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ANRIL in Chromosome 9p21 may be Contributing to Human Aging and Modulates Gene Expression in Vascular Endothelial Cells

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Abstract

The chromosome 9p21 region is the strongest susceptibility locus for Cardiovascular Disease (CVD) and a hot-spot for multiple disease-associated polymorphisms. The locus encodes for a long non-coding RNA, ANRIL. The disease-associated variants in the region have been correlated with ANRIL expression, suggesting ANRIL mediates these associations.

Since most of diseases associated with the locus are common diseases of aging, we sought to investigate the relation between ANRIL expression and age. We measured ANRIL expression by RT-PCR in three groups: 70 years-old, 80 years-old and 90 years-old subjects. ANRIL expression increased dramatically between 70 to 80 years-old.

In addition, we profiled the regulatory effects of ANRIL knock-down in endothelial cells. The artificial depletion of ANRIL caused the down-regulation of an upstream regulator of senescence, IL1A, and other inflammatory genes. Conversely, it produced the up-regulation of the transcription factor KLF2, which has athero-protective properties.

Our results suggest ANRIL has a pro-atherogenic effect by inducing inflammatory and senescence-related molecules in endothelial cells. Aggravated senescence and inflammation are characteristic of aging and might be mediated by the age-related changes in ANRIL expression. ANRIL is a promising target for future therapies for many aging-related diseases.

Keywords: Aging; Atherosclerosis; ANRIL; Senescence; Endothelial dysfunction; Chromosome 9p21; Cardiovascular diseases

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Introduction

The advent of the genomic era has led to the identification of hundreds of loci affecting the susceptibility to disease. An unexpected locus at chromosome 9 (region p21) was highlighted as the strongest susceptibility locus for Cardiovascular Disease (CVD) by genome-wide association studies [1,2]. Despite being a gene desert, this genetic region is extraordinarily dense in disease-associated polymorphisms. Besides its association with CVD, the locus has been linked to a wide range of conditions such as type 2 diabetes [2,3], Alzheimer disease [4], glaucoma [5], endometriosis [6] and periodontitis [7]. Variants in this region have also been associated with several cancers: leukemia [8], breast cancer [9], basal cell carcinoma [10], melanoma [10], pancreatic carcinoma [11], ovarian cancer [12] and glioma [13].

The disease-associated region overlaps the sequence of a long non-coding RNA, ANRIL and lies in the vicinity of the tumor suppressor genes CDKN2A and CDKN2B. CDKN2A encodes for two proteins, p16 and ARF, using alternative reading frames. Since the CDKN2A/ARF/CDKN2B locus has a well-established role in cell proliferation, apoptosis and senescence [14], the connection of the locus with cancer is not surprising. However, the association of the locus with cardiovascular disease

Table 1: Complete list of genes evaluated in the profiling assay, fold change and p-value.

