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REVIEW

Translational medicine

Emerging roles for RNA-binding proteins as effectors and regulators of cardiovascular disease

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The cardiovascular system comprises multiple cell types that possess the capacity to modulate their phenotype in response to acute or chronic injury. Transcriptional and post-transcriptional mechanisms play a key role in the regulation of remodelling and regenerative responses to damaged cardiovascular tissues. Simultaneously, insufficient regulation of cellular phenotype is tightly coupled with the persistence and exacerbation of cardiovascular disease. Recently, RNA-binding proteins such as Quaking, HuR, Muscleblind, and SRSF1 have emerged as pivotal regulators of these functional adaptations in the cardiovascular system by guiding a wide-ranging number of post-transcriptional events that dramatically impact RNA fate, including alternative splicing, stability, localization and translation. Moreover, homozygous disruption of RNA-binding protein genes is commonly associated with cardiac- and/or vascular complications. Here, we summarize the current knowledge on the versatile role of RNA-binding proteins in regulating the transcriptome during phenotype switching in cardiovascular health and disease. We also detail existing and potential DNA- and RNA-based therapeutic approaches that could impact the treatment of cardiovascular disease in the future.

Keywords Cardiovascular disease • Post-transcriptional gene regulation • RNA-binding proteins • Alternative splicing • RNA therapeutics

Introduction

The modulation of cellular phenotype is intimately intertwined with organ function, repair upon injury, and the pathophysiology of disease.[1–4] The cardiovascular system possesses numerous cell types, such as vascular smooth muscle cells (VSMCs), endothelial cells (ECs), monocytes and macrophages and cardiomyocytes, that are conferred with the capacity to undergo phenotypic switches in response to acute or chronic injury that serve to limit tissue damage and restore proper cardiovascular function.[3,5,6] However, these reparative cellular phenotypes can also drive the onset, persistence, and exacerbation of cardiovascular disease (CVD; *Figure 1*). An example of this phenomenon is the pre-stenotic fibroproliferative response of medial VSMCs as a result of endothelial denudation of the coronary artery after percutaneous coronary interventions or in coronary artery bypass grafts due to procedure-related stress factors.[3,7–9] In contrast to VSMCs, the cardiomyocyte adaptation to injury is characterized by an increase in cell size (hypertrophy), enhancement of protein synthesis, and more pronounced organization of the sarcomere.[10] Another class of environment-induced phenotypic switches that are critical in CVD pathogenesis are inflammatory cells. In particular, the differentiation of monocyte subsets into various highly plastic macrophage phenotypes profoundly impacts atherosclerotic lesion development and progression.[5,11–14]

Despite the knowledge that cellular phenotype switching is pivotal for CVD development, the treatment of CVD has primarily focused on

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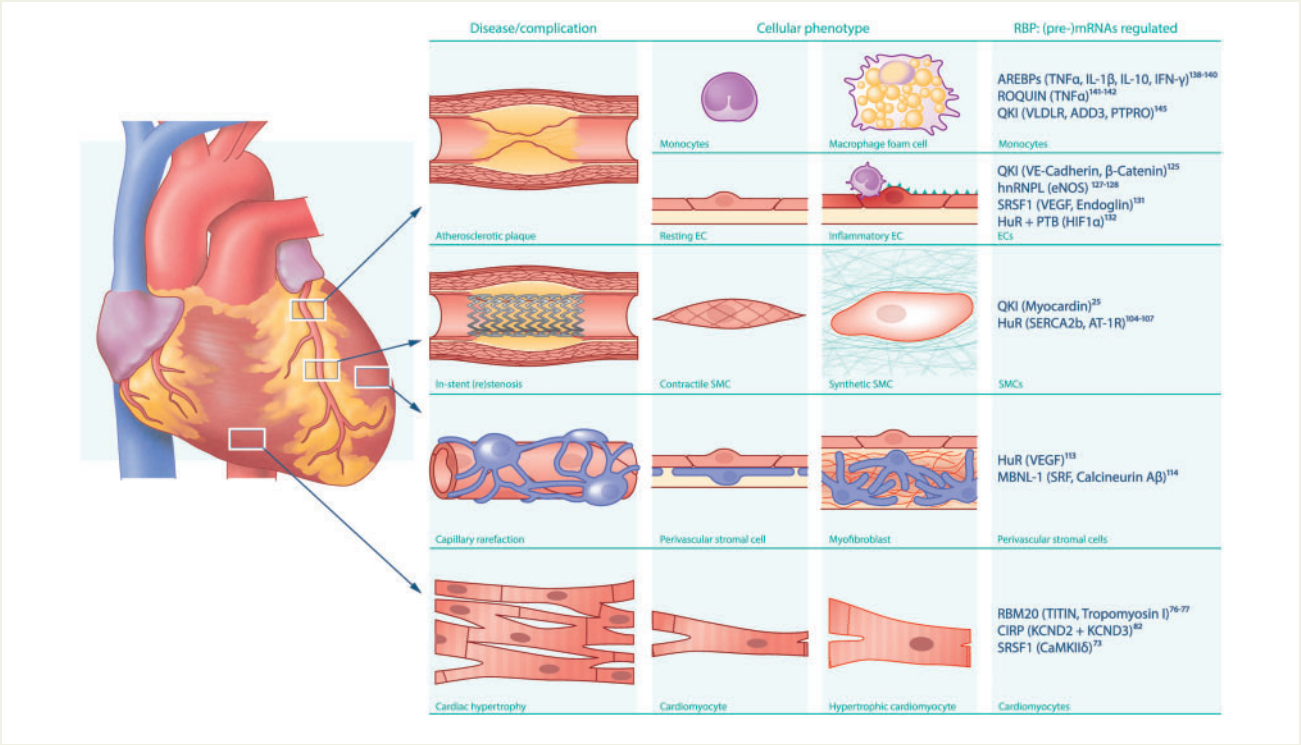

