RNA BIOLOGY
2020, VOL. 17, NO. 10, 1391–1401
https://doi.org/10.1080/15476286.2020.1771519

Taylor & Francis
Taylor & Francis Group

RESEARCH PAPER

Splice variants of lncRNA RNA ANRIL exert opposing effects on endothelial cell activities associated with coronary artery disease

Hyosuk Cho [a,b,c], Yabo Li [b,c], Stephen Archacki[b,c], Fan Wang [b,c], Gang Yu [b,c,d], Susmita Chakrabarti [b,c], Yang Guo [b,c], Qiuyun Chen[b,c], and Qing Kenneth Wang [a,b,c]

[a]Department of Genetics and Genome Sciences, Case Western Reserve University School of Medicine, Cleveland, OH, USA; [b]Department of Cardiovascular and Metabolic Sciences, Lerner Research Institute, Cleveland Clinic, Cleveland, OH, USA; [c]Department of Molecular Medicine, Cleveland Clinic Lerner College of Medicine of Case Western Reserve University, Cleveland, OH, USA; [d]Key Laboratory of Molecular Biophysics of the Ministry of Education, College of Life Science and Technology and Center for Human Genome Research, Huazhong University of Science and Technology, Wuhan, P. R. China

ABSTRACT

Each gene typically has multiple alternatively spliced transcripts. Different transcripts are assumed to play a similar biological role; however, some transcripts may simply lose their function due to loss of important functional domains. Here, we show that two different transcripts of lncRNA gene *ANRIL* associated with coronary artery disease (CAD) play antagonizing roles against each other. We previously reported that *DQ485454*, the short transcript, is downregulated in coronary arteries from CAD patients, and reduces monocyte adhesion to endothelial cells (ECs) and transendothelial monocyte migration (TEM). Interestingly, the longest transcript *NR_003529* is significantly upregulated in coronary arteries from CAD patients. Overexpression of *ANRIL* transcript *NR_003529* increases monocyte adhesion to ECs and TEM, whereas knockdown of *NR_003529* expression reduces monocyte adhesion to ECs and TEM. Much more dramatic effects were observed for the combination of overexpression of *NR_003529* and knockdown of *DQ485454* or the combination of knockdown of *NR_003529* and overexpression of *DQ485454*. The antagonizing effects of *ANRIL* transcripts *NR_003529* and *DQ485454* were associated with their opposite effects on expression of downstream target genes *EZR, CXCL11* or *TMEM106B*. Our results demonstrate that different transcripts of lncRNA can exert antagonizing effects on biological functions, thereby providing important insights into the biology of lncRNA. The data further support the hypothesis that *ANRIL* is the causative gene at the 9p21 CAD susceptibility locus.

ARTICLE HISTORY
Received 17 February 2020
Revised 9 April 2020
Accepted 29 April 2020

KEYWORDS
lncRNA; *ANRIL* (*CDKN2B-AS1*); *EZR*; *TMEM106B*; coronary artery disease (CAD) and myocardial infarction (MI); monocyte adhesion to endothelial cells; transendothelial migration of monocytes

Introduction

Coronary artery disease (CAD) and its complication myocardial infarction (MI) are the most common heart disease that causes 400,000 deaths each year in the US alone [1]. CAD is caused by accumulation or build-up of cholesterol and fatty lipids, leading to the development of plaques in the coronary arteries, a process referred to as atherosclerosis [2–4]. Atherosclerosis is initiated by monocyte adhesion to the endothelium of coronary arteries upon inflammatory stimulation or endothelial injury, followed by the transmigration of monocytes across the endothelial layer into the intima [2–4]. The monocytes then differentiate into macrophages, which further develop into lipid-laden foam cells known as a fatty streak, the hallmark of atherosclerosis [2–4]. For largely unknown reasons, some arterial beds are more prone to atherosclerosis than others, and coronary arteries are most often involved [5]. Moreover, genetic factors play a critical role in the development of atherosclerotic CAD. Genome-wide association studies (GWAS) have identified genomic variants at more than 150 loci that confer risk of CAD, including the chromosome 9p21.3 locus that contain a long noncoding RNA gene *ANRIL* [6].

Noncoding RNAs (ncRNAs) are a class of short and long transcripts that act as genetic, epigenetic and translational regulators for multiple biological processes and the development of some human diseases [7]. Examples of ncRNAs include microRNAs (miRNAs), circular RNAs, and long noncoding RNAs (lncRNAs) that are more than 200 base pairs in length [8]. Although the function of most lncRNAs remains unknown, some of these molecules are also implicated in various biological processes [9–11]. LncRNAs can regulate gene expression through a variety of mechanisms, such as epigenetic modifications of DNA, alternative splicing, post-transcriptional regulation, mRNA stability and translation [9–11]. Some lncRNAs are involved in *cis*-inactivation of larger genomic regions by epigenetic mechanisms [9–11]. Notably, many lncRNAs are known to regulate the expression of genes by a *trans* mechanism [11]. LncRNAs can act as scaffolds to bringing together multiple proteins to form ribonucleoprotein complexes [12]. In some cases, lncRNAs can function as molecular 'decoys' for transcription factors, other regulatory proteins, and miRNAs [11]. Interestingly, lncRNAs can positively or negatively control the expression of their target genes. For example, we recently showed that lncRNA *ANRIL* can positively regulate the expression of *ANHAK1, CLIP1, CXCL11, ENC1, EZR, LYVE1* and *WASL*, but negatively

CONTACT Qing Kenneth Wang wangq2@ccf.org; Qiuyun Chen chenq3@ccf.org Department of Cardiovascular and Metabolic Sciences, Lerner Research Institute, Cleveland Clinic, Cleveland, OH 44195, USA

regulates expression of *TMEM100* and *TMEM106B* [13], although the underlying molecular mechanism(s) are unknown.

Recent studies suggest that lncRNAs are involved in development of atherosclerosis and CAD. Knockout (KO) of lncRNA *MeXis* gene impaired cholesterol metabolism and accelerated atherosclerosis in mice via an LXR-MeXis-Abca1 axis [14]. For lncRNA *MALAT1*, heterozygous *Malat1*$^{\pm}$ KO mice showed increased atherosclerosis with an *ApoE*$^{-/-}$ KO background due to massive immune system dysregulation [15]. The lncRNA *ANRIL* gene is located within the most robust GWAS locus for CAD on chromosome 9p21.3 locus [16,17]. Many variants in *ANRIL* show significant association with risk of CAD [18]. Previously, we identified the most abundant transcript of *ANRIL* in ECs as *DQ485454*, and showed that *DQ485454* was significantly downregulated in coronary arteries from CAD patients compared with normal arteries [13]. *DQ485454* overexpression significantly reduced monocyte adhesion to endothelial cells (ECs) and transendothelial migration of monocytes (TEM), two critical cellular processes involved in the initiation of CAD [13]. The siRNA-mediated knockdown of *ANRIL* expression showed the opposite effects [13]. Mechanistic studies showed that overexpression of *ANRIL* downstream target genes *CLIP1, EZR*, and *LYVE1* reversed the effects of *ANRIL* KD on monocyte adhesion to ECs and TEM. These data suggest that *ANRIL* regulates EC functions directly related to CAD, and support the hypothesis that *ANRIL* is the causal gene for CAD at the 9p21 genetic locus. Moreover, in addition to ECs, *ANRIL* was also shown to regulate proliferation of vascular smooth muscle cells, a property also relevant to the development of atherosclerosis and CAD [18].

Despite the fact that accumulating evidence implicates *ANRIL* in development of CAD, much more work is needed to make a stronger case. Moreover, *ANRIL* shows extensive alternative splicing, which results in more than 20 different transcripts. The function of the longest *ANRIL* transcript, *NR_003529*, remains to be further defined. Therefore, in this study, we characterized the expression levels of *NR_003529* in CAD vs. non-CAD coronary arteries, and examined the functional effects of overexpression or knockdown of *NR_003529* on EC functions involved in the pathogenesis of CAD. Moreover, we investigated the interaction of *NR_003529* with *DQ485454* in EC functions. Identifying functions of different transcripts of *ANRIL* may be a key to identify new lncRNA-based molecular mechanisms contributing to the development of CAD.