Gene	p-value	Fold Regulation	Gene	p-value	Fold Regulation
KLF2	2E-06	3.1969	FGA	0.05399	1.7257
ITGA2	0.00013	-1.7186	IL2	0.05399	1.7257
PPARG	0.00017	1.9032	LPA	0.05399	1.7257
IL1A	0.00019	-4.0692	IL3	0.05466	3.0928
CTGF	0.00054	-2.1715	CCL5	0.06458	-1.369
ACE	0.00062	-1.8278	ITGAX	0.06624	1.5224
LDLR	0.00078	1.6878	IFNG	0.07257	2.7909
SELE	0.00082	-2.6738	IL5	0.07765	2.6438
FAS	0.00128	2.3482	THBS4	0.09799	3.3131
PPARA	0.00147	1.567	TNFAIP3	0.13282	-1.363
SELPLG	0.00175	-2.0507	NPY	0.14054	2.4602
APOA1	0.0025	2.7863	TNC	0.14639	1.8634
CD44	0.00259	1.6146	SERPINB2	0.14719	1.4744
SOD1	0.00367	1.3894	MSR1	0.15413	1.4969
PDGFB	0.00367	-1.9307	LIF	0.15637	2.0787
FN1	0.00385	1.6705	ICAM1	0.16811	1.2213
HBEGF	0.00463	-1.4015	VWF	0.19566	1.2014
ITGA5	0.005	1.4471	CSF1	0.21024	1.1341
CCL2	0.005	-2.9783	NFKB1	0.22561	1.1555
CCR2	0.00552	2.339	COL3A1	0.22817	1.6569
NOS3	0.00584	1.6354	BCL2A1	0.25503	-1.2541
PLIN2	0.00785	1.4254	IL4	0.27902	2.1318
LAMA1	0.00925	2.2442	MMP1	0.33355	-1.0746
FABP3	0.01016	2.1412	KDR	0.37302	-1.1247
APOB	0.0103	2.2035	ELN	0.37612	2.4518
CCR1	0.01106	3.1425	ENG	0.39746	1.0614
SERPINE1	0.01239	-1.5441	VEGFA	0.41618	-1.1531
PDGFRB	0.01295	3.0706	ABCA1	0.45133	1.1222
CDH5	0.01414	1.3032	ITGB2	0.47186	1.204
IL1R1	0.0154	1.3097	TGFB1	0.58253	1.107
VCAM1	0.01562	-2.9565	TGFB2	0.58644	1.2036
BCL2L1	0.01778	1.4201	SPP1	0.589	-1.4907
BIRC3	0.0193	-2.2341	PDGFA	0.61319	1.0544
FGF2	0.0212	1.4153	IFNAR2	0.73028	1.0243
PPARD	0.03048	1.3508	PTGS1	0.84014	-1.0093
EGR1	0.03073	2.464	SELL	0.84071	1.0054
NR1H3	0.03489	1.491	RXRA	0.8483	1.0339
LPL	0.03519	1.2899	TNF	0.88009	1.0499
BID	0.03651	-1.3483	CSF2	0.9385	-1.0289
MMP3	0.04152	1.6372	CFLAR	0.94555	-1.0184
APOE	0.04329	1.8978	BCL2	0.95449	1.0112
BAX	0.04391	1.3344	IL1R2	0.97525	1.0421

and several other conditions of relatively distant etiology is certainly intriguing. Atherosclerosis progression is accompanied by changes in proliferation and apoptosis rates in the artery wall suggesting the involvement of CDKN2A/B in the 9p21 association. However, most of the CVD-associated alleles have been correlated with ANRIL expression [15,16], favoring ANRIL as the most likely mediator of this association.

ANRIL has been involved in the regulation of CDKN2A/B expression in several reports [16-18], however, distant regulatory targets have also been described [19]. The role of ANRIL in the

