR is simply a by-product of increased intracellular levels or is functionally active in disease pathology is difficult to definitely demonstrate. However, circulating levels of miR 143 and 145 are associated with in-stent restenosis and as such have been proposed as biomarkers\(^57\). The correlation of SMILR and high CRP further supports its expression in low grade chronic inflammatory settings as well as proliferative settings. Further large clinical cohorts will be required to ascertain if SMILR has prognostic potential in inflammatory vascular disease, and if so, whether it provides enhanced prognostic value over current biomarkers.

Vessel re-narrowing after surgical intervention and atherosclerosis remain significant clinical problems and HA/HAS2/SMILR have emerged as key components of these pathological processes. Administration of an siRNA targeting SMILR could be used to prevent re-narrowing after surgical intervention for acute coronary syndrome. Using siRNAs has been proven to be effective in phase I clinical trials. Davis et al. recently showed a dose-dependent increase of siRNA delivered via nanoparticles and observed a reduction in the specific mRNA target\(^58\). However, we must remain cautious, since early clinical trials in the setting of vein graft failure suggested that antisense oligonucleotides directed against E2F (edifoligide) held promising for the
treatment of vein graft failure and atherosclerosis, but the subsequent phase-three PREVENT IV study yielded largely disappointing results\(^5\). However, these studies do demonstrate that the surgical setting of coronary artery bypass grafting provides the ideal clinical setting to evaluate the clinical efficacy of these targets by gene therapy, given that the vein can be transduced \textit{ex vivo} at the time of surgery. Administration of siRNA directly to the vessel would obviate the need to administer siRNA systemically and thus reduce the possibility of off target effects.

Unfortunately, there are no clear homologues of \textit{SMILR} in the mouse. It would, however, be important to determine if other pre-clinical models of human vascular disease contain \textit{SMILR} homologues, once this information becomes available.

Taken together, these observations broaden our awareness of the complex interplay between lncRNA and protein coding genes. The emergence of lncRNAs as regulators of gene expression will undoubtedly alter our understanding of the complex regulation network of pathological VSMC proliferation in vascular disease and may provide a means to specifically target VSMC or identify patients at risk of major adverse vascular outcomes.

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Conflict of Interest Disclosures: None.

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Clinical Perspective

Long noncoding RNAs (lncRNAs) are a relatively new class of discovered RNA molecules that possess important regulatory functions. The rapidly expanding catalogue of lncRNAs holds promise that in the near future lncRNAs might become relevant to vascular disease clinically, as possible biomarkers of cardiovascular events and for targeted treatment of disease. Our work indicates that dysregulation of key lncRNAs may have profound implications in regulating vascular smooth muscle cell function. In addition, we detected the release of this lncRNA in plasma samples and correlated with inflammatory CReactive Protein (CRP) levels, highlighting new methods and possibilities for improved detection. The emergence of lncRNAs as regulators of gene expression and vascular function will undoubtedly alter our understanding of the complex regulation network of cell function underpinning clinical vascular disease.
Figure Legends:

Figure 1. RNA sequencing shows IL1α and PDGF induction of inflammatory and cell cycle pathways. (A): Study design for determination of the transcriptome in quiescent and stimulated HSVSMC. HSVSMC were treated for 72 h, RNA quality assessed and subjected to RNA-seq following the Tuxedo pipeline for analysis. (B): Known inflammatory microRNA, miR146a, is upregulated by IL1α (n=4). **P<0.01 vs. 0.2% condition. Multiple comparison one-way ANOVA. (C): BrdU incorporation as an indirect marker of proliferation was assessed in all patients. (n=3). **P<0.01 vs. 0.2% condition. (D): Biotype distribution of all transcripts identified by RNA-seq analysis generated from HSVSM cells treated with IL1α and PDGF, cutoff at FPKM>0.1 (E): Venn diagram indicating overlap of protein coding genes with altered expression (analysed using EdgeR, FDR<0.01) across each treatment.

Figure 2. Identification of differentially expressed LncRNAs in HSVSMC treated with IL1α and PDGF. (A): Heatmaps showing order of differentially expressed transcripts within the 4 patient samples before and after IL1α/PDGF treatment. LncRNA selected for validation marked by * (B): Heatmap representing the fold change of the 5 LncRNAs selected for validation. Heatmaps represent data from RNA-seq pipeline.

Figure 3. Treatment with IL1α and PDGF significantly altered LncRNA expression and showed distinct expression within vascular cell types. (A): Graphs indicate fold change of LncRNA from RNA-seq data and subsequent validation by qRT-PCR (n=4). * P<0.05, ** P<0.01, *** P<0.001 vs 0.2% condition. (B): Basal and stimulated expression of LncRNAs 2 and 7 within HSVEC and
Figure 4. Localisation of SMILR. (A): RNA FISH analysis of SMILR, cytoplasmic UBC mRNA and nuclear SNORD3 RNA in HSVSMC. Magnification x630 for all panels. UBC and SNORD3 used for confirmation of cellular compartments. (B): Quantification of LncRNA molecules per cell in indicated conditions. Greater than 5 images were selected at random from each condition and at least 4 cells counted in each image.

