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ALUminating the path of atherosclerosis progression: chaos theory suggests a role for Alu repeats in the development of atherosclerotic vascular disease

Short running head: Alu repeated elements and the mechanisms of atherosclerosis .

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ABSTRACT

Atherosclerosis (ATH) and Coronary Artery Disease (CAD) are chronic inflammatory diseases with an important genetic background which derive from the cumulative effect of multiple common risk alleles, most of them located in genomic non-coding regions. These complex diseases behave as non-linear dynamical systems that show a high dependence on their initial conditions, so that long-term predictions of disease progression are unreliable. One likely possibility is that the non-linear nature of ATH could be dependent on non-linear correlations in the structure of the human genome. In this review we show how Chaos theory analysis highlighted genomic regions that shared specific structural constraints that could have a role in ATH progression. These regions were shown to be enriched in repetitive sequences of the Alu family, genomic parasites which colonized the human genome, which show a particular secondary structure and have been involved in the regulation of gene expression. We also review the impact of Alu elements on the mechanisms that regulate gene expression, especially highlighting the molecular mechanisms by which the Alu elements could alter the inflammatory homeostasis. We devise especial attention to their relationship with the lncRNA ANRIL, the strongest risk factor for ATH, their role as miRNA sponges, and their ability to interfere with the regulatory circuitry of the NF-κB response. We aim to characterize ATH as a non-linear dynamic system in which small initial alterations in the expression of a number of repetitive elements are somehow amplified to reach phenotypic significance.

KEYWORDS:
Atherosclerosis, Cardiovascular Disease, Chaos Theory, non-coding RNAs, Alu-elements, NF-κB, miRNA, miRNA sponges.

LIST OF ABBREVIATIONS:
AS-RNAs: antisense transcripts from coding regions
ASVD: Atherosclerotic Vascular Disease
ATH: Atherosclerosis
BM-EPCs: Bone Marrow-derived Endothelial Progenitor Cells
CAD: Coronary artery disease
cirRNAs: circular ncRNA
CKD: Chronic Kidney Disease
EC: Endothelial Cells
eQTL: Expression Quantitative Trait Locus
ECM: Extracellular matrix
ESRD: End Stage Renal Disease
GWAS: Genome-Wide Association Studies
LINE: Long Interspersed Nuclear Element
ncRNA: non-coding RNA
lncRNA: long non-coding RNA
lincRNAs: long intergenic ncRNAs
MACE: Major Adverse Cardiovascular Event
miRNA: micro RNA
MP: Microparticles
ncRNAs: non-coding RNAs
NO: Nitric Oxide
piRNAs: Piwi-interacting RNAs
RMC: Renal Microcirculation
ROS: Reactive Oxygen Species
SMC: Smooth-Muscle Cells
SNPs: Single-Nucleotide Polymorphisms
SINE: Short Interspersed Nuclear Element

LIST OF GENES
Ang II: Angiotensin II
ACE: Angiotensin Converting Enzyme
ANRIL/CDKN2B-AS1: Antisense Noncoding RNA in the INK4 Locus, CDKN2B AntiSense 1
BAFFR: B cell-Activating Factor belonging to the TNF Family Receptor
BCR: B Cell Receptor
ETA: endothelin A
ETB: endothelin B2
FOXO: Forkhead box O transcription factor
HIF: Hypoxia-Inducible Factor
HIF-PHDs: HIF-Prolyl Hydroxilase Domain enzymes
HRMs: Hypoxia-Regulated miRNAS
IKK: IκB Kinase
LTbR: LymphoToxin beta Receptor
MMP-9: Matrix Metalloproteinase-9

NEMO: NF-κB Essential Modulator
NIK: NF-κB Inducing Kinase
NF-κB: Activated Nuclear Factor-kB
NAD(P)H: Nicotinamide Adenine Dinucleotide Phosphate
PCAM1: Platelet Endothelial Cell Adhesion Molecule
PODXL: Podocalyxin
PRC: Polycomb Repressive Complexes
PRRs: Pattern-Recognition Receptors
RAAS: Renin-Angiotensin Aldosterone System
RANK: Receptor Activator of NF-κB
RYBP: RING1 and YY1 Binding Protein
STAT1: Signal Transducers and Activators of Transcription-1
TCR: T Cell Receptor
TLRs: Toll-like receptors
TNFAIP3: TNF alpha Induced Protein 3
TNFR: Tumor Necrosis Factor Receptors
TSSs: Transcription Start Sites
TWEAK: TNF related WEAK inducer of apoptosis
VLA-4: Very Late Antigen-4
VCAM-1: Vascular Cell Adhesion Molecule-1

1.- THE COMPLEX CAUSALITY OF CHRONIC KIDNEY DISEASE CALLS FOR NEW BIOMARKERS OF DISEASE PROGRESSION AND RISK TO ADVERSE CARDIOVASCULAR EVENTS

Major adverse cardiovascular events (MACEs) are the main cause of death in patients with Chronic Kidney Disease (CKD), although these are extremely difficult to predict. Consequently, validation of MACE biomarkers is urgently required to follow disease progression while new therapies have to be developed to offer new, personalized treatments to CKD patients. Globally, new concepts and ideas have to be added the research mainstream on CKD to ultimately lead to new approaches on MACE prevention.