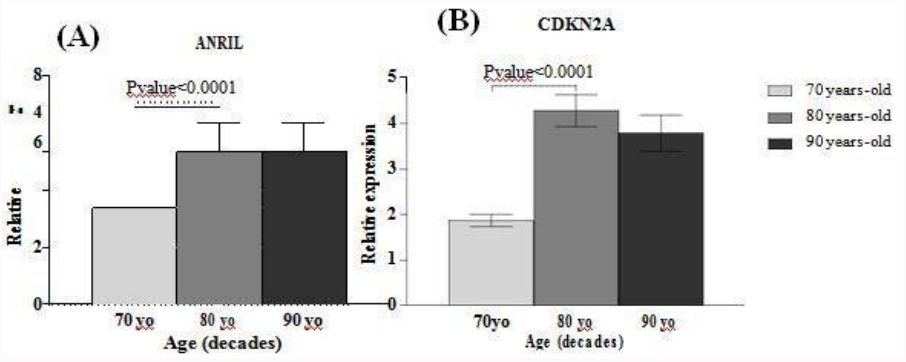
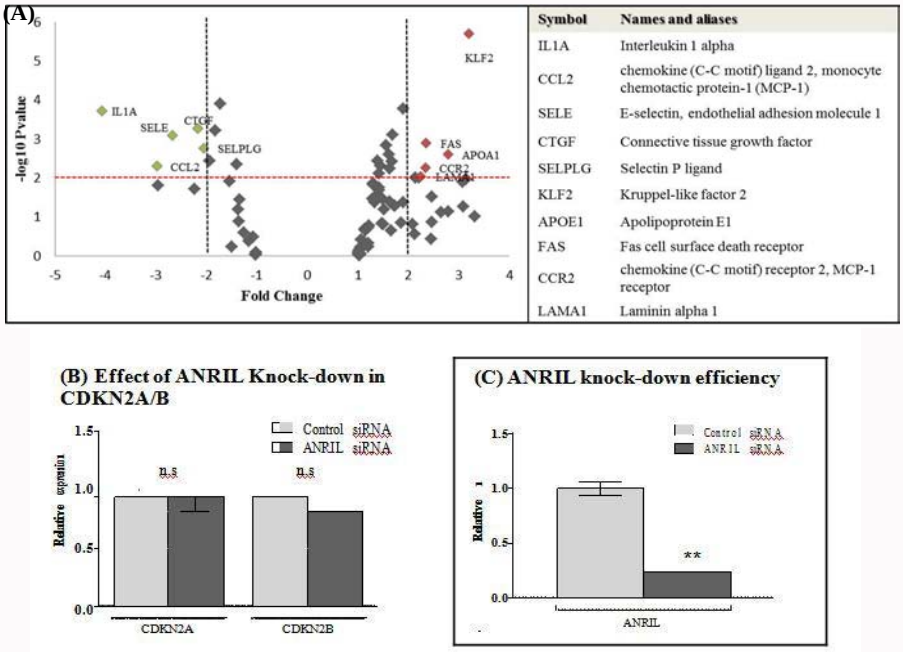


Figure 1: Relationship between ANRIL expression and aging.
To evaluate the relationship between ANRIL expression and aging, we tested 136 subjects from the SONIC study. Due to experimental design, volunteers were recruited based on their age, the 70 years-old group were subjects between 69 to 71 years old; the 80 years-old group were subjects aging between 79 to 81 years old and the 90 years-old were between 89 to 91 years old. Expression changes of ANRIL (A) and CDKN2A (B) through the age groups were detected using RT-PCR. P-values were determined by t-test and error bars indicate standard error of the mean.



Symbol	Names and aliases
IL1A	Interleukin 1 alpha
CCL2	chemokine (C-C motif) ligand 2, monocyte chemotactic protein-1 (MCP-1)
SELE	E-selectin, endothelial adhesion molecule 1
CTGF	Connective tissue growth factor
SELPLG	Selectin P ligand
KLF2	Kruppel-like factor 2
APOE1	Apolipoprotein E1
FAS	Fas cell surface death receptor
CCR2	chemokine (C-C motif) receptor 2, MCP-1 receptor
LAMA1	Laminin alpha 1

Figure 2: Most relevant ANRIL regulatory effects.
(A) The most significant regulatory effects of ANRIL knock-down detected in our profiling assay are displayed in this figure. The red dashed line represents the p-value threshold of 0.01 and both black dashed lines delimitate the 2-fold change in expression. The down-regulated genes are showed in green and up-regulated genes in red. The table presented in the right shows the list of the complete gene name and aliases of the most significant regulatory targets of ANRIL revealed by our profiling assay. (B)

regulation of histone modification is not unprecedented for long non-coding RNAs. Several long non-coding transcripts act as binding platforms for chromatin modifying complexes into their targets [20].

Here, we report the effects of ANRIL depletion in several genes critical for inflammation and endothelial senescence and the first evidence of a correlation ANRIL expression with aging.