Figure 5. Functional regulation of SMILR. (A): Schematic diagram showing specific sites of inhibition. HSVSMC were pre-treated for 60 minutes with the indicated concentration of the inhibitors. Following exposure to vehicle or 10 ng/ml IL1 or 20 nM PDGF for 24 h expression of SMILR was determined by qRT-PCR. (B): SMILR expression following MEKK1 inhibition. ***P<0.01 vs 0.2% media, ### P<0.001 vs IL1/PDGF treatment. Repeated measures ANOVA. n=3. (C): SMILR expression following p38 inhibition. Repeated measures ANOVA. ***P<0.01 vs 0.2% media, ### P<0.001 vs IL1/PDGF treatment alone. n=3. (D): SMILR expression in conditioned media from HSVSMC cultured in 0.2%, IL1 or PDGF conditions. Unpaired t test * P<0.05 vs. 0.2% (n=4). (E): Confirmation of the effect of siRNA targeting SMILR in HSVSMC using qRT-PCR (n=3). One way ANOVA ***P<0.001 vs. 0.2% control. ### P<0.001 vs IL1 + PDGF treatment. (F): IL1/PDGF induced proliferation classed as 100% for analysis across patient samples, knockdown of SMILR inhibits EdU incorporation in HSVSMC (n=3) One way ANOVA vs Si Control. ## P<0.01 (G): qRT-PCR analysis of SMILR expression following infection with either an empty lentivirus (LV-E) or lentivirus containing SMILR sequence (LV-S) at
an MOI of 25 (n=3) and MOI 50 (n=3) ***P<0.001 vs. relevant empty control assessed via multiple comparison ANOVA.

**Figure 6.** *SMILR* regulates proximal gene HAS2 in chromosome 8. (A): Schematic view of the 8q24.1 region showing lncRNAs and protein coding genes over the 5,000,000 bp region from Ensemble. (B): Regulation of protein coding and non-coding genes within the selected region following IL1α and PDGF treatment, heatmap depicts expression of genes found with RNA-seq in 4 patient samples. (C): Dotted line marks region containing *SMILR* lincRNA and closest protein coding genes HAS2 and ZHX2. (D): Expression of proximal gene HAS2 - modulated in the same manner as *SMILR* with IL1α and PDGF treatment (n=3). Un-paired t-test ***P<0.001 vs. 0.2% (E-F): Additional HAS isoforms are differentially modulated by IL1 and PDGF (n=3). Un-paired t-test ***P<0.001 vs. 0.2% (G-I): Validation of RNA seq data for lncRNAs SMILR and HAS2-AS1 by qRT-PCR (n=3). *<0.05 and ** P<0.01 vs 0.2%, Un-paired t test. (J): Inhibition of SMILR expression via dsiRNA treatment significantly inhibits HAS2 expression determined by qRT-PCR **P<0.01 vs. Si Control. Un-paired t test (n=3). (K-N): SMILR inhibition had no effect on proximal genes ZHX2 or HAS2-AS1 nor additional HAS isoforms, HAS1 or HAS3 (n=3). Un-paired t test.

**Figure 7.** Uptake [18F]-Fluoride and [18F]-FDG within plaque and normal artery and changes in non-coding RNA expression within carotid plaques. Axial images demonstrating unilateral (A, B) or bilateral [18F]-Fluoride carotid uptake (D, E). Image C is a multi-planar reformat of B. Axial images demonstrating [18F]-FDG carotid uptake (F, G). H shows the Siemens Biograph Clinical PET/CT system used for imaging. White arrows indicate carotid radio-ligand uptake.
(H-K): Uptake of tracer (L): MicroRNA profile of atherosclerotic plaque and paired healthy carotid controls \((n=6)\) assessed by qRT-PCR (paired students t test). Expression of \textit{SMILR} (M), \textit{HAS2} (N) and \textit{HAS2-AS1} (O) within atherosclerotic plaque \((n=5)\). analysed via qRT-PCR analysis, *** \(P<0.001\), ** \(P<0.01\) and * \(P<0.05\) assessed by paired students t test.

**Figure 8.** SMILR is detectable within plasma samples and correlates with patient CRP levels.

(A): SMILR expression is increased in patients with higher CRP levels \((n=13 \text{ CRP}<2, n=13 \text{ CRP}2-5 \text{ and } n=15 \text{ CRP}>5\), *\(P<0.05\), **\(P<0.01\) via One-way ANOVA). (B): Correlation between SMILR expression and CRP levels (linear regression \(P<0.0001\)).
**Figure 1**

