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Review article

Flow-induced reprogramming of endothelial cells in atherosclerosis

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Abstract

Atherosclerotic diseases such as myocardial infarction, ischaemic stroke and peripheral artery disease continue to be leading causes of death worldwide despite the success of treatments with cholesterol-lowering drugs and drug-eluting stents, raising the need to identify additional therapeutic targets. Interestingly, atherosclerosis preferentially develops in curved and branching arterial regions, where endothelial cells are exposed to disturbed blood flow with characteristic low-magnitude oscillatory shear stress. By contrast, straight arterial regions exposed to stable flow, which is associated with high-magnitude, unidirectional shear stress, are relatively well protected from the disease through shear-dependent, atheroprotective endothelial cell responses. Flow potently regulates structural, functional, transcriptomic, epigenomic and metabolic changes in endothelial cells through mechanosensors and mechanosignal transduction pathways. A study using single-cell RNA sequencing and chromatin accessibility analysis in a mouse model of flow-induced atherosclerosis demonstrated that disturbed flow reprogrammes arterial endothelial cells in situ from healthy phenotypes to diseased ones characterized by endothelial inflammation, endothelial-to-mesenchymal transition, endothelial-to-immune cell-like transition and metabolic changes. In this Review, we discuss this emerging concept of disturbed-flow-induced reprogramming of endothelial cells (FIRE) as a potential pro-atherogenic mechanism. Defining the flow-induced mechanisms through which endothelial cells are reprogrammed to promote atherosclerosis is a crucial area of research that could lead to the identification of novel therapeutic targets to combat the high prevalence of atherosclerotic disease.

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Key points

- Atherosclerosis preferentially develops in curved and branching regions of arteries, which are sites of disturbed blood flow and low-magnitude oscillatory shear stress.
- Disturbed flow delivers low-magnitude oscillatory shear stress to endothelial cells, which causes endothelial cells to adopt pro-atherogenic functions and gene transcription programmes.
- Endothelial cells detect shear stress magnitudes and patterns through mechanosensory proteins and organelles and transmit these signals into intracellular changes via mechanotransduction pathways.
- Advances in omics approaches and experimental models have helped to identify numerous novel potential therapeutic targets for atherosclerosis.
- Disturbed-flow-induced reprogramming of endothelial cells (which we term FIRE) promotes endothelial inflammation, endothelial-to-mesenchymal transition and endothelial-to-immune-cell-like transition during atherogenesis.
- Genes, proteins and pathways involved in FIRE are promising targets for anti-atherogenic therapies.

Introduction

Atherosclerosis is a multifactorial and chronic inflammatory disease of the arteries, in which fibrofatty plaques develop in the arterial wall[1]. As advanced plaques develop, the arterial wall stiffens, the arterial lumen narrows and, occasionally, plaques rupture or are eroded, resulting in severe clinical consequences, including myocardial infarction, ischaemic stroke and peripheral artery disease, which are the leading causes of death worldwide[2].

Dysfunction and inflammation of endothelial cells have a crucial role in the initiation and progression of atherosclerosis[3]. Endothelial cells lining the inner layer of the blood vessels are in direct contact with the blood and become dysfunctional and inflamed in response to various risk factors, such as hypercholesterolaemia, diabetes mellitus, hypertension, smoking and ageing, especially at specific atherosclerosis-prone regions associated with disturbed blood flow. At these sites of endothelial inflammation, circulating monocytes bind to endothelial cells and transmigrate into the subendothelial space, differentiating into macrophages. These regions also show increased permeability to circulating LDL cholesterol, which becomes oxidized in the subendothelial space and is ingested by nearby macrophages, thereby promoting foam cell development and triggering a vicious cycle of inflammation and macrophage accumulation[1]. In addition, vascular smooth muscle cells (VSMCs) in these regions transdifferentiate into synthetic phenotypes and migrate to the subendothelial layer and proliferate, contributing to arterial wall thickening[4]. In addition, some VSMCs transdifferentiate into foam cells[5], eventually leading to the formation of fatty streaks and fibrofatty plaques (Fig. 1a). As atherosclerotic plaques grow outwardly and inwardly, mature and progress, some plaques rupture, causing major cardiovascular events, such as myocardial infarction and stroke[5,6].

Although atherosclerotic risk factors such as hypercholesterolaemia, hyperglycaemia and hypertension are systemic, plaques preferentially develop in a focal manner in curved and branching regions of the arteries associated with disturbed blood flow[7]. Disturbed flow in these regions is characterized by the delivery of low-magnitude oscillatory shear stress (OSS) to the endothelial cell surface[7–9]. Endothelial cells detect various shear stress patterns and magnitudes through mechanosensing receptors (mechanosensors), which translate these mechanical cues into cell signalling (mechanosignal transduction) and subsequent structural and functional responses[10]. Advanced omics analyses have demonstrated that flow potently regulates nearly all facets of endothelial cell biology and pathobiology, from individual molecules and genes to structures and functions of the entire cell. Flow regulates endothelial cell transcriptomic and epigenomic landscapes at a genome-wide scale in vivo and in vitro, altering endothelial cell function, proliferation, survival and differentiation. Whereas stable blood flow, with the characteristic high-magnitude, unidirectional laminar shear stress (ULS) observed in straight, non-branching regions of the vasculature, promotes atheroprotective endothelial cell homeostasis, disturbed flow promotes pro-atherogenic endothelial cell responses, including endothelial dysfunction.