Vascular dynamics is a highly dynamical system that shows time-dependent functional changes which are critically dependent on the interaction with different physical forces. These can be extra-tissular (as blood flow oscillations, arterial pressure, etc.) or intra-tissular (as calcifications, tissue thickness, presence of white cell infiltrates, etc.), but all of them show an unpredictable evolution caused by the appearance of minor interferences with the potential to produce unforeseen outcomes. Furthermore, vascular dynamics could be considered as a "chaotic" system whose evolution demonstrates a very sensitive dependence on its initial conditions and follows an uncertain, non linear progression [1-3], and there are a number of reports that back this hypothesis In this regard, Bruschke et al., showed that atherosclerosis (ATH) progression in coronary arteries was a highly unpredictable process which followed a nonlinear course [4] while other studies showed that arteries exhibited a non-linear, elastic behavior [5]. Furthermore, carotid arteries exhibit nonlinear variations of circumferential stress and tangent elastic moduli within the normal pressure range [6], and the evolution of buckling pressures of arteries under pulsatile pressure conditions could be accurately described by a non-linear model of elasticity [7]. Lastly, non-linear models have been also used to study the effects of luminal stenosis (and the plaque morphology) on plaque stability [8] or the interaction between the elastic layer (ECM cap) and the rigid calcified cells [9].

Most of the above studies have characterized the chaotic behavior of ATH by uniquely taking into account the role of the blood flow physics in the onset and progression of the disease. Nevertheless, there are hints that the non-linear progression of ATH disease could be also derived from specific features in the genome of the tissues involved in ATH, although it is difficult to envision how differential dynamics could be generated in a structure so homogeneous and tightly controlled as the mammalian nucleus. In this regard, Xiao et al., used a nonlinear prediction method, derived from chaos theory, to analyze the sequence of the β-globin locus and highlighted a subset of genomic sequences with novel deterministic structures and nonlinear correlations essentially different to those of exonic and intronic sequences (see [10], and section 3 for a more in deep discussion on the topic). These sequences corresponded to members of the Alu family of repeated elements, i.e. short DNA sequences [11] involved in the regulation of gene expression [12], that are pervasively transcribed in a number or physiological and clinical conditions [13-15] and have been associated to human diseases and genetic disorders [16, 17].

The non-linear structural features of Alu elements make worth studying the potential involvement of these repeated sequences in the generation of a chaotic progression during ATH development. In this review, we will study the possible relationship of Alu repeats with the onset and evolution of ATH, specifically focusing in different structural and/or functional features of Alu elements that could contribute to the non-linear progression of ATH, such as: (*i*)- the potential of Alu transcripts to act as miRNA sponges and hence to impact on *general levels* of mRNA expression, (*ii*)- their ability to generate new regulatory networks through retrotransposition to gene regulatory sites, and (*iii*)- their disruptive effect on the function of the CVD-associated lncRNA *ANRIL*, one of the strongest risk factors known for ATH.

2.- Alu REPEATED ELEMENTS: UNWANTED PLAYERS IN THE GENE EXPRESSION GAME.

One of the surprises that have arised from the systematic sequencing of different transcriptomes has been the discovery of a plethora of transcribed non-coding RNAs (ncRNAs) which corresponded to genomic regions previously known as "junk DNA". Without being exhaustive, these ncRNAs have been classified in 3 basic categories: (*i*) housekeeping RNAs (rRNAs, tRNAs, snRNAs and snoRNAs), (*ii*) short ncRNAs (less than 200-300 nt) that include microRNAs (miRNAs), Piwi-interacting RNAs (piRNAs), and retrotransposon-derived ncRNAs such as SINEs (Short Interspersed Nuclear Elements), and (*iii*) long non-coding RNAs (lncRNAs, greater than 200 nt), which can

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be further divided into intronic long intergenic ncRNAs (lincRNAs), antisense transcripts from coding regions (AS-RNAs, which do not encode proteins), circular ncRNA (cirRNAs), LINES (Long Interspersed Nuclear Elements) etc, [18].

At the genomic DNA level, the most numerous group of genes encoding ncRNAs is that of the Alu repeats, members of the SINE family. Alu elements are dimeric, over 300 bp-long, retrotransposons composed by two arms separated by an A-rich stretch. Alu repeats also include a bipartite Polymerase III internal promoter at the 5' end of left arm and a short poly-A tail at the 3' end of the right arm [12] (Figure 1). Alu repeats can be considered as highly successful genomic parasites which have colonized the human genome through cycles of retrotranscription (RNA to cDNA), insertion (cDNA into genomic DNA) and transcription (DNA to RNA), to the extent that approx. one million copies can be currently detected in the human genome [19]. This means that over 10% of the human genome is composed by Alu repeats, especially present in gene-rich regions, and that circa 30% of human genes harbour a copy of an Alu element [20]. Based on their evolutionary history, Alu elements have been classified in 12 subfamilies [21] from which only one is currently transpositionally active while the others have been inactivated, mainly by 5' truncation but also by sequence degeneration [22]. Alu elements are non-autonomous, so that their reverse transcription and integration in the genome requires the protein machinery of other autonomous retrotransposons such as LINEs (Long Interspersed Nuclear Elements), although under normal conditions these are also repressed in the human genome, mostly through promoter methylation [23,24], thus contributing to the silencing of Alu elements.

It has been known for long that Alu elements do play significant roles in the regulation of gene expression as well as in the maintenance of genomic integrity. Since Alu elements are usually in the neighbourhood of gene-rich regions, these repeated elements have been shown to impact the regulation of gene expression at the transcriptional as well as the post-transcriptional level through a number of different mechanisms [25]. At the transcriptional level, Alu repeats can be transcribed by RNA polymerase III (Pol III) from its endogenous internal promoter in response to cellular stress, giving rise to "free" Alus in the form of individual Alu RNA sequences which can repress RNA polymerase II-mediated transcription by binding to the Pol II initiation complex [12,26]. Much interestingly, a number of "immobilized" Alu elements have been detected in mature mRNAs, usually in their 5' or 3' UTRs [12] , with their levels being subjected to regulation since there are reports of their downregulation in cancer cells [27], and concatemers of individual Alu-RNAs of yet unknown function have been detected and cloned also in cancer cells [15].