A. HSVSMC treated with 10ng/ml IL1α, 20ng/ml PDGF or combination for 72h (4 Patients)

RNA quality assessment

**Bowtie**
General purpose short read aligner

**TopHat**
Aligns RNA-seq reads to the transcriptome – discovers splice sites

**Cufflinks**
Assembles transcripts and compares transcripts to annotation

**Edge R**
Performs pairwise differential gene expression for all sample group combinations

Filtered LncRNA
LogFC>2, FDR<0.01, FPKM>1

Coding Genes

**Classification**

Overlapping
Intergenic
Sense
Antisense

B. miR 146a expression

miR 146a expression normalized to UBC

C. BrdU proliferation

D. IL1α + PDGF

**D**

IL1α + PDGF

**E**

IL1α

76

6

127

PDGF

246

190

217

IL1α + PDGF

480

**Treatment**

| Total protein coding genes |
|---------------------------|
| FDR=0.01 |
| IL1α | 518 |
| PDGF | 540 |
| IL1α+ PDGF | 1133 |
A

Figure 2

Up regulated IncRNA

Down regulated IncRNA

Downloaded from http://circ.ahajournals.org/ at UNIV OF EDINBURGH on May 9, 2016
Figure 3
Figure 4
Figure 5
Figure 8
Smooth Muscle Enriched Long Non-Coding RNA (SMILR) Regulates Cell Proliferation
Margaret D. Ballantyne, Karine Pinel, Rachel Dakin, Alex T. Vesey, Louise Diver, Ruth Mackenzie, Raquel Garcia, Paul Welsh, Naveed Sattar, Graham Hamilton, Nikhil Joshi, Marc R. Dweck, Joseph M. Miano, Martin W. McBride, David E. Newby, Robert A. McDonald and Andrew H. Baker

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SUPPLEMENTAL MATERIAL

Supplemental Methods

Tissue and Cell culture

Endothelial media supplemented 20% FCS (Life Technologies, Paisley, UK). HSVSMC media supplemented with 15% foetal bovine serum (FBS) (PAA laboratories, Yeovil, UK), 2 mM L-Glutamine (Invitrogen, Paisley, UK) 50 μg/ml penicillin (Invitrogen) and 50 μg/mL streptomycin (Invitrogen). All cells were used between passages 3-5.

HSVSMC BrdU and EdU Incorporation assay

HSVSMC proliferation was quantified using a DNA bromodeoxyuride (BrdU) incorporation assay (Millipore, Watford, UK), or EdU according to the manufacturer's instructions. Briefly, cells were plated and quiesced in 0.2% FCS media for 48 h prior to stimulation. Cells were stimulated with either 10 ng/mL IL1α, 20 ng/mL PDGF or a combination of both for the stated times. For BrdU experiments, 6 h after stimulation cells were incubated with BrdU and for EdU experiments EdU was added at the point of stimulation for the remaining time to allow cell proliferation. BrdU: after removing the culture medium, the cells were fixed followed by incubation with anti-BrdU antibody which binds the incorporated DNA. After adding the substrate solution, the immune complexes were detected using a plate reader set at dual wavelength of 450/550 nm, Victor (Perkin Elmer, Waltham, USA). EdU: following stimulation, cellular RNA was extracted as described earlier or fixed in 70% ethanol for EdU FACs analysis. EdU incorporation was quantified using Click-it EdU Proliferation assay with an Alexa Fluor 594 antibody according to the manufacturer's protocol (Life Technologies).
MEKK1 and P38 inhibitor studies

For inhibitor studies, HSVSMC were plated and quiesced for 48 h. One hour prior to stimulation, cells were incubated with either 10, 15 or 20 µM AZD6244 (MEKK1 inhibitor, Selleckchem, Suffolk, UK) or 5, 10 or 20 µM P38 (SB 203580). Cells were then maintained in either 0.2% media or stimulated with a combination of IL1α and PDGF for 24 h before RNA isolation.

5' and 3' RACE

5' 3' Rapid amplification of cDNA ends \(^1\) was performed to determine the full length transcript of SMILR using the SMARTer RACE 5'/3' Kit (Clontech, Saint-Germain-en-Laye, France) according to manufacturer's instructions. Nested PCR was used to ensure only specific 5' and 3' products were detected (PCR Primer sequence – Suppl. Table 1). Following cloning into supplied cloning vector, products were sequenced.

Fluorescent in situ hybridisation

Custom RNA-FISH tiled probe sets were generated to all exons of SMILR. RNA FISH utilises “branch tree” technology. Briefly, a target specific probe set, containing 40 oligo probes, hybridises to the target mRNA as 20 oligo pairs. Each oligo pair forms a required platform for assembly of the signal amplification structure (tree) through a series of sequential hybridisation steps. Each fully assembled structure, covers a space of 40-50 nt of the target RNA, and has the capacity for 400-fold signal amplification. Therefore, a typical RNA probe set (containing 20 oligo pairs) has the capacity to generate 8,000-fold signal amplification. Due to this technology the company confirms single-molecule RNA sensitivity, thus each fluorescent signal corresponds to an individual IncRNA molecule.
Control SNORD3 and UBC were used as housekeepers to determine spatial location of SMILR (Panomics, Affymetrix, California, US). RNA-FISH was performed according to manufacturer’s instructions (ViewRNA™ cell FISH) with minor modifications for both cell and tissue experiments. For cellular analysis, HSVSMC ± IL1α/PDGF were grown on 16-mm coverslips to 80% confluency, washed in PBS and fixed in 4% paraformaldehyde supplemented with 1% glacial acetic acid. Following detergent QS permeabilisation and 1:6000 protease digest, coverslips were incubated with a combination of UBC and SMILR probe sets or UBC. Probe set buffer was used as a negative control and SNORD3 as confirmation of nuclear permeabilisation. Following probe hybridisation, cover slips were incubated with branched tree technology pre amplifier for 1 h and amplifier for 30 min. Cover slips were finally incubated with fluorescent probes, mounted onto glass slides using Prolong gold anti-fade with DAPI mounting medium (Life Technologies).

