Identification of lncRNA regulators of stiffness-dependent cell responses

Ingenuity Pathway Predicted Categories	p-value	# Molecules	MALAT1	NEAT1	DLEU2
Cell Cycle	3.63E-27	538			
Cellular Growth and Proliferation	4.22E-26	910	X		
Cancer	4.01E-24	2057	X	X	X
Organismal Injury and Abnormalities	4.01E-24	2099	X	X	X
Cellular Assembly and Organization	4.48E-23	592	X	X	
DNA Replication, Recombin., Repair	4.48E-23	454			
Cell Death and Survival	3.86E-22	873	X		
Organismal Survival	2.88E-18	608	X	X	
Cellular Development	6.54E-18	806	X		
Post-Translational Modification	1.54E-16	80			
Protein Synthesis	1.54E-16	91			
Cellular Movement	2.71E-12	575	X		
Cellular Function and Maintenance	2.78E-12	431			
Cell Morphology	2.34E-11	528			
Tissue Development	1.40E-10	483			
Cardiovascular Sys Dev. and Func.	3.20E-10	331			
Organismal Development	6.58E-10	698			
Tumor Morphology	5.50E-09	203			
Nucleic Acid Metabolism	2.04E-08	135			
Small Molecule Biochemistry	1.01E-07	113			
Tissue Morphology	1.78E-07	479			
Embryonic Development	2.17E-07	412			
Organ Development	2.17E-07	196			
Organ Morphology	2.17E-07	153			
Skeletal and Muscular Disorders	1.03E-06	115	X		
RNA Post-Transcriptional Modification	1.47E-06	78	X		
Cardiovascular Disease	1.54E-06	416			

Table I. Identification of lncRNA regulators of stiffness-dependent cell responses (Predicted Categorical Pathways). Top categorical pathways identified in Ingenuity Pathway Analysis. Enrichment p-value and number of molecules for each category are listed. An (X) denotes that the corresponding lncRNA is represented within that canonical pathway. Only three lncRNAs were represented for the top enriched pathways listed (i.e. no other lncRNAs were annotated to these pathways in the ingenuity database).

Ingenuity Pathway Predicted Functions	p-value	MALAT1	NEAT1
Proliferation of cells	4.22E-26	X	
Necrosis	3.86E-22	X	
Cell death	1.72E-21	X	
Apoptosis	2.34E-20	X	
Organismal death	2.88E-18	X	X
Morbidity or mortality	3.94E-18	X	X
Metastasis	1.63E-13	X	
Cell movement	2.71E-12	X	
Migration of cells	2.25E-11	X	
Invasion of cells	3.13E-10	X	
Smooth muscle tumor	1.06E-06	X	
Processing of RNA	1.47E-06	X	
Cell transformation	2.7E-06	X	
Organization of organelle	9.24E-06	X	
Formation of nucleus	1.21E-05		X
Metastasis of cells	3.29E-05	X	
Splicing of RNA	3.55E-05	X	
Leiomyomatosis	0.00012	X	

Table II. Identification of lncRNA regulators of stiffness-dependent cell responses (Predicted Functions). Top predicted functions identified in Ingenuity Pathway Analysis that contained lncRNAs annotated to those functions. Enrichment p-value and for each category are listed. An (X) denotes that the corresponding lncRNA is represented within that predicted function. MALAT1 and NEAT1 are the only lncRNAs linked to these cellular functions in the ingenuity database.

FIGURE LEGEND (SUPPLEMENTAL EXCEL FILE)

Table III. Correlated stiffness-sensitive lncRNA-protein coding gene pairs. Table depicts the significantly correlated stiffness-sensitive lncRNAs-stiffness-sensitive protein coding gene (PCG) pairs. Protein coding genes and lncRNA genes are listed along with their log2 fold change (log2FC > 0 represents decreased expression by stiffness). The chromosome for both lncRNA and protein coding gene, coordinates for the lncRNA transcriptional start site, distance between gene pairs, Spearman's correlation coefficient, and correlation p-value, and stiffness-fold change and adjusted p-value (false discovery rate) for each lncRNA and protein coding gene are listed.