Figure 1 RNA-binding proteins (RBPs) control cellular phenotype changes that determine cardiovascular health and disease. Schematic depicting cardiovascular diseases or complications (left panels) and associated cellular phenotype changes that influence their development (middle panels). During these phenotypic conversions, RBPs have been established to mediate post-transcriptional events that dramatically impact (pre-)mRNA fate (right panels), including alternative splicing, stability, localization, and translation. In atherosclerosis, this involves the adhesion of monocytes and their conversion to macrophages at sites where the endothelium is damaged (top). The adoption of a fibroproliferative phenotype by VSMCs or perivascular stromal cells leads to vascular (re)stenosis and capillary rarefaction, respectively, whereas cardiac hypertrophy is associated with myocyte enlargement that results in thickening of the ventricular walls of the heart to ensure sufficient cardiac output.

combating proteins responsible for generating unfavourable lipid profiles. An excellent clinical example of this biology is represented by statins, which aside from their lipid-lowering capacity, skew ECs to an atheroprotective phenotype by promoting their anti-inflammatory and anti-thrombotic properties. Mechanistically, statins transcriptionally repress NF-κB signalling[15] while simultaneously inducing shear-responsive transcription factor Krüppel-Like Factor 2 (KLF2), driving expression of anti-inflammatory markers such as trombomodulin and eNOS.[16,17] Simultaneously, statins activate microRNA (miRNA) expression profiles that are essential for the maintenance of EC health by enhancing transcription and activity of anti-apoptotic and pro-angiogenic AKT signalling pathways.[18,19] Therefore, adaptations in cellular function in disease settings are associated with dynamic changes at the transcriptional and post-transcriptional levels, suggesting that therapeutic targeting of factors that drive these processes could shift cellular phenotype from a disease-advancing to a regenerative state (*Figure 1*).[20–22]

RNA-binding proteins (RBPs) are rapidly emerging as pivotal players in this biological script, as they are intimately involved in co-ordinating all aspects of (patho)physiological RNA processing and gene expression.[22–25] In the past decade, extensive work has elucidated the sequence to which many of these RBPs bind,[26–31] enabling one to identify putative RNA species specifically targeted by individual RBPs. Importantly, this knowledge, when coupled with the recent development of sophisticated delivery methods[32] for RNA-based therapeutics,[33,34] provides the interesting possibility of modifying the transcriptome by altering RBP expression or activity, or targeting specific RBP-mediated events, making it possible to direct molecular pathways involved in disease pathogenesis.

The RNAissance: new insights into genomic complexity

Remarkably, the human and roundworm genomes (and other less complex organisms) encode similar numbers of genes (approximately 20 000),[35] indicating that the number of encoded genes does not directly determine organismal complexity. In the slipstream of the development and utilization of revolutionary tools that enabled scientists and clinicians to sequence (human) genomes and transcriptomes, came the realization that organismal complexity evolved with an increased capacity to regulate gene expression at the post-transcriptional level. Following the human genome project, attempts to better understand our genome culminated in the Encylopaedia of DNA Elements (ENCODE), a project designed to identify the 'functional elements' in the human genome.[36] This worldwide collaborative effort exponentially expanded our understanding of regulatory elements in our genome that affect human health and disease, and

divided the genome more definitively into protein-coding and non-protein-coding transcribed portions of our genomic DNA.[36] Previously, it was established that about 1.2% of the human genome codes for protein-encoding mRNA precursors, whereas an astounding majority contains information allowing for the generation of a large variety of non-protein-coding RNA species, including microRNAs (miRs), long non-coding RNAs, piwi-interacting RNAs, small nucleolar RNAs, and small nuclear RNAs.[37,38] These non-protein-coding RNAs are widely considered to control the activation or repression of gene expression in response to, e.g. developmental and environmental cues such as aging, metabolic stress, cancer and inflammation,[39–44] whereas the cellular functions of an extensive list of other non-protein-coding RNA species are currently unclear. This review will focus on the regulatory role of RBPs and events they catalyse, as the biology on non-coding RNAs in CVD has been detailed in numerous outstanding reviews.[38,44–48]

RNA-binding proteins: directors of post-transcriptional regulation

Concomitant with their transcription, nascent pre-mRNA molecules are covered with a myriad of RBPs that collectively form ribonucleoprotein structures (RNPs). The dynamic formation of these RNPs determines all facets of RNA fate, including splicing, stability, cellular localization, and rate of translation (*Figure 2*). It is estimated that the human genome encodes more than 700 RBPs.[26] These RBPs have been divided into families based on evolutionarily conserved RNA-binding motifs that confer the capacity to bind target RNAs in a sequence-specific fashion.[28,29,49,50] RBPs interact with target (pre-)mRNAs at the 5′- and 3′-untranslated regions (UTRs), as well as at non-coding (intronic) and coding (exonic) regions. Importantly, the region to which a given RBP binds on a target (pre-)mRNA generally influences the event that is catalysed, illustrating how these proteins dynamically and spatially impact gene expression (*Figure 2*). Finally, RBPs serve as global and cell-type-specific regulators of gene expression, where competition for access to target RNAs is determined by expression levels of individual RBPs in health and disease.[51–53]

Given that splicing of pre-mRNAs markedly impacts mature mRNA fate and the protein repertoire in healthy and diseased cells, it is important to provide a brief overview of splicing biology. Firstly, pre-mRNAs containing sequence encoding protein generally require RBP-guided excision of intronic sequences by (i) constitutive splicing; and/or (ii) alternative splicing, which when combined can lead to functionally distinct mature mRNAs.[52,54,55] Splicing requires the dynamic assembly of RBPs into the spliceosome, a highly organized intra-nuclear structure consisting of RBPs and small RNA complexes, called heterogeneous ribonucleoprotein particles (hnRNPs).[55–57] The spliceosome also plays a central role in alternative splicing,[52] as the binding of RBPs to consensus sequences proximal to exons limits spliceosomal accessibility to 5′- and/or 3′-splice junctions, promoting the formation of splice variants (*Figure 2*).[20,23,52,54] ‘Alternative’ splicing occurs in an estimated 80–90% of protein-coding genes, with recent estimates indicating that this process is responsible for generating more than 200 000 unique protein-coding transcripts in humans.[58] Interestingly, although the core spliceosomal proteins are expressed in almost all individual cell types, including anucleate platelets,[59] it is the differential expression and modifiable activity of RBPs that has been pinpointed as defining tissue-specific splicing patterns.[20,54,60] Clinically relevant examples of alternatively spliced transcripts that influence CVD risk are Troponin T,[61] SERCA2a/b,[62] and CETP.[63]