**Image acquisition**

Images acquired on a Zeiss 510 confocal system. At least 5 images were taken per condition. Parameters for acquisition and post analysis were identical for all conditions. Images were Z stacked to confirm nuclear localisation.

**Dicer substrate siRNA (dsiRNA) mediated transfection**

Double stranded dicer substrate siRNA targeting SMILR and Si-control were synthesised (Integrated DNA Technologies, Leuven, Belgium). The Si-control does not target any sequence in the human, mouse, or rat transcriptomes. Transient transfection was performed with Lipofectamine 2000 (Life Technologies). Cells were transfected with either 25 nM Si-SMILR or Si-Control. Six hours post transfection, cells were quiesced for 48 h and stimulated for a further 48 h with 0.2% media containing IL1α/PDGF.
**Lentiviral mediated infection**

Lentiviral vectors were produced by triple transient transfection of HEK293T cells with a packaging plasmid (pCMVΔ8.74), a plasmid encoding the envelope of vesicular stomatitis virus (VSVg) (pMDG) (Plasmid Factory, Bielefeld, Germany) and pLNT/SFFV-MCS plasmid employing polyethylenimine (PEI; Sigma-Aldrich, St Louis, USA) as previously described. Lentiviral titres were ascertained by TaqMan quantitative real-time PCR (qRT-PCR) using the following primer/probe sequences: forward, 5'-TGTGTGCCCAGTCTGTTGTGT-3'; reverse, 5'-GAGTCCTGCGTAGAGACG-3'; probe, 5'-(FAM)-CAGTGGCGCCGAACAGGGA-(TAMRA)-3'. SMILR was cloned into the pLNT/SFFV-MCS (kind gift from Adrian J. Thrasher, London, UK) plasmid using Platinum taq polymerase, according to manufacturer’s instructions, to create pLNT/SFFV-MCS-SMILR. A confluent monolayer of smooth muscle cells were plated and infected with a multiplicity of infection of either 25 or 50, neither of which induced any form of toxicity in our cells. Following 24 h infection, media was changed to 0.2% for a further 48 h. Cells were then stimulated and EdU incorporation or SMILR expression investigated as above.

**Detection of LncRNA in exosomes secreted from HSVSMC**

SMILR expression in conditioned media utilising both ultracentrifugation and exosome isolation kits. RNA extraction of exosome free HSVSMC media was performed using a standard volume (15 mL). The conditioned media was centrifuged at 2000 g at 4°C for 10 min and then at 12000 g for 45 min to remove all cell debris. The supernatant was filtered (0.22 µm) followed by ultracentrifugation at 110 000 g, 4°C for 90 min (Optima L-80 XP ultracentrifuge Beckman coulter) to obtain microvesicles (MV) and exosomes and exosome free media compartments. Additional experiments were performed utilising the Total exosome isolation kit (Life technologies) following the manufacturer’s instructions. The presence of microvesicles and exosomes was verified using the Nanosight technology.
For exosomes and microvesicles, 700 µL of Qiazol (Qiagen) was added and 3 µL of c.elegans total RNA at 25 ng/µL and the RNA was extracted using miRNEasy mini kit (Qiagen) as previously described. For the exosome free media compartment, RNA was extracted from 2 mL and following the same protocol as describe in the manuscript. SMILR relative expression was determined in theses 2 compartments by qRT-PCR.

In Vivo Studies Atherosclerosis Studies: Patients, Imaging and Sampling

Carotid cohort

Patients with symptomatic carotid artery stenosis (≥50% by NASCET criteria) scheduled to undergo carotid endarterectomy were recruited from neurovascular clinics at the Royal Infirmary of Edinburgh between January 2013 and April 2014. Exclusion criteria included a modified Rankin score of 3, insulin-dependent diabetes mellitus, women of child-bearing age not receiving contraception, severe chronic kidney disease (eGFR <30 mL/min/1.73 m²), known iodine-based contrast media allergy, prior ipsilateral carotid intervention, prior neck irradiation, and inability to provide informed consent. Patients underwent a standard baseline clinical assessment including blood sampling (for standard clinical haematological and biochemical indices, including C reactive protein, and plasma RNA analysis) before undergoing separate [18F]-fluoride and [18F]-fluorodeoxyglucose ([18F]-FDG) positron emission tomography combined with computed tomography (CT) scans with the use of a hybrid scanner (Biograph mCT, Siemens Medical Systems, Erlangen, Germany). Both of these tracers have been used by our group and others for plaque imaging and highlight high-risk actively calcifying and inflamed or hypoxic atherosclerotic plaques.