Results

Upregulation of the longest transcript of *ANRIL, NR_003529,* in coronary arteries from CAD patients

We recently reported that *ANRIL* transcript *DQ485454* was significantly downregulated in CAD coronary artery tissue samples [13]. However, it is unknown whether the expression level of the full-length or the longest transcript *NR_003529* is also changed in CAD coronary artery tissue. We investigated the transcript-specific expression of the reported major transcripts of *ANRIL, NR_003529, DQ485454*, and circ*ANRIL*7-5, with the tissue samples from CAD patients vs. non-CAD controls (Fig. 1A). Using quantitative RT-PCR (qRT-PCR) analysis, we found that in contrast to *DQ485454*, the expression level of *ANRIL* transcript *NR_003529* (detected using primers in exons 12 and 13b) was significantly upregulated by 2.5-fold in CAD patients compared to that of the non-CAD controls (Fig. 1B). *ANRIL* has more than 20 different transcripts, and it was technically challenging to design unique primers for individual transcripts and examine their expression levels using qRT-PCR analysis. However, we found that many transcripts share exons 5–7 and our qRT-PCR analysis revealed that the overall expression level across all transcripts of *ANRIL* sharing exons 5–7 was significantly increased in CAD coronary artery tissue compared to non-CAD tissue (Fig. 1C). Interestingly, we also found that the expression level of circ*ANRIL* containing exons 7–5 was significantly downregulated in CAD coronary artery tissue compared to the healthy controls (Fig. 1D). Similarly, the expression level of *DQ485454* (detected using primers in exons 11–13) was significantly downregulated in CAD coronary arteries as previously reported [13] (Fig. 1E). Quantitative RT-PCR analysis also revealed that the expression level of transcripts containing exon 19 (Fig. 1F) was significantly increased in CAD coronary artery tissue compared to non-CAD tissue. These data suggest that *ANRIL* transcripts show differential expression patterns in CAD coronary arteries. Specifically, *ANRIL* transcripts *NR_003529* and *DQ485454* show significant upregulation and downregulation in coronary artery tissue samples from CAD patients, respectively.

Overexpression of *ANRIL* transcript *NR_003529* in endothelial cells increases monocyte adhesion and transmigration

Based on the finding of significant upregulation of *NR_003529* transcript in CAD tissue, we hypothesized that *NR_003529* is involved in the development of CAD. To test the hypothesis, we overexpressed *NR_003529* transcript in different ECs, including human coronary artery ECs (HCAECs), human umbilical vein ECs (HUVECs), and an EC cell line, EA.hy926 cells, by transient transfection with a *NR_003529* expression plasmid. Our qRT-PCR analysis showed that the expression level of *NR_003529* was increased by 13–17 fold depending on the type of ECs (Fig. 2A). Moreover, our qRT-PCR analysis showed that the overexpression was specific to *NR_003529* and did not affect the expression of transcript *DQ485454* (Fig. 2B). The transfected ECs were then used for assays for monocyte adhesion to ECs and transendothelial migration of monocytes, two key processes involved in the initiation of atherosclerosis during the pathogenesis of CAD [5,19,20]. We used THP-1 cells as monocytes in these assays. As shown in Fig. 2C, specific overexpression of *NR_003529* in HCAECs and EA.hy926 cells, but not HUVECs, significantly increased monocyte adhesion to ECs (Fig. 2C) and transendothelial migration of monocytes (Fig. 2D). The data suggest that overexpression of *ANRIL* transcript *NR_003529* confers a risk role in some ECs in development of CAD.

Knockdown of *NR_003529* expression in endothelial cells reduces monocyte adhesion and transmigration.

To validate the finding of *NR_003529* overexpression on EC functions involved in CAD, we performed similar functional assays but using ECs with siRNA knockdown of *NR_003529*

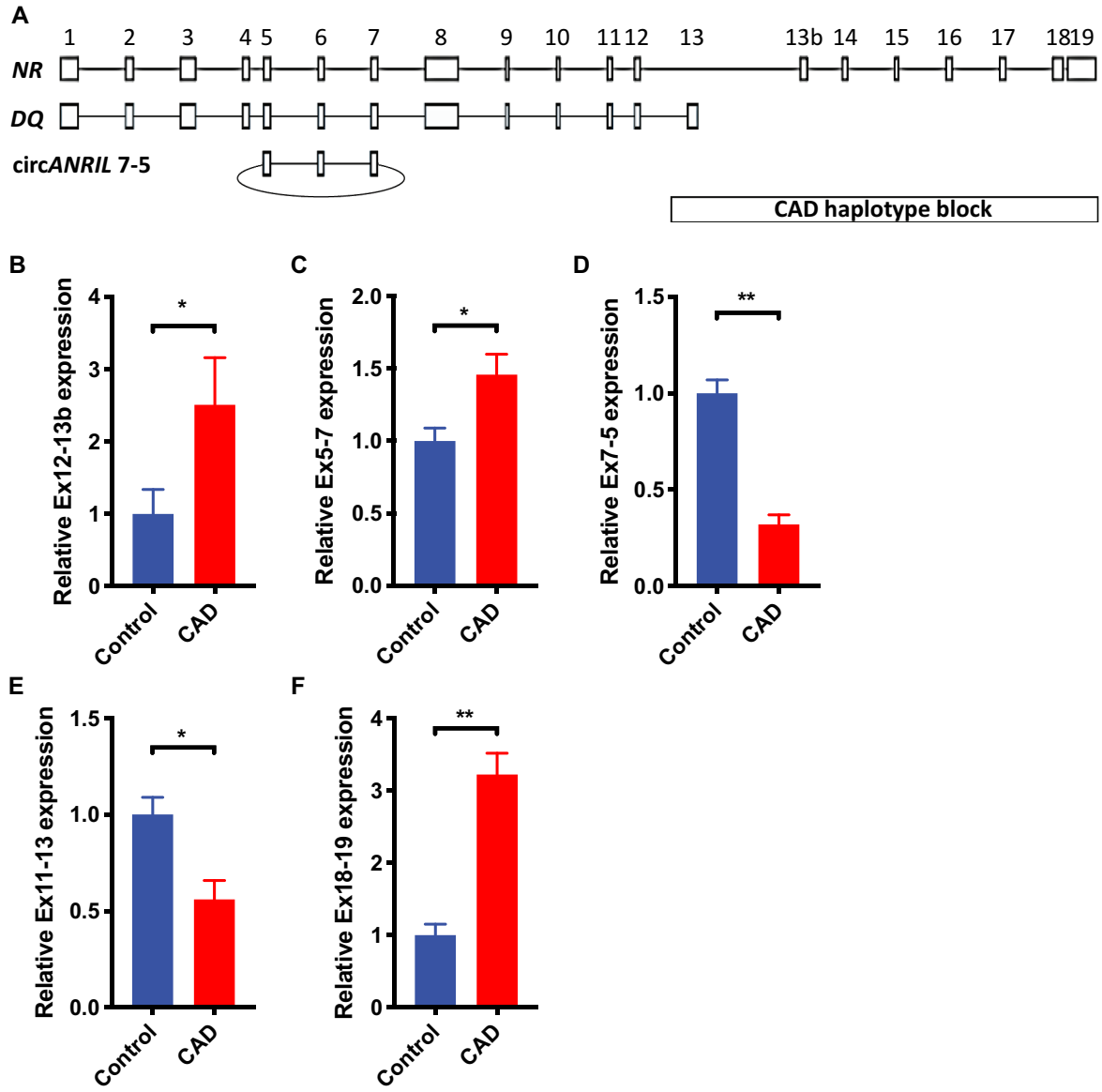

Figure 1. Increased expression of the longest *ANRIL* transcript *NR_003529* (NR) in coronary arteries from CAD patients vs. non-CAD controls. Coronary arteries from 6 CAD patients and 6 age-matched non-CAD individuals were studied with real-time RT-PCR analysis. (A) Schematic diagram for the structure of *ANRIL* transcripts *NR_003529* and *DQ485454*, and *circANRIL* with exons 5–7. The location of the CAD haplotype block is shown. (B-E) The relative abundance of different transcripts of *ANRIL* in CAD patients vs. non-CAD controls as represented by expression of (B) exons 12–13b for the detection of *NR_003529*, (C) exons 5–7 for the detection of both *NR_003529* and *DQ485454*, (D) exons 7–5 for the detection of circ*ANRIL7-5*, and (E) exons 11–13 for the detection of *DQ485454*. (F) exons 18–19 for the detection of 3′ end of *NR_003529* $*p < 0.05$ and $**p < 0.01$ vs. vector, $N = 6$.

expression. Our qRT-PCR analysis showed that transient transfection of *NR_003529*-specific siRNA reduced its expression by 75–80% in HCAECs, HUVECs, and EA.hy926 cells (Fig. 3A). We also ensured that the *NR_003529* knockdown did not affect the expression level of *ANRIL* transcript *DQ485454* (Fig. 3B). In the assays for THP-1 monocyte adhesion to ECs and transendothelial migration of monocytes, we found that knockdown of *NR_003529* expression in HCAECs, HUVECs, and EA.hy926 cells significantly reduced the function of ECs in monocyte adhesion (Fig. 3C) and transmigration (Fig. 3D). These data further show that *ANRIL* transcript *NR_003529* plays an important role in EC functions involved in the pathogenesis of CAD.

Combination of *NR_003529* overexpression and *DQ485454* knockdown in HCAECs dramatically increases monocyte adhesion and transmigration.

