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Omics-based approaches in understanding mechanosensitive endothelial biology and atherosclerosis

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Abstract

Atherosclerosis is a multifactorial disease that preferentially occurs in arterial regions exposed to disturbed blood flow (d-flow). The mechanisms by which d-flow induces atherosclerosis involve changes in the transcriptome, methylome, proteome, and metabolome of multiple vascular cells, especially endothelial cells. Initially, we begin with the pathogenesis of atherosclerosis and the changes that occur at multiple levels owing to d-flow, especially in the endothelium. Also, there are a variety of strategies used for the global profiling of the genome, transcriptome, miRNA-nome, DNA methylome, and metabolome that are important to define the biological and pathophysiological mechanisms of endothelial dysfunction and atherosclerosis. Finally, systems biology can be used to integrate these 'omics' datasets, especially those that derive data based on a single animal model, in order to better understand the pathophysiology of atherosclerosis development in a holistic manner and how this integrative approach could be used to identify novel molecular diagnostics and therapeutic targets to prevent or treat atherosclerosis.

INTRODUCTION

It has been known for decades that specific flow patterns in branched and curved regions of the vasculature are associated with the formation of atherosclerotic plaques. The underlying reasons for this preferential localization of plaques have been intensely studied ever since. Historically, researchers have isolated various cell types in the arterial wall for further study of specific cellular mechanisms. A variety of *in vitro* models have been developed for these studies. Additionally, *in vivo* models of atherosclerosis have been developed to study the pathology of this disease on a systemic level. However, up until recently, single genes, proteins, and pathways were identified from these studies. With the advent of new technologies, such as microarrays, RNA sequencing, and mass spectrometry, large amounts of data have generated thousands of genes, pathways, and RNAs that are potentially involved in atherogenesis.

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The endothelium plays a key role in atherogenesis, as endothelial cells (ECs) are the first point of contact with the blood and any blood-borne molecules or cells. Furthermore, ECs are known to respond to the physical force generated by blood flow. The study of the interplay between these physical forces and living cells is known as mechanobiology and is still a relatively young field. Therefore, studying ECs in atherosclerosis with the aforementioned techniques is a crucial first step in linking the complex web of events associated with atherosclerosis to disturbed blood flow (d-flow). Finally, the ultimate goal of studying ECs with omics approaches is to integrate the datasets and translate our knowledge of mechanobiology into specific targets that can be developed into biomarkers or therapeutics for athero-sclerosis (Figure 1)

ATHEROSCLEROSIS AND SPATIAL LOCALIZATION OF PLAQUES

Atherosclerosis is an inflammatory disease of the arterial wall and is the most common cause of death in the world.[1] The arterial wall consists of three layers: the innermost intimal layer composed of ECs, the medial layer composed of smooth muscle cells (SMCs), and the outermost adventitial layer composed of fibroblastic cells. The endothelium is a monolayer of cells that function as the barrier between the blood and the rest of the vessel wall. Atherosclerosis is initiated by inflammation in the endothelial layer, which allows the endothelium to become more permeable. As the endothelium becomes more permeable, circulating lipids (such as cholesterol) bound to low-density lipoproteins (LDL) accumulate in the intima. Once these lipids infiltrate the intima, immune cells (particularly monocytes) transmigrate into the intima as well. Once these immune cells come into contact with these LDL-bound lipids, they are transformed into foam cells. The foam cells secrete cytokines to recruit more immune cells, recruit and activate SMCs, and generally promote the formation of lesions. The presence of lesions, or plaques, is the defining feature of atherosclerosis.[2] After the plaque is formed, the resultant outcome is occlusion of the artery and a loss of blood flow and oxygen to downstream regions and organs. This can occur by either the development of a plaque so large that it becomes occlusive or the formation of a smaller plaque which is vulnerable and erodes so that the endothelium is denuded and an occlusive thrombus forms.[3–5]

Atherosclerotic plaques tend to develop in specific regions of the vasculature that experience flow separation, such as at sites of curvature, branching, or cross-sectional expansion.[6] At these regions, the flow departs from pulsatile, unidirectional flow to create flow-separation zones including flow reversal, oscillatory flow, and turbulence known as d-flow.[7–10] These sites include the abdominal aorta, the carotid bifurcation, and the lesser curvature (LC) of the aorta.[6] Caro et al. were the first to show that lesions develop directly upstream of these flow dividers in regions of low, oscillating wall shear stress (OS).[11,12] However, this observation has been validated by many other investigators as well.[13–19] Wall shear stress (WSS), which is the force exerted by the blood flowing tangential to the surface of the blood vessel, is a major determinant of endothelial function and gene expression. In healthy humans, the WSS in the common carotid artery ranges from 9.5 to 15 dyn/cm^2 with an average of 11.6 dyn/cm^2, whereas in the brachial artery, common femoral artery, superficial femoral artery, infrarenal aorta, and suprarenal aorta the values are much lower, with averages of 6.5, 4.3, 4.4, 0.2, and 7.3 dyn/cm^2, respectively.[20–36] Not only does the shear

stress vary widely with location, the geometry of the vessel also plays a major role in the WSS. This heterogeneity has been studied in mice. In the murine aortic arch, the WSS reaches only up to 150 dyn/cm^2 in the inner curvature (LC), whereas the straighter greater curvature (GC) reaches up to 600 dyn/cm^2. Furthermore, in the GC, the velocity vectors are generally in the same direction, whereas the LC has velocity vectors in multiple directions.[37] Thus, not only does the magnitude of shear stress widely vary within the artery but the directionality also varies. This heterogeneity in WSS promotes differential gene expression. This can be clearly seen in a murine model of atherosclerosis known as the partial carotid ligation (PCL) model.[38] When the left carotid artery (LCA) is ligated, the WSS drops from approximately 110 to 30 dyn/cm^2 and thus contributes to pathogenesis of atherosclerosis.

ATHEROSCLEROSIS AND THE ENDOTHELIUM

Endothelial Regulation by Flow

The main functions of the endothelium are to maintain a barrier between the blood and underlying tissues, recruit immune cells to sites of injury, and to form new blood vessels. Each of these endothelial functions can be modulated by WSS and dysregulation of these functions contributes to the pathogenesis of atherosclerosis. One of the earliest functions to be studied was endothelial permeability and the effects of WSS on individual transport pathways (tight junctions, adherens junctions, vesicles, and leaky junctions).[39] It was found that endothelial permeability decreases after prolonged exposure.[40,41] Furthermore, it was found that this phenomenon is due to increases in nitric oxide (NO) production from chronic shear stress, which decrease permeability.[42–44] Mechanistically, it was first proposed that leaky junctions around cells in a state of apoptosis or mitosis provide the major pathway for transport of large molecules such as LDL across the endothelium.[45] More recent studies have also shown a strong correlation between apoptosis, proliferation, and LDL permeability of endothelial monolayers in culture.[46] Physiologically relevant shear stress decreases EC proliferation[47–49] and apoptosis.[50–52] Also, EC apoptosis is triggered by lack of shear stress[53] and EC proliferation increases dramatically within 48 h after reduction of shear stress,[54] whereas OS induces cell turnover.[18] Ultimately, chronic differences in shear stress affect LDL transport into the vessel wall and that low shear stress may be expected to increase LDL permeability.[55–58] Taken together, these studies suggest that the long-term downregulation of permeability is antiatherogenic in that LDL is not allowed to penetrate the wall.[46] Furthermore, it has been shown in a mouse model of atherosclerosis that endothelial permeability is increased in atheroprone regions due to the degradation of the endothelial extracellular matrix (ECM) by matrix metalloproteinases (MMPs).[59] Additionally, alterations in the endothelial ECM lead to stiffening of the intimal layer, which also affects endothelial permeability.[60]

Not only does d-flow negatively impact endothelial permeability, numerous studies have shown that immune cell recruitment (monocyte adhesion) to the endothelium has been enhanced in d-flow regions due to the presence of increased chemoattractants and adhesion molecules.[61,62] Furthermore, endothelial transcription profiles taken in these d-flow regions from mice[63] and pigs[64] indicate that in general, ECs exhibit a proinflammatory phenotype when exposed to d-flow. Hajra et al. found that the subunits of the proinflammatory nuclear

transcription factor NFkB (nuclear factor kappa B) (p65, IkBα, IkBβ) were upregulated in areas of the proximal aortas of mice that were prone to lesion formation.[63] NFkB was only activated in a minority of the cells basally, but was highly activated when stimulated with LPS or hypercholesterolemia. Later, Passerini et al. found that in ECs isolated from the inner aortic arch of pigs, which experiences d-flow, versus the descending thoracic aorta, which experiences unidirectional laminar flow/laminar shear stress (LS), exhibit a general upregulation of several inflammatory cytokines and NFkB elements.[64] Taken together, these studies indicated that the endothelium in these regions has a proinflammatory phenotype. Finally, d-flow has also been shown to increase migration and angiogenesis.[65,66] Specifically, our laboratory found that LS inhibits EC migration and angiogenesis.[65,66] Overall, d-flow on endothelium induces proatherosclerotic changes that impact endothelial leakiness, stiffness, and the ability to form new vessels.