For [18F]-fluoride imaging, a target dose of 250 MBq was administered intravenously. Scanning took place after a 60-min delay. Following an attenuation-correction CT scan (non-enhanced, low dose 120 kV, 50 mAs) PET imaging was performed in static mode covering 2 bed positions (15 min each) with the superior bed centered over the carotid bifurcation.
Following PET acquisition, a CT carotid angiogram was performed without moving the patient (Care Dose 4D, 120 kV, 145 mA, rotation time 0.5 s, pitch 0.8).

[18F]-FDG PET/CT was performed on a separate day. A target dose of 125 MBq was administered intravenously and scanning commenced after a 90-min delay. PET/CT acquisition was identical to [18F]-Fluoride save for a longer bed time of 20-min and a pre-scan fast of 6 h. Static images were reconstructed using the Siemens Ultra-HD algorithm (time of flight + True X) with corrections applied for attenuation, dead time, scatter, and random coincidences.

PET tracer uptake was quantified using an OsiriX workstation (OsiriX version 3.5.1 64-bit; OsiriX Imaging Software, Geneva, Switzerland). PET/CT image data were reviewed for evidence of tracer uptake, image quality and registration. The CT angiogram was examined to establish plaque presence, location and characteristics. Regions of interest were then drawn on three adjacent 3-mm PET slices to incorporate the internal carotid artery plaque. Three ROI were then drawn around adjacent healthy portions of carotid artery and the lumen of the SVC to derive control values for “normal” arterial uptake and the blood pool respectively. Arterial standardized uptake values (SUV) were recorded and also indexed to blood pool activity thus giving a target-to-background-ratio (TBR).

At the time of surgery, plaques were collected immediately following excision and photographed. Two-millimeter diameter core biopsy specimens for RNA analysis were taken from regions of focally high uptake on PET and from normal tissue at the periphery of the endarterectomy specimen. These, along with the main specimen, were immediately frozen and placed in an -80°C fridge for subsequent batch analysis. Patient characteristic found in Suppl. Table. 5.
Assessment of IncRNA in human plasma

A standard volume of each plasma sample (300 µL) was used to extract RNA. Five volumes of QIAzol lysis reagent (Qiagen) was added per extraction and supplemented with spike-in RNA controls: 3.5 µL of miRNeasy Serum/Plasma Spike-In Control at 1.6 x 10^8 copies/µL (C. elegans miR-39 miRNA mimic; Qiagen) and 3 µL of c.elegans total RNA at 25 ng/µL. Following 5-min incubation at RT, chloroform was added at equal volumes to the starting sample. Following centrifugation (15 min; 8000 g; 4°C) the clear upper aqueous phase was used to isolate RNA as above.
**Supplemental Figures**

| LncRNA Name | Chromosomal Location | Ensembl ID | Forward Primer sequence | Reverse Primer sequence |
|-------------|----------------------|------------|-------------------------|-------------------------|
| SMILER      | chr8:123426571-123440790 | RP11-94A24.1 | ACCTGGAGGCTCTTTGGGAGT | TTGCAGACACCTTCACAAACA   |
| LncRNA 4    | chr15:68591128-68593343 | RP11-709B3.2 | AAAAACTGCCACCTGTGACC  | TTTGCTAGGCTCTGGGGAAG     |
| LncRNA 6    | chr8:121066919-121068440 | RP11-760H22.2 | CTGCAATGGAGAGACAGGAAT | AAAGCTGAAACCCCATGAAGTCATTG |
| LncRNA 7    | chr3:177534653-177617012 | RP11-91K9.1  | TGGCTAAGGAGGAGGCTATC  | CACGGTGCTGCAACACTTTTA    |
| LncRNA 8    | chr7:35756084-35774497  | AC018647.3  | CCAAGGATGAGCACAAAAA   | AAAGGTCAGAGTCTTGA        |
| SMILER RACE |                      |            | GATACCAGCAAGCTTTTGGCAACATGAGGATGACGCTGGAT | GATTAGGCAAGATTTTCTCACAGCATGCTCTGAGGGACATT   |

**Supplemental Table 1: Sybr green primer sequences.** Exon spanning lncRNA primers were designed to each lncRNA to ensure no genomic DNA was assessed during qRT-PCR.
Supplemental Table 2: IL1α stimulation Ingenuity Pathway analysis. Top 10 disease and functional pathways predicted to be altered by IPA in HSVSM cells stimulated with IL1α.