Gene	Distance (kB)	Spearman R	Correlation p-value	Type
BANF1	-504	-0.80	1.8E-04	PCG
MRPL11	-969	-0.79	2.7E-04	PCG
SAC3D1	457	-0.79	2.9E-04	PCG
NEAT1	75	0.78	3.5E-04	lncRNA
EIF1AD	-504	-0.77	4.4E-04	PCG
DRAP1	-421	-0.77	5.0E-04	PCG
MUS81	-359	-0.76	6.8E-04	PCG
POLA2	236	-0.75	7.6E-04	PCG
FAU	375	-0.74	9.3E-04	PCG
FIBP	-391	-0.74	1.1E-03	PCG
SNX32	-336	-0.73	1.2E-03	PCG
LTBP3	-61	0.72	1.7E-03	PCG
CFL1	-364	-0.72	1.8E-03	PCG
CDCA5	414	-0.71	2.0E-03	PCG
FOSL1	-403	-0.71	2.0E-03	PCG
SF3B2	-553	-0.69	2.8E-03	PCG
RP11-869B15.1	712	0.69	3.1E-03	lncRNA
BATF2	501	-0.66	5.7E-03	PCG
B3GNT1	-850	0.64	7.7E-03	PCG
FAM89B	-75	0.64	8.1E-03	PCG