RNA-binding proteins are also critically involved in embryonic development of the cardiovascular system.[64] The Mouse Genome Information (MGI) database provides a comprehensive list of reported gene ‘knockout’ mice and their associated phenotypes.[65–67] Our assessment of this database uncovered a significant proportion of mice with defects in cardiac- and/or vascular development as a result of validated RBP loss (*Table 1*). As foetal gene expression or splicing programmes are often recapitulated in adult disease settings,[68] we elected to analyse the relative expression, extracted from publically available data sets deposited in the NCBI Geo dataset server, of validated RBPs in several specialized cell-types of the cardiovascular system. This analysis clearly illustrates that a vast number of RBPs are abundantly expressed and display cell-type-specific expression profiles (*Figure 3*, see Supplementary material online, *Figure S1* and *Table S1*). The heatmap in *Figure 3* clearly shows the expression levels of individual RBPs discussed in the review, whereas Supplementary material online, *Figure S1* shows the relative expression of more than 300 RBPs in numerous cell-types relevant in CVD (Supplementary material online, *Table S1* provides all the raw data used to generate these heatmaps).

Here below, we will focus on some of the more recent developments and insights into RBP biology gained primarily from human and mouse studies. Collectively, they illustrate the versatility of RBPs in regulating key aspects of cardiovascular health and disease.

RNA-binding proteins in cardiomyocytes: preserving heart function and aiding in post-natal heart remodelling

Insight into the differential expression of RBPs in the adult heart and their critical regulatory role in cardiomyocyte pathophysiology has in part been derived from studies assessing alternative splicing patterns during foetal heart development[69–71] and the discovery that cardiomyocyte dysfunction is associated with a reversion to foetal mRNAs and protein isoforms.[68] In fact, foetal transcripts of key sarcomeric genes, including cardiac troponin T, cardiac troponin I, myosin heavy chain 7, and filamin C-γ were found to be enriched in the setting of human ischaemic cardiomyopathy, idiopathic dilated cardiomyopathy (DCM) and aortic stenosis. Of note, in these aortic stenosis samples, patient inclusion was based on high or low ejection fraction (EF <50%), prompting the authors to postulate that splicing defects could precede heart dysfunction.[72] Interestingly, RBPs have also been found to critically regulate splicing during cardiac remodelling post-natally.[69,73] The RBPs Celf1 and Muscleblind1 (MBNL) were found to guide alternative splicing patterns in mice required immediately after birth for the effective organization of transverse tubules and calcium handling.[69] Further along the developmental timeline, Serine/Arginine-Rich Splicing Factor 1 (SRSF1; also known as ASF/SF2) was found to guide the alternative splicing patterns required for

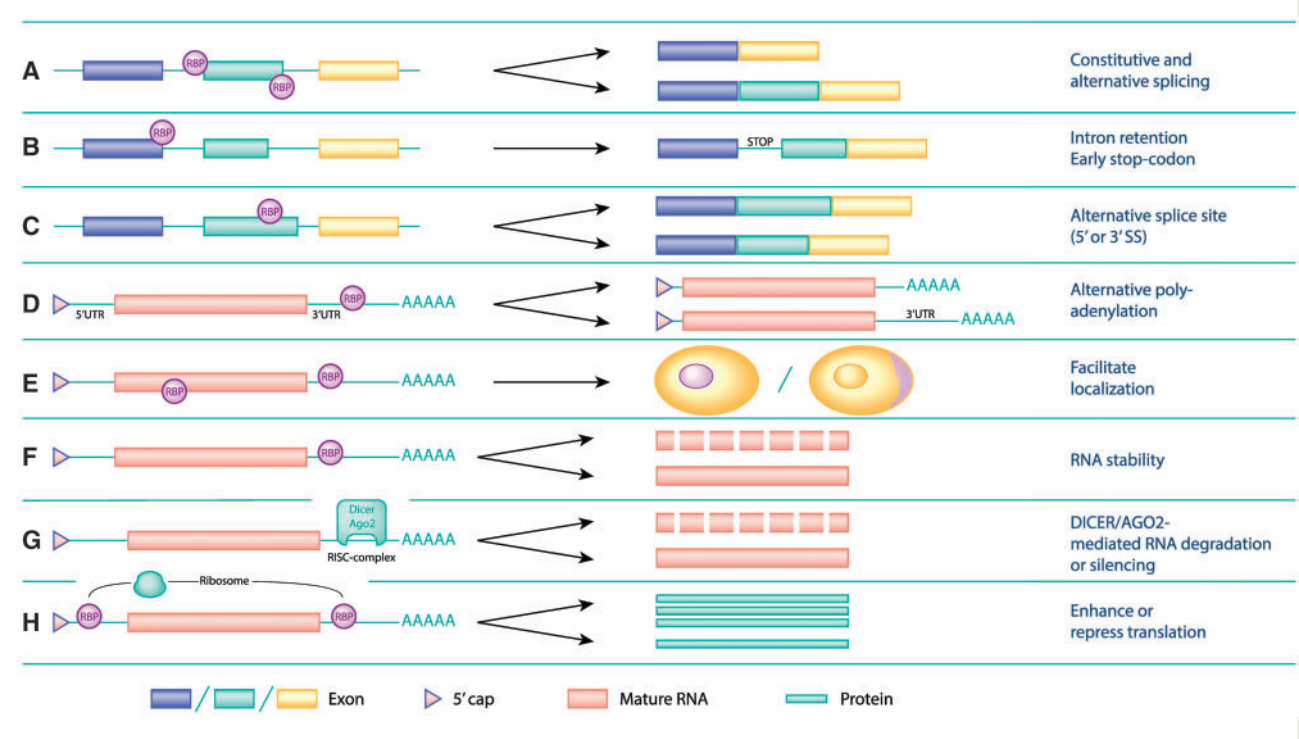

Figure 2 RNA-binding proteins (RBPs) serve as critical effectors and regulators of RNA fate by guiding gene expression post-transcriptionally. The spatial interaction of RBPs with RNA species impacts the post-transcriptional event catalysed, be it: modifications of pre-mRNAs that alter mature mRNA composition including splicing (*A–C*) and alternative polyadenylation (*D*); subcellular localization (*E*); RNA stability (*F, G*); or ribosome-mediated translation of mature mRNAs (*H*). By co-ordinating these events, RBPs are intimately involved in determining the cellular transcriptome, and thereby impact cellular phenotype and function in health and disease settings.