Although lipid-lowering drugs such as statins and PCSK9 inhibitors are highly efficient in reducing blood cholesterol levels and cardiovascular disease burden[11], atherosclerotic diseases continue to be leading causes of death worldwide, highlighting the need for novel anti-atherogenic drugs targeting non-lipid, pro-atherogenic pathways. In this context, the CANTOS trial[12] demonstrated that inhibition of vascular inflammation using the IL-1β inhibitor canakinumab significantly reduced atherothrombotic events in patients with previous myocardial infarction compared with placebo, in a cholesterol-independent manner. Although canakinumab was not approved by the FDA owing to an increase in fatal infections with canakinumab treatment in the trial, the findings demonstrated that targeting an inflammatory pathway could be a novel and effective anti-atherogenic therapy. Similarly, genes, proteins and pathways regulated by flow (flow-sensitive) that control pro-atherogenic endothelial cell dysfunction and inflammation could be promising novel therapeutic targets for atherosclerosis. To this end, in this Review, we discuss the current literature on flow-sensitive genes, proteins and pathways, including the emerging concept of disturbed-flow-induced reprogramming of endothelial cells (FIRE), involved in endothelial dysfunction and atherosclerosis.

Atherosclerosis preferentially develops at sites of disturbed flow

Vascular haemodynamics

The vascular endothelium is in direct contact with the blood in the arterial lumen and forms a protective barrier between the blood and the outer vascular wall[13,14]. The vascular endothelium is constantly exposed to haemodynamic forces: normal (transmural) stress and circumferential stress in the arterial wall resulting from blood pressure, and tangential shear stress on the endothelial surface due to blood flow (Fig. 1b). Whereas transmural pressure and circumferential stress in the vessel wall mainly affect and regulate medial VSMCs, fluid shear stress mostly affects endothelial cells, potently regulating their function[13–16].

Shear stress on endothelial cells

Shear stress is the frictional force derived from blood viscosity and flow rate that acts tangentially on the endothelial surface[13,16,17]. Due to complex vascular geometries and haemodynamic conditions, shear stress levels and directional patterns vary greatly in different regions

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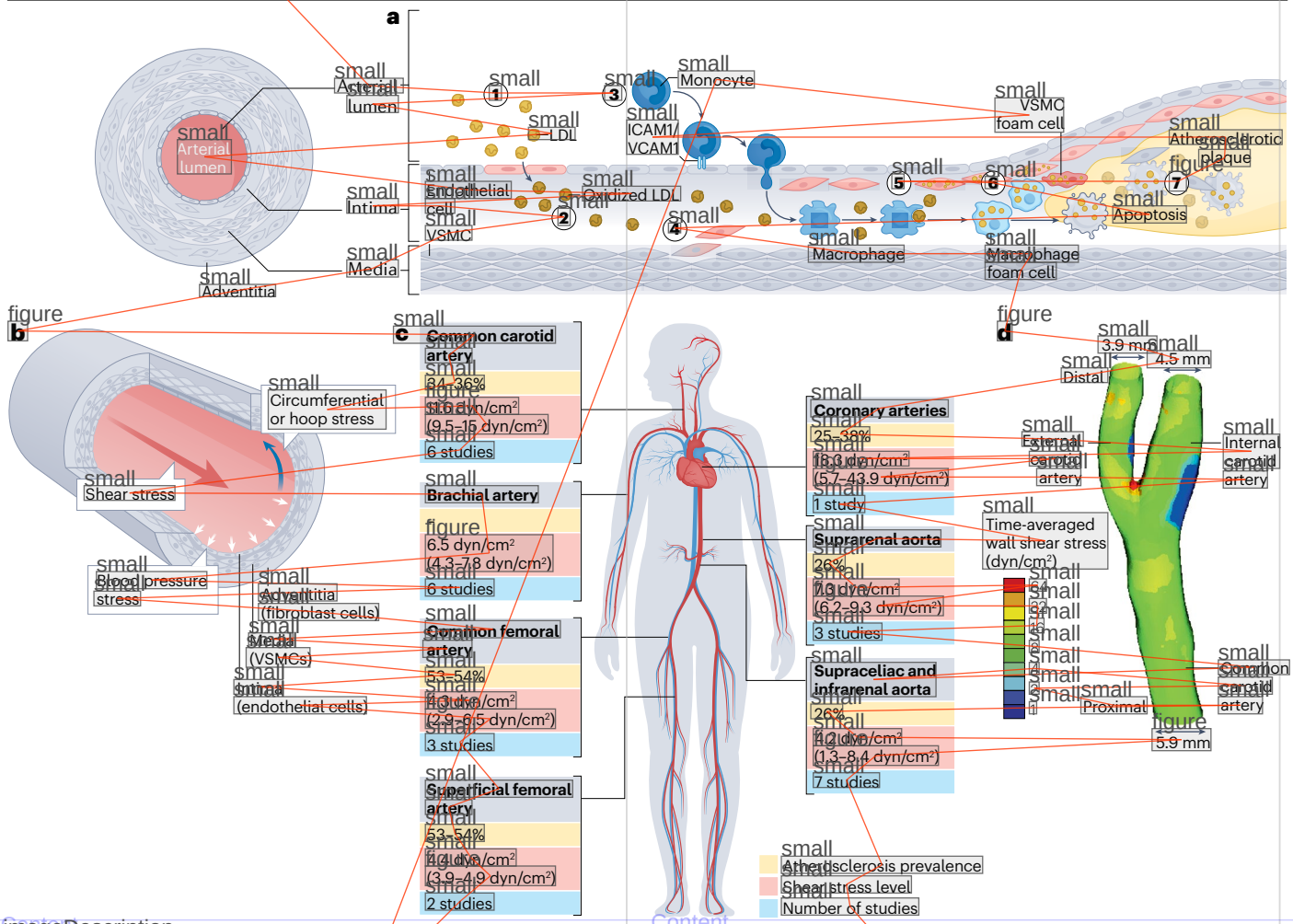