Table IV. Genes correlated to MALAT1 expression. Table depicts correlated genes within 1000kB of MALAT1 on chromosome 11 and correlation p-value < 1e-2.
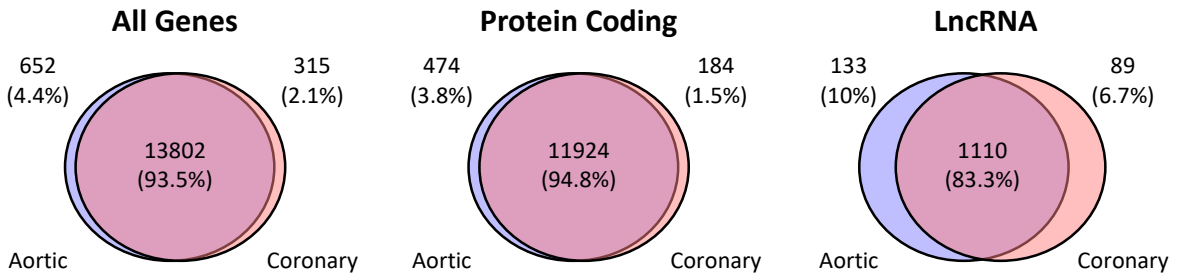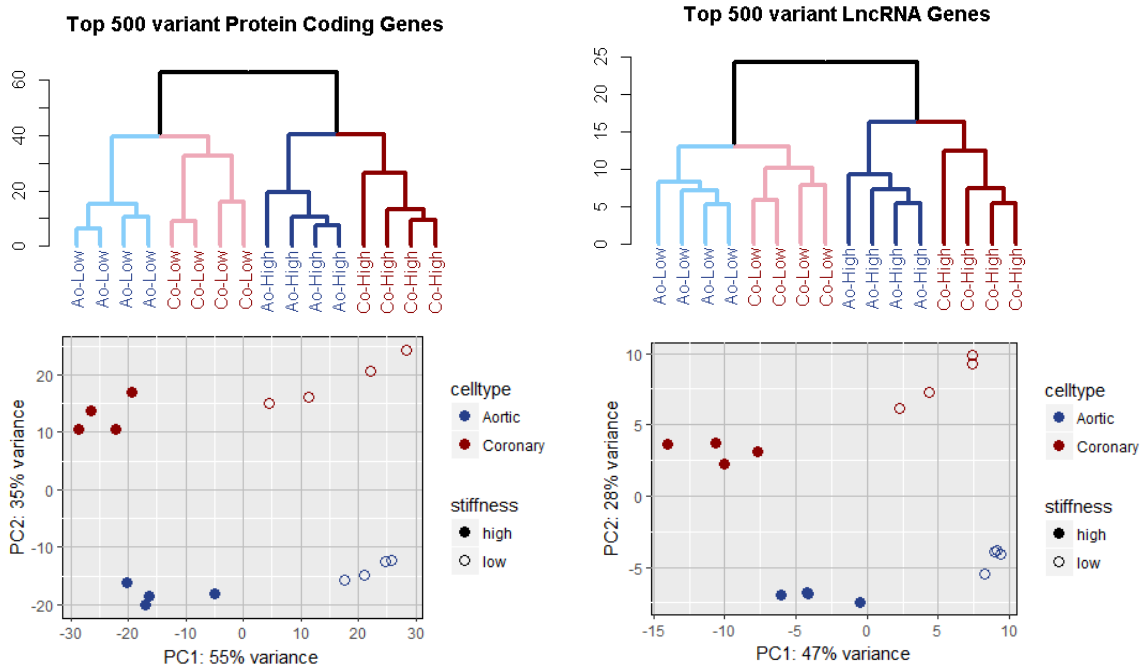
Figure I. (A) Comparison of expression overlap of human aortic tissue and coronary artery tissue from GTEx. Venn diagrams depicting the overlap of expressed genes (defined as RPKM > 0.7) in aortic and coronary tissue expression data. (B) Principal component and hierarchical clustering analysis of Ao and Co VSMCs using the top variant protein coding genes and top variant lncRNA genes.
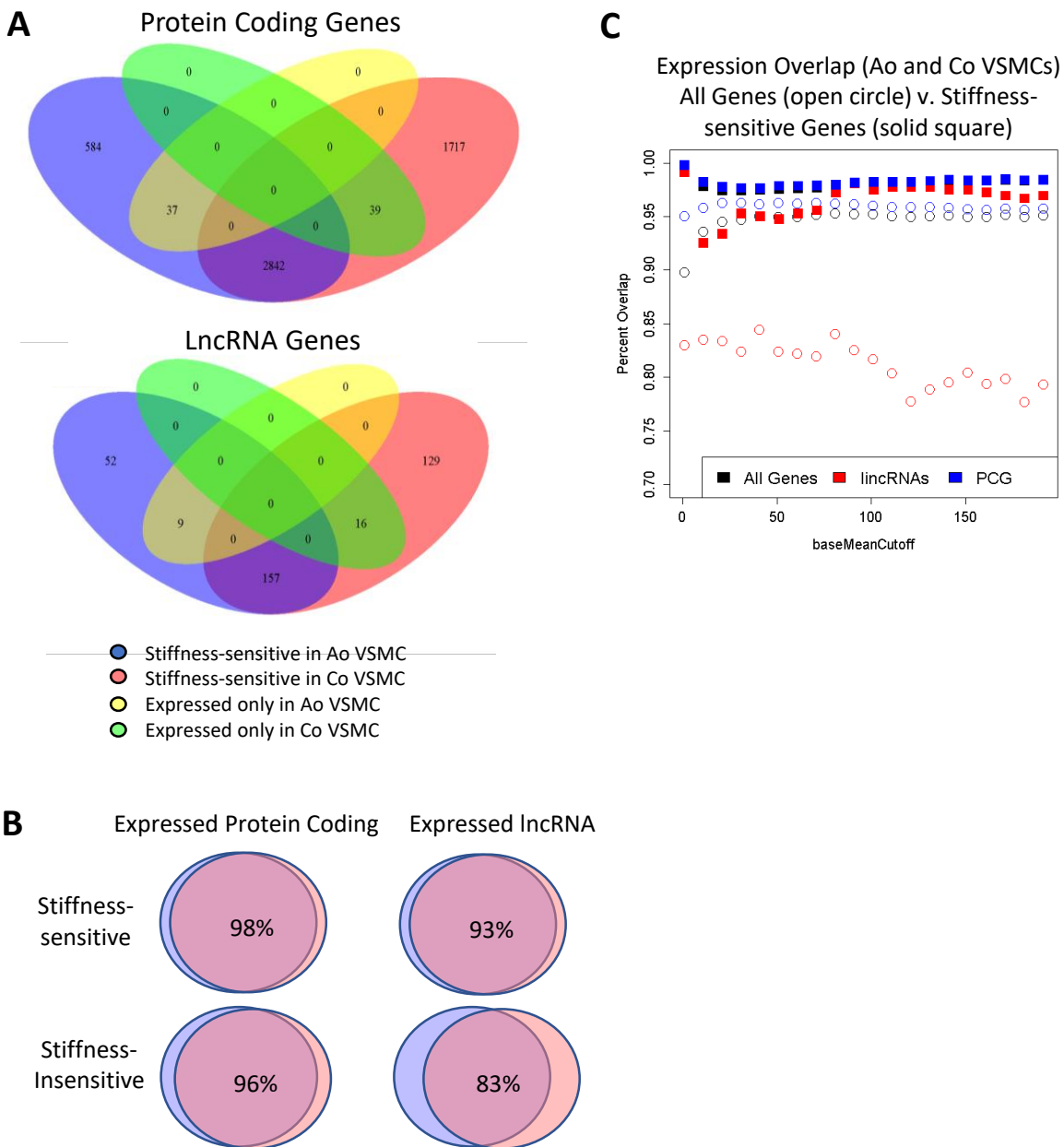
Figure II. Cell-specific expression of stiffness-sensitive genes. (A) Venn diagrams depicting the number of cell-specific stiffness-sensitive genes (by FDR<0.01) that are cell-specific-expressed (by normalized count > 8) or expressed in both Ao and Co VSMCs. (B) Overlap of expressed stiffness-sensitive genes (identified in either Ao or Co VSMCs) in Ao or Co VSMCs. 