In human coronary artery tissue samples from CAD patients, we showed that the *NR_003529* transcript of *ANRIL* was significantly upregulated (Fig. 1A), whereas the expression level of the *DQ485454* transcript was significantly downregulated [13]. Therefore, we were interested in determining the effect of *NR_003529* overexpression and *DQ485454* knockdown on EC functions relevant to CAD. We co-transfected HCAECs with an expression plasmid for *NR_003529* and two different siRNAs. Our qRT-PCR analysis showed that the co-transfection led to the

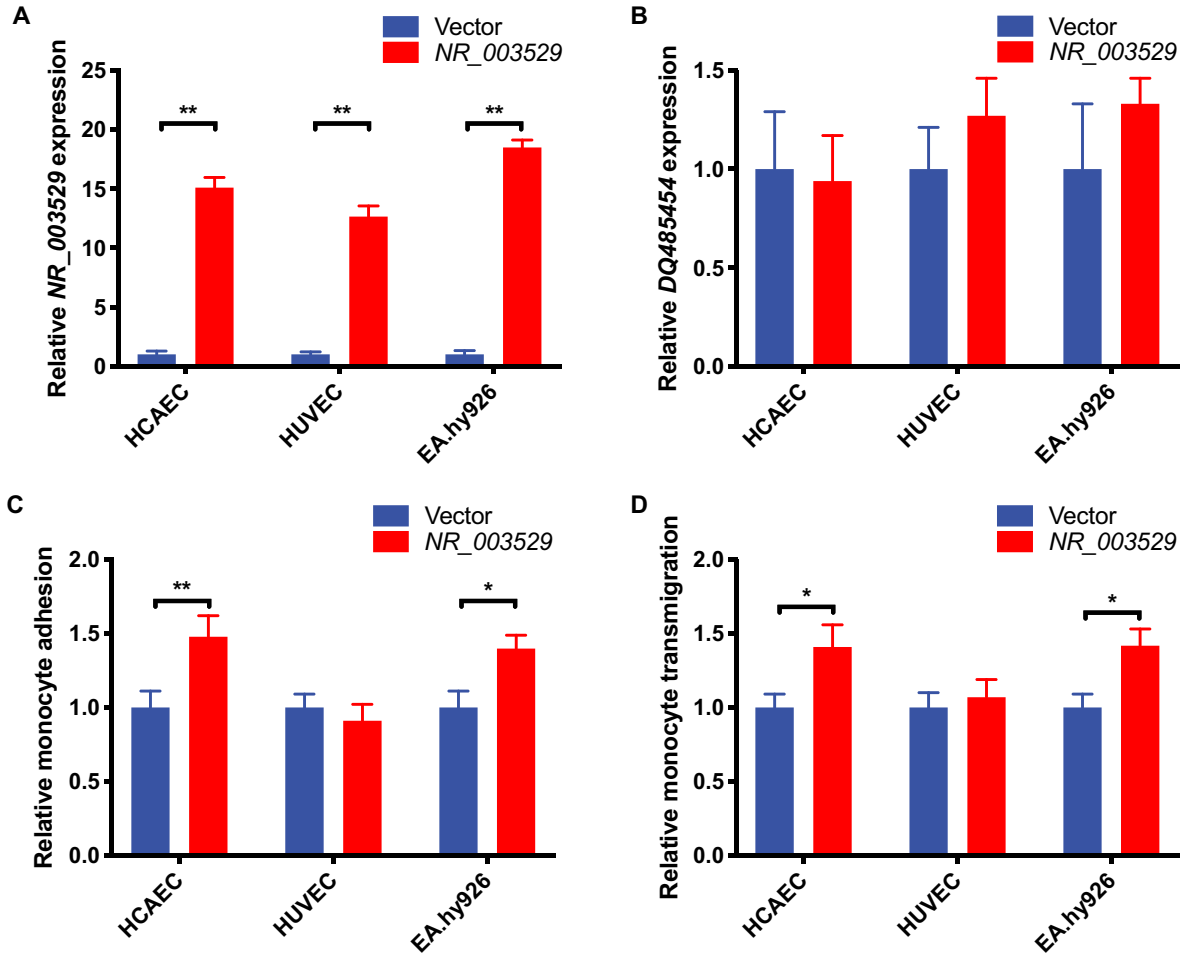

Figure 2. Overexpression of *NR_003529* in HCAEC and EA.hy926 cells increases monocyte adhesion and transendothelial migration. (A) Expression of the *NR_003529* transcript of *ANRIL* in HCAECs, HUVECs, and EA.hy926 cells transiently transfected with an expression plasmid for *NR_003529* vs. pcDNA vector as quantified by qRT-PCR. (B) Expression of the *DQ485454* transcript of *ANRIL* in HCAECs, HUVECs, and EA.hy926 cells transiently transfected with an expression plasmid for *NR_003529* vs. pcDNA vector as quantified by qRT-PCR. (C) Relative adhesion of monocytes to HCAECs, HUVECs, and EA.hy926 cells transiently transfected with an expression plasmid for *NR_003529* vs. pcDNA vector. (D) Relative transmigration of monocytes across a layer of HCAECs, HUVECs, and EA.hy926 cells transiently transfected with an expression plasmid for *NR_003529* vs. pcDNA vector. $*p < 0.05$ and $**p < 0.01$ vs. vector, $N = 5$.

overexpression of *NR_003529* by 10–12 fold in HCAECs (Fig. 4A), and specific knockdown of *DQ485454* by 70–75% with two separate siRNAs (Fig. 4B). Then, we utilized the transfected HCAECs for assays for THP-1 monocyte adhesion to ECs and transendothelial migration of monocytes. As shown in Fig. 4C, *NR_003529* overexpression as well as siDQ1 and siDQ2 all significantly increased monocyte adhesion to HCAECs by about ~1.5 fold, however, the combination of *NR_003529* overexpression with siDQ1 or siDQ2 dramatically increased monocyte adhesion to HCAECs by about 2.7 fold. Similar observations were made for transendothelial migration of monocytes (Fig. 4D). These data suggest that there is a synergistic effect between *NR_003529* overexpression and *DQ485454* knockdown on EC functions involved in the pathogenesis of CAD.

Combination of *NR_003529* knockdown and *DQ485454* overexpression in HCAECs dramatically decreases monocyte adhesion and transmigration.

We further investigated the effects of *NR_003529* knockdown and *DQ485454* overexpression on functions of HCAECs (Fig. 5A and 5B). As shown in Fig. 5C, knockdown of *NR_003529* expression using siRNAs siNR1 and siNR2 as well as overexpression of *DQ485454* all significantly decreased monocyte adhesion to HCAECs by about 25%, however, the combination of overexpression of *DQ485454* with either *NR_003529* siNR1 or siNR2 dramatically decreased monocyte adhesion to HCAECs by about 80%. Similar observations were made for transendothelial migration of monocytes (Fig. 5D). These data suggest that there is a synergistic effect between *NR_003529* knockdown and *DQ485454* overexpression on EC functions involved in the pathogenesis of CAD.

Transcript-specific regulation of downstream target genes by *ANRIL* transcripts *NR_003529* and *DQ485454*

To determine why *ANRIL* transcript *NR_003529* regulates EC functions in an opposite direction from *DQ485454*, we assessed their effects on the expression of downstream target genes, including *CLIP1*, *EZR*, *LYVE1*, *TMEM100*, *TMEM106B*, *CXCL11*, and *ENC1* identified by global gene expression arrays [13]. As previously reported [13], our qRT-