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Fig. 1 | Atherosclerosis preferentially develops at sites of disturbed flow. **a**, Stages of atherosclerotic plaque development: LDL particles infiltrate into the subendothelial space in areas of endothelial dysfunction (1); oxidized LDL promotes inflammation (2); circulating monocytes (3) and vascular smooth muscle cells (VSMCs) from the media (4) migrate towards the region of inflammation; macrophages and VSMCs ingest oxidized LDL particles (5) and, eventually, transform into lipid-laden foam cells (6), contributing to the development of atherosclerotic plaques (7). **b**, The haemodynamic forces acting on the artery wall are blood pressure, circumferential stress and shear stress. The three layers of artery wall are the intima (which contains endothelial cells), the media (with VSMCs) and the adventitia (which contains fibroblasts). **c**, Common sites of atherosclerosis development, with the associated prevalence of plaques in middle-aged adults reported in the AWHS and PESA studies and the shear stress level average and ranges, based on available literature[18,19]. **d**, Time-averaged shear stress levels in the left carotid bifurcation in a healthy individual show that the lateral wall of the internal carotid, a common site of atherosclerosis development, experiences low and oscillating shear stress from disturbed flow. Panel **d** adapted from ref. 14, Elsevier.

of the vasculature[13,14,16,17] (Fig. 1c). In straight, non-branching regions of human arteries, viscous forces of blood flow predominate over inertial forces, leading to stable, unidirectional laminar flow, which delivers high-magnitude ULS (~15 dyn/cm^2) to endothelial cells[13,16]. Conversely, in curved and branching regions, inertial forces become more prominent, leading to disturbed, multidirectional oscillatory flow that delivers low-magnitude OSS (approximately ±4 dyn/cm^2) to the endothelial cell surface[14,17] (Fig. 1d). The terms 'stable flow' and 'disturbed flow' are typically used in in vivo studies, whereas most in vitro studies use ULS and OSS to describe the experimental flow conditions to which endothelial cells are exposed. To reduce potential confusion and simplify these interchangeable terms, we use stable flow or disturbed flow in this Review, whenever feasible.

The clinical significance of blood flow is that atherosclerosis preferentially develops in curved or branching vascular regions exposed to disturbed flow conditions in the presence of additional risk factors such as hypercholesterolaemia and diabetes. For example, atherosclerotic plaques form preferentially in the lateral wall of the internal carotid artery at the carotid bifurcation, the lesser curvature of the aortic arch, and the proximal portion of the left anterior descending coronary artery[18,19].

Animal models of flow-induced atherosclerosis

Clinical observations strongly suggest a correlation between disturbed flow and sites of atherogenesis, but whether disturbed flow directly causes atherosclerosis remained unknown until it was proven by

experimental studies in animal models. Studies using the partial carotid ligation (PCL) and the shear stress-modifying constrictive carotid cuff mouse models directly demonstrated the effect of disturbed flow, or low flow, on atherosclerosis development[20,21]. In the PCL model, three of the four caudal branches of the left common carotid artery (LCA) are surgically ligated without manipulating the LCA itself. The PCL surgery induces disturbed flow in the LCA with characteristic low-magnitude OSS patterns (Fig. 2a). In *Apoe*$^{-/-}$ mice or C57BL/6 mice overexpressing PCSK9 fed a high-fat diet to induce hypercholesterolaemia, the PCL surgery causes rapid development of atherosclerosis in the entire length of the LCA within 2–3 weeks[20,22,23]. Importantly, in this mouse model, the contralateral right carotid artery (RCA) continues to be exposed to stable flow and does not develop atherosclerotic plaques, serving as an ideal control in the same animal[20,22,23] (Fig. 2a). The constrictive carotid cuff model involves the implantation of a shear stress-modifying cast over a portion of the RCA (Fig. 2b), which exposes that portion of the RCA to three different shear stress regimes that translate into atherosclerosis-inducing patterns in hypercholesterolaemic *Apoe*$^{-/-}$ mice fed a Western diet: low-magnitude, stable flow in the region proximal to the cast, which induces the development of vulnerable