93% of ALL-SS lncRNAs (identified as stiffness-sensitive in either Ao or Co VSMCs) are expressed (normalized count > 8) in both Ao and Co VSMCs compared to 83% of stiffness-insensitive lncRNAs. More stiffness-sensitive genes are expressed in both cell types than stiffness-insensitive genes. (C) Percent overlap as in panel B but comparing stiffness-sensitive vs. all genes per subtype over various expression cut-offs (DESeq2 normalized count from 0 – 200).
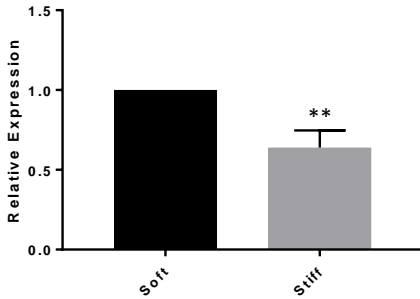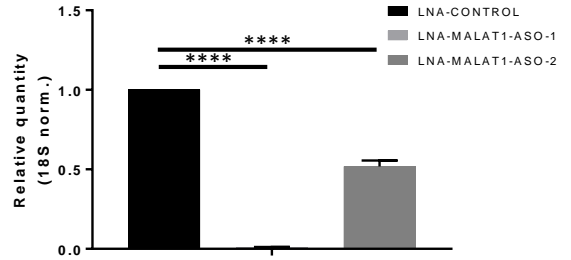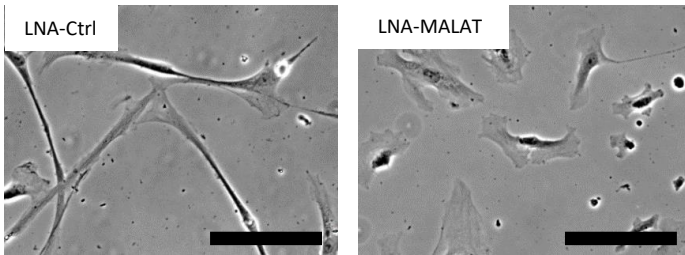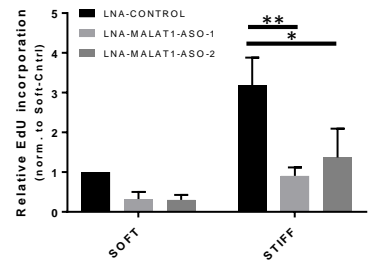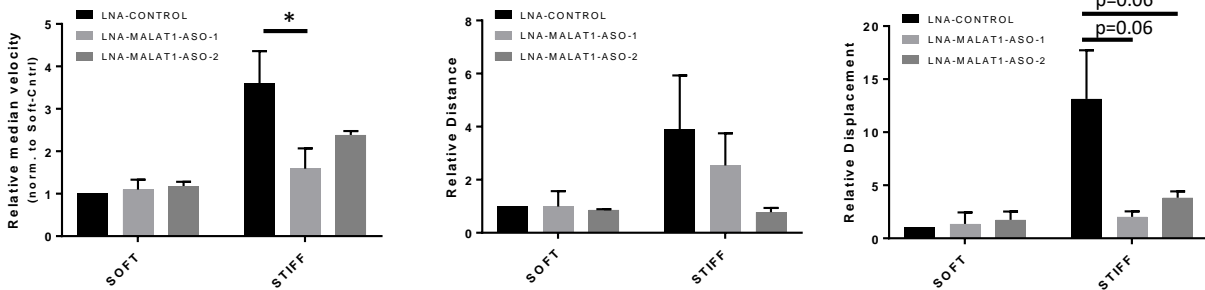