| Categories                        | Disease or Function Annotation                  | p-value  |
|-----------------------------------|------------------------------------------------|----------|
| Cellular Movement                 | cellular movement                               | 1.3x10^-36 |
| Cell Death and Survival           | necrosis                                        | 1.6x10^-33 |
| Cellular Growth and Proliferation | proliferation of cells                          | 2.4x10^-28 |
| Organismal Development            | angiogenesis                                    | 2.3x10^-26 |
| Cancer                            | growth of tumour                                 | 6.7x10^-25 |
| Connective Tissue Disorder        | arthropathy                                      | 8.1x10^-24 |
| Inflammatory Disease              | chronic inflammatory disorder                    | 1.4x10^-22 |
| Cellular Movement                 | leukocyte migration                              | 1.9x10^-23 |
| Inflammatory Response             | Inflammatory response                            | 4.3x10^-23 |
| Gastrointestinal Response         | Digestive system cancer                          | 6.9x10^-23 |
### 0.2% VS PDGF

| Categories                          | Disease or Function Annotation                        | p-value       |
|-------------------------------------|-------------------------------------------------------|---------------|
| Cellular Growth and Proliferation   | proliferation of cells                                | \(2.5 \times 10^{-29}\) |
| Cell Death and Survival             | apoptosis                                             | \(3.3 \times 10^{-25}\) |
| Cellular Movement                   | migration of cells                                    | \(5.3 \times 10^{-28}\) |
| Cardiovascular System Development   | development of the cardiovascular system              | \(2.3 \times 10^{-26}\) |
| Organismal Development              | angiogenesis                                          | \(7.1 \times 10^{-21}\) |
| Cellular Development                | proliferation of tumour cell lines                    | \(8.5 \times 10^{-21}\) |
| Cancer                              | cancer                                                | \(1.5 \times 10^{-20}\) |
| Cell Cycle                          | mitosis                                               | \(1.7 \times 10^{-17}\) |
| Cell Morphology                     | morphology of cells                                   | \(5.3 \times 10^{-16}\) |
| Tissue Development                  | growth of connective tissue                           | \(6.7 \times 10^{-16}\) |

**Supplemental Table 3: PDGF stimulation Ingenuity Pathway analysis.** Top 10 disease and functional pathways predicted to be altered by IPA in HSVSM cells stimulated with PDGF.
**Supplemental Table 4: IL1α + PDGF stimulation Ingenuity Pathway analysis.** Top 10 disease and functional pathways predicted to be altered by IPA in HSVSM cells stimulated with IL1α and PDGF.

| Categories                      | Disease or Function Annotation       | p-value   |
|---------------------------------|--------------------------------------|-----------|
| Cellular Growth and Proliferation| proliferation of cells                | 1.0x10^-45 |
| Cell Death and Survival         | apoptosis                             | 7.2x10^-44 |
| Cancer                          | cancer                               | 1.2x10^-37 |
| Cellular Movement               | migration of cells                    | 2.3x10^-34 |
| Gastrointestinal Response       | digestive system cancer              | 7.0x10^-30 |
| Cellular Development            | proliferation of tumour cell lines    | 8.5x10^-28 |
| Reproductive System Disease     | tumour                               | 4.8x10^-27 |
| Cell Cycle                      | Cell cycle progression                | 6.2x10^-26 |
| Cardiovascular System development| morphology of cells                  | 2.2x10^-24 |
| Cardiovascular System development| angiogenesis                         | 4.8x10^-24 |
Supplemental Figure 1: Spike-in method of *C. elegans* total RNA in whole HSVSMC media. (A): Dose response effect of *C. elegans ama-1* expression. Expression determined by qRT-PCR and results displayed as 1/Ct. Number at the top of each histogram corresponds to Ct values. (B): Specificity of products analysis by melting curve. (C): Specificity of products analysed using agarose gel. The cDNA amplicon size has been resolved by migration on a 2% agarose gel using 100 bp ladder. (D): Correlation between quantity spike-in and *ama-1* expression. *C. elegans ama-1* expression follow a logarithmic function: \( y = -1.231\ln(x) + 30.406 \) with a coefficient of correlation \( r^2 = 0.9668 \). (E): Reproducibility of the technique. Following RNA extraction after 75 ng of spike-in total *C. elegans* RNA, *ama-1* expression was determined by qRT-PCR and the results have been displayed as Ct.
Supplemental Figure 2: Assessment of RNA-seq data. (A): Biotype distribution of all transcripts identified by RNA-seq analysis generated from HSVSM cells treated with IL1α and PDGF, cutoff at FPKM>0.1 (B): IPA analysis of top protein coding genes following IL1α and PDGF treatment.
Supplemental Figure 3: Identification of differentially expressed LncRNAs in HSVSMC treated with IL1α and PDGF. Transcripts differentially expressed between 0.2% and stated treatment (p<0.01), pie chart indicates the relative percentage, and tables present numbers, of each biotype differentially expressed. LncRNAs differentially expressed between 0.2% vs IL1α/PDGF can be subdivided based on IncRNA biotype. Groups include intervening IncRNA (lincRNA), antisense, overlapping and processed transcripts.
Supplemental Figure 4: Heat map of most significantly dysregulated intervening lncRNAs across all treatment groups. Heat map shows most significant changes in intervening lncRNA 0.2% vs IL1+PDGF treatment. LincRNA cut off using FDR<0.01, FPKM>1.
Supplementary Figure 5: Dissociation curves and gel products of PCR reactions indicating single PCR products. (A-H) Dissociation curves and gels for each lncRNA primer set. Primers were tested under 0.2% and IL1+PDGF conditions. Each gel also contains lanes containing −ve RT and H2O samples.
Supplemental Figure 6: LncRNA tissue and cell specificity analysis. (A): Expression of LncRNAs in a healthy tissue panel. Expression determined by qRT-PCR and results displayed as 1/ΔCT. (B): Expression of IncRNAs in unstimulated HSVSMC, HSVEC or HCAVSMC.
Supplemental Figure 7: Validation of additional SMILR primers. (A-C): Assessment of SMILR via qRT-PCR expression via 3 independent primer sets. The number on top of graphs represent Ct values obtained under 0.2% and dual stimulated conditions.
Supplemental Figure 8: Temporal regulation of IncRNA 2 assessed by qRT-PCR. HSVSMC were stimulated with IL1α, PDGF of a combination for the stated time points. RNA was extracted and expression determined by qRT-PCR.
Supplemental Figure 9: Visual representation of full SMILR transcript. (A): Grey boxes indicate the predicted SMILR sequence obtained from UCSC genome browser (RP11-94A24.1). Black boxes represent additional 316 basepair sequence obtained via 3' RACE of SMILR transcript. ***P<0.001, **P<0.01 and *P<0.05 vs 0.2% in each time point (1 way ANOVA). (B): Full length sequence of lncRNA 2.
A