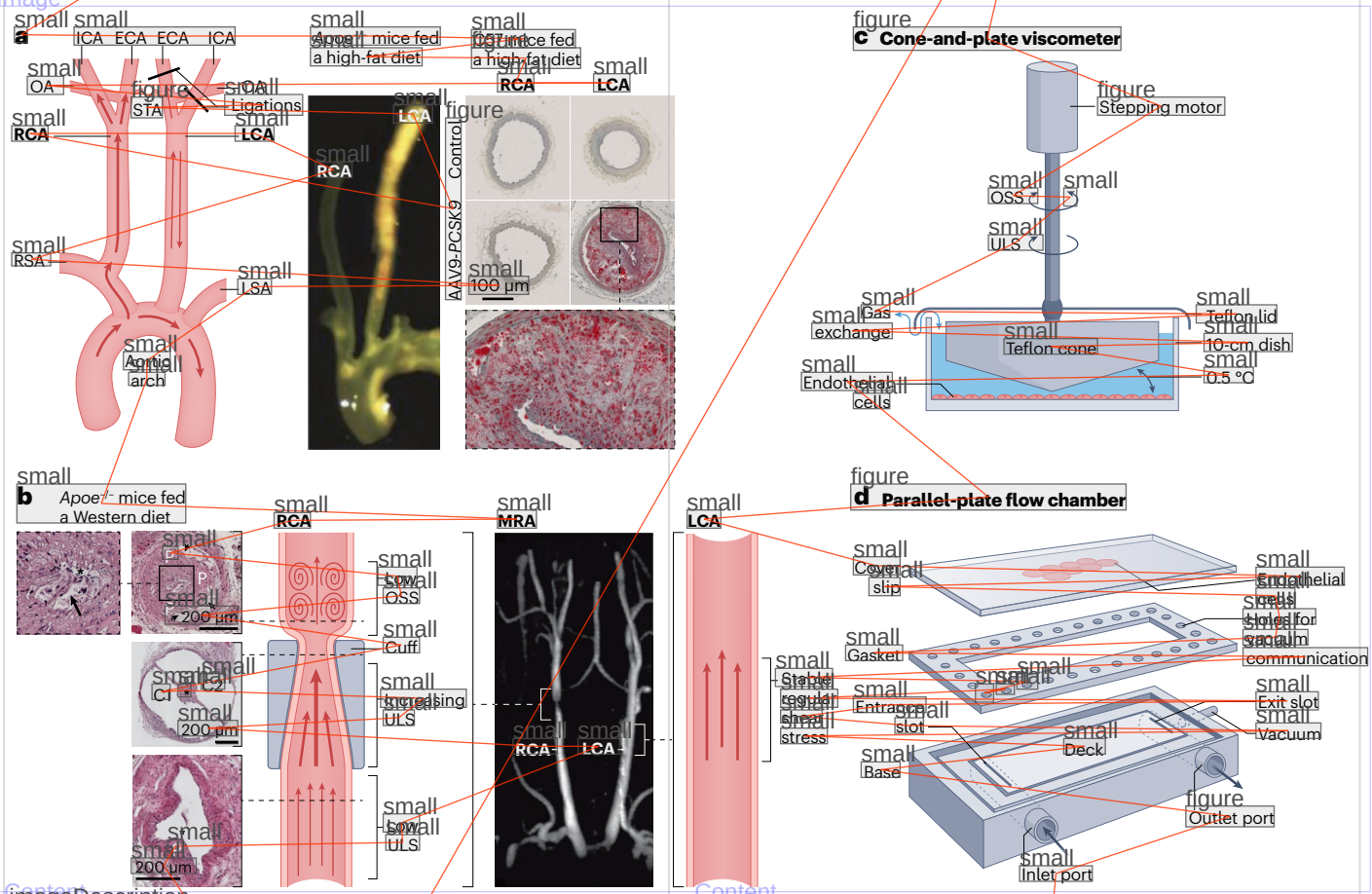

Fig. 2 | Models of atherosclerosis induced by disturbed flow. a, Schematic representation of the partial carotid artery ligation mouse model of atherosclerosis (left panel). The external carotid artery (ECA), occipital artery (OA) and internal carotid artery (ICA) are surgically ligated (black lines) to induce disturbed flow in the left carotid artery (LCA), which promotes atherosclerosis development in the LCA in hypercholesterolaemic conditions, such as in *Apoe*$^{-/-}$ mice fed a high-fat diet (central panel)[20,143] and mice with adeno-associated virus (AAV)-mediated *PCSK9* overexpression fed a high-fat diet (right image, middle-right and bottom panels; shown by oil-Red-O staining)[22]. By contrast, even in hypercholesterolaemic conditions, the right carotid artery (RCA), which is exposed to stable flow, does not develop atherosclerosis (right image, middle-left panel). Mice without AAV-*PCSK9*-induced hypercholesterolaemia do not develop atherosclerotic plaques in the RCA or the ligated LCA (right image, top panels). **b**, Schematic representation of the shear-modifying constrictive cuff model. Implanting a constrictive cuff (white bracket) on the RCA shown in the magnetic resonance imaging angiogram (MRA) exposes endothelial cells to low-magnitude, unidirectional, laminar shear stress (ULS) in the proximal region of the cast, high-magnitude ULS within the cuff and low-magnitude oscillatory shear stress (OSS) in the distal region of the cuff. In hypercholesterolaemic conditions, such as in *Apoe*$^{-/-}$ mice fed a Western diet for 8 weeks, the low-magnitude OSS induces atherosclerotic plaque (P) development with a large lipid core (black arrows) in the carotid artery, as shown by haematoxylin and eosin staining. The vessel lumen is indicated by an asterisk. **c**, Schematic representation of a cone-and-plate viscometer. **d**, Schematic representation of a parallel-plate flow chamber. Endothelial cells are exposed to differential shear stress with the use of a rotating Teflon cone in the cone-and-plate viscometer and with computer-generated hydrostatic pressure in the parallel-plate system. C1 and C2, cuffs; LSA, left subclavian artery; RSA, right subclavian artery; STA, superior thyroid artery. Panel **a** left drawing adapted from ref. 20, APS; central image adapted from ref. 143, CC0; and right images adapted from ref. 22, Elsevier. Panel **b** adapted from ref. 24 (Kuhlmann, M. T., Cuhlmann, S., Hoppe, I., Krams, R., Evans, P. C., Strijkers, G. J., Nicolay, K., Hermann, S., Schäfers, M. Implantation of a carotid cuff for triggering shear-stress induced atherosclerosis in mice. J. Vis. Exp. (59), e3308, https://doi.org/10.3791/3308 (2012)). Panel **c** adapted from ref. 17, Elsevier. Panel **d** adapted from ref. 33, Elsevier.