Mechanosensing and Mechanotransduction

There are a variety of mechanoreceptors on the surface of ECs that are capable of detecting and responding to shear stress stimuli. After activation of any one of these mechanoreceptors, a complex network of several intracellular pathways is triggered in a process known as mechanotransduction. These pathways are activated simultaneously and/or crosstalk with each other. These pathways lead to regulation of a variety of mechanosensitive elements, which ultimately induce or suppress gene expression. Recently, Tzima and coworkers identified an ECM-specific, mechanosensitive signaling pathway that regulates endothelial compliance vessel integrity.[67] The role of major mechanical sensors, including platelet endothelial cell adhesion molecule 1 - (PECAM1), the glycocalyx, caveolins, cytoskeletal structures, integrins, angiotensin type 1 (AT1) receptor (AT1R), and the nucleus in the regulation of endothelial function, has been discussed elsewhere.[68]

Once the shear stress stimulus is applied, many of the triggered pathways converge on the mitogen-activated protein kinase (MAPK) pathway and the phosphatidylinositol-3-OH kinase (PI3K)/Akt pathways.[69] These shear-responsive pathways activate the MAPK and PI3K/Akt pathways differentially in response to LS and OS.[70] The MAPK pathway in particular can be activated through integrins, among others. Briefly, integrins activated by mechanical stimuli phosphorylate and activate a complex of kinases, adaptor proteins, and guanine nucleotide exchange factors, which ultimately lead to the activation of Ras. When Ras becomes activated, this leads to the activation of MAPKs. Extracellular signal-regulated kinase (ERK)1/2, members of the MAPK family, then activate transcription factors.[71] Furthermore, mechanosensitive membrane proteins can activate the MAPK pathway through various protein mediators such as protein kinase C (PKC)[71] or through reactive oxygen species (ROS).[72] Furthermore, the PI3K/Akt pathway can converge with the same integrins that the MAPKs interact with and can lead to activation of endothelial nitric oxide synthase (eNOS).[73,74]

In LS, atheroprotective genes become upregulated.[75–77] Particularly, eNOS becomes phosphorylated and activated by Akt via a PI3K-dependent pathway[78,79] and leads to an antiatherogenic phenotype in ECs.[80] As opposed to LS, in OS, ECs express proinflammatory cytokines such as monocyte chemoattractant protein (MCP)-1[73] and inflammatory cell

adhesion molecules such as VCAM1 and ICAM1.[81] The chemokine MCP-1 contains a phorbol ester (TPA)-responsive element (TRE) in its promoter region, which was also found to be shear-sensitive and regulated through MAPKs.[73] Similarly, VCAM1 and ICAM1 also contain these shear-responsive elements in their promoters.[81] Furthermore, ECs express other proinflammatory, shear-sensitive proteins such as nicotinamide adenine dinucleotide phosphate oxidase[77] and bone morphogenetic protein (BMP4).[82] Two of the main transcription factors that are responsible for the upregulation of many of these proinflammatory genes such as ICAM1, VCAM1, and E-selectin are the activator protein complex (AP-1) and NFkB complex[83–89] and has been discussed elsewhere.[68] Ultimately, these findings have demonstrated that LS upregulates 'atheroprotective' genes and downregulates 'proatherogenic' genes, while OS results in the opposite phenomenon. However, although the PI3K/Akt and the MAPK pathways have been well documented, other pathways still remain to be discovered.

Models of Flow and Shear Stress to Study Endothelial Function and Atherosclerosis

In Vitro Models—One of the most characterized *in vitro* models to study the effect of physiological or pathophysiological shear stress on ECs is the cone-and-plate viscometer.[49,90] In this system, fluid shear stress is applied to ECs in a standard culture dish with media by a rotating cone. This design was later modified to include a speed-controlled motor with variable rotational velocities.[91] However, more recently, our laboratory has developed a cone-and-plate system that can be housed in a standard incubator and programmed shear stress profiles [stable (s-flow)/(LS) is 10 dyn/cm^2 and d-flow/OS is ±5 dyn/cm^2] can be controlled by a computer.[92,93] An alternative model is the parallelplate flow chamber.[94,95] In this system, fluid is driven through a flow chamber either by the hydrostatic pressure between the two reservoirs, which produces steady flow, or via cam-driven clamps upstream of the chamber, which produces pulsatile flow. The flow chamber consists of a polycarbonate plate, a rectangular Silastic gasket, and a glass slide (or cover slip) containing the ECs held together by a vacuum maintained at the periphery of the slide. Although the cone-and-plate and the parallel-plate flow chamber systems are the most commonly used *in vitro* shear systems, microfluidic chambers have become more recently used as they allow for high-throughput experiments. This method was pioneered and commercialized by Schaff et al.[96–98]

Ex Vivo Models—While *in vitro* models study the effect of shear stress on ECs in isolation, *ex vivo* models allow for the inclusion of a limited number of related factors, such as the ECM.[99] Initial *ex vivo* models consisted of explanted artery segments cannulated at the ends. In these setups, the arteries were perfused with a controlled intraluminal pressure, flow pulsatility, and directionality in a culture medium bath.[100,101] Through an *ex vivo* model of porcine carotid arteries, Gambillara et al. showed that OS reduced eNOS expression.[101] Lu and Kassab also used porcine arteries to show NO levels drop after exposure to flow reversal.[102] Furthermore, an *ex vivo* model for murine carotid arteries was developed by Gleason et al.[103] In this model, defined mechanical stress can be applied to the arteries based on a computer controller.

In Vivo Models—There are also *in vivo* models used to study the broad effects of shear stress on the development of atherosclerosis.[99] One of the first models of atherosclerosis in mice was the hypercholesterolemia model developed by Paigen et al.[104–107] This model induced hypercholesterolemia in the C57BL/6J mouse strain by genetic mutation and high-fat diet. The two most widely used mutations are the apolipoprotein E (ApoE) disruption[108–110] and the LDL receptor deletion.[111] The most common diets are Paigen's diet, which includes cholate,[104] and the Western diet, which does not include cholate and is less inflammatory.[109,112] More recent models of atherosclerosis that study the effects of shear stress on atherogenesis use the hypercholesterolemic model in conjunction with surgical intervention. These include the constrictive perivascular cuff model in mice[113–115] and the ligation models in mice. In the perivascular cuff model, the constricted region experiences higher shear stress, whereas the proximal section is exposed to d-flow and thus develops atherosclerotic plaques. Both complete ligations and incomplete ligations are also commonly used models. In the complete ligations, the carotid artery is ligated and this induces vascular remodeling, neointimal hyperplasia, and atheroma formation.[16,115–125] In this model, there is no WSS on the endothelium. However, as this injures the artery, this model does not isolate the role of shear stress alone in atherosclerosis. Finally, among the incomplete ligations, first studied in pigs,[126] we have robustly developed a PCL model.[38] In this model, three of the four branches of the LCA are ligated, which leads to bidirectional flow mimicking d-flow.