SMILR Raw RNA-seq Reads – Control (0.2%) and stimulated (IL1 + PDGF)
B  HAS2 Raw RNA-seq Reads – Control (0.2%) and stimulated (IL1 + PDGF)
Supplemental Figure 10: Raw sequencing profiles generated utilising tophat files, constructed on integrative genome viewer (IGV). (A): Raw sequencing reads of SMILR under both basal and dual stimulated (IL1 + PDGF) conditions n=4. (B): Raw sequencing reads of HAS2 indicating a similar expression pattern following stimulation. (C): Raw sequencing reads of SMILR under stimulated conditions – expanded (D): Northern analysis of miR146a and SMILR RNA. U6 shown as loading control.
Supplemental Figure 11: Exosome isolation from HSVSMC conditioned media. (A): Size evaluation using the Nanosight of exosomes and MV isolated using the Total exosome isolation kit from 0.2% conditioned media. Sizes obtained between 70 and 600 nm. (B): Quantification of miR-143 in exosomes/MV isolated using the Total exosome isolation kit from 0.2% conditioned media. (C): SMILR expression analysed by qRT-PCR in exosomes/MV and exosomes/MV free media compartment from IL-1α + PDGF conditioned media. (D): Agarose gel of qRT-PCR products obtained in C; 1: exosomes/MV compartment, 2: exosomes/MV free media. (E): melting curves and gel electrophoresis of SMILR primer set in conditioned media. (F): SMILR expression from conditioned media following control lentivirus or SMILR lentivirus infection of cells. (G): Subsequent proliferation of quiesced cells following 48h incubation with lentivirus conditioned media.
Supplemental Figure 12: Proliferation of HSVSMC 0.2% vs IL1 + PDGF treatment. P<0.05 students t test.
Supplemental Figure 13: (A): Confirmation of siRNA mediated down regulation of SMILR using second siRNA targeting a separate sequence of SMILR. (B): Confirmation of knockdown of SMILR using second siRNA. Analysed by 
students t-test ***P<0.001 vs SiControl. (C-D): qRT-PCR analysis of interferon gamma associated mRNA OAS1 and IRF7. (E-F): qRT-PCR validation of HAS1 and HAS3 regulation by IL1α and PDGF. One way ANOVA *P<0.05. (G-I): Validation of siSMILR using second siRNA targeting different section of the lncRNA.
Supplemental Figure 14: Effect of HAS2 and HAS2-AS1 knockdown on SMILR expression. (A): Knockdown of HAS2 or HAS2-AS1 both reduced HAS2 expression. (B): Knockdown of neither HAS2 nor HAS2-AS1 affected SMILR expression levels. (C): Knockdown of HAS2 –AS but not HAS2 significantly reduced HAS2-AS1 levels. (D-F): Overexpression of SMILR did not affect HAS1-HAS3 nor HAS1-AS1 expression levels.
Supplemental Table 5 - Baseline Patient Characteristics – Carotid Cohorts.