plaques; high-magnitude, stable flow within the casted region, with no plaque development; and disturbed flow in the region distal to the cast, which induces the development of stable plaques[21,24]. This mouse model demonstrates flow-dependent atherosclerosis development within a single carotid artery[21,24] (Fig. 2b). Both animal models have been used in numerous laboratories worldwide and are extremely valuable tools to study flow-dependent endothelial cell function and atherogenic mechanisms in vivo. One major difference between the models is that in the PCL model, atherosclerotic plaques form throughout the entire length (~1 cm) of the LCA, whereas the contralateral RCA remains healthy and serves as a control. Therefore, this model provides sufficient endothelial samples from the LCA and RCA from the same animal to conduct omics studies, such as single-cell RNA sequencing (scRNA-seq) or bulk RNA sequencing, unlike the cuff model, which provides relatively smaller amounts of endothelial samples[15].

In addition to these mouse models of disturbed flow-induced atherosclerosis, the use of zebrafish has emerged as a genetically tractable model to examine early events of atherogenesis[25,26]. Combined with genetic manipulation approaches to reduce blood flow, zebrafish models provide further evidence that disturbed flow causes atherosclerosis under hypercholesterolaemic conditions[27–31]. An interesting question is whether disturbed flow-induced atherosclerosis occurs with other risk factors, such as diabetes and hypertension, independently of hypercholesterolaemia.

In vitro flow models

Numerous in vitro models of shear stress, including the cone-and-plate viscometer, the parallel-plate flow chamber and the microfluidic channel have been developed and have been reviewed previously[14,32–34]. These in vitro bioreactors expose endothelial cells to various shear conditions and can be used to determine the detailed mechanisms of shear stress-dependent endothelial function in a well-defined biomechanical environment (Fig. 2c,d).

Blood flow regulates endothelial function

Endothelial cells in vivo are constantly exposed to various haemodynamic factors, especially shear stress associated with blood flow, which potently regulates nearly all facets of endothelial cell function. Blood flow regulates vascular tone; endothelial barrier permeability; angiogenesis; endothelial cell proliferation, death, differentiation, senescence, metabolism, inflammation and morphology; and extracellular matrix remodelling[13,14,16]. Defining the mechanisms by which stable flow protects endothelial homeostatic function and disturbed flow induces endothelial dysfunction is crucial for understanding the pathogenesis of flow-dependent atherosclerosis and developing novel therapeutic approaches.

Blood flow potently regulates endothelial barrier function. Stable flow protects endothelial barrier permeability, whereas disturbed flow promotes endothelial barrier dysfunction[13,14,16]. Stable flow regulates endothelial cell permeability by promoting tight junction stability via control of occludin expression and attachment to the actin cytoskeleton, as well as control of adherens junction integrity via phosphorylation and degradation of VE-cadherin[35–37]. Disturbed flow increases both endothelial cell proliferation and apoptosis via multiple mechanisms, including downregulation of the expression of the tumour suppressor protein p53 and inhibition of the anti-apoptotic kinase AKT[38–41]. Stable flow induces autophagy through a sirtuin 1-dependent and FOXO1-dependent pathway, providing a cytoprotective mechanism for endothelial cells[42]. Disturbed flow induces endothelial cell senescence through a p53-dependent and sirtuin 1-dependent mechanism, thereby reducing endothelial cell migration and disrupting arterial repair mechanisms[43].

Blood flow also regulates metabolism and redox reactions in endothelial cells. Whereas stable flow reduces endothelial glucose uptake and metabolic activity as well as the expression of genes encoding proteins involved in glycolysis, disturbed flow promotes glycolytic metabolism, causing a markedly different metabolomic profile and increased mitochondrial fission[44–47]. Disturbed flow also increases the endothelial production of reactive oxygen species (ROS) and oxidative stress[48–51]. This process is mediated by bone morphogenetic protein 4 (BMP4), which induces increased production of superoxide via NADPH oxidases and endothelial nitric oxide synthase (eNOS) uncoupling-dependent mechanisms[48–53]. Endothelial ROS generation in response to disturbed flow increases vascular oxidative stress and LDL oxidation in the context of atherosclerosis and hypertension[51,54–57].