maintaining electrical conductivity in mouse cardiomyocytes during juvenile to adult transition, where in particular defects in CaMKIIδ splicing resulted in severe excitation–contraction coupling defects, triggering a hypercontractile phenotype.[73]

In the adult heart, changes in splicing patterns have also been shown to perturb cardiomyocyte function. The expression levels of several crucial splicing factors (SF1, ZRSR2, SRSF4, and SRSF5) are potently repressed in dysfunctional, low EF cardiomyocytes, and display tight correlations with high EF cardiomyocytes (see Supplementary material online, *Figure S1*). Other studies have identified that a reduction in expression levels of RNA-binding motif protein 20 (RBM20; or MATR3) can lead to DCM in humans. RBM20 is most abundantly expressed in the human heart.[74–76] Common single nucleotide polymorphisms (SNPs) in a RBM20 exonal hotspot were found to significantly associate with an increased risk of DCM due to altered RBM20 expression in humans.[74,75] This finding enabled Guo *et al.*[77] to discover that these genetic variants impair RBP20 function in rats, directly affecting the splicing of a myriad of pre-mRNAs involved in ion homeostasis, sarcomere organization, and diastolic function, such as titin, tropomyosin I, and PDZ- and LIM-domain 5. Genome-wide analyses of RBM20 RNA targets revealed that the protein represses splicing by binding introns proximal to alternatively spliced exons. These studies nicely illustrate how RBPs can orchestrate pre-mRNA processing, thereby serving as molecular switches in gene networks with essential cardiac functions.

The arrhythmias and dilated cardiomyopathies observed in type I myotonic dystrophy (DM1) also illustrate how dysregulated splicing can be causal for heart disease. Recently, expansion of CUG trinucleotides in the 3′ UTR of the DM protein kinase mRNA was found to result in the nuclear retention of the mRNA in affected human cardiomyocytes.[78] Using genetically modified mice that similarly accumulate nuclear CUG-repeat-containing DM mRNAs, this CUG-repeat expansion was found to trigger sequestration of the RBPs CUG-binding protein 1 and CUG-binding protein 2, impairing their ability to participate in splicing programmes required for the maintenance of physiological cardiomyocyte function.[78]

Alongside splicing, RBPs also critically control mRNA transcript abundance and translation of mature mRNAs into protein. For example, expression (and splicing) of the voltage-gated sodium channel SCN5A has recently been described to be regulated by the RBP MBNL1 in cardiomyocytes,[79,80] whereas the RBP PCBP2 was found to inhibit angiotensin II-induced hypertrophy of cardiomyocytes by promoting GPR56 mRNA degradation.[81] Finally, a short QTc interval and abbreviated action potential were observed in cardiomyocytes derived from cold-inducible RNA-binding protein (CIRP)-deficient rats.[82] This phenotype was triggered by an increased transient-outward potassium current due to decreased translation of the KCND2 and KCND3 mRNAs. CIRP binding to mature RNAs enhanced the translation of these essential ion channel subunits, illustrating how loss of this RBP is causal for defective voltage-gated