|                                | Carotid (n=7)       |
|--------------------------------|---------------------|
| **Age in years, mean (SD)**    | 63 (13.8)           |
| **Men, n (%)**                 | 4 (57)              |
| **BMI (kg/m²), mean (SD)**     | 26.3 (5.8)          |
| **Systolic blood pressure (mmHg), mean (SD)** | 141.1 (22.5) |
| **Diastolic blood pressure (mmHg), mean (SD)** | 88.4 (16.6) |
| **Presenting syndrome, n (%)** |                     |
| Stroke                         | 2 (29)              |
| TIA/Amaurosis fugax             | 5 (71)              |
| **Cardiovascular history, n (%)** |                    |
| Ischemic heart disease         | 3 (43)              |
| Myocardial Infarction          | 1 (14)              |
| **Risk Factors, n (%)**        |                     |
| Hypertension                   | 5 (71)              |
| Diabetes                       | 1 (14)              |
| Hypercholesterolemia           | 7 (100)             |
| Current smoker                 | 3 (43)              |
| **Medication, n (%)**          |                     |
| Aspirin                        | 2 (29)              |
| Clopidogrel                    | 5 (71)              |
| Anti-coagulant                 | 1 (14)              |
| Statin                         | 7 (100)             |
| ACEi/ARB                       | 3 (43)              |
| B-blocker                      | 2 (29)              |
| **Hematology, mean (SD)**      |                     |
| Hemoglobin                     | 137.0 (23.1)        |
| White cell count               | 8.1 (1.8)           |
| Platelet count                 | 284 (66)            |
| **Serum biochemistry, mean (SD)** |               |
| Creatinine (mmol/L)            | 90 (21.1)           |
| Total cholesterol (mmol/L)     | 4.7 (1.3)           |
Supplemental Table 6 - Baseline Patient Characteristics – CRP matched plasma samples. Values are represented in mean ± SEM with p values calculated by one-way ANOVA or by Fisher’s exact test for categorical variables.

|                  | group 1: crp<2 (n=13) | group 2: 2<crp<5 (n=13) | group 3: crp>5 (n=15) | p values |
|------------------|-----------------------|--------------------------|------------------------|----------|
| Age (years)      | 48.5 ± 1.8            | 48.5 ± 1.9               | 50.7 ± 2.1             | 0.66     |
| CRP (mg/L)       | 1.24 ± 0.15           | 3.56 ± 0.28              | 7.09 ± 0.48            | p<0.0001 |
| Systolic BP (mmHg) | 123 ± 2.9          | 131.2 ± 6.5              | 137.5 ± 4.6            | 0.12     |
| Diastolic BP (mmHg) | 77.5 ± 1.9         | 76.2 ± 2.0               | 79.0 ± 2.7             | 0.68     |
| BMI (kg/m²)      | 26.0 ± 0.5            | 28.7 ± 1.3               | 29.6 ± 1.7             | 0.14     |
| WHR              | 0.96 ± 0.02           | 1.00 ± 0.02              | 0.99 ± 0.02            | 0.23     |
| cIMT (mm)        | 0.64 ± 0.03           | 0.59 ± 0.03              | 0.64 ± 0.04            | 0.47     |
| Smoking status, n (%) |                |                           |                        | 0.015    |
| Never smoker     | 61.5                  | 61.5                     | 60.0                   |          |
| Ex-smoker        | 15.4                  | 38.5                     | 0.0                    |          |
| Current          | 23.1                  | 0.0                      | 40.0                   |          |
| SIMD quintile, n (%) |                |                           |                        | 0.111    |
| 1                | 30.8                  | 0.0                      | 0.0                    |          |
| 2                | 23.1                  | 7.7                      | 6.7                    |          |
| 3                | 7.7                   | 23.1                     | 40.0                   |          |
| 4                | 7.7                   | 15.4                     | 13.3                   |          |
| 5                | 30.8                  | 53.8                     | 40.0                   |          |
Supplemental Figure 15: Primer validation and quality control in plasma samples. (A): Melting curve for SMILR in plasma. (B): Agarose gel of qPCR product. (C): Water melting curve.
A  The Pearson correlation:

|       | crp | trans dct |
|-------|-----|-----------|
| Crp   | 1.0000 |          |
| Transdct | 0.5719  | 1.0000    |
|       | 0.0001 |           |

So \( r = 0.5719 \), \( r^2 = 0.327 \), \( p < 0.001 \)

B  If we take out the two outliers (the two highest dCts):

\[
pwcorr crp transdct, sig
\]

|       | crp | trans dct |
|-------|-----|-----------|
| Crp   | 1.0000 |          |
| Transdct | 0.389  | 1.0000    |
|       | 0.0144 |           |

\( r = 0.389 \), \( r^2 = 0.151 \), \( p = 0.014 \)

Supplemental Figure 16: Statistical analysis of SMILR vs. CRP correlation. (A): Pearson correlation of SMILR vs. CRP utilising all data points. \( R = 0.5719 \), \( r^2 = 0.327 \) and \( P < 0.001 \). (B): Pearson correlation of SMILR vs. CRP omitting the 2 highest outlying points. \( R = 0.389 \), \( r^2 = 0.151 \), \( p = 0.014 \).
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