Disturbed flow potently induces endothelial cell inflammation and transdifferentiation of endothelial cells, which have crucial roles in the initiation and progression of atherosclerosis. Disturbed flow induces endothelial inflammation by increasing the expression of endothelial adhesion molecules (VCAM1, ICAM1 and E-selectin), which mediate monocyte adhesion to endothelial cells[16]. Activation of nuclear factor-κB (NF-κB) signalling is crucial in disturbed flow-induced inflammation[58]. In addition, disturbed flow promotes the expression of cytokines and chemokines such as IL-1, IL-6 and CCL5 (refs. 58,59). Disturbed flow can induce the transdifferentiation of endothelial cells to mesenchymal cells (endothelial-to-mesenchymal transition; EndMT) and immune-like cells (endothelial-to-immune cell transition; EndIT)[15]. The pathophysiological importance of EndMT in disturbed flow-induced atherosclerosis has been demonstrated, but the validation and relevance of EndIT in atherosclerosis remain to be determined[60]. Increased endothelial cell turnover under disturbed flow conditions also coincides with increased transcription of genes encoding angiogenic factors and with increased neovascularization[61–63].

Morphologically, endothelial cells adopt an elongated, fusiform shape and align to the direction of flow under stable flow conditions[32,64]. By contrast, endothelial cells under disturbed flow or no-flow static conditions adopt a polygonal, 'cobblestone' shape without uniform alignment[65,66]. These morphological changes are accompanied by actin cytoskeleton remodelling from a pattern of bands encircling the periphery of the cell to a pattern of thick, central stress fibres aligned in the direction of shear stress[67,68]. These cytoskeletal changes alter intercellular stress and cellular traction forces, affecting subsequent cellular strain and status[69,70]. In addition, shear stress induces subcellular structural changes, such as changes in nuclear shape and relocation of the Golgi apparatus towards the upstream flow direction[71,72].

Mechanosensors and mechanotransduction

Endothelial cells transduce flow signals into intracellular changes through the processes of mechanosensing and mechanosignal transduction. Endothelial cells recognize fluid shear stress through mechanosensors located in the apical and basal surfaces of the cell, in cell–cell junctions and intracellularly[73] (Fig. 3). On the apical surface, the mechanosensors include plasma membrane proteins, such as the cation channels PIEZO1 and P2X purinoreceptor 4, NOTCH1, protein kinases, G protein-coupled receptors and plexin D1, as well as membrane-associated structures, such as caveolae, the glycocalyx and primary cilia[74–87]. PIEZO1 is an inward-rectifying calcium channel present in many cell types that opens in response to mechanical force[88]. In endothelial cells, PIEZO1 mediates shear stress magnitude-dependent increases in