Figure III. MALAT1, a lncRNA regulator of stiffness-dependent cell responses in hTERT-immortalized Co VSMCs. (A) MALAT1 expression validation by quantitative-PCR in primary Co and Ao VSMCs (n = 7, **, p < 0.005). (B) MALAT1 knockdown using LNA-GapmeRs in hTERT-Co VSMCs. (C) Phase image of hTERT-Co VSMCs cultured on tissue culture plastic for 48 hours in SmGm-2 after MALAT1 knockdown. Scale bar = 100um. (D) hTERT-Co VSMCs cultured on soft and stiff hydrogels for 48 hours with serum containing EdU. Values are normalized to non-targeting control LNA-GapmeR on soft hydrogels (n = 5, * p<0.05; ** p<0.005). (E) Normalized median velocity, total distance (path-dependent), and total displacement (path-independent) migrated in hTERT-VSMCs over 5 hours. Values are normalized to velocity and distance in non-targeting control LNA-GapmeR on soft hydrogels. (n=2-4, * p<0.05, Two-way ANOVA; Holm-Sidak's multiple comparisons test).
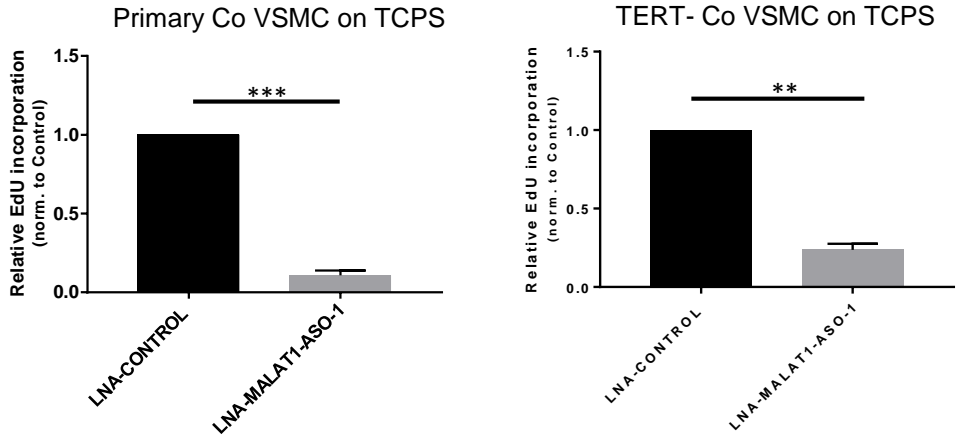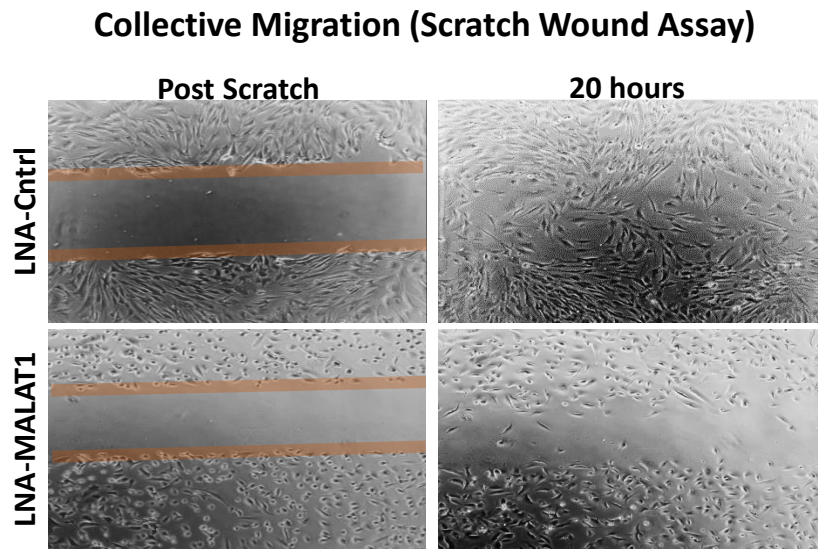
A