IDENTIFICATION OF MECHANOSENSITIVE MOLECULES BY SYSTEMS BIOLOGICAL ANALYSES

Understanding the underlying mechanisms governing the pathophysiology of atherosclerosis is the key to unlocking potential therapies for this widespread disease. In recent years, many resources have been devoted to unraveling basic mechanisms. However, because the nature of the disease is complex and multifactorial, different research techniques must be combined to integrate molecular pathways, cellular functions, and systemic responses involved in atherogenesis. Therefore, researchers are now using high-throughput technologies to identify molecular changes at the DNA, RNA, and protein levels and with the help of computational tools, these datasets are now being integrated to more fully model biological processes as interconnected and regulated networks. Here, we briefly review how a high-throughput 'omics' approach, such as epigenomics, transcriptomics, proteomics, and metabolomics, can be used to explore the mechanisms of d-flow-induced endothelial inflammation and atherosclerosis (Figure 2).

Epigenomics Approach

Genes can be regulated by a variety of mechanisms, one of which is through epigenetic modifications. Epigenetic modifications alter the structure of DNA and, consequently, gene expression without affecting the genetic sequence. This is mainly accomplished by altering the conformation of the DNA.[127] Some of the most widely studied epigenetic modifications include DNA methylation, histone modifications (methylation and acetylation), as well as posttranscriptional regulation by microRNAs (miRNAs).[128]

Transcriptionally active chromatin is known as euchromatin and is associated with acetylated histones and unmethylated DNA, whereas condensed/transcriptionally inhibited DNA is known as heterochromatin and is associated with histone deacetylation and specific repressive methylation sites such as trimethylated–histone 3 lysine (H3K) 9 and 27. Heterochromatin also features heavy DNA methylation, which is generally accepted to be suppressive of transcription when present in the promoter region. However, DNA methylation can occur anywhere in the gene body and the resultant outcome of methylation in these regions is not well studied or understood. On the other hand, DNA demethylation has been shown to play an important role in gene regulation and progression of disease. For example, a recent study by Liu et al. has identified Ten-eleven translocation-2 (TET2), which removes methyl groups from DNA, as a master regulator of arterial SMC plasticity and implicated its downregulation in the pathogenesis of atherosclerosis.[129–135]

Histone Modifications in Atherosclerosis—Histone modifications have been implicated in the pathogenesis of atherosclerosis and there are reports describing the relationship between d-flow and histone modifications. Multiple groups have reported that LS promotes histone acetylation in ECs by activating histone acetyltransferases (HATs), such as ribosomal S6 kinase-2 (RSK-2), mitogen and stress-activated kinase-1 (MSK-1), and cAMP-responsive element-binding protein (CREB)/CREB-binding protein (CBP) complexes, activating histone phosphorylation, and inhibiting histone deacetylases (HDACs).[130,136] Wang et al. showed that steady laminar blood flow blocks HDAC-mediated inhibition of myocyte enhancer factor-2 (MEF2), which in turn allows the expression of the antiatherogenic genes Klf2 and eNOS. In agreement with these prior findings, Lee et al. showed that d-flow leads to overexpression of HDACs, which block the antiinflammatory effect of nuclear factor (erythroidderived 2)-like 2 (Nrf2) and MEF2.[130] HDACs are grouped into two main classes, Class I (HDACs 1/2/3) and Class II (HDACs 5/7). Enzymes in both classes have been shown to play functional roles in EC inflammation and atherosclerosis.

These initial studies suggest that histone acetylation in response to LS is atheroprotective, whereas deacetylation by OS is proatherogenic. However, there have been some conflicting reports regarding HDACs in atherosclerosis. Specifically, HDAC3 suppression was reported to cause less endothelial inflammation, yet was also reported to increase EC apoptosis, which can be proatherogenic. Similarly, H3/4 acetylation, H3 phosphorylation, and HDAC inhibition have been reported to induce inflammatory cytokine release by ECs exposed to oxidized LDL. Moreover, HDAC7 was reported to suppress endothelial proliferation, which can also be proatherogenic. Additionally, HDAC2 expression was reduced in human coronary artery atherosclerotic lesions (reviewed elsewhere[137]). In conclusion, it appears that the exact function of different HDACs and HATs in atherosclerosis is dependent on environmental factors, cell types, and particular enzyme subclasses.

DNA Methylation—One of the most widely studied epigenetic changes is DNA methylation. It involves the addition of a methyl group to cytosine. DNA methylation most frequently occurs in cytosine–guanine (CG) base pairs and in sites with CG clusters called CpG islands. In mammalian cells, methylation is mediated by one of three DNA methyltransferases: DNA methyltransferases (DNMTs) 1, 3A, or 3B. DNA methylation at

the promoter site is hypothesized to inhibit gene expression by two different mechanisms: either by physically preventing transcription factors from binding directly to the promoter or by recruiting repressive complexes with histone-modifying enzymes that alter the DNA conformation by altering histones. The association between DNA methylation and histone modification is not very well understood and is being expansively studied.[138–142]

DNA Methylation in Atherosclerosis—One of the earliest reports of aberrant DNA methylation in atherosclerosis, by Lund et al., describes differential methylation patterns in both leukocytes and the aortas of ApoE knockout mice fed a high-fat diet. In this report, they also find that atherogenic lipoproteins promote global DNA hypermethylation in a human monocyte cell line.[143] To date, multiple groups have reported a link between atherosclerosis and methylation. Specifically in humans, ApoE knockout mice, and New Zealand White rabbits, there is increased global hypomethylation in atherosclerosis, which leads to SMC hypertrophy.[144,145] However, Zaina et al. reported that atherosclerotic lesions in diseased human aortas were hypermethylated across many genomic loci in comparison with healthy controls. They further identified several differentially methylated genes that are associated with atherosclerosis and have been shown to play an important role in SMC and EC function. These include HOXA6, HOXA9, MIR23b, PDGFA, PLAT, PRRX1, and PXDN.[146] Taken together, these studies indicate that there is differential methylation in many cell types that are involved in the pathogenesis of atherosclerosis.

DNA Methylation in Disturbed Flow—Recently, our laboratory and others have discovered that d-flow can alter the expression of DNMTs and thus affect DNA methylation patterns using independent *in vitro* and *in vivo* models of ECs and atherosclerosis in d-flow.[147,148] Initially, Jiang et al. showed that d-flow causes increased expression of DNMT3A, which leads to the downregulation of an important antiatherosclerotic gene Kruppel-like Factor 4 (Klf4).[149] Subsequently, Zhou et al. found that DNMT1 expression increases in ECs under d-flow conditions both *in vitro* and in a rat model.[150]

However, independently of Zhou et al., our group showed that DNMT1 is increased in ECs subjected to d-flow conditions, thus indicating that d-flow may impact the methylation pattern of ECs and thereby impact the transcription of mechanosensitive genes.[150] In order to examine this hypothesis, Dunn et al. integrated a genome-wide microarray previously developed in the laboratory,[151] which uses endothelial-enriched RNA derived from our murine PCL to identify EC genes differentially expressed by d-flow-induced atherosclerosis, with a reduced representation bisulfite sequencing (RRBS) array to identify the methylation patterns of the whole endothelial genome and which genes were suppressed by this methylation. Although RRBS is known to have certain limitations, mainly due to the use of restriction enzymes that can miss some CpG sites, it is a well-accepted and cost-effective technique to study common CpG sites and CpG islands.[152] Techniques with broader genomic coverage of CpG sites, such as MethylC-seq, can be used to study genome-wide methylation but are more expensive, involve more sequencing, and the results generally agree with the more cost-effective RRBS.[153] After integrating these two arrays, Dunn specifically determined which genes are downregulated by d-flow and also hypermethylated promoter regions. Using this systems biological approach, 11 genes were identified: HoxA5,

Klf3, Tmem184b, Adamtsl5, Cmkrl1, Pkp4, Acvrl1, Dok4, Spry2, Zfp46, and F2rl1. Inhibition of DNMT1 by 5-aza-2′-deoxycytidine (5Aza) was able to reverse the hypermethylation of those genes and thus restore their expression.[148] Interestingly, 5 of the 11 genes (HoxA5, Klf3, Cmklr1, Acvrl1, and Spry2) share a cAMP response element (CRE)-binding site in their promoters.[148]