On the other hand, a number of reports have shown that Alu elements have the potential to also modify the maturation process of mRNAs. In this sense, many Alu elements are detected in intronic regions [28] where they can provide new signals to originate alternative mRNA splicings or even exon skipping [29]. On the other hand, intronic Alus can be incorporated to mature mRNAs as a "bona fide" exon by itself (Alu exonization) or after retaining part of the neighbor intron (Alu-dependent intronic retention) [30]. Finally, Alu elements have been also considered as probable miRNA targets, likely acting as "miRNA sponges" [31] (see Section 6.2), and have been involved in regulating the function of circular RNAs [20], or in the regulation of mRNA stability [11]. In resume, due to their high number of copies, their internal promoters and their embedding in mature transcripts, Alu elements can impact on most of the mechanisms regulating RNA expression.

3.- MULTIFRACTAL AND CHAOS THEORY ANALYSIS OF THE HUMAN GENOME POINT OUT TO THE INVOLVEMENT OF Alu ELEMENTS IN THE DEVELOPMENT OF COMPLEX, NON-LINEAR HUMAN DISEASES

2.1 Chaos theory provides tools to for the analysis of global genomic signatures

The human genome is one of the most intricate molecular machines known and many different approaches have been used to study and analyze its complexity. Chaos theory and the chaos game representation (CGR) are two mathematical tools widely used to characterize highly complex systems. CGR, an implementation of chaos theory and chaotic dynamics, is an algorithm that has been used for the graphical representation of DNA sequences [32]. The output of a CGR representation is a scatter plot in which each point of the plot corresponds to one base of the sequence, thus producing a complex picture of the DNA sequence in which local and global patterns of sequential structure can be defined [32]. In this sense, the CGR would represent either statistical properties of base frequencies as intrinsic properties of the DNA sequence itself [32]. The main interest of CGR plots and its developments [33] rely in that these reduce complex DNA sequences into visual patterns that facilitate comparative studies of genomic signatures, as well as the analysis of characteristic sequence motifs [34]. In this way, CGR plots have been used to determine the degree of variability within and between genomes [35] or to compare two entire genomes for mismatches, insertions, deletions or shuffles [36-37].

Images produced by the CGR can be further studied with methods derived from fractal geometry (*multifractal* analysis) [35]. This approach has been used in different genomic analysis [38], among them to differentiate between coding and non-coding DNA sequences in humans [39] or bacteria [40], to predict promotor regions [41] to characterize whole genomes in C. elegans and humans [42,43] , of protein families [44,45], to allow the fast comparison of microbial genomes [46] or the study of the high order of the chromatin structure [43] .

2.2 Mathematical analysis of the human genome highlights features of non-linear correlations in the Alu family of genomic parasites

One likely possibility is that the non-linear nature of ATH could be due to a number of non-linear causes, and in Section 1 we discussed a number of ATH-related physical features in which disease progression was not directly proportional to the strength of the physical insult. On the other hand, we also hypothesized that evolution of ATH could be dependent on non-linear correlations in the structure of the human genome. The first report on the existence of these non-linear correlations in the human genome come from the work of Xiao et al, who used a chaos theory-derived nonlinear prediction method to differentiate among "random" and "non-random" (deterministic) DNA sequences [10]. In a first analysis, the authors studied the β-globin locus, which encodes six globin genes (with their exons and introns) and is enriched in Alu repeated sequences. They demonstrated that exonic and intronic sequences at the β-globin locus did not show significant deviation from randomness, while on the contrary most of the Alu sequences studied presented similar deterministic structures. This result suggested the existence of a structural determinants common to the different Alu elements. A further analysis showed that these non-linear correlations (deterministic structures) in the Alu elements were due to their dimeric composition, indicating that these could be based on the base-pairing of the two Alu-arms [10]. This intriguing result was somehow confirmed by further work by Moreno et al., which reported that the human genome displayed a multifractal behaviour, i.e. was rich in highly polymorphic sequences organized in a large number of combinations, which was strongly dependent in the content of Alu-S, the older and most abundant of the Alu families [49]. As already stated, Alu repeats have repeatedly colonized the human genome [11], but only a few Alu master genes remain transpositionally active [47]. Consequently, Alu elements are highly heterogeneous due to sequence divergences caused by the different evolutionary times in which they were retrotransposed into the genome, so that we can distinguish "old" Alus (classified in the Alu-J, Alu-S families) from "younger" Alus (Alu-Ya, Alu-Yb families) by specific sequential features [48].

The nonlinear correlations reflected distinctive biological functions of Alu repeats due to their dimeric structure, which showed differences among old (Alu-J, Alu-S) and young (Alu-Ya, Alu-Yb) subfamilies. These differences were likely related to distinctive traits of their 3-dimensional folding, which were not present in other genomic regions [10]. In this sense, the secondary structure of Alu elements is very complex, being formed by two independent 7SL RNA-like folding units (components of the Signal recognition Particle, SRP) plus a domain of interaction between the two Alu arms [48, 50]. These Alu sub-domains are sequentially and structurally heterogeneous due to sequence divergence [48] , thus making the Alu family of sequences a potential generator of sequential and structural diversity.

In this context, in the next sections we will address the question of how highly heterogeneous Alu elements can impact on mechanisms regulating gene expression to facilitate ATH progression, and more specifically with those that could support a non-linear developmental basis for this disease.