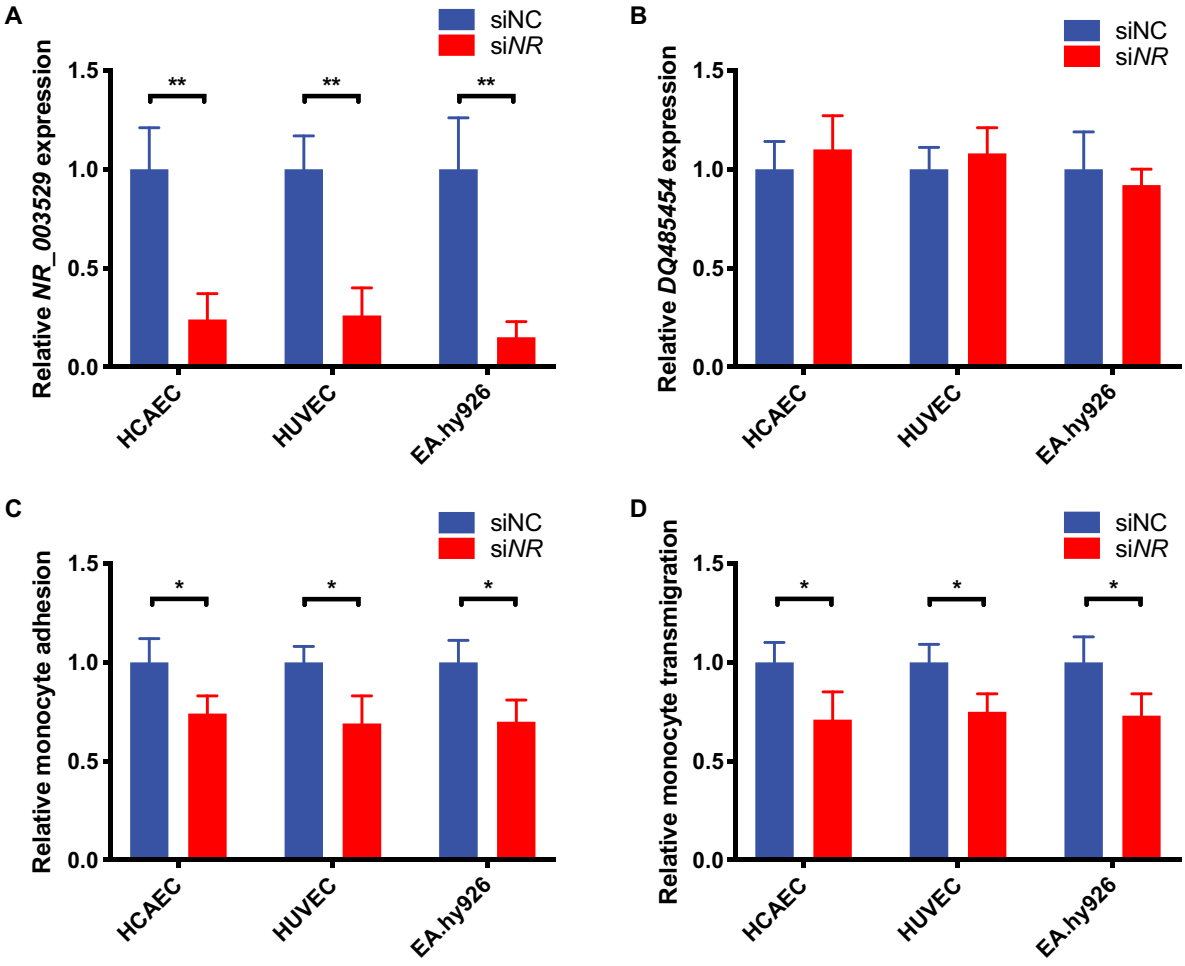

Figure 3. Knockdown of *NR_003529* in HCAEC, HUVECs and EA.hy926 cells reduces monocyte adhesion and transmigration. (A) Expression of the *NR_003529* transcript of *ANRIL* in HCAECs, HUVECs, and EA.hy926 cells transiently transfected with *NR_003529*-specific siRNA (siNR) vs. negative control siRNA (siNC) as quantified by qRT-PCR. (B) Expression of the *DQ485454* transcript of *ANRIL* in HCAECs, HUVECs, and EA.hy926 cells transiently transfected with *NR_003529*-specific siRNA (siNR) vs. negative control siRNA (siNC) as quantified by qRT-PCR. (C) Relative adhesion of monocytes to HCAECs, HUVECs, and EA.hy926 cells transiently transfected with *NR_003529*-specific siRNA (siNR) vs. negative control siRNA (siNC). (D) Relative transmigration of monocytes across a layer of HCAECs, HUVECs, and EA.hy926 cells transiently transfected with *NR_003529*-specific siRNA (siNR) vs. negative control siRNA (siNC). *$p < 0.05$ and **$p < 0.01$ vs. vector, $N = 5$.

PCR analysis showed that overexpression of *DQ485454* significantly increased the expression of *CLIP1*, *EZR*, *LYVE1*, *CXCL11*, and *ENC1*, but reduced the expression of *TMEM100* and *TMEM106B* in HCAECs (Fig. 6A). However, overexpression of *NR_003529* significantly decreased the expression of *EZR* and *CXCL11*, but increased the expression of *LYVE1* and *TMEM106B* in HCAECs (Fig. 6A). Knockdown of *NR_003529* expression significantly increased the expression of *EZR* and *CXCL11* in HCAECs (Fig. 6B). Together, these data suggest that *ANRIL* transcripts *NR_003529* and *DQ485454* affect EC functions involved in CAD in an opposite direction by differentially regulating the expression of *EZR, CXCL11*, and probably *TMEM106B*.

Discussion

Genomic variants in and near *ANRIL* on chromosome 9p21 were significantly associated with multiple diseases, including CAD, type 2 diabetes, several forms of cancer, aortic aneurysms, and others. For CAD, the 9p21 locus is the first locus identified by GWAS in 2007, and appears to be the most robust CAD locus with a recent association *P* value of 10^{-223} [21]. *ANRIL* shows a complex pattern of alternative splicing, resulting in more than 20 different isoforms of transcripts (Fig. 1A). There are two major groups of transcripts for *ANRIL* [22], one group ending with exon 19 such as the longest transcript *NR_003529*, and the other group ending with exon 13 such as *DQ485454* (Fig. 1A). However, little is done with the characterization of different transcripts for their disease-associated or tissue-specific expression and functions. Such studies are essential for full understanding of the functionality of *ANRIL* and its role in the pathogenesis of human diseases. In this study, we focused on two major *ANRIL* transcripts in endothelial cells, *NR_003529* and *DQ485454*. We found that the two transcripts showed opposite expression patterns in coronary arteries from CAD patients as compared with non-CAD tissue samples. Our qRT-PCR analysis showed that *NR_003529* expression was significantly upregulated in CAD arteries (Fig. 1B), whereas *DQ485454* expression was significantly downregulated in CAD arteries [13]. Similarly,