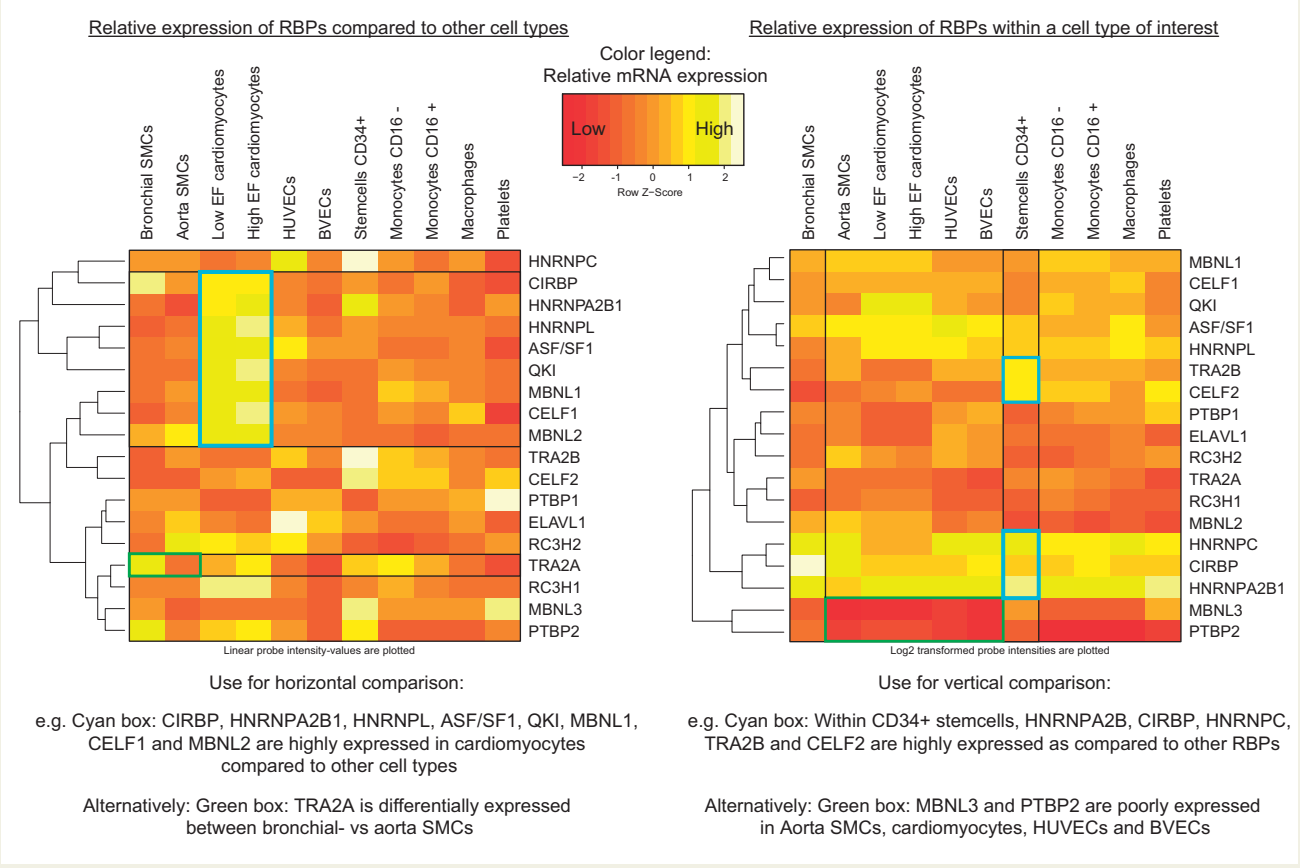

Figure 3 RNA-binding protein (RBP) expression levels in various healthy and diseased vascular cells. Heatmap depicting relative expression levels 317 RBPs in various cell types that are associated with cardiovascular health and disease. Red and white shading depict low and high relative mRNA expression levels, respectively. The heatmap was generated using online available data sets (NCBI Geo dataset server) which were all run on Affymetrix GeneChip® Human Genome U133 Plus 2.0 arrays to allow inter-array comparison (data set references as follows: bronchial SMCs;[161] aortic SMCs;[162] low and high EF cardiomyocytes;[163] HUVECs;[164] BVECs; $CD34^+$ stem cells;[165] $CD16^-$ and $CD16^+$ monocytes;[166] macrophages;[167] platelets[168]). The data were subjected to robust multi-array averaging (RMA) for normalization using the Affymetrix expression console. Subsequently, RNA-binding proteins were selected by cross-referencing the online RBP-database as published by Ray *et al.*,[29] whereafter either linear or Log2-transformed probe-intensities were plotted using R-studio software including the gplots algorithm for heatmap generation.

potassium channel function and reduced bioelectric activity in mammalian hearts.[82]

Collectively, these studies define an important co-ordinating role for RBPs in foetal, juvenile, and adult hearts, while also demonstrating how altered RBP levels can impact cardiac function in health and disease.

RNA-binding proteins in vascular smooth muscle cells and perivascular stromal cells: governors of cellular phenotype and function

Upon vascular injury, VSMCs undergo a well-established phenotypic shift from a contractile to a fibroproliferative, migratory state (*Figure 1*). This physiological response aids in repair of damaged vessels.[3] This VSMC-mediated damage response aids in the resolution of initial damage,[3,5] but is also tightly linked with pathophysiological situations including coronary artery disease, vascular(re)stenosis, atherosclerosis, and peripheral arterial disease.[83–85] Surprisingly, the RBPs that co-ordinate vital splicing events in genes involved in this phenotype switch, such as SM-myosin heavy chain,[86] myosin light chain kinase,[87,88] smoothelin,[89,90] tropomyosin,[91,92] (meta)vinculin,[93] calponin,[94] and caldesmon,[95,96] are largely unknown.

Studies investigating the consequences of knockout of the RBP Quaking (QKI) revealed an embryonic lethal phenotype.[97–99] In keeping with the aforementioned frequent developmental defects in the cardiac and vascular systems of RBP knockout mice, $QKI^{-/-}$ mice displayed an inability to form vitelline vessels, along with defects in pericyte ensheathment of nascent vessels and pericardial effusion.[97–99] More recently, QKI was found to play a critical role in the human, adult vasculature,[25] where VSMC dedifferentiation in response to vessel injury was associated with increased QKI protein

Table 1 RNA-binding protein deficiencies in mice resulting in vascular and/or cardiac developmental defects (pre- and post-natal)

RBP	Binding domain	MGI ID	Phenotype description	References
Vascular phenotype				
QKI	KH	3033861	Lack of SMαA in blood vessel, vascular remodelling incomplete, decreased complexity of brain vasculature, abnormal heart morphology, pericardial effusion, irregular vitelline artery	98
ANKRD17	KH	1932101	Haemorrhages, impaired vascular smooth muscle cell development, impaired vascular integrity, and growth retardation	169
SHARPIN	RanBP ZF	1856699	Perturbed angiogenesis, tortuous dilated capillaries in dermis	170
ZFP36	CCCH ZF	2652418	Reduced blood pressure, vascular inflammation, reduced relaxation upon acetylcholine, EC dysfunction	171, 172
ZFP36L2	CCCH ZF	4360890	Overt gastrointestinal haemorrhage, decreased leucocyte number	173
G3BP1	RRM	3604716	Intracranial haemorrhaging	174
HNRNPD	RRM	3693617	Kidney haemorrhage, altered macrophage function	175
MSI1	RRM	2450917	Intracerebral haemorrhage	176
ELAV1/HuR	RRM	5316082 3847912	Decreased angiogenesis after hind limb ischaemia, abnormal placental labyrinth vasculature	177, 178
TRA2B	RRM	4450921	No vasculogenesis	179
UHMK1	RRM	3832867	Accelerated neointima and more VSMCs after femoral wire injury	180
PABPC4	RRM	4364054	Decreased circulating cholesterol, HDL, and free fatty acid levels	181
Cardiac phenotype				
PPARGC1A	RRM	3511352 3522468	Increased or decreased heart weight, accelerated cardiac dysfunction after aortic constriction, decreased cardiac output, decreased heart rate	182, 183
RCAN2	RRM	3641543	No cardiac hypertrophy upon phenylephrin/angiotensin II infusions, protection against volume overload, increased myocardial damage after ischaemia reperfusion injury	184
SRSF2	RRM	3036846	Extensive fibrosis, myofibril disarray, dilated cardiomyopathy evident after 5 weeks, decreased ventricle muscle contractility	185
PPARGC1B	RRM	3757705	Decreased heart rate elevation after dobutamine	186
SRSF1	RRM	3766573	Hypoplastic pulmonary trunk, signs of tetralogy of fallot complex, ventricular septal defects, overriding aortic valve, transposition of great arteries, suppulmonary stenosis	187
SPEN	RRM	2667509	Defects in the formation of the cardiac septum and muscle	188