4.- NEW NON-CODING RNA BIOMARKERS FOR ATHEROSCLEROSIS PROGRESSION

4.1 *ANRIL* (*Antisense Noncoding RNA in the INK4 Locus*): a long non-coding RNA at 9p21, the gene-desert locus which harbors the strongest risk factor for atherosclerosis

Coronary artery disease (CAD) has an important genetic background [51] and the increased susceptibility to CAD has been associated to a number of genetic variants [52]. Much interestingly, different genome-wide association studies (GWAS) have identified a strong association between the risk of coronary artery disease and a large intergenic locus at chromosome 9p21, spanning over 58 kb of DNA [53]. This genomic region includes several SNPs in tight linkage disequilibrium which disrupt predicted transcription factor binding sites involved in key physiological processes [54]. Much surprisingly, the 9p21 risk locus is a protein coding gene-free region, which otherwise encodes a long non-coding RNA (lncRNA) called *ANRIL/CDKN2B-AS1* (*Antisense Noncoding RNA in the INK4 Locus* or *CDKN2B AntiSense 1*), which has been identified as the strongest genetic factor associated to cardiovascular morbidity and mortality [55], also correlating with atherosclerosis severity [56]. At 9p21, *ANRIL* is found at the "far neighbourhood" (over 100 kbp) of the cyclin-dependent kinase inhibitor gene cluster *p15/CDKN2B-p16/CDKN2A-p14/ARF*, although the first exon of ANRIL is located next to the promoter of the *p14/ARF* gene and overlapped with two exons of *p15/CDKN2B* [57], and to date no other transcript has been detected in this genomic region.

Long ncRNAs have been implicated in the regulation of most of the mechanisms of gene expression, either transcriptional, or postranscriptional (translational), as well as with the control of mRNA stability, pre-miRNA processing and chromatin structure [58], and consequently the mechanisms by which *ANRIL* would exert its effects are expected to be also many and diverse [59]. In this sense, it has been reported that in different diseases *ANRIL* behaves as a miRNA sponge by targeting miR-199a [60], miR-125a [61], miR-186 [62], or miR-323 [63] among others. Furthermore, *ANRIL* has been also described as a regulator of the signaling pathways ATM-E2F1 [64], VEGF [65], and NF-kB [66].

Another mechanism by which ANRIL would act is through the regulation of the cell cycle by interfering with the expression of the *p15/CDKN2B-p16/CDKN2A-p14/ARF* locus. In this sense it has been reported that ANRIL over-expression correlated with the down-regulation of *p16(INK4a)* [67], and *p15(INK4b)* [68] in different pathologies. Furthermore, *ANRIL* over-expression was shown to up-regulate a number of genes involved in the proliferation, adhesion and apoptosis in monocytes [56], while the depletion and mutagenesis of *ANRIL* reversed trans-regulation of these genes and normalized cellular functions [56].

A number of *ANRIL* splicing isoforms have been described [56] , and expression of these containing exons proximal to the *INK4/ARF* locus has been correlated with an increased risk of arteriosclerotic vascular disease [69,70]. *ANRIL* risk-alleles have been also associated with the inflammatory response, since these were shown to disrupt a binding site for the transcriptional factor STAT1 (Signal Transducers and Activators of Transcription-1) which mediated the transcriptional response to γ-IFN [71]. Furthermore, it has been also suggested that risk SNPs would alter the profile of *ANRIL* isoforms at the splicing level or by generating circular forms of *ANRIL*, which would impact on the expression of the neighbouring *p15/CDKN2B-p16/CDKN2A-p14/ARF* locus [72]. In this context, our group has reported that the *ANRIL* SNP rs10757278 (GG) doubled the risk for MACE in patients with CKD on haemodialysis through a yet unknown mechanism [73].

A recent report revealed an unsuspected functional relationship among *ANRIL* and members of the Alu family of repeated sequences, by which these last added a new regulatory tier to the ANRIL activity which impacted in the ability of cells to adhere and proliferate and facilitated atherosclerosis progression [56] (see Section 6 and Figure 2A).

4.2- De-regulation of miRNA expression and atherosclerosis progression

Like in many other complex diseases, multiple genes predispose to the vascular phenotype in ATH, and number of experimental models have highlighted a direct link between altered microRNA (miRNA) expression and the onset of ATH progression and cardiovascular disease [74-76]. MiRNAs, are a class of short ncRNAs (20-22 nucleotides long) that regulate the stability of most coding transcripts by binding to the 3'UTR of target mRNAs, hence playing a critical role in the coordination of different physiological and pathological processes. The interaction between miRNAs and mRNAs is highly complex and not completely understood [76]. Thus, miRNAs effects on individual genes can be modest, but have the potential to alter cellular responses via a coordinated effect on multiple targets. In addition, miRNA/ mRNA interaction may reflect a partial complementarities thus increasing the number of the off-target effects [77].

A number of serum miRNAs whose specific expression patterns could be associated to cancer, diabetes or cardiovascular diseases have been detected [78-79] making them potential biomarkers for disease detection. In this sense, there are sound evidences that miRNAs control vascular inflammation, with miR-21, miR-126, and miR-155 being reported as regulators of vessel remodeling [80], and miR-21and miR-155 being involved in foam-cell formation [76,81]. Furthermore, a number or reports showed that lipid uptake by macrophages was regulated by miR-9, miR-125a-5p and miR-155 [82], while miR-33, miR-106, miR-122 and miR-144 controlled lipid homeostasis and miR-758 targeted transcripts involved in cholesterol metabolism and fatty acid oxidation [75]. On the other hand, miR-17-5p, miR-20a, miR-106a and miR-424 have been shown to regulate monocyte/macrophage differentiation [80], and miR-92a, the cluster miR-17-92 and let-7 were involved in angiogenesis [82]. Furthermore, it has been also established a functional link between hypoxia and the expression of a specific group of miRNAs [83], among them miR-125b, a multifunctional miRNA involved in the regulation of apoptosis, proliferation and maintenance of stem cell homeostasis [84]. Interestingly, mature miR-125b is transcribed from two different loci, miR-125b-1 at 11q24.1 and miR-125b-2 at 21q21.1 [85], and upregulation of miR-125b has been seen to increase the risk to coronary heart disease [86]. Expression of miR-125b has been also related to the ATH process through its ability to down-regulate the expression of podocalyxin (PODXL), an adhesion molecule of endothelial cells [87]. In this context, we have recently described the up-regulation of miR-125b in an experimental model of atherosclerosis progression, as well as in human atherosclerotic plaques [88]. Those changes were reversed upon CD40 silencing [88].