Integrating gene expression and differential methylation data on the genomic scale in order to identify novel gene targets is a powerful method to understand complex diseases such as atherosclerosis.[147,148,150] The beauty of using such powerful tools is that the results of such *in silico* analyses can always be validated back in the laboratory and can ultimately lead us to potential therapeutic targets as well as biological markers for early detection of disease.[154,155]

Transcriptomics Approach

The transcriptome encompasses both coding and noncoding RNA genome-wide and includes mRNA, miRNA, and long noncoding RNA. The expression of these RNAs is primarily identified using microarray analyses. Microarrays are widely used to study the underlying mechanisms of diseases and have also been extensively used to study the expression profiles of atherosclerotic-prone or atherosclerotic vessels[156] in humans[157–159] and in experimental animals.[160–164] Furthermore, advanced techniques like laser-capture microdissection have enabled scientists to isolate specific subregions within atherosclerotic lesions, such as the plaque area, the media, or the adventitia for microarray analysis.[165] Furthermore, methods have been developed to isolate enriched populations of particular cell types, such as ECs[38,166,167] or macrophages[168] for subsequent genome-wide expression profiling.

Interestingly, recent tools have been developed to aid in the integration of multiple omics datasets. Specifically, next-generation sequencing technology and data from ENCyclopedia of DNA Elements (ENCODE) have recently been used to integrate transcriptomics as well as epigenomics[169] in order to provide valuable information regarding the regulation of gene expression.[170] ENCODE not only includes acetylation and methylation information but also DNAseseq, RNA-seq, and CHIP seq data from a variety of human cell types.[171] In addition to the development of ENCODE for human DNA, there is a Mouse ENCODE project,[172] which is definitely of particular interest owing to the importance of mouse models when exploring a wide variety of diseases including atherosclerosis. Together, all of these techniques work to enhance our understanding of cell type-specific functions in atherogenesis. As the use of a transcriptomic approach in atherosclerosis has been reviewed elsewhere,[156,165,173–175] here we will focus on how this approach has been used to identify mechanosensitive genes in the endothelium and the role these genes play in endothelial dysfunction and atherosclerosis.

mRNA-Omics—One of the earliest studies was conducted in HUVECs. McCormick et al. studied changes in gene expression of sheared HUVECs using microarray and identified that genes responsible for cell proliferation, differentiation, vascular tone, ECM, RNA degradation, thrombosis, chemotaxis, and inflammation were differentially regulated.[176,177]

Later, Chen et al. investigated the effects of 24 h of shear stress on HAECs by microarray and identified that genes related to inflammatory cytokines, cell proliferation, ECM/cytoskeleton remodeling, and signal transduction were altered by long-term LS. Ultimately, these changes acted to keep ECs quiescent under laminar flow.[178] Furthermore, using a custom-designed microarray, Dekker et al. showed that the majority of flow-regulated endothelial genes are also influenced by increased cytokine levels, which results in cross-talk between flow and inflammatory-mediated downstream signaling mechanisms. They also identified a flow-sensitive endothelial-specific transcription factor LKLF.[179] Additionally, Ohura et al. showed that LS, but not OS, decreased DNA synthesis and cell cycle regulators in ECs and that OS affects genes responsible for vascular remodeling, such as endothelin-1, transforming growth factor β (TGFβ), collagen type IV, and ephrin A1.[180]

Following these microarray studies, Viemann et al. integrated the collective information from these array datasets and found that endothelial subtype heterogeneity and limited quantity of RNA samples were the important limitations for studying the global gene expression using arrays.[181] Moreover, these datasets were obtained from *in vitro* ECs, which do not necessarily show the same genotypic/phenotypic correlation as the ECs experiencing LS or OS in *in vivo* conditions. Similarly, while comparing the data obtained from *in vivo, ex vivo*, and *in vitro* endothelial gene expression, we found that many flow-sensitive genes appear to be lost or dysregulated when the ECs are cultured *in vitro*.[59,151] Although these *in vitro* studies have provided critical insights into shear-sensitive mechanisms, it cannot be assumed that *in vivo* flow-sensitive vascular responses will directly translate to *in vitro* flow-sensitive genes. Therefore, it is critical to study how the arterial endothelium responds to different flow conditions *in vivo*. To this end, flow-dependent, site-specific endothelial gene expression changes have been studied in pigs.[166,182] However, an experimental model with reproducible modulation of flow conditions that rapidly leads to atherosclerosis has been one of the main factors limiting *in vivo* studies. To address this concern, a perivascular collar model was developed (as described elsewhere)[183–187] and more recently our laboratory developed the mouse PCL model (described previously).[38] Using endothelial RNAs isolated from the flow-disturbed LCA and the undisturbed RCA in the PCL mouse model, we reported a microarray study that identified novel mechanosensitive genes (mRNAs) in the mouse carotid endothelium.[59,65]

miRn-Omics—Recently small noncoding RNAs, miRNAs, have emerged as important regulatory RNAs that have been implicated in gene expression regulation.[188] miRNAs interact with the 3′ untranslated region (UTR) of specific target mRNAs in a sequence-specific manner, resulting in mRNA degradation or translational inhibition.[188] With the advancements in the field and availability of miRNA microarray platforms, many research groups used this ‘omics’ approach to identify flow-sensitive regulators of endothelial transcriptome. It has been previously demonstrated that LS and OS differentially regulate the expression of miRNAs in ECs. Initially, the majority of flow-sensitive miRNAs has been identified and characterized using cultured ECs that were subjected to LS or OS conditions. Chien and coworkers were the first to report flow-sensitive miRNAs (miR-19a and 23b) in cultured ECs. Later, using an omics approach on cultured ECs, Weber et al. showed that miR-21 is induced in ECs by shear stress and modulates apoptosis and eNOS activity.[189]

Likewise, Wang and coworkers showed that miR-19a suppresses the expression of cyclin D1 under LS.[190] Furthermore, our laboratory showed that miR-663 is one of the most flow-sensitive miRNAs that is upregulated by OS in a microarray of cultured ECs exposed to LS or OS for 24 h.[191]

However, some *in vivo* studies have been conducted. Davies and coworkers reported miR-10a as the first flow-sensitive miRNA identified directly from the porcine endothelium *in vivo*.[166,192] Furthermore, our laboratory identified two important and novel d-flow-induced miRNAs, miR-712 and miR-205, by using the PCL model described above.[59,65] These miRNAs play a critical role in regulation of the MMPs by regulating their upstream inhibitors (TIMP3 and RECK). Using a simplistic systems biology approach, we integrated the knowledge from these two datasets, and we identified key hub genes and important gene networks that are crucial in the pathophysiological process of atherosclerosis.[59] Further mining of these two important datasets (d-flow-altered miRNAs and d-flow-altered genes) could provide additional insight into the underlying mechanisms of d-flow-induced atherosclerosis.

Inc-Omics—In recent years, increasing evidence suggests that noncoding RNAs play important roles in the regulation of tissue homeostasis and pathophysiological conditions. In addition to small noncoding RNAs (miRNAs), longer transcripts (>200 nucleotides long transcripts), namely long noncoding RNAs (lncRNAs), can also modulate gene expression and signaling pathways at various stages. With the advancement of RNA sequencing technologies, studies have characterized the expression of lncRNAs under normal physiological conditions and in disease states of atherosclerosis. There have been several studies aimed at identifying and underlining the specific roles of lncRNAs in a tissue-specific manner in vascular biology. Initially, Lnc-Ang362 was identified as a lncRNA from rat vascular SMCs in response to angiotensin II (AngII) using transcriptome and epigenomic profiling. Also, miRNAs miR-221 and miR-222, which are associated with SMC proliferation and neointimal hyperplasia in response to vascular injury, are co-transcribed with Lnc-Ang362. Interestingly, knockdown of Lnc-Ang362 reduces the expression of these miRNAs and vascular SMC proliferation. Importantly, eNOS (NOS3), which regulates SMCs via NO, can be regulated by lncRNAs. Specifically, natural antisense transcript (NAT) to NOS3, NOS3 antisense (NOS3-AS), is induced by hypoxia in ECs and regulates NOS3 expression in a posttranscriptional manner under normoxic and hypoxic conditions.[193] Overexpression of NOS3-AS reduces NOS3 expression while inhibition shows an opposite effect. In addition, lncRNAs are implicated in inflammation and the innate immune response as well. Recently, lncCox2, a lncRNA proximal to Cox2 gene, identified using whole transcriptome analysis shows that lncCox2 can mediate both activation and repression of inflammatory gene sets.[193–196] Taken together, these findings suggest that lncRNAs are emerging players in endothelial dysfunction and atherosclerosis.