Primary Co VSMC on TCPS

TERT- Co VSMC on TCPS

B

Collective Migration (Scratch Wound Assay)

Figure IV. MALAT1 knockdown inhibits EdU incorporation and wound closure in Co VSMCs on tissue-culture plastic (TCPS). (A) Primary and hTERT-immortalized Co VSMCs grown on tissue culture plastic with serum containing EdU for 48 hours following LNA-GapmeR knockdown of MALAT1 (n = 4, ** p<0.005, *** p<0.0005 (B) Phase images of the closure of the scratch wound in hTERT-immortalized Co VSMCs at 20 hours post-scratch.
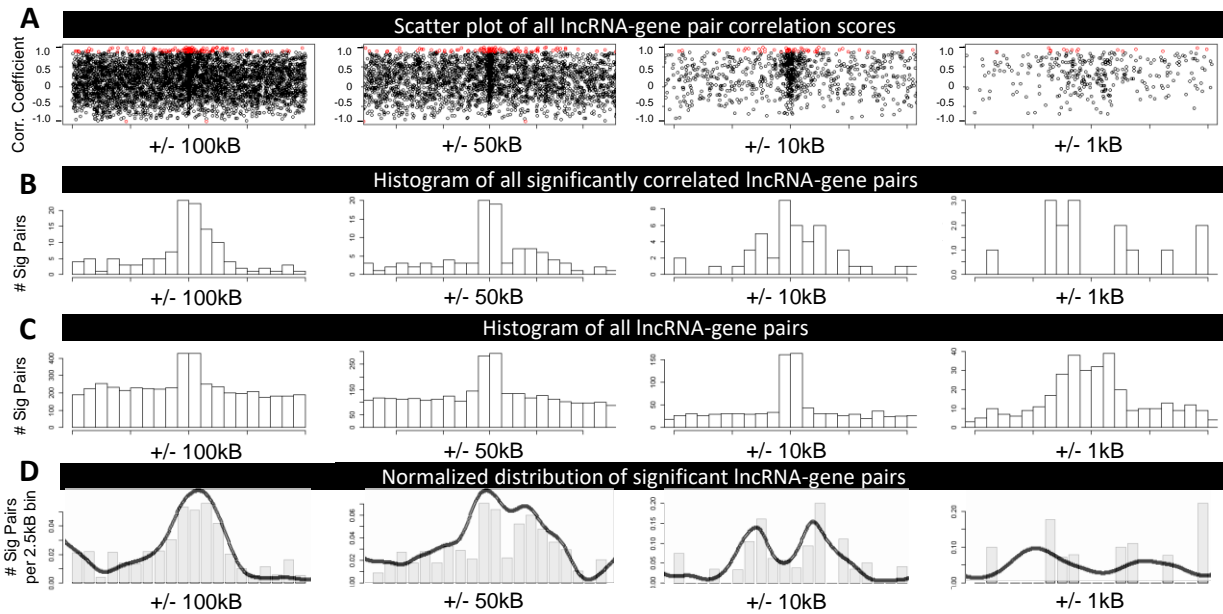
Figure V. Increased lncRNA-gene pair correlation with pair proximity. Correlation distributions of the top variant 1000 lncRNAs expressed Ao and Co VSMCs over different distances (A) Scatter plot of all lncRNA-gene pair correlation scores. Each dot position represents the distance between a lncRNA and nearby gene (x-axis) and the Spearman's correlation coefficient (y-axis). All possible lncRNA-gene pairs within the same chromosome were correlation tested and all lncRNAs are centered at the 0-position on the x-axis. Color of each dot represents significance after correction for multiple-hypothesis testing (black dots are insignificant, red dots are correlations with p < 1e-5). (B) Histogram depicting the distance distribution of significantly correlated lncRNA-gene pairs. (C) Histogram depicting the distance distribution of all correlation tested lncRNA-gene pairs. (D) Normalized distributions of significantly correlated lncRNA-gene pairs. The number of significant lncRNA-gene pairs per bin (distance from lncRNA) is normalized by the number of total lncRNA-gene pairs within that bin