5.- GENOMIC Alu ELEMENTS IN SICKNESS AND IN HEALTH

The structure of the human genome is highly complex. Protein-encoding regions represent only over 2% of the entire genome, and are immersed in a “dark matter” of non-coding sequences [89]. Formerly considered as "junk DNA", these regions include a number of transcribed non-coding RNAs as well as different mobile elements as endogenous retrovirus, DNA transposons and RNA-mediated retrotransposons, which have shaped the human genome along the evolution, and with many of them having specific roles in gene regulation [90]. The role of these mobile elements in transcriptional regulation is so important that the term *mobilome* has been coined to describe transposable elements involved in the coordination of gene expression as well as in the engineering of transcriptional networks [91].

As already stated, approx. one million of Alu elements have colonized the human genome by retrotransposition, mostly to gene-rich areas. This process of invasion, not only has had a major impact on the structure of the human genome in normal conditions (health), but also has shaped the genomic landscape for disease. A first consideration is that the retrotransposition process requires or would benefit of an "open" accessible chromatin structure to proceed, so that retrogressed elements would show a trend to concentrate in regulatory or gene rich regions. In this sense, a recent work by Gu et al, who used technologies based on chromosome conformation capture, showed that density of Alu elements correlated strongly and positively with functional DNA elements like enhancers and promoters [92]. Furthermore, there are also functional evidences of the integration of Alu elements in human genomic regulatory regions. Thus, and without being exhaustive (since this is not the aim of this review), a T-cell-specific enhancer, which contained an Alu element, was located in the last intron of the human CD8 alpha gene [93] while other repetitive Alu elements in its neighbourhood had the ability to form a cruciform structure that regulated the function of the *CD8* alpha enhancer [94] , and the human growth hormone (*HGH*) gene was shown to contain a functional silencer element within an Alu repeat in its 3'-flanking region [95]. This close involvement of Alu elements with regulatory regions has lead several authors to propose that retrogressed Alu repeats could be the source for new functional sites, as Pol II transcription factor binding sites, which would contribute to the generation of new regulatory networks [31], cryptic/alternative splice sites [96], or nuclear receptor binding sites [97] , so that Alu elements could be considered as a large reservoir of potential regulatory functions which contributed to the evolution of the mechanisms regulating gene expression [29], or even of new functional genes [98].

On the other hand, Alu elements have been also related with the onset of a number of human diseases [30, 16] by different mechanisms: by causing genetic deletions and duplications [99], through insertional mutagenesis [100] or by contributing to the alteration of the methylation patterns of DNA [101]. In this sense, genomic regions highly enriched in Alu elements are considered as intrinsically unstable since these can be targeted by the homologous recombination machinery, due the high homology among Alu sequences to cause diseases [102]. Thus, to give a few examples, a retroinserted Alu element was shown to be causative of neurofibromatosis 1 (NF1) by inactivating a downstream exon during splicing and consequently shifting the reading frame of the *NF1* gene [103], a deletion which occurred between two Alu-repetitive sequences in the same orientation was shown to inactivate the low-density lipoprotein (*LDL*) receptor gene in Korean patients suffering familial hypercholesterolemia (FH) [104] , and Alu-mediated recombinations (leading to exon skipping) were implicated in the origin of Hunter disease [105, 106].

6. Alu ELEMENTS PLAY MULTIPLE ROLES IN THE PROGRESSION OF THE ATHEROSCLEROSIC DISEASE

6.1 ANRIL and Alu elements: two for tango.

As stated above, *ANRIL* (*Antisense Noncoding RNA in the INK4 Locus*) is a lncRNA-encoding genomic locus which harbours the strongest risk alele for atherosclerosis known to date [107]. Much interestingly, a recent work has highlighted a link among the regulation of *ANRIL* function and the presence of Alu elements [56]. In this work, authors first performed an expression analysis to characterize the pattern of *ANRIL* isoforms expressed in human PBMCs and in the monocyte cell line MonoMac, while subsequent analysis of *ANRIL* expression in CAD patients and controls demonstrated that *ANRIL* expression was significantly increased in those samples harbouring the risk allele. Much interestingly, *ANRIL* over-expression caused the upregulation of other mRNA transcripts related to cell adhesion, growth and proliferation, an effect that could be reversed by down-regulating *ANRIL* with a specific siRNA [56]. The mechanism by which *ANRIL* was able to regulate the expression of a number of genes in trans required the binding to *ANRIL* of Polycomb-group proteins PRC1/PRC2, CBX7, and SUZ12, among others, which were recruited to the promoters of their target genes upon *ANRIL* over-expression. Bioinformatic analysis of *ANRIL* and of the promoter regions of *ANRIL*-targeted genes highlighted the common presence of Alu elements in both suggesting that *ANRIL* might bind to chromatin through interaction via the Alu motif to guide PRC proteins to *ANRIL*-regulated genes to modify their expression. In this way, *ANRIL* over-expression would increase cell proliferation and adhesion and decrease apoptosis, thus modulating pro-atherogenic cell functions, while, on the contrary, *ANRIL* silencing reversed trans-regulation and normalized cellular functions [56] (Figure 2A).

On the other hand, the *ANRIL* murine ortologous is encoded in chromosome 4 [108] although the locus is not fully conserved between mice and human [109]. Interestingly, a murine mutant showing a 70 kb deletion of non-coding DNA at the *Anril* locus, which included the risk allele, showed a markedly decreased expression of *Cdkn2a* and *Cdka2b*, as well as an increased proliferation and diminished senescence of primary aortic smooth-muscle cells (SMCs) in culture [108], a fact strongly supporting the hypothesis that the *ANRIL* locus could be implicated with the pathogenesis of CAD. Nevertheless, the fact that mice do not have "bona fide" Alu elements [110] but the structurally related B1 elements (see [111], for a recent review), makes it difficult to determine the mechanism of action and compare it with that of the human *ANRIL*.