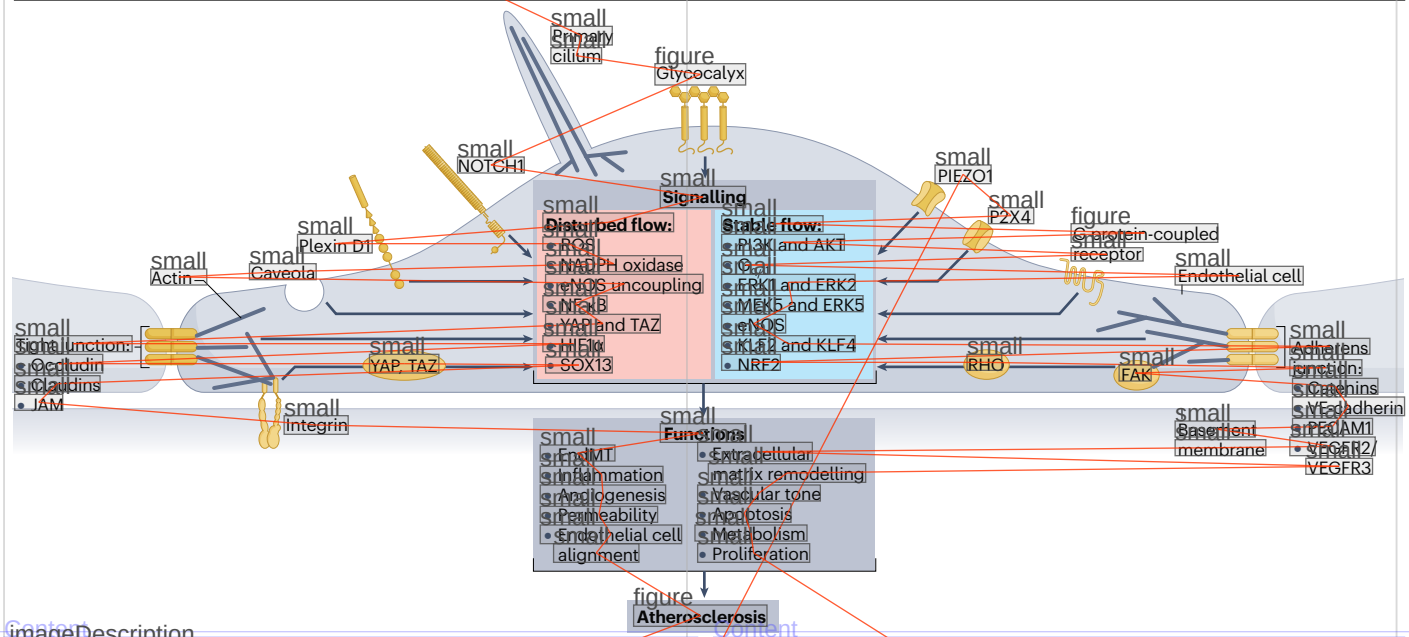

Fig. 3 | Mechanosensors and mechanosignal transduction pathways in endothelial cells. The apical surface of the endothelial cell contains protein mechanosensors, such as plexin D1, NOTCH1, PIEZO1, P2X4 and G protein-coupled receptors (such as GPR68), as well as mechanosensitive cell structures, such as caveolae, primary cilia and the glycocalyx. Cell–cell junctions contain the mechanosensory complex comprising VE-cadherin, platelet endothelial cell adhesion molecule (PECAM1), vascular endothelial growth factor receptor 2 (VEGFR2) and VEGFR3. The basal surface of endothelial cells contains integrin mechanosensors. Mechanosignal transduction pathways include the PI3K–AKT pathway, ERK1–ERK2 pathway, YAP–TAZ pathway and the RHO signalling pathway. Many mechanosignal transduction pathways result in activation of transcription factors including Krüppel-like factor 2 (KLF2) and KLF4, nuclear factor-κB (NF-κB) and hypoxia-inducible factor 1α (HIF1α). EndMT, endothelial-to-mesenchymal transition; eNOS, endothelial nitric oxide synthase; FAK, focal adhesion kinase; JAM, junctional adhesion molecule; NRF2, nuclear factor erythroid 2-related factor 2; ROS, reactive oxygen species; SOX13, transcription factor SOX13.

intracellular calcium levels, influencing flow-induced, anti-atherogenic and pro-atherogenic responses, such as cell alignment[50,87,89]. NOTCH1 mediates stable flow-induced cellular alignment, suppression of cell proliferation and maintenance of cell–cell junctional integrity, and protects against atherosclerosis development[81]. These effects have been suggested to be controlled by tension-induced NOTCH1 signalling and modulation of intracellular calcium levels[81]. However, another study showed that shear stress-mediated activation of the NOTCH1 response requires PIEZO1, warranting additional clarification regarding the mechanosensory role of NOTCH1 (ref. 76). G protein-coupled receptors, such as the proton-sensing GPR68, undergo conformational changes in response to flow in endothelial cells, mediating shear-induced calcium influx[53,90]. Plexin D1, potentially in connection with the junctional mechanosensory complex, mediates flow-dependent atherosclerosis development by regulating calcium uptake, phosphorylation of vascular endothelial growth factor receptor 2 (VEGFR2), AKT, eNOS, ERK1 and ERK2, as well as induction of the major flow-sensitive transcription factors, Krüppel-like factor 2 (KLF2) and KLF4 (ref. 82).

In cell–cell junctions, platelet endothelial cell adhesion molecule (PECAM1), VE-cadherin and VEGFR2 form the junctional mechanosensory complex, which mediates integrin activation, cellular alignment and eNOS activation in response to stable flow via PI3K–AKT signalling[69]. On the basal surface of endothelial cells, flow induces conformational activation and expression changes in extracellular matrix-binding integrin mechanosensors[91,92]. Stable flow activates the integrin αvβ3, causing increased binding to the extracellular matrix and inactivation of downstream RHO signalling[91]. In addition, the actin cytoskeleton in the cytosol has been shown to serve as a mechanosensory structure[93].

Flow mechanosensing in endothelial cells activates numerous early-to-intermediate (seconds to minutes) cell signalling pathways that lead to long-term (hours to days) atheroprotective or pro-atherogenic processes. However, it is important to note that both pro-atherogenic disturbed flow and atheroprotective stable flow often activate many of the same early-to-intermediate signalling pathways, especially in in vitro studies. For example, stable flow transiently activates NF-κB signalling without leading to long-term endothelial inflammation, whereas disturbed flow induces persistent NF-κB activation resulting in endothelial inflammation[94]. The mechanisms that distinguish these flow-pattern-dependent activation pathways, especially in vitro, are not well understood and remain crucial knowledge gaps that need to be filled. This uncertainty is due in part to the common experimental strategy of conducting in vitro studies with endothelial cells cultured under no-flow conditions that are suddenly subjected to stable flow or disturbed flow. Under these conditions, early-to-intermediate responses might overlap with common adaptative changes in response to altered mechanical cues regardless of flow patterns and magnitudes. With this caveat in mind, we review the literature that provides crucial knowledge in understanding flow-dependent endothelial cell mechanosignal transduction pathways.

PIEZO1

PIEZO1 mediates both atheroprotective and pro-atherogenic flow-dependent endothelial cell responses[74,80,85,95]. PIEZO1 mechanosensing of stable flow induces intracellular calcium influx, leading to ATP

release and eNOS activation and the production of the atheroprotective and vasorelaxant nitric oxide from endothelial cells[80,85]. The PIEZO1-induced effect on ATP is mediated by the $P2Y_2$ and $G_{\alpha q/11}$ pathways, which activate AKT, which in turn activates eNOS[96,97]. PIEZO1 also mediates pro-atherogenic endothelial responses of disturbed flow. Disturbed flow stimulates NF-κB activity and endothelial inflammation via PIEZO1–$G_{\alpha q/11}$-mediated integrin activation, which in turn activates the focal adhesion kinase (FAK)[74]. Endothelial cell-targeted deletion of *Piezo1* in *Ldlr*-knockout mice inhibited atherosclerotic plaque development in disturbed flow regions, suggesting a pro-atherogenic role of PIEZO1 in endothelial cells[74].