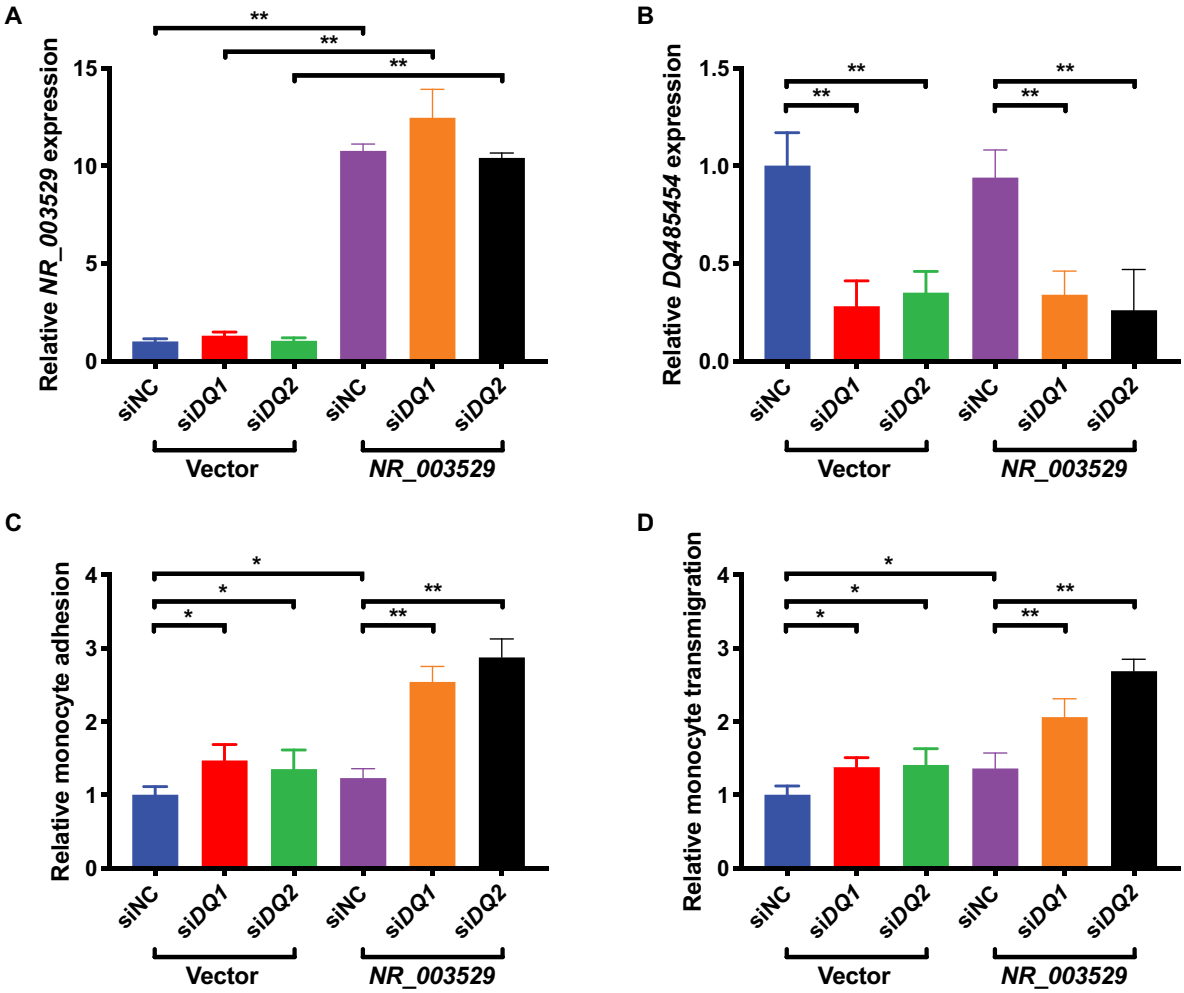

Figure 4. Overexpression of *NR_003529* together with knockdown of *DQ485454* in HCAECs dramatically increases monocyte adhesion and transmigration. (A) Expression of the *NR_003529* transcript of *ANRIL* in HCAECs transiently co-transfected with *DQ485454*-specific siRNAs (siDQ1, siDQ2) vs. negative control siRNA (siNC) and the overexpression plasmid for *NR_003529* vs. pcDNA vector as quantified by qRT-PCR. (B) Expression of the *DQ485454* transcript of *ANRIL* in HCAECs transiently co-transfected with *DQ485454*-specific siRNAs (siDQ1, siDQ2) vs. negative control siRNA (siNC) and the overexpression plasmid for *NR_003529* vs. pcDNA vector as quantified by qRT-PCR. (C) Relative adhesion of monocytes to HCAECs transiently co-transfected with *DQ485454*-specific siRNAs (siDQ1, siDQ2) vs. negative control siRNA (siNC) and the overexpression plasmid for *NR_003529* vs. pcDNA vector. (D) Relative transmigration of monocytes across a layer of HCAECs transiently co-transfected with *DQ485454*-specific siRNAs (siDQ1, siDQ2) vs. negative control siRNA (siNC) and the overexpression plasmid for *NR_003529* vs. pcDNA vector. $^{*}p < 0.05$ and $^{**}p < 0.01$ vs. vector, $N = 5$.

we found that the expression of all transcripts ending with exon 19 were significantly upregulated in CAD arteries, where the expression of all transcripts ending with exon 13 were significantly downregulated (Fig. 1D and 1E). These data suggest that different *ANRIL* transcripts exhibit disease-specific expression patterns in CAD.

Consistent with their opposite expression patterns, *NR_003529* and *DQ485454* antagonize each other functionally (Fig. 2–5). In two endothelial cell assays relevant to the pathogenesis of CAD, *NR_003529* increased monocytes adhesion to ECs (Fig. 2C) and transendothelial migration of monocytes (Fig. 2D) when overexpressed in ECs. Knockdown of *NR_003529* expression reduced monocytes adhesion to ECs (Fig. 3C) and transendothelial migration of monocytes (Fig. 3D). Interestingly, the finding of upregulation of *NR_003529* and downregulation of *DQ485454* in CAD arteries showed a synergistic effect in dramatically increasing monocytes adhesion to ECs (Fig. 4C) and transendothelial migration of monocytes (Fig. 4D). Increased monocyte adhesion to ECs and transendothelial migration of monocytes are involved in promoting the initiation and development of atherosclerosis and CAD. These data further highlight the importance of molecular and functional characterization of different transcripts of *ANRIL*. More importantly, our data further support the hypothesis that *ANRIL* is the disease-causing gene at the 9p21 CAD locus.

We previously performed microarray global gene expression profiling with ECs transfected with *ANRIL* siRNA vs. scramble siRNA, and identified important downstream target genes of *ANRIL*. We showed that ANRIL can positively regulate the expression of *ANHAK1, CLIP1, CXCL11, ENC1, EZR, LYVE1* and *WASL*, but negatively regulates expression of *TMEM100* and *TMEM106B* [13]. Mechanistic studies showed that overexpression of *ANRIL* downstream target genes *CLIP1, EZR*, and *LYVE1* reversed the effects of *ANRIL* KD on monocyte adhesion to ECs and TEM [13]. Recently, we showed that knockdown of *TMEM106B*, but not of

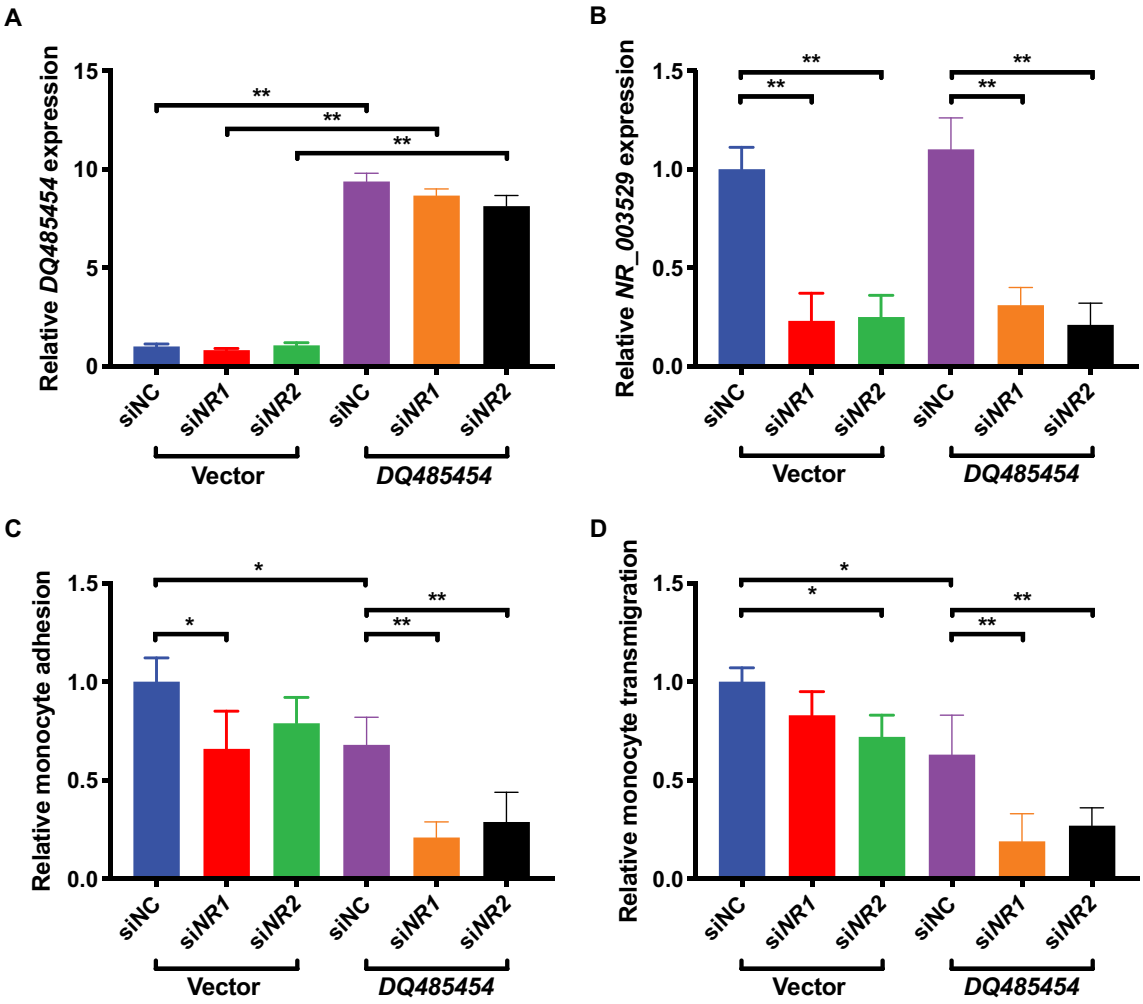

Figure 5. Overexpression of *DQ485454* together with knockdown of *NR_003529* in HCAECs dramatically reduces monocyte adhesion and transmigration. (A) Expression of the *NR_003529* transcript of *ANRIL* in HCAECs transiently co-transfected with *NR_003529*-specific siRNAs (siNR1, siNR2) vs. negative control siRNA (siNC) and the overexpression plasmid for *DQ485454* vs. pcDNA vector control as quantified by qRT-PCR. (B) Expression of the *DQ485454* transcript of *ANRIL* in HCAECs transiently co-transfected with *NR_003529*-specific siRNAs (siNR1, siNR2) vs. negative control siRNA (siNC) and the overexpression plasmid for *DQ485454* vs. pcDNA vector as quantified by qRT-PCR. (C) Relative adhesion of monocytes to HCAECs transiently co-transfected with *NR_003529*-specific siRNAs (siNR1, siNR2) vs. negative control siRNA (siNC) and the overexpression plasmid for *DQ485454* vs. pcDNA vector. (D) Relative transmigration of monocytes across a layer of HCAECs transiently co-transfected with *NR_003529*-specific siRNAs (siNR1, siNR2) vs. negative control siRNA (siNC) and the overexpression plasmid for *DQ485454* vs. pcDNA vector. *$p < 0.05$ and **$p < 0.01$ vs. vector, $N = 5$.