6.2- Interaction of Alu-RNAs and miRNAs creates complex regulatory networks that are altered in disease

As described above, many Alu elements in the human genome have functional RNA Pol III promoters (termed A/B boxes) in their 5' end which give them the ability to be transcribed autonomously and independently of the RNA Pol II transcriptional machinery [12, 26]. Much interestingly, it has been recently described that Alu sequences can serve as promoters for RNA-Pol III- dependent (Pol II-independent) miRNA transcription [112], and over 50 miRNAs have been detected within Alu and other known repetitive elements [113]. In this sense, bioinformatic analysis has shown that 5% of intronic miRNA genes contained functional upstream Pol III-dependent Alu regulatory elements (A/B boxes) which would allow miRNA expression independently of the host gene (Pol II-dependent) transcription, thus explaining discordant expression between a miRNA and its host gene [114], although currently only a few Pol III-dependent miRNAs have been functionally characterized [112].

On the other hand, there are several reports on the mutual functional interference between Alu elements and miRNAs resulting in the inactivation of one of these transcripts. Thus, almost 30 human miRNAs exhibited short-seed homology with highly conserved Alu sequence elements located at the 3' UTRs of human mRNAs, suggesting that these Alu sequences could serve as microRNA targets [115], and Daskalova et al.

showed that the majority of the Alu sequences inserted in 3'UTRs of the human genes analyzed carried strong potential target sites for over 50 different miRNAs [116]. More recently, Di Ruocco et al., have shown that Alu RNA induced epithelial-to-mesenchymal transition (EMT) in colorectal cancer cell lines by acting as a molecular sponge of miR-566 [117]. Furthermore, miR-15a-3p and miR-302d-3p, which are elevated in the stress response, were shown to target RAD1, GTSE1, NR2C1, FKBP9 and UBE2I exclusively within Alu elements [118], and targeting of Mdm2 and Mdm4 by miR-661, which causes their down-regulation with the subsequent augment of p53 activity and inhibition of cell cycle progression in p53-proficient cells, was shown to occur whithin Alu elements [119]. In this context, and although much more work has to be done on the interaction among Alu RNAs and miRNAs, it is currently accepted that individual Alu RNAs could behave as molecular sponges for miRNAs involved in atherosclerosis progression and impact in disease development (Figure 2B).

6.3 Alu elements are common binding sites for transcription factors such as NF-κb and may impact on gene expression of the inflammatory response.

NF-κB proteins are critical regulators of the immune response with a substantiated role in ATH progression in animal models of ASVD [120] and in human ASVD [121, 122] patients. Activated NF-κB has been localized in vascular endothelial cells (VEC), smooth muscle cells (SMCs) and lymphocytes in the vasa-vasorum of abdominal aortas with atherosclerotic plaques from deceased patients [122]. NF-κB can be activated through two different, canonical and non-canonical, pathways. In the canonical pathway, NF-κB nuclear factors (RelA or p65, RelB, c-Rel, p50 and p52) are maintained inactive in the cytoplasm by interacting with IκB inhibitory proteins. Receptor triggering (PRRs, TNFRs, TCR, BCR, etc.), activates IKKs which cause IκB phosphorylation and their subsequent proteasomal degradation, thus facilitating the nuclear translocation of NF-κB RelA/p50 and the subsequent activation of NF-κB downstream target genes [123]. In the alternative, non-canonical NF-κB pathway, NIK (NF-κB inducing kinase) activates IKKκ, which phosphorylates and process p100 to p52, inducing the formation of a transcriptionally active RelB/p52-complex [123]. In this context, we have recently described, the up-regulation of the activator *IKK*κ and the down-regulation of the *IKBα* inhibitor during disease progression in the experimental ApoE$^{-/-}$ model of ATH [88] suggesting a role of a canonical NF-κB activation in ATH progression.

NF-κB nuclear factors (RelA, RelB, c-Rel) bind to the consensus κB site (5'-GGGRNYYYCC-3') in the promoters or enhancers of target genes [123]. Much interestingly, NF-κB has been shown to also bind to many non-consensus sites [124] of which near 10% have been detected in Alu-repetitive elements (termed Alu-κB elements) [125]. Although, only a few of them have been directly correlated with changes in the expression of the associated genes, it has been suggested that these Alu-κB elements could perform other cell type-specific functions, as sequestering transcriptionally inert NF-κB molecules to allow competent factors to activate target genes but preventing excessive targeting and superactivation of promoters [125].This data suggest that Alu elements combined with other nearby cis-acting elements might play an important role in expanding the repertoire of NF-κB binding sites to engage new genes into NF-κB-dependent regulatory networks (Figure 2C). Furthermore, genome-wide Chip.seq in different individuals and cell lines has demonstrated that binding NF-κB sites are polymorphic and can differ by over 7.5% among individuals. Most of these differences were in SNPs in intergenic regions and correlated with differences in gene expression, indicating that polymorphic variation in binding sites could have functional consequences [126]. Although no data is available on how much of that binding variation occurs in Alu elements, it could be expected that significant differences in transcription factor binding sites as well as in gene expression between individuals could be due to polymorphic Alu repeats.