Results

Expression of ANRIL is associated with chronological age

We investigated the relationship of ANRIL expression with aging in our 136 subjects' sample. Since previous studies have reported a robust correlation between CDKN2A, an ANRIL neighbor, with aging [21,22]; we also evaluated CDKN2A expression. ANRIL and CDKN2A expression were highly correlated (Pearson correlation coefficient, r=0.8482, p-value <0.0001) and both transcripts expression increased between the 70 to 80 years-old groups but it did not show further increase between 80 to 90 years-old groups as shown in Figure 1.

The increased expression level of CDKN2A observed in the transition from the seventh decade to the eighth is consistent with previous reports; however, none of the studies we reviewed evaluated CDKN2A expression in subjects older than 80 years old. In our sample, CDKN2A remained unaltered from the eighth to the ninth decade. We also evaluated a potential connection between ANRIL and atherosclerotic plaque. Measurements of the Intima-Media Thickness (IMT) of the Common Carotid Artery (CCA) were performed in all subjects and were used as a criterion of atherosclerosis development.

We found no significant correlation between ANRIL expression

Table 2: Blood donors sample characteristics.

Age groups	CCA Stenosis (presence/absence)	Female /Male	Age
70 years old	23/36	32/28	70.38 (± 1.01)
80 years old	26/13	22/17	79.95 (± 0.76)
90 years old	36/1	23/14	89.94 (± 0.89)

CCA: Common Carotid Artery

and CCA stenosis. However, the distribution of CCA stenosis cases was not uniform among age groups, due to a dramatic increase in incidence of CCA stenosis in older individuals. The bias caused by the differential distribution of cases and the sample size may have limited the detection of such a correlation.

Profiling of genetic effects of ANRIL

To identify the functional consequences of ANRIL silencing in endothelial cells, we used a RT-PCR-based gene expression profiling assay. We knocked-down ANRIL in HUVECs using a customized siRNA oligonucleotide and the effects of the siRNA mediated knock-down were evaluated using a RT-PCR array, which measured the expression level of 84 atherosclerosis-relevant genes in the ANRIL exon-1 targeted siRNA and the control scrambled-siRNA-treated HUVECs.

The genes deregulated as a consequence of ANRIL knock-down using a threshold of 0.01 P-value and more than a 2-fold change are presented in Figure 2, and the complete list including fold changes and p-values of all the genes studied is shown in Table 1.

The most down-regulated gene, IL1A, is a well-established marker and trigger of endothelial senescence [23]. Remarkably, SERPINE1, which is another indicator for endothelial senescence [24], was also significantly down-regulated in our RT-profiler results (Table 1) but did not pass the 0.01 p-value threshold.

Although, some of the genes have controversial effects in atherosclerosis progression, most of the down-regulated genes are pro-inflammatory, pro-atherogenic genes. IL1A is a pro-inflammatory cytokine that plays negative roles in atherosclerosis [25], diabetes and neurodegeration [27]. Vascular adhesion molecules, CCL2, SELE and VCAM1 were down-regulated (P-value<0.05, see Table 1), they participate in the recruitment of leukocytes and contribute to the development of the atheroscle [26] rotic plaque [28,29]. CTGF has a complex role in endothelial function, but evidence suggests it enhances infiltration of inflammatory cells and promotes atherogenesis [30]. Conversely, the two most up-regulated genes (KLF2 and APOA1) have been reported to have vasoprotective properties [31-32].

Since ANRIL is a putative regulator of the CDKN2A/B locus, we tested the samples used in the profiling assay for CDKN2A/B expression. We did not detect any regulatory effect of ANRIL upon CDKN2A/B transcripts in these samples, indicating that the expression changes were independent from CDKN2A/B and produced by ANRIL trans effects.

Senescence markers are also down-regulated by ANRIL in aortic endothelial cells

We considered aortic endothelial cells are a model that reflects the context of atherosclerosis development better than HUVECs. Based on that, we sought to determine if the effect of ANRIL upon endothelial senescence markers was also observed in Aortic Endothelial Cells (AoECs).