TMEM100, reversed the increased monocyte adhesion to ECs and TEM induced by knockdown of *DQ485454* [23]. Furthermore, overexpression of *TMEM106B* reversed the decreased monocyte adhesion to ECs and TEM induced by *DQ485454* overexpression [23]. *TMEM106B* expression was significantly upregulated by >2-fold in CAD coronary arteries, and genomic variants in *TMEM106B* (but not in *TMEM100*) showed significant association with CAD ($p = 1.9 \times 10^{-8}$) in the UK Biobank samples [23]. We also identified significant gene-gene interaction between *ANRIL* variant rs2383207 and *TMEM106B* variant rs3807865 ($p = 0.009$) [23]. These studies suggest that *ANRIL* downstream target genes *CLIP1*, *EZR*, *LYVE1*, and *TMEM106B* are particularly important in regulating the functions of *ANRIL* in ECs involved in the development of CAD. To identify a molecular mechanism underlying the differential antagonizing functions of transcripts *NR_003529* and *DQ485454*, we examined *ANRIL* downstream genes whose expression was affected by *DQ485454* [13]. Overexpression of *NR_003529* significantly reduced expression of *EZR* and *CXCL11*, and increased expression of *TMEM106B*, whereas overexpression of *DQ485454* showed opposite expression patterns (Fig. 6A). On the other hand, knockdown of *NR_003529* expression significantly increased the expression of *EZR* and *CXCL11* (Fig. 6B). As CAD patient coronaries showed upregulation of *NR_003529* and downregulation of *DQ485454*, we propose that reduced *EZR* expression and increased *TMEM106B* expression are more critically involved in EC functions regulated by *ANRIL*.

In addition to more than 20 different linear *ANRIL* transcripts, multiple circular forms of *ANRIL* (circ*ANRIL*) were reported [24–26]. Holdt et al showed that the expression of circ*ANRIL* with exons 5-6-7 was detected in vascular smooth muscle cells and macrophages in human atherosclerotic

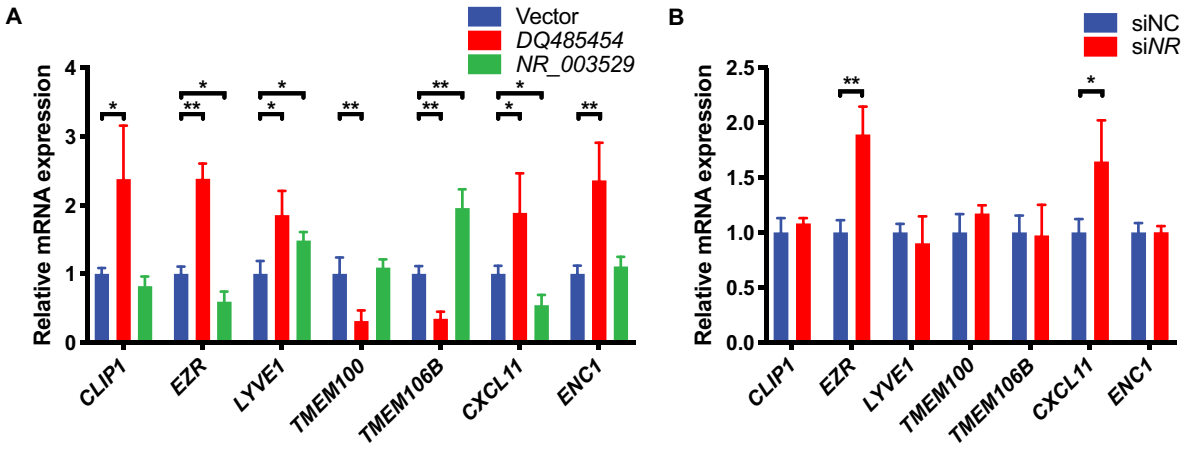

Figure 6. Expressions of *EZR, CXCL11*, and *TMEM106B* are differentially regulated by different transcripts of *ANRIL*. (A) Relative mRNA expressions of seven downstream genes of *ANRIL* (*CLIP1, EZR, LYVE1, TMEM100, TMEM106B, CXCL11*, and *ENC1*) in HCAECs transfected with the overexpression plasmid for either *DQ485454* or *NR_003529* transcripts of *ANRIL* vs. pcDNA3.1 vector control as determined by qRT-PCR. (B) Relative mRNA expressions of the seven downstream genes of *ANRIL* in HCAECs transfected with the *NR_003529*-specific siRNA (siNR) vs. negative control siRNA (siNC) as determined by qRT-PCR. *$p < 0.05$ and **$p < 0.01$ vs. vector, $N = 5$.

plaques, and a CAD protective haplotype was associated with increased expression of circ*ANRIL* in peripheral blood mononuclear cells, whole blood and endarterectomy samples [25]. Interestingly, we showed that the expression level of circ*ANRIL* was significantly less expressed in CAD coronary arteries than in non-CAD tissue samples (Fig. 1). Together, all these data suggest that reduced circ*ANRIL* expression is associated with risk of CAD, whereas increased circ*ANRIL* expression is associated with a protective role in CAD. Burd et al showed that the expression of both linear and circular *ANRIL* at the proximal end was correlated with CAD risk genotype of rs10757278, whereas distal *ANRIL* transcripts with exons 18 and 19 were independent to the risk genotype [24]. Moreover, some SNPs near exon 15 were predicted to affect *ANRIL* splicing [24]. The interesting findings support the hypothesis that different CAD genotypes (risk, protective) may differentially affect the expression of transcripts *NR_003529* and *DQ485454* by affecting *ANRIL* expression and/or splicing, resulting in the opposite expression patterns of the two transcripts in CAD coronary arteries. Future studies with large sample sizes may be able to test the interesting possibility.

There are similarities and differences between *ANRIL* transcripts *DQ485454* and *NR_003529* (Fig. 1A). *DQ485454* is 2,659 bp in length and contains 13 exons, whereas *NR_003529* is 3,857 bp long and has 19 exons (Fig. 1A). *DQ485454* and *NR_003529* share exons 1 to 12. *DQ485454* contains a unique exon, exon 13, whereas *NR_003529* contains an alternatively spliced exon 13, exon 13b, and 6 other exons from exon 14 to 19 (Fig. 1A). Therefore, an alternative splicing event at and after exon 12 is a key for generation of two different transcripts *DQ485454* and *NR_003529*. The molecular mechanism for the differential expression of *NR_003529* and *DQ485454* in coronary artery tissue samples from CAD patients is unknown. One possibility is that different CAD-relevant splicing regulatory proteins act at the exon 12/exon 13 junction and the exon 12/exon 13b junction, respectively, to either inhibit or enhance alternative splicing, resulting in differential expression of *DQ485454* and *NR_003529* in CAD tissue samples. Alternatively, as noted above, a risk CAD genotype at the junction between exon 12 and exon 13 may inhibit splicing, resulting in reduced *DQ485454* expression, whereas a different risk CAD genotype at the junction between exon 12 and exon 13b enhances splicing, resulting in increased *NR_003529* expression in CAD tissue samples.

The molecular mechanism by which different *ANRIL* transcripts *NR_003529* and *DQ485454* differentially regulate the expression of its downstream genes *EZR, CXCL11*, and *TMEM106B* in endothelial cells is not clear. *ANRIL* can regulate expression of adjacent genes by facilitating the recruitment of the chromatin-modifying complex to promoter/regulatory regions via interaction with the components of PRC1/2, such as EZH2, SUZ12, and CBX7 [27–30]. The unique sequences of *NR_003529* (exon 13a, 14–19) and *DQ485454* (exon 13) may affect the interaction between *ANRIL* and different components of PRC1/2, and thus, regulate the expression of downstream genes in an opposite direction. Alternatively, as with many other lncRNAs, *ANRIL* can act as a sponge for some microRNAs such as let-7a [31], miR-9 [32], miR-7-5p [33], miR-127 [34], miR-181b [35], miR-181a [36,37], and potentially many other unreported miRNAs. The unique sequences of *NR_003529* (exon 13a, 14–19) and *DQ485454* (exon 13) may bind different miRNAs, and differentially regulate the expression of downstream target genes in endothelial cells.