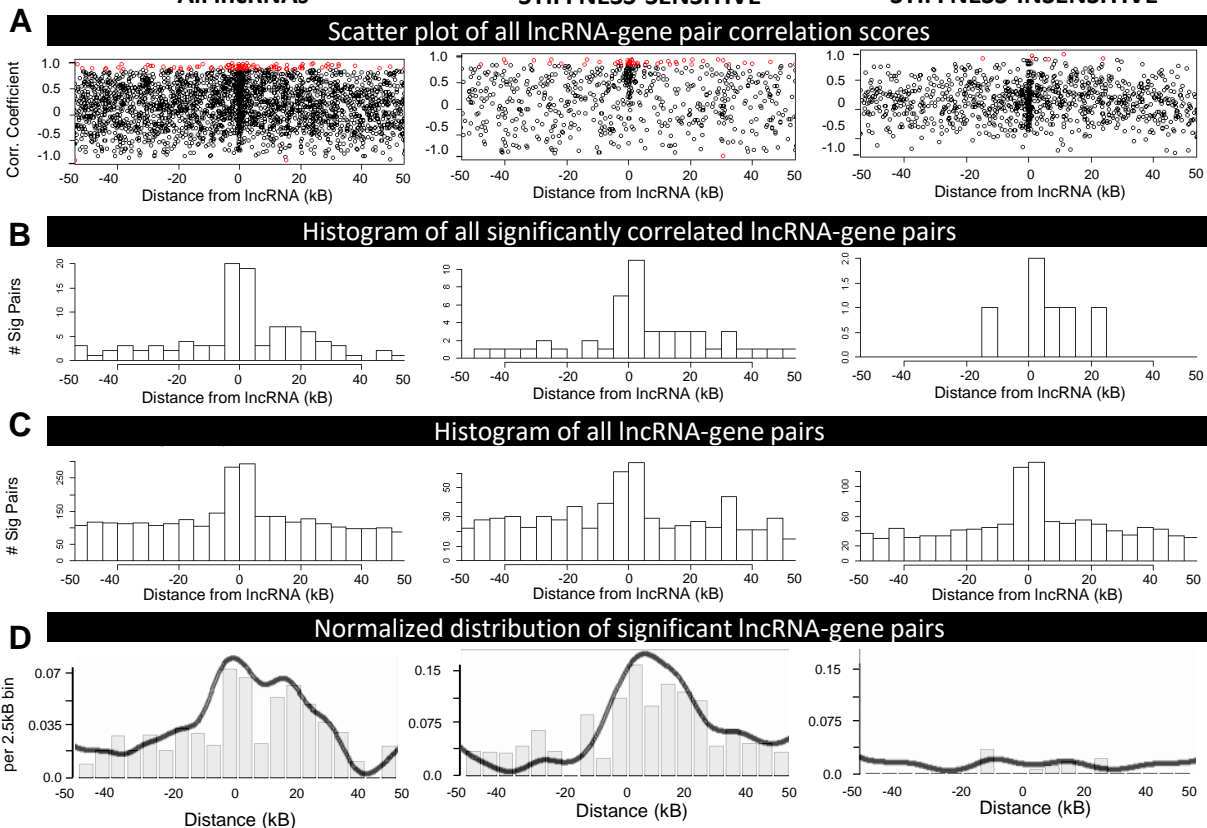
Figure VI. Increased lncRNA-gene pair correlation with pair proximity and stiffness-sensitivity. Correlation distributions of all expressed Ao and Co VSMCs lncRNAs (first column), combined Ao and Co VSMCs stiffness-sensitive lncRNAs (second column), stiffness-insensitive lncRNAs (third column). (A) Scatter plot of all lncRNA-gene pair correlation scores. Each dot position represents the distance between a lncRNA and nearby gene (x-axis, within a +/- 50kb range) and the Spearman's correlation coefficient (y-axis). All possible lncRNA-gene pairs within the same chromosome were correlation tested and all lncRNAs are centered at the 0-position on the x-axis. Color of each dot represents significance after correction for multiple-hypothesis testing (black dots are insignificant, red dots are correlations with p < 1e-5 for all lncRNAs, p < 4e-5 for stiffness-sensitive lncRNAs, p < 2e-5 for stiffness-insensitive lncRNAs). (B) Histogram depicting the distance distribution of significantly correlated lncRNA-gene pairs. (C) Histogram depicting the distance distribution of all correlation tested lncRNA-gene pairs. (D) Normalized distributions of significantly correlated lncRNA-gene pairs. The number of significant lncRNA-gene pairs per bin (distance from lncRNA) is normalized by the number of total lncRNA-gene pairs within that bin.