We examined the expression of IL1A and SERPINE1 which are

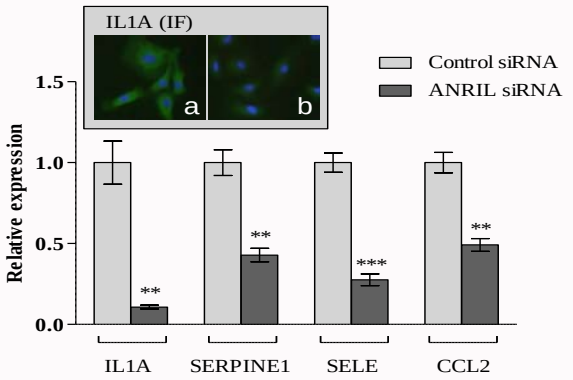


Figure 3: ANRIL regulatory effects in Aortic Endothelial Cells (AoEC). siRNA inhibiting ANRIL in AoEC cells were assayed for endothelial senescence markers, IL1A and SERPINE1 expression and inflammatory genes SELE and CCL2 expression using RT-PCR. RNA extraction was performed 48h post-transfection. Expression level of each transcript is expressed in relation to the control siRNA treated cells (arbitrarily set at 1.0) and error bars indicate standard error of the mean. IL1A protein was visualized in-situ using immuno-fluorescence, green fluorescence corresponds to IL1A and nuclei are visualized in blue using DAPI staining. (a) Visualization of IL1A protein in cells treated with the scrambled siRNA control. (b) Visualization of IL1A protein cells treated with the ANRIL targeted siRNA which showed almost undetectable levels of IL1A protein.

bona fide biomarkers of endothelial senescence. Two of the top pro-inflammatory genes, CCL2 and SELE, detected in our profiling assay were also strongly down-regulated in AoEC. CCL2 (also known as monocyte chemotactic protein 1) and SELE (E-selectin) have potent pro-inflammatory properties.

Experimental Procedure

Study sample

For the RNA expression analysis, we obtained whole blood samples from 136 volunteers from Asago City, Hyogo Prefecture, Japan (sample characteristics in Table 2) who were participants in "SONIC" (Septuagenarians, Octogenarians, Nonagenarians Investigation with Centenarians) Study. The 70 years-old group were 60 subjects between 69 to 72 years old; the 80 years-old were 39 subjects aging between 79 to 81 years old and the 90 years-old were 37 individuals between 89 to 92 years old. The available information about the subjects is presented in Table 2. The intention of this experimental design in the recruitment of the subjects was to make apparent the expression changes through decades.

Carotid ultrasonography using GE LOGIQ Book X-P was performed to measure the Intima-Media Thickness (IMT) of the Common Carotid Artery (CCA) for the evaluation of human atherosclerosis in the study subjects. We defined the criteria for CCA atherosclerosis as: maximal IMT ≥1.1 mm [33].

All participants gave written informed consent to participate in the genetic analyses and in all other procedures associated with the study. The ethics committee of Osaka University approved the study protocol.

Cell culture

For the profiling assay human umbilical endothelial cells, passage 6 (n=3), were incubated for 48 hours with ANRIL targeted siRNA. The confirmatory tests for ANRIL expression, CDKN2A and CDKN2B were performed using same cells, conditions and passage number than the RT-profiler assay. For the confirmation of the effects

detected in the profiling assay we used human aortic endothelial cells incubated during the same time and conditions. Both human umbilical and aortic endothelial cells were grown in the defined EGM-2 (Lonza) containing 2% FBS and growth factor supplements.