ANRIL appears to be a primate-specific lncRNA. Sequence analysis did not detect an ortholog with a high similarity in the mouse genome to date. A lncRNA gene *Gm12610* in the mouse genome was previously referred to as *Anril* (https://www.ncbi.nlm.nih.gov/nuccore/NR_132431.1) because it is located at the *Cdkn2a/Cdkn2b* locus on mouse chromosome 4. However, we performed BLASTN analysis and found no significant similarity between *Gm12610* and human *ANRIL* transcript *NR_003529* or between *Gm12610* and *DQ485454*. Therefore, no *in vivo* knockout studies can be performed in animals to investigate the causal effect of *ANRIL* on atherosclerosis. Although the two functional assays used in this study, monocytes adhesion to ECs and transendothelial migration of monocytes, mimic the initiation processes involved in the development of atherosclerosis, they are

in vitro assays and care should be taken when extrapolating the data to human CAD *in vivo*. Nevertheless, the data from two other studies provide evidence to support that *ANRIL* transcripts *NR_003529* and *DQ485454* are relevant to the pathogenesis of CAD in humans. First, we studied human coronary artery tissues from CAD patients and control individuals that are directly relevant to the pathogenesis of CAD, and found that *ANRIL* transcripts *NR_003529* and *DQ485454* were significantly upregulated and downregulated in coronary artery tissue samples from CAD patients, respectively (Fig. 1). Second, our genome-wide association studies (GWAS) showed that *TMEM106B*, one of the downstream genes of *ANRIL*, is a new susceptibility gene for CAD, and interacts with *ANRIL* to modulate the risk of CAD [23]. We further showed that overexpression of *NR_003529* significantly increased the expression of *TMEM106B* in HCAECs (Fig. 6A), whereas overexpression of *DQ485454* significantly reduced the expression of *TMEM106B* in HCAECs (Fig. 6A). The data suggest that the *ANRIL-TMEM106B* regulatory axis is involved in the development of CAD.

NR_003529 knockdown significantly reduced THP-1 monocyte adhesion (Fig. 3C) and transendothelial migration of monocytes (Fig. 3D) in all three different types of ECs, including HCAECs, HUVECs, and EA.hy926 cells. However, overexpression of *NR_003529* significantly increased monocyte adhesion to ECs (Fig. 2C) and transendothelial migration of monocytes (Fig. 2D) in HCAECs and EA.hy926 cells, but not in HUVECs. The lower transfection efficiency of HUVECs for plasmid DNA (40% vs. 50% for HCAEs and 70% for EA.hy926) may be an underlying cause. Moreover, the transfection efficiency of plasmid DNA into ECs is lower than of siRNAs.

In summary, this study reveals an interesting finding that two different transcripts of *ANRIL, NR_003529* and *DQ485454*, show an opposite expression pattern in coronary arteries from CAD patients, and exhibit an antagonizing role in EC functions involved in the initiation and development of CAD. Functionally, *NR_003529* regulates the expression of *ANRIL* downstream genes *EZR, CXCL11*, and *TMEM106B* in an opposite direction against *DQ485454*, which may explain their antagonizing roles in EC functions. Our data further support the hypothesis that *ANRIL* is the disease-causing gene at the 9p21 CAD locus, and provide important insights into the biology of lncRNA ANRIL. The results highlight the importance of molecular characterization of different transcripts of a lncRNA for their expression and functions in human disease.

Materials and methods

Human subjects

This study involved human coronary artery tissue samples from six CAD patients and six age-matched non-CAD study subjects reported by us previously [13,23,38]. The mean age was 51 ± 4 years for both CAD and control groups. This study was approved by the Cleveland Clinic Institutional Review Board (IRB) on Human Subject Research and written consent was obtained from the subjects. This study abides by the Declaration of Helsinki principles.

Plasmids and siRNAs

A mammalian expression plasmid for *ANRIL* transcript *DQ485454*, referred to as pcDNA3.1-*ANRIL* (*DQ485454*), was described previously [13]. The pcDNA3.1-*ANRIL* (*NR_003529*) plasmid was originally created by cloning the full-length *ANRIL* transcript into Xho I and Hind III restriction sites of the pcDNA3.1 vector as previously described by us [39].

The siRNAs used in the study were ordered from Integrated DNA Technologies (Skokie, IL) and Lincode control siRNA was ordered from GE Dharmacon (Lafayette, CO). The specific sequences of the *ANRIL* siRNAs are as follows:

si*DQ1*: AUCUGUGUUUUUGUCCAAUGUCCTT;
si*DQ2*: GAAUGAUACCUGCUUCUCUAUCUTG;
si*NR1*: CAUGCUCCCUCCCCUCAUUGAGGTT;
si*NR2*: CACAGAUGCCUAACGCACUAUGGTA.

Cell culture and transfection

HCAECs (ATCC, USA) and HUVECs (Lonza, USA) were cultured in a phenol-red-free endothelial growth medium (ATCC, USA) supplemented with 2% foetal bovine serum (FBS), 0.4% human fibroblast growth factor, 0.1% human epidermal growth factor, 0.1% vascular endothelial growth factor, 0.1% insulin-like growth factor, 0.1% ascorbic acid, 0.1% heparin, 0.1% gentamicin/amphotericin-B and 0.04% hydrocortisone (all from ATCC, USA). EA.hy926, a human umbilical vascular EC line (ATCC, USA), was cultured in Dulbecco's Modified Eagle Medium (DMEM) with 10% FBS. Human THP-1 monocytes were obtained from the ATCC and cultured in RPMI 1640 supplemented with 10% FBS. All cell cultures were maintained at 37°C in a humidified atmosphere with 5% CO_2.

Transient transfection of ECs with mammalian expression plasmids and siRNAs was performed using non-liposomal TransIT-X2 Dynamic Delivery System (Mirus Bio, Madison, WI) according to the manufacturer's instructions.

Monocyte adhesion to EC assays

Monocyte adhesion to ECs assays were performed as previously described [13]. Five independent experiments were performed.

Assays for transendothelial migration of monocytes

TEM was performed as previously described [13]. Five independent experiments were performed.

Real-time RT–PCR analysis

Total RNA samples were isolated from the transfected cells and coronary arteries using TRIzol reagent (Thermo Fisher Scientific) according to the Manufacturer's instructions. cDNA synthesis and RT-PCR analysis were performed as described by us previously [19,20]. The RT-PCR analysis was performed in a final volume of 20 µl containing 10 µl Power SYBR Green PCR Master Mix kit (Applied Biosystems, USA), 0.4 µl of each primer at 10 pmol/µl, and 2 µl of the cDNA solution using an Applied Biosystems Prism 7900HT

(Applied Biosystems, USA) system. Divergent primers were used to detect circANRIL. qRT-PCR primer sequences for *CLIP1, EZR, LYVE1, TMEM100, TMEM106B, CXCL11*, and *ENC1* genes were provided previously [13] and primer sequences for the remaining genes are as follows:

ANRIL 12 F: AACCTGAGCAGCTGGGACTA
ANRIL 13bR: TGTGTCCATAGCACCTTCCA
ANRIL 5 F: TGAAAAACACACATCAAAGGAGA
ANRIL 7 R: TCACGAGGTCGAGAGTTCAA
ANRIL 11 F: CAGAAACCACATCCCTTGGA
ANRIL 13 R: CCTTTTATCACCCAGCTTCG
ANRIL 18 F: CTGCTACATGGAGGCTAGGG
ANRIL 19 R: GGTTCTGCCACAGCTTTGAT
circ*ANRIL* 7 F: CTCCCAAAGTGCTGGGATTA
circ*ANRIL* 5 R: ATCTGGTGGCCAGAAAACAG

Raw data were analysed using Sequence Detection System (SDS) Software v2.4 (Applied Biosystems, USA) and compared by the ΔΔCt method as previously described by us [19,20]. Results are expressed relative to the housekeeping *GAPDH* transcript and normalized to untreated cells. Each sample was analysed in triplicate. Each experiment was repeated at least three times.

Statistical analysis

Data are presented as mean ± standard deviation (SD). Statistical analysis was performed using a Student's *t*-test (paired and two-tailed) or using analysis of variance (ANOVA) using GraphPad Prism 8.3.1 software. $p < 0.05$ was used as a cut-off value to determine statistical significance of the data.

Author contributions

Study design: Q.K.W., Q.C., and H.C.; Experiments: H.C.; Data analysis and critical scientific inputs: H.C., Y.L., S.A., F.W., G.Y., S.C., Y.G., Q.C., and Q.K.W.; Manuscript preparation: H.C., Q.C., and Q.K.W.; Comments and revision of the manuscript: all authors; Study supervision: Q.C. and Q.K.W.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This work was supported by NIH/NHLBI grants R01 HL121358 (Q.K.W), R01 HL126729 (Q.K.W), and R01 HL138465 (Q.K.W. and Q.C.).