6.4 A polymorphic Alu insertion controls the renin-angiotensin system

It is known that elevated levels of angiotensin II (Ang II) contribute to vascular disease, and that kidney plays a critical role in the regulation of the Renin-Angiotensin-Aldosterone System (RAAS) [127]. Kidney renin is released into blood where it cleaves circulating angiotensinogen into angiotensin I, which is subsequently transformed in Ang II by the angiotensin converting enzyme (ACE) produced in the vascular endothelium. Ang II may accelerate ATH through activation of factors such as NF-κB, adhesion molecules, TGF-β or endothelin-1, thereby inducing vascular growth, cell migration and inflammation [128]. In addition, Ang II is a potent stimulus for prooxidant enzymes leading to an increase in reactive oxygen species (ROS) production, and consequently increased oxidative stress. On the contrary, blocking RAAS has demonstrated beneficial effects for the treatment of cardiovascular and renal disease [129].

Alu elements are involved in the regulation of RAAS, and consequently in the progress of renal [130] and cardiovascular diseases [131], by virtue of a polymeric Alu insertion in the intron 16 of the ACE gene which gives rise to two different alleles: the "insertion allele" (I allele) and the "deletion allele" (D allele). The insertion of the Alu element (I allele) in the ACE gene resulted in an ORF shift which caused the premature termination of the ACE protein and originated a protein with a single active site in the N-terminal domain [29]. Homozygous D/D individuals have plasma ACE levels about twice as high as those homozygous I/I individuals [132], and diminished levels of tissue ACE [133]. Surprisingly, the Alu repeat was found to also upregulate the transcriptional activity of ACE promoter [134]. However, association studies on the I/D polymorphisms of ACE gene and cardiovascular outcomes are still controversial due to the lack of powered studies and the existence of interactions with other genes or environmental factors [135].

7. CONCLUDING REMARKS AND FUTURE TRENDS

CKD-dependent atherosclerosis is a very complex disease whose development requires the functional integration of a number of gene networks in different organs and tissues. Although great efforts have been devised to explore the landscape of molecular alterations which underlie the development of ATH, and despite the wealth of knowledge generated, we still have a limited vision of most of the mechanisms involved in ATH development, their interactions or their mutual interferences. Clearly, new approaches for ATH research are required to integrate new tiers of information and new regulatory layers. In this sense, chaos theory and the study of non-linear dynamic systems offers new conceptual approaches and insights to better understand highly complex problems [136]. Chaotic systems, which can be defined as deterministic but not predictable, are characterized by their exquisite sensitivity to their initial conditions which will develop by following the trajectories of strange attractors of fractal nature [137]. A number of authors have proposed a chaotic component for the development of atherosclerosis [138-140], hypothesis that has been confirmed by work by Xiao et al, who used a chaos theory-derived nonlinear prediction method to highlight deterministic (non-random) structures in the repetitive Alu elements caused by their dimeric composition, a likely basis for a non-linear regulatory behaviour [10]. These results were further backed by Moreno et al., who used a multifractal approach to study the human genome, showing that multifractality was strongly correlated with the presence of repeated elements of the Alu family [49]. These works made conceptual links among disease (ATH), the overall structure of the human genome, and focused our attention in the functional involvement of the repeated elements of the Alu family in ATH progression. In conclusion, the advent of the genomic revolution has highlighted the involvement of non-coding DNAs and RNAs in human diseases. Here we have reviewed data on the role of two of these, the lncRNA ANRIL and the family of Alu repeated elements on ATH onset and progression. Although much work remains to be done on these (and other) non-coding elements, it is becoming clear that the natural history of human disease is no longer a question of proteins and coding genomic regions, and that non-coding regions do have an important role in disease development.

DECLARATIONS:

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

Please contact author for data requests.

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The contribution of each authors were as follows: MH conceived and drafted the manuscript, EN conceived and drafted the manuscript, JC provided intellectual content of critical importance to the work, and JT helped to draft the manuscript. All authors read and approved the final manuscript.

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FIGURE LEGENDS

Figure 1. Structural features of Alu repeated sequences.

Shown are the distinctive elements that conform an Alu monomer. The two arms linked by an A-rich sequence, the bipartite A,B boxes) Pol III promoter and the poly-A tail. Graphic not drawn to scale.

Figure 2. Possible mechanisms by which Alu repeated sequences impact on ATH progression.

(A). Alu elements regulate the function of ANRIL, the strongest risk factor for atherosclerosis and CVD.

ANRIL RNA, which harbors Alu elements, is transcribed and recruits the Polycomb Repressive Complexes 1 and 2 and interacts with other genes via an "Alu/Alu" or "Alu/other site" direct interaction, thus facilitating PRC1/2 to regulate their expression. Taken from [56]. Graphic not drawn to scale.

(B). Alu-RNAs could behave as miRNAs sponges to create complex regulatory networks that are altered in disease.

Shown are the main elements implicated in the Alu/miRNA regulatory loop: Alu genes, free Alu-RNAs, miRNA genes and miRNAs. The postulated Alu/miRNA interaction does not consider the folding of the Alu elements nor the existance of Alu [miRNA] binding proteins that could impact on the interaction. STRESS stands for any stimuli that upregulates transcription of free Alu elements as glucocorticoids [141], HIV infection [142], adenovirus type 5 [143], or type 2 infection [144], herpex simplex virus infection [145] or heat-shock [146].Graphic not drawn to scale.

(C). Several Alu elements are binding sites for transcription factors such as NF-κb and may impact on gene expression of the inflammatory response.

Retrogressed Alu elements can function as NF-κb binding sites thus expanding the set of genes co-regulated by NF-κb in the inflammatory response (se main text for details). Shown are the main elements implicated in the Alu/ NF-κb regulatory loop: Alu genes, free Alu-RNAs, Alu cDNAs and their retrogression to gene regulatory regions. STRESS definition as in (B). Graphic not drawn to scale.