RT-PCR based profiling assay

RT-PCR array for atherosclerosis related pathways was performed using RT2-Profiler PCR Array (Human Atherosclerosis, PAHS-038Z) from SABiosciences. The PCR array uses a SYBR Green-based real-time PCR for multiple gene profiling. Ninety-six-well plates containing gene-specific primer sets for 84 relevant human atherosclerosis-related genes and 5 housekeeping genes were used. Treatment and control samples, in quadruplicates, were evaluated using the profiling array. Thermal cycling was carried out following the manufacturer's instruction using ABI Prism 7900HT. Gene expression was normalized to internal controls to determine the fold change in gene expression between test and control samples by ΔΔCT method using the software provided by the manufacturer.

siRNA-mediated ANRIL knock-down

A custom silencer select siRNA® (Ambion) was designed to target ANRIL in its exon 1 (siRNA(5'->3'): GAAUGUCAGUUUUGA ACUAtt). A scrambled siRNA control (universal negative control siRNA #1 Ambion) was used as a control for all the experiments. Lipofectamine RNAimax was purchased from Invitrogen and used as a transfection reagent according to the manufacturer's instructions. Knock-down efficiency of ANRIL was measured by RT-PCR. The cells were incubated for 48 h before RNA extraction for profiling assay and RT-PCR.

RT-PCR

ANRIL transcript expression was measured using a made-to-order ANRIL expression assay (Hs04259476_m1) covering exons 1-5. RT-PCR for the expression quantification of IL1A, CCL-2, SELE, SERPINE1, CDKN2A and CDKN2B was conducted using commercial Taqman expression assays and ABI Prism 7900HT. All experiments were performed in triplicates, excepting the RT-PCR based profiling assay which was performed in quadruplicates. Standard curve method was used for the quantification and 18S was chosen as housekeeping gene for the normalization.

RNA extraction

For the functional analysis using HUVEC and AoEC RNA extraction was performed using RNeasy kit (Qiagen).

For the patient blood expression analysis, we performed RNA extraction using paxgene blood RNA kit (Qiagen). We used a pool of 5 subjects RNA to prepare the standard curve. These subjects were chosen due to higher RNA concentration after RNA extraction.

Immunofluorescence

AoEC cells were either treated with control siRNA or ANRIL siRNA for 48 hr before fixation with 4% paraformaldehyde. Cells were permeabilized using 0.2% Nonidet P-40 (Sigma-Aldrich) in 5% skim milk and 50nm HEPES for 30 minutes and incubated with anti-IL1A (ABCAM, ab7632) at a 1/100 dilution for 1 hour at room temperature. The secondary antibody was Alexa-Fluor 488 conjugated anti-rabbit IgG used at a 1/1000 dilution.

Statistical analysis

Expression association results were analyzed using JMP10 (SAS) and Prism 5 for Windows (version 5.02, GraphPad Software Inc.). To evaluate the effect of age in ANRIL and CDKN2A expression, the results were analyzed using analysis of variance (ANOVA) and the t-test. P-values lower than 0.05 were considered significant. To remove the effect of age we used a nominal logistic fit model, and data from multiple age groups were analyzed using Chi-square likelihood ratio test. To analyze the results of the profiling assay we used the software provided by the assay manufacturer.

Discussion

The chromosome 9p21 association with cardiovascular disease is not only the strongest and more consistent association detected for CVD, but it is also particularly interesting because it is independent from traditional cardiovascular risk factors. The mechanism underlying the association seems to involve a dimension of the disease previously unknown, and most importantly, not targeted by current treatments.

Evidence suggests that the CVD-risk alleles influence ANRIL expression and the regulation of ANRIL could be behind the 9p21 association. We were not able to establish an association between ANRIL expression and the presence of atherosclerotic plaque in our sample, possibly due to the limited size of the sample. However, the effect of age in ANRIL expression was a significant and novel finding, particularly interesting because the diseases associated with the locus are associated with aging.

We found a strong increase in ANRIL expression between the 70 and the 80-years-old group. Under the assumption that ANRIL promotes inflammation and is associated with aging, the lack of further increase between 80 to 90-years-old groups might be explained by an earlier death of the subjects expressing the higher levels of ANRIL, from diseases related with aging (average life-span in Japan is 80 and 86 in males and females respectively). In addition, the increased incidence of cancer in advance age could distort CDKN2A and ANRIL expression pattern in this age group.