RNA-binding protein deficiency is associated with cardiovascular developmental defects. Defective vascular and cardiac development as a result of RBP loss in various mouse models are detailed in this table. The RBPs have been grouped based on their RNA-binding domains for clarity. Data were obtained from the Mouse Genome Information (MGI) database from Jackson Laboratories and RBP knockout mice were selected by cross-referencing the online RBP database as published by Ray *et al.*[29] Human RBP names were extracted from http://cisbp-rna.ccbr.utoronto.ca/bulk_archive.php (1 December 2016), thereafter cross-referenced with the MP:0005385 mammalian phenotype 'cardiovascular phenotype system' from the MGI database to screen for a CVD defect.

levels. This augmentation of QKI enhanced the direct interaction with the Myocardin pre-mRNA (Myocd), driving an alternative splicing event that alters Myocd protein balance to a distinct isoform (named Myocd_v1) that activates proliferative gene expression profiles in VSMCs [via serum response factor (SRF) and myocyte enhancing factor 2-binding domains].[25,100–102] After injury resolution in mice, QKI protein levels subside and Myocd reverts to an isoform that solely interacts with SRF (Myocd_v3), enhancing expression of contractile apparatus proteins and the restoration of VSMC contractile function.[25] Interestingly, the well-established role of Myocd in the human heart, and abundant expression of QKI in cardiomyocytes (*Figure 3*), suggests that QKI could similarly regulate cardiomyocyte-mediated remodelling of the heart following injury.

Increased expression of the RBP HuR was also observed in the setting of neointimal hyperplasia, vein graft specimens, and fibromuscular dysplasias of the human kidney, which all represent clinical manifestations of enhanced VSMC proliferation.[103] Furthermore, HuR was found to stimulate VSMC-mediated vasoconstriction, as the interaction of this stabilizing RBP with the 3′ UTR of the sarco/endoplasmic reticulum Ca^{2+} pump (SERCA2b)[104] and angiotensin receptor type 1 (AT-1R) mRNAs enhanced Ca^{2+} influx and angiotensin II binding, respectively.[105–107] Interestingly, Paukku *et al.*[107] identified that subjects with type 2 diabetes-associated hyperinsulinaemia have increased HuR protein levels, leading to enhanced AT-1R protein levels in VSMCs. This provided novel mechanistic insight into factors that increase CVD risk in patients with type II diabetes, namely via activation of the renin–angiotensin–aldosterone system.[107] Given the

considerable attention gained for the combinatorial use of angiotensin-converting enzyme-inhibitors with AT-1R blockade for the effective management of blood pressure,[108,109] these studies collectively illustrate how RBPs intracellularly impact VSMC-mediated vasoconstriction by determining cell-surface availability of the angiotensin receptor.

In keeping with the central role that perivascular stromal cells play in ensheathing nascent microvessels and stabilizing existing microvessels, it is critical for these cells to regulate the expression of cell–cell adhesion proteins, along with growth factors that stimulate the maintenance of these interactions.[110,111] Moreover, it is well-established that CVD is associated with the disappearance of microvessels (microvessel rarefaction), as a result of progressive perivascular stromal cell loss that is associated with a pro-fibrotic phenotypic shift to a myofibroblastic state.[112] This phenotypic conversion is tightly coupled with capillary destabilization, pathological angiogenesis, and ultimately microvascular rarefaction. Several RBPs have been intimately linked with maintenance of EC–perivascular stromal cell interactions, or mediating a shift to the destabilizing myofibroblast phenotype, including HuR, which was found to drive excessive angiogenesis by stabilizing the vascular endothelial growth factor (VEGF) mRNA.[113] Muscleblind (MBNL-1) was also shown to bind to the 3′ UTR of the SRF mRNA, enhancing expression of this transcription factor that guides perivascular stromal cells differentiation into myofibroblasts in mice.[114] Moreover, MBNL-1 was also found to directly influence alternative splicing of calcineurin Aβ[114], a protein phosphatase that activates T-cell-mediated responses to injury. Importantly, the resultant constitutively active Calcineurin Aβ1 isoform was found to be enriched in both mouse cardiac fibroblasts[114] and cardiomyocytes after myocardial infarction (MI).[115]

These findings suggest that CVD progression could be limited by developing strategies that target RBPs such as HuR, MBNL-1, and QKI or splicing events mediated by these proteins, with the goal of rendering VSMCs quiescent.

RNA-binding proteins in endothelial cells: alternating between function and dysfunction

The activation of ECs by inflammatory stimuli triggers endothelial dysfunction and accelerates CVD onset.[116,117] This pro-atherogenic state is greatly increased in patients with risk factors such as diabetes, renal failure, hypercholesterolaemia, and high blood pressure. Statins have proved to effectively ameliorate endothelial dysfunction in combination with their lipid-lowering effects.[16]

Similar to VSMCs, ECs play a critical role in regulating vascular tone, as EC activation is tightly coupled with a decrease in nitric oxide (NO) bio-availability that triggers vasoconstriction.[118–120] The expression and activity of eNOS, the main enzyme responsible for synthesizing free NO by ECs, is modulated by the shear-responsive and atheroprotective transcription factor KLF2.[17,121–124] KLF2 mediates these effects by binding to the promoter region of shear-responsive genes, including the RBPs QKI and HuR.[125,126] Further evidence that RBPs directly impact eNOS expression, and thereby activity, has been derived from computational analyses[27,28] and experimental studies investigating RBP function in human ECs. Another means by which RBPs impact eNOS biology is through hnRNP L, a protein that by co-ordinating eNOS pre-mRNA alternative splicing triggers the generation of a truncated, dominant negative eNOS isoform.[127,128] Despite evidence indicating that these alternative eNOS isoforms affect NO production, the pathophysiological relevance and consequences on EC function in patients with CVD are at present unknown. Nonetheless, these studies pinpoint an important role for RBPs in maintaining the quiescent EC phenotype.