Proteomics Approach

Traditional techniques to understand changes at the protein level are mainly based on immunological detection methods such as Western blots and ELISAs. But these techniques can identify only one or a few proteins at a time and heavily depend on the abundance of

protein of interest in the sample and the availability of specific antibodies, thus limiting their feasibility for comprehensive analyses.[76] Using mass spectrometry-based strategies, changes in large numbers of proteins in response to shear stress can be examined in greater detail. Proteomic studies on cultured ECs involving the analysis of ECs from human and other animal sources are summarized below.

Previous studies identified that a point mutation in the 5′-flanking region of the eNOS gene, −786T→C, renders its transcription insensitive to LS. Consequently, human ECs homozygous for the eNOS mutant variant (CC) do not respond to shear stress and thus the lack of NO contributes to EC dysfunction.[197–199] Additionally, reduced NO production indirectly affects the expression of other proteins in CC genotype cells. In fact, a total of 14 proteins were identified to be differentially expressed. These proteins were primarily involved in the NO-dependent endoplasmic reticulum stress response. Furthermore, the antioxidant gene manganese-containing superoxide dismutase (SOD-2) expression increased in the CC genotype ECs and possibly contributed to an antiatherosclerotic phenotype.[200]

NO signaling not only affects protein expression but also directly interacts with susceptible cysteine residues in proteins, resulting in S-nitrosylation. S-nitrosylation is an important posttranslational modification that plays a role in the modulation of cardiovascular function via the regulation of mitochondrial metabolism, intracellular Ca^{2+} handling, protein trafficking, and cellular defense against apoptosis and oxidative stress.[201–203] Huang et al. analyzed the changes in S-nitrosylation of reactive cysteine residues present in endothelial proteins post LS.[204] Using a similar approach, Huang et al. showed that increased S-nitrosylation on cytoskeletal proteins is critical for adaptation and remodeling of the endothelium in response to LS.[205] Wang et al. also used a proteomics approach in BAECs to identify the effect of shear stress at multiple time points and confirmed that many previously identified proteins change as a result of LS within a few minutes to a few hours.[76]

Furthermore, researchers have now begun to investigate the effect of pulsatile shear stress.[206–208] In ECs, exposure to pulsatile shear stress for 18 h conferred protection against tumor necrosis factor α (TNF-α)-induced apoptosis through an NO-independent mechanism that relied on *de novo* protein synthesis.[207] Specifically, pulsatile shear stress provides antioxidative and anti-inflammatory benefits to ECs partly through the induction of sirtuin 1 - (SIRT1). A recent study has indicated that pulsatile shear stress induces an upregulation of SIRT1 and Ca^{2+}/calmodulin-dependent protein kinase kinase β (CaMKK-β) phosphorylation of SIRT1 at Ser-27 and Ser-47 based on nano-LC–MS/MS. The role of CaMKK-β in SIRT1 activation was later validated in mice lacking CaMKK-β or endothelial SIRT1, as these mice show a remarkable increase in atherosclerosis.[206] Taken together, these studies indicate that LS and pulsatile shear stress provide protection to ECs.

AMP-activated protein kinase (AMPK) is a crucial regulatory protein that has been implicated in a variety of cellular processes. Given its role in the cell, unsurprisingly, AMPK regulates key mechanosensitive proteins such as eNOS. Specifically, AMPK has been shown to phosphorylate eNOS at Ser1177/1179 directly.[209] Furthermore, AMPK is required for adiponectin-induced eNOS phosphorylation and subsequent NO production in cultured ECs.[210] The regulation of eNOS by AMPK can also be induced by statins.[211] Additionally,

it was shown that AMPK regulates eNOS in a shear-dependent manner. Specifically, AMPK Thr172 phosphorylation was increased with shear stress in cultured ECs as well as the phosphorylation of eNOS at Ser1179.[212] Furthermore, AMPK can directly phosphorylate eNOS at Ser633 in response to shear stress, statins, and adiponectin and is crucial for NO bioavailability.[213]

AMPK not only regulates eNOS directly but also regulates the key mechanosensitive transcription factor Klf2, which also regulates eNOS. AMPK inhibition significantly blocked pulsatile shear-induced KLF2 expression and the phosphorylation of ERK5 and MEF2. The expression of KLF2 was also significantly reduced in knockout mice.[214] Also, AMPK regulates SIRT1, which is an NAD(+)-dependent deacetylase. Pulsatile LS increases SIRT1 levels and activity and SIRT1 levels are higher in mouse thoracic aortas as opposed to the arch. LS increases SIRT1–eNOS association and eNOS deacetylation. It was found that pulsatile-shear-induced AMPK phosphorylation of eNOS is needed to prime SIRT1-induced deacetylation of eNOS to enhance NO production. Furthermore, knockout mice had higher repressive acetylation in eNOS.[215] Furthermore, AMPK, in conjunction with Akt, can account for the differences in cell cycle regulation arising from LS and OS.[216] Specifically, LS transiently activated both AMPK and Akt, but OS activated only Akt. Functionally, AMPK phosphorylation in LS counteracted Akt, thus the promitotic protein S6K, was inhibited and the cell cycle was arrested in G0/G1 in LS. Furthermore, there was less S6K phosphorylation in the mouse thoracic aortas, which experiences LS, than the aortic root, experiencing OS.

Importantly, AMPK promotes endothelial homeostasis through eNOS/NO bioavailability, and AMPK also exerts its atheroprotective effects by preventing endothelial inflammation. Specifically, AMPK exerts shear-sensitive, anti-inflammatory effects through PARP-1 and Bcl-6. Bcl6 (B-cell lymphoma-6 protein) is a corepressor for inflammatory mediators such as VCAM1 and MCP-1. Poly(ADP ribose) polymerase 1 (PARP-1) is proinflammatory, in part through its binding at the Bcl-6 intron 1 to suppress Bcl-6 expression. PARP-1 dissociation from the Bcl-6 intron 1 prevents endothelial inflammation. It was found that phosphorylation of PARP-1 Ser-177 by pulsatile shear-induced AMPK is responsible for the induction of Bcl-6.[217] AMPK also exerts its antiinflammatory effects by inhibiting the NLRP3 inflammasome. The NLRP3 inflammasome is activated in d-flow regions through the activation of sterol response element-binding protein (SREBP2).[218] AMPK inhibits SREBP1c and 2 through Ser372 phosphorylation, which inhibits SREBP cleavage, nuclear translocation, and transcriptional activity.[219] Whereas Akt, which is activated by d-flow, positively regulates SREBP40 through direct phosphorylation and transcriptional activation via mTORC1.