Our previous work and others highlighted the subsequent ANRIL-mediated regulation of CDKN2A/B as a possible underlying mechanism of atherogenesis [16-18]. However, the findings presented here, support an important role of trans effects of ANRIL in mediating atherosclerosis and other conditions. A recent study has identified ANRIL trans-acting regulatory functions and targets, using a human cell line [19]. However, ANRIL functions in endothelial cells, a cell type directly involved in atherosclerosis pathogenesis, have remained unexplored.

The strongest regulatory effect of ANRIL silencing detected in our study was the repression of Interleukin 1α (IL1A). IL1A is a pro-inflammatory cytokine that induces senescence and its depletion extends the lifespan of endothelial cells [34]. Besides its contribution to vascular aging, it also has a detrimental effect in insulin secretion and induces the loss of beta-cells that leads to type 2 diabetes [26]. IL1A has also been linked to several conditions associated with this locus such as glaucoma [35] and Alzheimers disease [27]. On the other hand, the most up-regulated gene, KLF2 is a transcription factor that contributes to a vasoprotective phenotype in endothelial cells [31].

We previously profiled the effects of ANRIL depletion in VSMCs, observing a pattern of gene regulation very different from the one presented in this study [36]. Probably, ANRIL has distinct tissue-specific actions, highlighting the need of studying its function in the different cell types involved in chr9p21-associated diseases.

Healthy endothelial cells play an important vasculo-protective role in the vessel wall. Age-associated changes in the vascular endothelium characterized by the expression of inflammatory cytokines and adhesion molecules are known to promote atherosclerosis development [37,38]. Our results suggest that age-related changes of ANRIL expression may contribute to inflammation and early-aging of the vascular endothelium.

ANRIL is a putative repressor of the CDKN2A/B locus in fibroblasts and VSMC [16,17,39]. These genes, particularly CDKN2A, are known to mediate senescence, suggesting an anti-senescence role of ANRIL. However, our findings altogether speak against this model in endothelial cells. As a consequence of ANRIL silencing, the profiling assay depict a cascade of anti-inflammatory and anti-senescence regulatory effects. IL1A is an up-stream senescence trigger. This cytokine also activates expression of CCL2 [40] which is consistent with our finding of both genes being down-regulated in the ANRIL-depleted cells. KLF2 transcriptionally induces endothelial Nitric Oxide Synthase (NOS3) expression and inhibits endothelial adhesion molecule E-selectin (SELE) and vascular cell adhesion molecule-1 (VCAM1) [41]. Interestingly, NOS3 is up-regulated and both VCAM1 and SELE are strongly repressed by ANRIL knock-down (Table 1).

The main limitation of the approach we used to evaluate ANRIL effects is that the profiler assay measures the expression of only 84 pre-selected genes. Our method gave us a glance of the regulatory effects of ANRIL in endothelial cells using well-established markers of atherosclerosis; however it does not allow the identification of bona fide ANRIL targets.

The locus has been linked to a wide set of disorders of aging, suggesting an interconnection with aging basic mechanisms. Moreover, aging is considered the major risk factor for atherosclerosis and other conditions linked to the 9p21 locus. Age-related up-regulation of ANRIL and our functional studies, suggest ANRIL mediates some cellular changes occurring during aging such as inflammation and exacerbated senescence.

Further study will be required to clarify the mechanism causing the correlation of ANRIL and aging reported here, and to determine if this expression pattern in peripheral blood cells is detected in other tissues. The elucidation of ANRIL effects in different cell types is necessary to have a complete view of the role of this transcript in atherosclerosis and the other age-related diseases associated with the locus. However, our results support a pro-inflammatory and pro-senescence role of ANRIL in endothelial cells.

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