Another hallmark of the healthy endothelium is the maintenance of barrier function, which requires the formation of tightly linked adherens junctions on adjacent ECs, ensuring the low permeability of the vessel to circulating solutes, proteins, and cells. Strikingly, reports regarding the post-transcriptional regulation of adherens junction proteins are limited. Recently, we discovered that the RBP QKI is highly expressed in quiescent human ECs *in vivo*, and that the specific abrogation of this RBP markedly impaired the capacity to form a high-resistance endothelial monolayer in human ECs and in mice.[125] Mechanistically, QKI appears to be essential for maintaining barrier function by interacting with quaking response elements in the 3′ UTRs of mature β-catenin and VE-cadherin mRNAs, ensuring sufficient translation to restrict vascular permeability.[125]

RNA-binding proteins have also been implicated in the post-transcriptional regulation of several other vital EC-derived factors, including VEGF,[129,130] endoglin,[131] and HIF1α[132]. Along with pivotal roles in tumour-accelerating angiogenesis,[133–135] changes in the abundance and splicing of these pre-mRNAs have also been linked with the development of CVD. An isoform of RBP76 (DRBP76/NF90) was found to bind to the 3′ UTR of the VEGF mRNA, enhancing VEGF production by human ECs,[130] whereas changes in SRSF1 levels in senescent ECs altered VEGF and endoglin pre-mRNA splicing.[131] More recently, a pivotal role for HuR in guiding angiogenesis has been strengthened based on the finding that it enhances translation of the human VEGF mRNA[129] while also working in unison with polypyrimidine tract binding protein (PTB) to enhance translation of HIF1α by binding to distinct sites on the human HIF1α mRNA, namely the 5′- and 3′ UTRs, respectively.[132] Although the mechanism by which these factors drive CVD is incompletely understood, a potential explanation linking their established role in cancer biology and CVD is that they could stimulate the formation of vasa vasorum in large vessels. This could accelerate lesion formation as it enhances the supply of essential nutrients and pro-atherogenic factors to sites of vessel injury.

RNA-binding proteins in monocytes and macrophages: co-ordinating inflammatory responses to injury

In acute and chronic disease settings, circulating monocytes are exposed to diverse stimuli that generally triggers their activation into pro-inflammatory phenotype,[136,137] followed by their homing to sites of tissue injury and differentiation into macrophages. As cellular differentiation is tightly coupled with the dynamic regulation of mRNA stability, splicing patterning, and mRNA localization, RBPs are ideally

positioned to post-transcriptionally co-ordinate events that determine monocyte and macrophage function. Indeed, AU-rich element binding proteins (ARE-BPs) have long been known to tightly control the expression of a plethora of cytokines and chemokines in monocytes and macrophages, including TNF-α, GM-CSF, M-CSF, IL-1β, IL-6, IL-10, and IFN-γ.[138] The RBP-mRNA interaction at AREs encoded in the 3′ UTR of these target (pre-)mRNAs[139] triggers rapid mRNA decay.[140] More recently, diversification of cytokine-regulating RBPs was made with the discovery that the Roquin RBPs specifically bind to stem-loop structures [termed constitutive decay elements (CDEs)] in the 3′ UTR of target mRNAs in mice.[141] The 3′ UTR of mouse TNF-α mRNA contains such a CDE, conferring Roquin with the capacity to bind to and destabilize the mRNA. Importantly, although these experiments were performed in mice, interaction of the human Roquin-1 and -2 isoforms with an mRNA containing a CDE was recently confirmed by X-ray crystallography.[142] As such, in concert with ARE-BPs, Roquins are likely responsible for ensuring a limited window of TNF-α expression in response to tissue injury in humans. These studies indicate that the induction, as opposed to targeting of certain RBPs, could serve as a novel approach for repressing the inflammatory component of atherosclerosis.[143]

Very recently, four SNPs were identified proximal to the QKI locus, revealing a nominally significant association with incident MI and coronary heart disease (CHD).[144] This two-stage full genome-wide association studies (GWAS) analysis of more than 64 000 individuals (with 3898 MI cases and 5465 CHD cases) pinpointed QKI as a novel predictive locus for incident CHD in prospective studies.[144] Although the authors did not detail the pathophysiological mechanism for this association, the recent discovery that QKI mRNA and protein are induced in macrophages of advanced human plaques, and that depletion of QKI protein in primary human monocytes significantly impaired: (i) monocyte adhesion and migration, (ii) differentiation into pro-inflammatory macrophages, and (iii) foam cell formation *in vitro* and *in vivo*, suggest that this RBP plays a central role in guiding inflammatory processes that accelerate CHD. Transcriptome analysis of monocytes and macrophages derived from a unique QKI haploinsufficient individual suggests that this phenotypic conversion is reliant on QKI-mediated changes in pre-mRNA splicing and mRNA transcript abundance.[145] Collectively, these studies indicate that RBPs such as QKI can post-transcriptionally guide pro-inflammatory macrophage identity and function.