Proteomics studies have also been conducted in OS conditions. Ai et al. analyzed the pathophysiological significance of SOD-2 in response to OS using LDL particles to assess protein nitration via peroxynitrite ($ONOO^-$). The analysis of apolipoprotein B-100 (apoB-100), the protein component of LDL, by LC–MS/MS revealed that OS increased the extent of LDL protein nitration in comparison to static controls. OS also induces oxidative stress as it enhances peroxynitrite ($ONOO^-$) formation through alteration of the O_2^- to NO ratio, leading to protein nitrotyrosination that further induces atherosclerosis.[220,221]

Not only have cellular proteins been analyzed by proteomics, but secreted proteins have as well. Our group conducted a protein array on proteins secreted from HUVECs exposed to LS or OS consisting of 68 human cytokines involved in angiogenesis. We identified that thrombospondin 1 (TSP-1) and angiopoietin 2 (Ang2) were highly upregulated in OS as opposed to LS.[66] The role of Ang2-induced angiogenesis in OS was then validated *in vitro* and *in vivo*.[66] Burghoff and Schrader also analyzed the secretome of ECs under static and shear stress (both LS and OS) conditions using a quantitative proteomics approach and found that out of a total of 240 secreted proteins, 101 were differentially regulated under shear stress. This finding highlights the impact of shear stress on the contribution of ECs to the regulation of vascular homeostasis.[222]

In summary, proteomic studies represent a cutting edge tool that can be used to understand the underlying mechanisms of atherosclerosis and identify novel disease-associated biomarkers that will provide specificity and sensitivity to diagnostics and improvement in prognostics. Investigations of endothelial dysfunction and atherosclerosis using direct proteomic studies have been very challenging in particular due to the heterogeneity of the vascular tissue and plaque composition. Presently, there is a limited insight into the endothelial proteome under shear stress. These studies have identified some key shear stress-responsive proteins that were not previously known and may pave the way for future investigations to understand the mechanisms linking cause and effect in atherosclerosis. Further research is needed in order to completely understand the specific role of these proteins in activation or inhibition of specific signaling pathways with regard to the response to shear stress.

Metabolomics Approach

Metabolites are small-molecule intermediates and products of cellular metabolism. Over the past several years, researchers have gleaned valuable information from determining the metabolic profile of organisms from blood plasma. With the advent of new platforms to analyze hundreds of biomolecules, a wealth of information regarding systemic metabolic change is within easy grasp. The most common method for metabolic profiling uses mass spectrometry. Metabolite information can be integrated with biological phenotypes and can improve our understanding of the metabolic basis of disease by illuminating both pathogenesis as well as the metabolic impact of disease development.

Initial metabolomics studies were conducted in mouse models of atherosclerosis. Mayr et al. conducted one of the first studies to identify differential expression of biomarkers in atherosclerosis. In this seminal report, it was found that there is a decreased ratio of alanine to pyruvate in ApoE knockout mice as well as a gender difference in the cholate metabolite trimethylamine oxide (TMAO) in mouse aortas.[223] Following this study, Chen et al. found that the fatty acid palmitate significantly contributed to atherogenesis through its effects on apoptosis and inflammation.[224] Ultimately, the study concluded that the development of atherosclerosis is linked to dysfunctional fatty acid metabolism. Furthermore, Cheng et al. found that in ApoE and LDL receptor knockout mice, a high-fat diet led to differences in the tricarboxylic acid cycle, fatty acid metabolism, and choline metabolism, notably the choline

oxidation pathway.[225] Additionally, the loss of the LDLR caused a marked reduction in the urinary excretion of betaine and dimethylglycine.

In addition to these murine atherosclerosis metabolic studies, there have been several studies run on human plasma to further understand the systemic metabolic effect of atherosclerosis. One of the first, reported by Wang et al., discovered that three metabolites of the dietary lipid phosphatidylcholine, choline, TMAO, and betaine, were enriched in a cardiovascular disease clinical cohort. In murine validation studies, they found that dietary supplementation of mice with choline, TMAO, or betaine promoted upregulation of multiple macrophage scavenger receptors linked to atherosclerosis, and supplementation with choline or TMAO promoted atherosclerosis. Furthermore, they found that the interaction of dietary choline with gut flora was playing a critical role in this process.[226]

Additional human studies have focused on not only atherosclerosis but also outcomes such as acute coronary syndrome (ACS) or ischemia. Martinez-Pinna et al. used GC-MS to demonstrate that ACS patients had decreased plasma citric acid, 4-hydroxyproline (4OH-Pro), aspartic acid, and fructose and increased lactate, urea, glucose, and valine.[227] Furthermore, Teul et al. conducted another study utilizing both GC-MS as well as ^{1}H-NMR focused on the analysis of patients with stable atherosclerosis and found that 24 metabolites were significantly altered in the atherosclerotic patients.[228] These metabolites included increased D-glucose, decreased D-fructose, decreased pyruvate, and decreased myoinositol. Finally, in a study by Stubiger et al., a targeted lipidomics approach was used to study the metabolic profiles of young patients that were genetically prone to hyperlipidemia as well as ACS. These findings included positive correlations between sphingomyelin (SM), a common mammalian cell membrane sphingolipid, and LDL-C, as well as lysophosphatidylcholine with VLDL-C.[229]

Although these studies were pioneering work in the field of metabolomics and atherosclerosis, our more recent study investigated the role of d-flow on the metabolite profile in order to elucidate specific mechanisms of d-flow-induced atherosclerosis. In this study, we used blood plasma samples from ApoE knockout mice collected 1 week after undergoing a PCL to induce d-flow. Mice receiving sham ligation were used as a control. A metabolome-wide association study showed that 128 metabolites were significantly altered in the ligated mice compared to the sham group. Of these, SM was the most significantly increased in the ligated mice, which is in agreement with the human studies by Stubiger et al. There were several metabolites associated with SM, 18 of which were positively correlated with SM and 41 of which were negatively correlated with SM. Furthermore, metabolic network analysis of these 59 SM-associated metabolites was performed using Meta-Core. From the analysis, 13 significant metabolic networks were discovered as being altered.[230] These results suggest that local signaling from d-flow can induce systemic metabolic changes associated with atherosclerosis (Figure 3).

CONCLUSIONS

This advanced review provides an overview of the effects of blood flow on EC signaling pathways and the application of various omics-based technologies to ECs and *in vivo*

models of atherosclerosis. Although the application of systems biology to the study of atherosclerosis is in its early stages, these studies are already providing novel insights into the development of atherosclerosis. We now understand that atherosclerosis is a complex and active process and that the ultimate clinical presentation results from the interaction of multiple cell types and organ systems. Owing to the underlying complexity of the disease, the study and treatment of atherosclerosis presents several fundamental challenges that the emerging discipline of systems biology is uniquely suited to address. Its practice begins with the acquisition of global sets of biological data at multiple hierarchical levels: DNA, RNA, protein, and metabolite abundance. However, the complete integration of these epigenomic, transcriptomic, proteomic, and metabolomic measurements to form signal transduction networks is still needed. However, this approach has been attempted in a mouse model of atherosclerosis and what we have learned so far is that the transcriptional, epigenomic, and metabolic reprogramming of ECs by d-flow initiates the inflammatory process underlying atherogenesis.[59,68,137,148,151,230]

While reviewing the literature, we noted that some signaling pathways were studied at a greater depth than the others, especially the proinflammatory pathways involving MAPK, NFkB, and ROS signaling. Likewise, KLF2 and KLF4 were the most studied transcription factors followed by NRF2, the ATF family, p38, and the JNK family. More recently, signaling pathways involving the Notch and Wnt signaling are also widely studied. However, recent pioneering studies seek to integrate our knowledge of mechanotransduction with gene expression. Specifically, Chien et al. and Shyy et al. showed that integrins and the associated small GTPase RhoA play important roles in the mechanotransduction process.[73,211,214,216,218,219] These studies elaborated how fluid shear stress can be transduced by a variety of mechanosensors that ultimately activate intracellular signaling pathways that modulate gene expression and cellular functions. Importantly, their findings revealed that shear stress activates SREBP1 and integrins in the endothelium.[218,231] Further, Shyy et al. discovered the multifaceted role of shear stress-regulated miRNAs in endothelial redox and inflammatory balance.[232] Importantly, they showed that atheroprotective flow patterns decrease miR-92a, which in turn increases KLF2 expression, thus maintaining endothelial homeostasis.[192] Taking cues from these studies, systems biology methods should be designed and tested in such a way that these intraomics and interomics interactions at multiple levels are highlighted and further enhanced in order to increase our understanding of mechanotransduction and gene expression in the endothelium and its role in atherosclerosis.