ORCID

Hyosuk Cho http://orcid.org/0000-0003-0592-9102
Yabo Li http://orcid.org/0000-0002-8954-7007
Fan Wang http://orcid.org/0000-0001-9806-5037
Gang Yu http://orcid.org/0000-0002-8496-8814
Susmita Chakrabarti http://orcid.org/0000-0003-1197-2780
Yang Guo http://orcid.org/0000-0001-7430-7038
Qing Kenneth Wang http://orcid.org/0000-0002-5639-8519

References

[1] Benjamin EJ, Virani SS, Callaway CW, et al. Heart disease and stroke statistics-2018 update: a report from the American Heart Association. Circulation. 2018;137:e67–e492.
[2] Lusis AJ. Atherosclerosis. Nature. 2000;407(6801):233–241.
[3] Shen GQ, Li L, Seidelmann DG, et al. An LRP8 variant is associated with familial and premature coronary artery disease and myocardial infarction. Am J Hum Genet. 2007;81(4):780–791. .
[4] Wang Q, Rao S, Shen GQ, et al. Premature myocardial infarction novel susceptibility locus on chromosome 1P34-36 identified by genomewide linkage analysis. Am J Hum Genet. 2004;74(2):262–271. .
[5] Archacki SR, Angheloiu G, Moravec CS, et al. Comparative gene expression analysis between coronary arteries and internal mammary arteries identifies a role for the TES gene in endothelial cell functions relevant to coronary artery disease. Hum Mol Genet. 2012;21(6):1364–1373.
[6] Clarke SL, Assimes TL. Genome-wide association studies of coronary artery disease: recent progress and challenges ahead. Curr Atheroscler Rep. 2018;20(9):47.
[7] Wolin SL, Maquat LE. Cellular RNA surveillance in health and disease. Science. 2019;366(6467):822–827.
[8] Sallam T, Sandhu J, Tontonoz P. Long noncoding RNA discovery in cardiovascular disease: decoding form to function. Circ Res. 2018;122(1):155–166.
[9] Nair L, Chung H, Basu U. Regulation of long non-coding RNAs and genome dynamics by the RNA surveillance machinery. Nat Rev Mol Cell Biol. 2020;21(3):123–136.
[10] Kopp F. Molecular functions and biological roles of long non-coding RNAs in human physiology and disease. J Gene Med. 2019;21(8):e3104.
[11] Wang KC, Chang HY. Molecular mechanisms of long noncoding RNAs. Mol Cell. 2011;43(6):904–914.
[12] Ribeiro DM, Zanzoni A, Cipriano A, et al. Protein complex scaffolding predicted as a prevalent function of long non-coding RNAs. Nucleic Acids Res. 2018;46(2):917–928. .
[13] Cho H, Shen GQ, Wang X, et al. Long noncoding RNA ANRIL regulates endothelial cell activities associated with coronary artery disease by up-regulating CLIP1, EZR, and LYVE1 genes. J Biol Chem. 2019;294(11):3881–3898. .
[14] Sallam T, Jones M, Thomas BJ, et al. Transcriptional regulation of macrophage cholesterol efflux and atherogenesis by a long noncoding RNA. Nat Med. 2018;24(3):304–312. .
[15] Gast M, Rauch BH, Nakagawa S, et al. Immune system-mediated atherosclerosis caused by deficiency of long non-coding RNA MALAT1 in ApoE−/− mice. Cardiovasc Res. 2019;115(2):302–314. .
[16] Kong Y, Hsieh CH, Alonso LC. ANRIL: a lncRNA at the CDKN2A/B locus with roles in cancer and metabolic disease. Front Endocrinol (Lausanne). 2018;9:405.
[17] Holdt LM, Teupser D. Long noncoding RNA ANRIL: lnc-ing genetic variation at the chromosome 9p21 locus to molecular mechanisms of atherosclerosis. Front Cardiovasc Med. 2018;5:145.
[18] Lo Sardo V, Chubukov P, Ferguson W, et al. Unveiling the role of the most impactful cardiovascular risk locus through haplotype editing. Cell. 2018;175(7):1796–810 e20. .
[19] Luo C, Pook E, Tang B, et al. Androgen inhibits key atherosclerotic processes by directly activating ADTRP transcription. Biochim Biophys Acta Mol Basis Dis. 2017;1863(9):2319–2332. .
[20] Luo CY, Wang F, Ren X, et al. Identification of a molecular signaling gene-gene regulatory network between GWAS susceptibility genes ADTRP and MIA3/TANGO1 for coronary artery disease. Bba-Mol Basis Dis. 2017;1863(6):1640–1653. .
[21] van der Harst P, Verweij N. Identification of 64 novel genetic loci provides an expanded view on the genetic architecture of coronary artery disease. Circ Res. 2018;122(3):433–443.
[22] Hubberten M, Bochenek G, Chen H, et al. Linear isoforms of the long noncoding RNA CDKN2B-AS1 regulate the c-myc-enhancer binding factor RBMS1. Eur J Hum Genet. 2019;27(1):80–89. .

[23] Li Y, Cho H, Wang F, et al. Statistical and functional studies identify epistasis of cardiovascular risk genomic variants from genome-wide association studies. J Am Heart Assoc. 2020;9(7):e014146. .
[24] Burd CE, Jeck WR, Liu Y, et al. Expression of linear and novel circular forms of an INK4/ARF-associated non-coding RNA correlates with atherosclerosis risk. PLoS Genet. 2010;6(12):e1001233.
[25] Holdt LM, Stahringer A, Sass K, et al. Circular non-coding RNA ANRIL modulates ribosomal RNA maturation and atherosclerosis in humans. Nat Commun. 2016;7(1):12429. .
[26] Sarkar D, Oghabian A, Bodiyabadu PK, et al. Multiple isoforms of ANRIL in melanoma cells: structural complexity suggests variations in processing. Int J Mol Sci. 2017;18(7):1378.
[27] Zhang EB, Kong R, Yin DD, et al. Long noncoding RNA ANRIL indicates a poor prognosis of gastric cancer and promotes tumor growth by epigenetically silencing of miR-99a/miR-449a. Oncotarget. 2014;5(8):2276–2292. .
[28] Sarkar D, Leung EY, Baguley BC, et al. Epigenetic regulation in human melanoma: past and future. Epigenetics. 2015;10(2):103–121.
[29] Meseure D, Vacher S, Alsibai KD, et al. Expression of ANRIL-polycomb complexes-CDKN2A/B/ARF genes in breast tumors: identification of a two-gene (EZH2/CBX7) signature with independent prognostic value. Mol Cancer Res. 2016;14(7):623–633. .
[30] Chi JS, Li JZ, Jia JJ, et al. Long non-coding RNA ANRIL in gene regulation and its duality in atherosclerosis. J Huazhong Univ Sci Technolog Med Sci. 2017;37(6):816–822.
[31] Wang Y, Cheng N, Luo J. Downregulation of lncRNA ANRIL represses tumorigenicity and enhances cisplatin-induced cytotoxicity via regulating microRNA let-7a in nasopharyngeal carcinoma. J Biochem Mol Toxicol. 2017;31(7). doi:10.1002/jbt.21904.
[32] Deng W, Chen K, Liu S, et al. Silencing circular ANRIL protects HK-2 cells from lipopolysaccharide-induced inflammatory injury through up-regulating microRNA-9. Artif Cells Nanomed Biotechnol. 2019;47(1):3478–3484.
[33] Shu L, Zhang W, Huang C, et al. lncRNA ANRIL protects H9c2 cells against hypoxia-induced injury through targeting the miR-7-5p/SIRT1 axis. J Cell Physiol. 2019;235(2):1175–1183.
[34] Liu B, Cao W, Xue J. LncRNA ANRIL protects against oxygen and glucose deprivation (OGD)-induced injury in PC-12 cells: potential role in ischaemic stroke. Artif Cells Nanomed Biotechnol. 2019;47(1):1384–1395.
[35] Guo F, Tang C, Li Y, et al. The interplay of LncRNA ANRIL and miR-181b on the inflammation-relevant coronary artery disease through mediating NF-kappaB signalling pathway. J Cell Mol Med. 2018;22(10):5062–5075. .
[36] Tan P, Guo YH, Zhan JK, et al. LncRNA-ANRIL inhibits cell senescence of vascular smooth muscle cells by regulating miR-181a/Sirt1. Biochem Cell Biol. 2019;97(5):571–580.
[37] He ZY, Wei TH, Zhang PH, et al. Long noncoding RNA-antisense noncoding RNA in the INK4 locus accelerates wound healing in diabetes by promoting lymphangiogenesis via regulating miR-181a/Prox1 axis. J Cell Physiol. 2019;234(4):4627–4640.
[38] Archacki SR, Angheloiu G, Tian XL, et al. Identification of new genes differentially expressed in coronary artery disease by expression profiling. Physiol Genomics. 2003;15(1):65–74. .
[39] Bai Y, Nie S, Jiang G, et al. Regulation of CARD8 expression by ANRIL and association of CARD8 single nucleotide polymorphism rs2043211 (p.C10X) with ischemic stroke. Stroke. 2014;45(2):383–388. .