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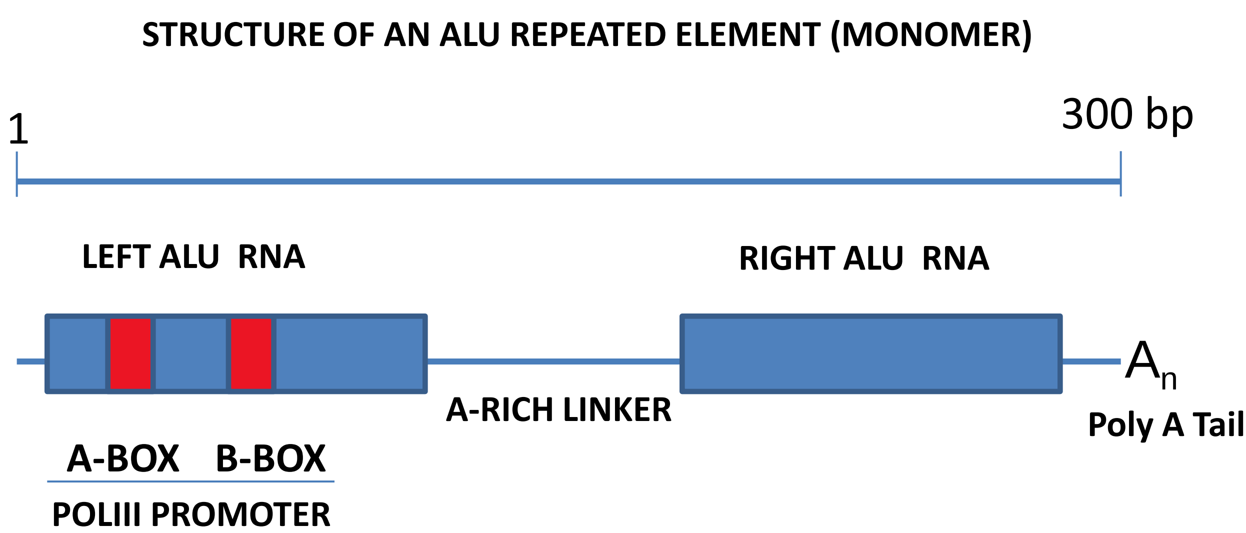

FIGURE 1

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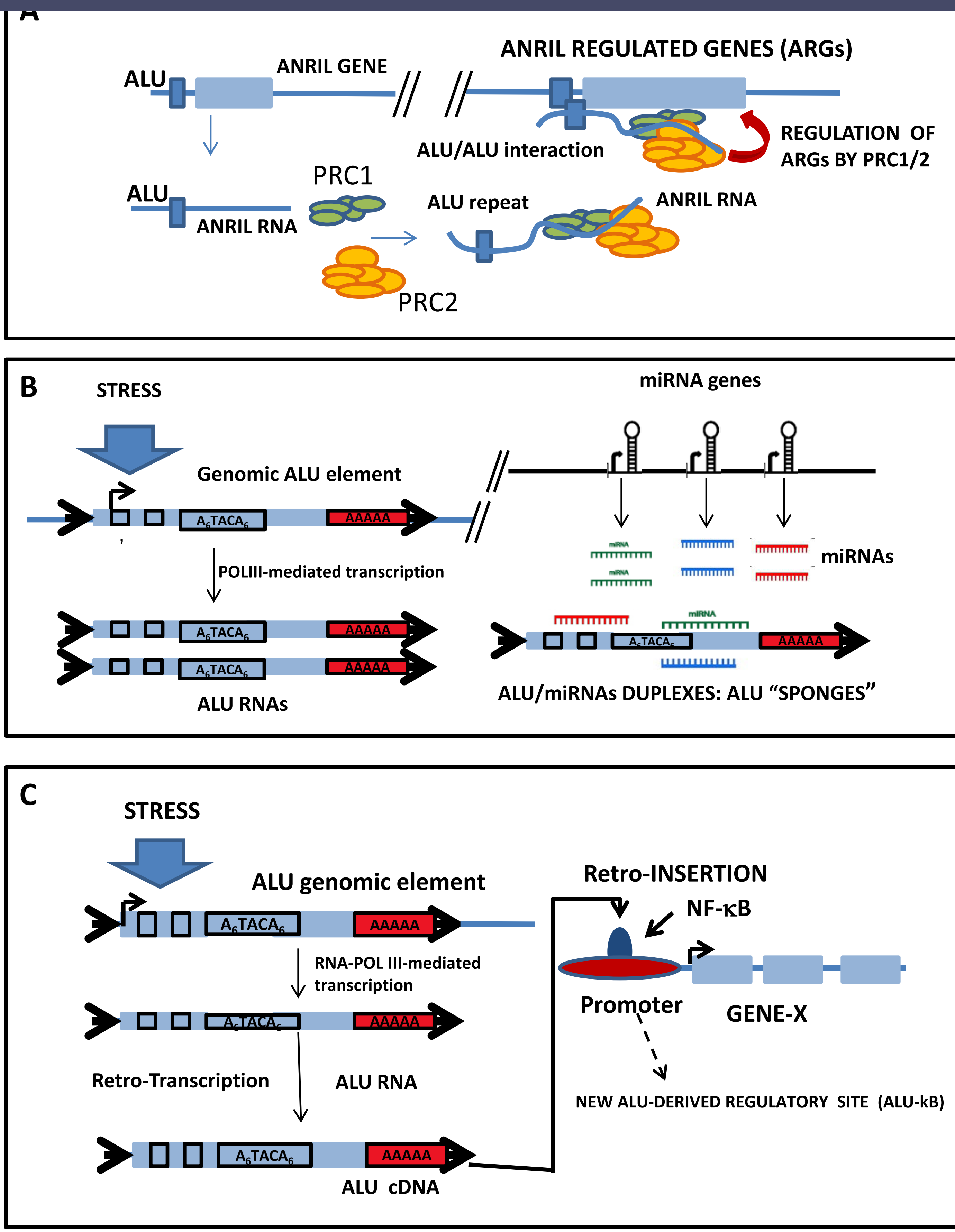

FIGURE 2