In conclusion, several groups have studied mechanosensitive pathways and over time, these studies became more reliable due to the maturation of the microarray, RNA sequencing, and mass spectroscopy platforms in terms of coverage, robustness, and reliability. All these omics techniques generate an enormous wealth of data, advocating for stronger computational methods to extract more information than before. Systems biology aims to model biological processes as networks, generating graphical maps consisting of nodes and edges representing the individual system components and their relations, respectively.[233,234] By combining multiple high-throughput datasets generated by *in vitro* and *in vivo* studies, with intensive literature mining, mathematical modeling can be developed to simulate network behavior of signaling event during the disease process. Repetitive optimization of

the model by additional, hypothesis-driven system perturbations and data integration is essential to increase its precision and its ability to accurately predict the dynamics of the system studied.[233,234] Although the development of such mathematical, disease-predicting models is still in its infancy, network analysis of high-throughput datasets has already proven a powerful tool to increase our insights into complex diseases. The network analysis tools and methods can be further extended and refined to accommodate complex designs spanning multiple tissues types across the blood vessel. This is particularly relevant to atherosclerosis, where available models of plaque formation and leukocyte infiltration necessarily involve a trade-off between ease of expression profiling and pathophysiological relevance.

Although the application of systems biology to the study of complex diseases is in its early stages, these studies are already providing novel insights into atherosclerosis and powerful tools to continue to decipher the intricacies of this disease. The promise of a systems approach includes disease prediction and prevention as well as personalized medicine. It is expected that the refinement, expansion, and cost reduction of high-throughput methods in all these fields will increase data generation even more. Combination of genomics with transcriptomics has proven to be very successful in identifying disease-associated genes and pathways. Ultimately, it is hoped that accurate network modeling of diseases can be used to predict therapeutic responses and potential side effects. However, the future success of systems biology requires a continuous expansion and refinement of tools for high-throughput data acquisition, storage, and integrative analysis.

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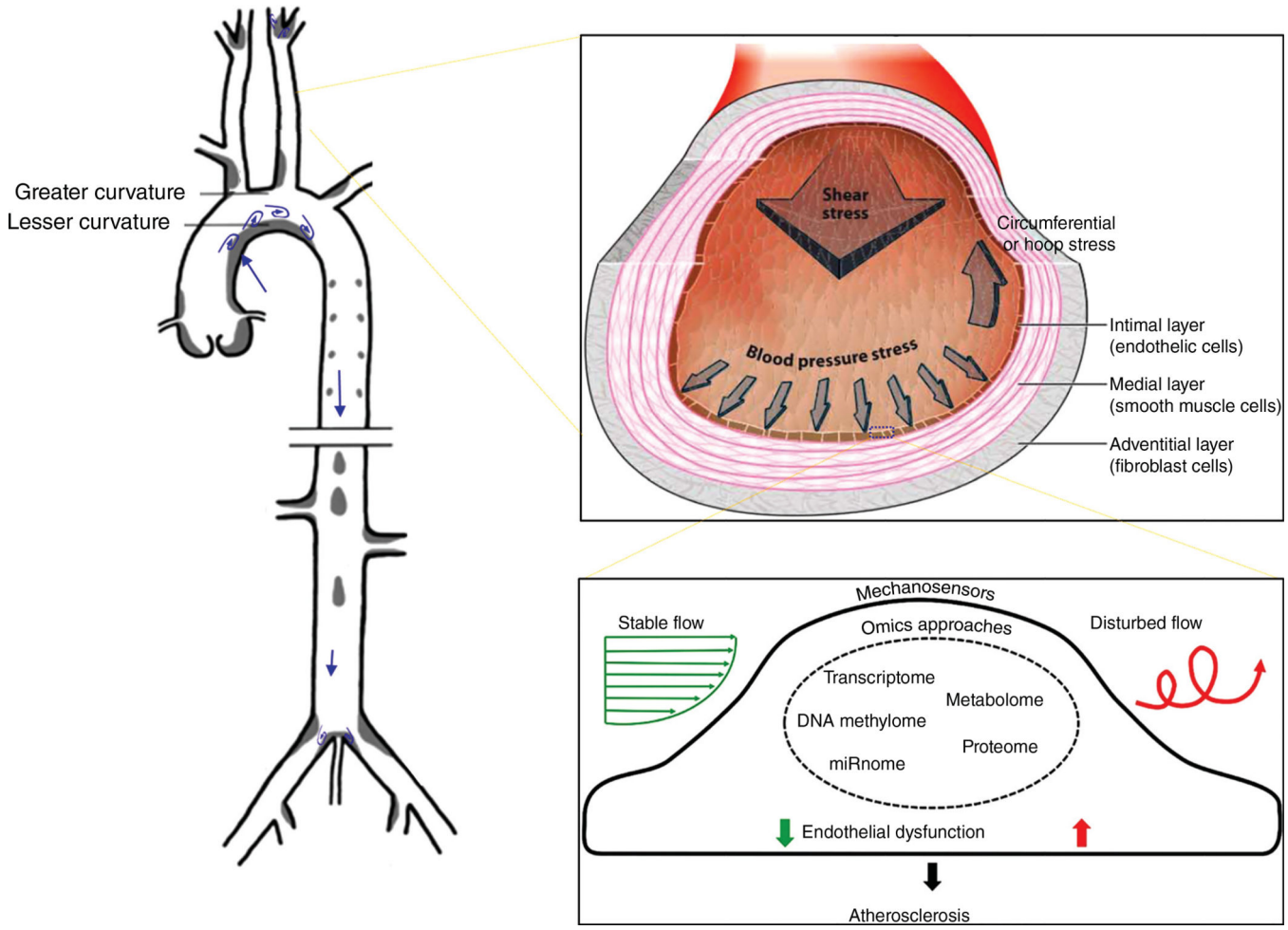

FIGURE 1.

Disturbed flow (d-flow) in human carotids, the aortic arch, and abdominal aorta is transduced through the arterial wall and initiates changes at multiple-omics level in the endothelium. Atherosclerosis tends to develop in regions of d-flow marked by blue arrows. d-Flow on the endothelial cells (ECs) lining the blood vessel wall leads to changes in the EC transcriptome, methylome, proteome, and metabolome that lead to endothelial dysfunction and atherosclerosis. (Reprinted with permission from Ref 235. Copyright 2004; Ref 58. Copyright 2014; Ref 68. Copyright 2016)