Therapeutic targeting of RNA-binding protein-mediated events: harnessing the power of RNA regulation in human disease

Strategies geared towards augmenting gene expression represent a powerful means of correcting decreases in the abundance of transcripts that encode proteins required to limit disease progression. Recently, adenoviral-associated virus (AAV) vectors have regained their status as a plausible means of achieving this therapeutic goal,[146] as these minimally immunogenic and non-integrative vectors can increase expression levels of selected genes by infecting both dividing and non-dividing cardiac cells based on the existence of several viral serotypes, whereas their small size allows for the efficient delivery to the myocardium via coronary arteries.[146,147] Alternatively, retrograde delivery into the coronary sinus and a surgical recirculation method have recently been implemented to enhance cardiomyocyte expression of SERCA2a in pigs and sheep, respectively. More recently, AAV has also been used to drive exogenous expression of heme oxygenase I[148] and VEGF-B[149] in pigs and dogs, respectively. These pre-emptive studies effectively limited cardiac ischaemia[148] and tachy-pacing induced heart failure,[149] respectively. Attempts to treat established CVD using AAV in the form of Mydicar (Celladon; now Eiger Biopharmaceuticals) initially yielded promising results for SERCA2a enzymatic replacement therapy in clinical trials.[150] However, the Phase IIb clinical trial failure of Mydicar is being attributed to an inability of the AAV to deeply penetrate the myocardial tissue mass, indicating that direct intramyocardial injection or coronary sinus delivery method could increase the likelihood of success in the future.[150] Importantly, AAVs could also be tailored to specifically encode beneficial splice variants of genes, such as the full-length SCN5A splice variant (as opposed to the truncated SCN5A splice variant), which could limit arrhythmias by maintaining cardiac Na^+ currents and thereby electric conduction velocity in the heart.[79,151] Furthermore, the introduction of promoter regions that induce expression solely in response to injury could broaden the applicability of AAVs as a means of correcting decreased cardiac-protective gene expression in a spatiotemporal fashion.

To combat increases in inflammatory and fibroproliferative gene expression commonly observed in CVD, short-interfering RNA-based approaches are currently being extensively employed in the (pre-)clinical forum (see RNA-based clinical trial review[34]). As their safety profile and mechanism by which they function are well-established, these could represent an excellent means of ameliorating RBP expression, although the hierarchical positioning of these proteins as global regulators of the transcriptome could elicit undesirable off-target effects. Therefore, computational mining of transcriptomic databases in cardiovascular centres worldwide could uncover splice variants that are enriched in diverse cardiovascular complications, enabling the design of small interfering RNAs (siRNAs) that could specifically target disease-advancing splice variants (thereby reducing production of encoded protein isoforms), representing a novel and highly effective means of targeting in a cell type-specific fashion. Although several methods are currently being employed to deliver siRNAs in humans,[34] the development of lipid-based formulations for the effective transport of siRNAs, miRNAs and antagomiRs is regarded as essential for the broad applicability of RNA-based therapeutic approaches in the clinical setting, and has been prioritized by the pharmaceutical industry.[32]

Aberrant splicing, as a result of genetic mutations that alter either RBP function or the splice sites these proteins recognize, is becoming increasingly recognized as a major contributor to human disease, including CVD.[152–154] The use of antisense oligonucleotides (AONs) to correct these RNA-based defects has been applied extensively at the drug developmental level,[33,155] conferring the capacity to skip one or more exons or restore/disrupt the transcript reading frame (*Figure 2*).[155,156] Importantly, these biotools have gained widespread attention as a result of their therapeutic potential for Duchenne's muscular dystrophy (drisapersen and etiplerisen; GlaxoSmithKline plc. and Sarepta Therapeutics Inc., respectively) and spinal muscular atrophy (nusinersen; Ionis Pharmaceuticals & Biogen Inc.). Of note, etiplerisen has recently been granted accelerated FDA approval

whereas nusinersen filing for FDA approval is imminent. Despite these successes in correcting splicing dysregulation in rare genetic diseases, ventures into the cardiovascular field are limited. This is particularly surprising given the identification of numerous CVD-associated splicing events, such as troponin T in cardiomyocytes,[157] oxidized low-density lipoprotein receptor 1 in macrophages,[154] VEGF in ECs,[158] and myocardin in VSMCs.[25] Recently, an AON-based approach was used to correct the A-band truncating mutation of Ser14450fsX4 in exon 326 of titin,[159] a protein that plays a critical role in sarcomere organization and passive elasticity in cardiomyocytes. Importantly, missense mutations in human titin, including truncations as described above,[159] have been found to responsible for 25% of familial DCM cases and 18% of sporadic DCM cases. By forcing excision of exon 326 in patient-specific cardiomyocytes *ex vivo*, myofibril assembly was improved, and similar studies with the truncation-correcting AON in mice revealed a correction of DCM phenotype.[159] Further evidence that AONs could represent a potent means of limiting CVD progression can be found in their recent application to correct autoimmunity in mice as a result of defective NLRP3.[160] Interestingly, the inflammasome protein complex plays a critical role in promoting cytokine maturation and inflammation in myeloid cells, including macrophages. As such, the *in vivo* correction of an alternative splice acceptor site[160] (as detailed in *Figure 2*) in macrophages by Thygesen *et al.* represents an important step towards similar AON-based interventions in human myeloid cells, and potential therapeutic application in humans.

Collectively, the continued development of DNA- and RNA-based approaches designed to alter the transcriptome could result in the generation of novel therapies that harness RBP-mediated processes, and significantly impact the treatment of CVD in the future.

Conclusions and future perspectives

In conclusion, cells undergo functional adaptations at sites of injury that serve to limit tissue damage and restore proper tissue function and structure. These remodelling and regenerative responses in affected cells are tightly coupled with dynamic changes in gene expression patterns that necessitate RBPs to determine the fate of nascent RNAs. In doing so, RBPs have emerged as potent effectors and regulators of cellular function in (patho)physiological settings. In light of our expanding insight into the diversity and complexity of protein-coding and non-protein-coding RNA transcripts, as well as the critical role played by an ever-expanding number of RBPs involved in processing these transcripts, our understanding of the human genome has broadened significantly. Importantly, this 'RNAissance' has unleashed a revolution in drug development, leading to numerous RNA-based therapies that are currently being explored in diverse pre-clinical animal studies and clinical trials. The potential inclusion of these novel therapeutic modalities could represent an important broadening of our medical arsenal in combating CVD in the 21st century.

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Supplementary material

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