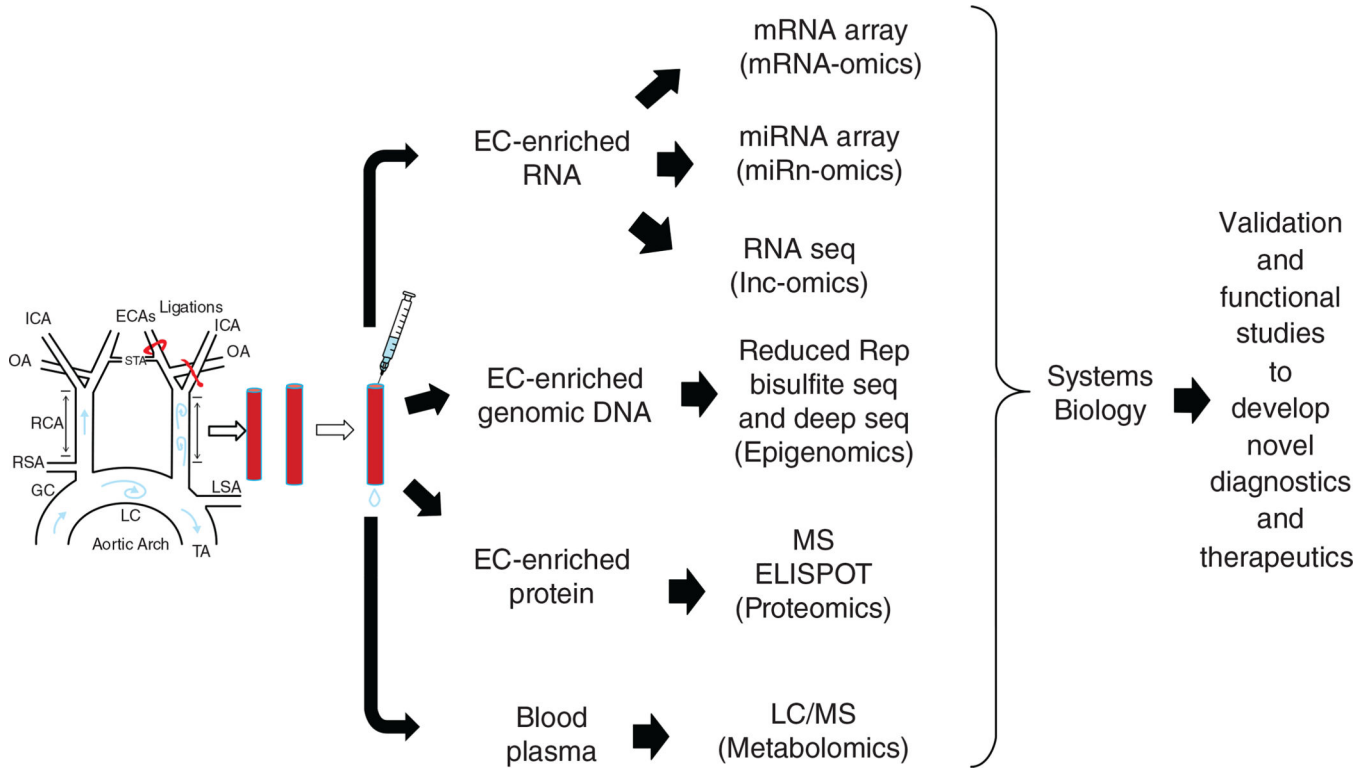

FIGURE 2.

Integration of the transcriptomics, methylomics, and metabolomics using datasets from one animal model. Using the partial carotid ligation model, endothelial-enriched RNA was collected and subjected to mRNA or miRNA microarrays in order to determine mechanosensitive genes and miRNAs. Furthermore, genomic DNA was collected in order to determine the status of methylation in many of these genes. Finally, blood plasma from the model was subjected to mass spectrometry in order to identify metabolites that are differentially expressed in the model. State-of-the-art techniques like mass spectroscopy (MS) could be used to profile the proteome using miniscule amount of proteins from the endothelium exposed to stable or disturbed blood flow. These datasets can be subjected to integrative systems biology to identify meaningful information that can lead to discovery of novel biomarkers and therapeutic candidates.

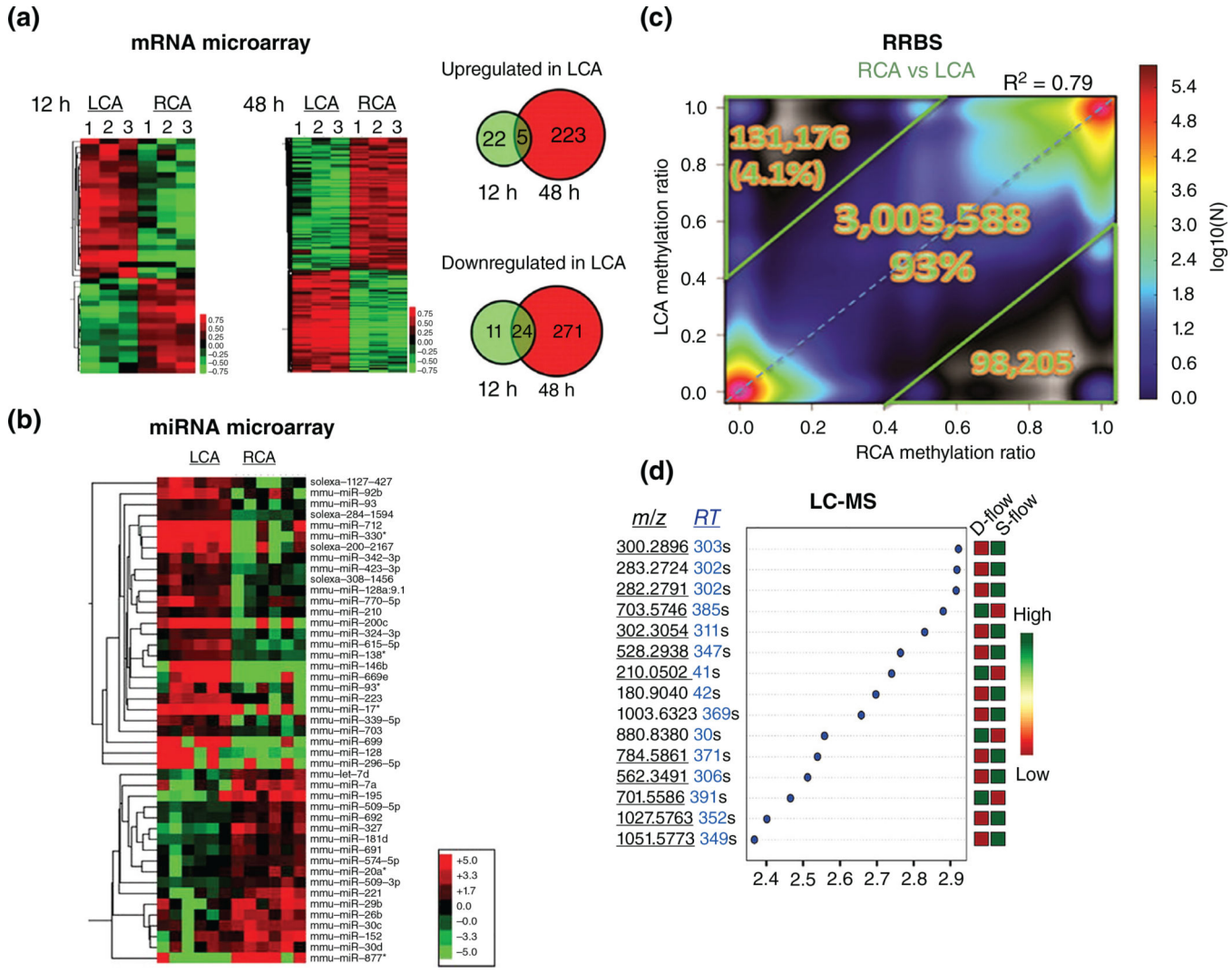

FIGURE 3.

Transcriptomics data, methylomics data, and metabolomics data from a single animal model of d-flow-induced atherosclerosis. Following partial carotid ligation, endothelial RNA was collected either 12 or 48 h after ligation and subject to a microarray. (a) Heat maps of single samples pooled from three different left carotid arteries (LCAs) or right carotid arteries (RCAs) show the number of genes affected by flow increase from 12 to 48 h. The Venn diagrams also show the temporal effects of d-flow on the number of upregulated or downregulated mechanosensitive genes. (Reprinted with permission from Ref 151. Copyright 2010). (b) Endothelial RNA collected 48 h postligation (pooled from three mice) was also analyzed by miRNA array. The heat map shows several miRNAs that are differentially regulated by flow. (Reprinted with permission from Ref 59. Copyright 2013). (c) Partially ligated animals, endothelial genomic DNA from 20 LCAs and RCAs each was pooled and the methylation status was determined by reduced representation bisulfite sequencing (RRBS). Shown is density heat-map correlation plot portraying the methylation status at each of 3,232,969 CG sites covered by the RRBS analysis. The numbers indicated in the upper, middle, and lower portions indicate CG sites hypermethylated, not altered

significantly, and hypomethylated, respectively, in the partially ligated LCA compared with the RCA. (Reprinted with permission from Ref 148. Copyright 2014). (d) Blood plasma was collected 7 days postligation and liquid chromatography and mass spectrometry (LC-MS) was performed in order to determine metabolites significantly altered by disturbed blood flow. Metabolomics analysis-identified top most significantly different 15 ions (top to bottom) are shown with variable importance in projection (VIP) scores and an expression heat map (green: high, red: low) from PLS-DA models. Underlined m/z indicated ions that were matched with known chemicals by Metlin metabolite search. (Reprinted with permission from Ref 230. Copyright 2014)