callout

Plexin D1

Plexin D1 is another mechanosensor that responds to both stable flow and disturbed flow, mediating both atheroprotective and pro-atherogenic responses, respectively. Knockdown of *Plxnd1*, which encodes plexin D1, in mouse endothelial cells inhibits atheroprotective signal transduction pathways, such as eNOS activation, cell alignment and KLF2 and KLF4 expression in response to stable flow[82]. Interestingly, *Plxnd1* knockout in endothelial cells also prevents pro-atherogenic inflammatory responses, including expression of VCAM1 and CCL2 in response to disturbed flow[82]. Endothelial cell-specific knockout of *Plxnd1* in mice prevented atherosclerosis development in arterial regions with disturbed flow but exacerbated plaque development in arterial regions with stable flow[82]. These results suggest that plexin D1 is a mechanosensor with dual functions depending on blood flow patterns.

Junctional mechanosensory complex

Stable flow stimulates eNOS activation through the mechanosensory complex formed by PECAM1, VE-cadherin and VEGFR2 or VEGFR3, which in turn activates the PI3K–AKT pathway[69]. Stable flow also induces integrin αvβ3 and NF-κB activation through the junctional mechanosensory complex[69]. However, *Pecam1* knockout in mice prevents endothelial inflammation and atherosclerosis in arterial regions with disturbed flow[69,98]. This finding suggests that PECAM1 is a mechanosensor that mediates pro-atherogenic effects of disturbed flow in vivo.

Integrins

Integrin activation in response to stable flow inactivates RHO GTPases through RHO-like GTPase signalling[99]. RHOA inactivation promotes YAP phosphorylation (at Ser127 and Ser381) in the cytoplasm to maintain an atheroprotective endothelial cell phenotype[100]. These interactions between integrins and RHO GTPases further activate RAC, leading to the assembly of the junctional mechanosensory complexes[101]. Additionally, the RHO GTPase CDC42 is polarized and activated in an integrin-dependent manner and subsequently regulates the polarity of the microtubule-organizing centre[102,103]. Under disturbed flow conditions, the cooperation between RGD-binding integrins (including α5β1 and αvβ3 integrins) and fibronectin has been shown to drive pro-inflammatory signal transduction involving the nuclear translocation of NF-κB, YAP and serine/threonine-protein kinase PAK[104–107].

Flow-sensitive transcription factors

Thus far, we have discussed mechanosignal transduction pathways occurring in an early-to-intermediate timescale, mediated by specific mechanosensors in response to flow in endothelial cells. These relatively acute responses lead to the regulation of downstream, long-term responses, including activation of transcription factors and transcriptional co-activators, such as KLF2 and KLF4, NF-κB, hypoxia-inducible factor 1α (HIF1α), YAP, TAZ and SOX13, which regulate gene expression profiles and cell function[73]. KLF2 and KLF4 are two of the most flow-sensitive master transcription factors regulating the expression of genes that control anti-atherogenic pathways induced by stable flow, including vasodilatation and antithrombotic and anti-inflammatory pathways[108–112]. KLF2 reduces the expression of pro-atherogenic genes by competing with NF-κB for transcriptional cofactor CBP–p300 and by promoting the translocation of nuclear factor erythroid 2-related factor 2 (refs. 113–115). Stable flow increases *KLF2* transcription by sequentially activating the members of the MAP kinase family MEKK3, MEK5 and ERK5, which in turn activates the transcription factors MEF2A and MEF2C[116]. By contrast, disturbed flow inactivates the ERK5 pathway, leading to the inhibition of *KLF2* expression[116]. In addition, *KLF2* expression is suppressed by the flow-regulated microRNA (miRNA), miR-92a[117–123].

NF-κB is a well-recognized transcription factor that is activated by flow. Nuclear translocation and activation of NF-κB in endothelial cells is increased transiently by stable flow and persistently by disturbed flow[94,124]. NF-κB target genes include those encoding VCAM1, ICAM1, E-selectin, HIF1α and numerous cytokines, all of which have a crucial role in atherosclerosis[94,125,126]. HIF1α is a pro-atherogenic transcription factor that is activated by disturbed flow[127–129]. HIF1α induces the expression of glycolytic enzymes such as HK2, PFKFB3 and PDK1 (ref. 130). YAP and TAZ — which are transcriptional co-activators induced by the Hippo signalling pathway and are involved in organ growth and development as well as various diseases such as cancer and atherosclerosis — are also regulated by both stable and disturbed flow[131–133]. Disturbed flow induces YAP and TAZ nuclear translocation and activation, leading to endothelial cell inflammation and cytoskeletal remodelling and atherosclerosis[131]. A study published in 2022 identified SOX13 as a novel flow-sensitive transcription factor. Disturbed flow represses the expression of *SOX13*, which in turn leads to a strong induction of pro-inflammatory cytokine and chemokine production, including CCL5 and CXCL10, resulting in endothelial inflammation[134].

Omics approaches to study endothelial cells

Omics-based analyses have become standard approaches to determine changes in endothelial cells in response to various flow and disease conditions. Unlike traditional reductionist approaches studying one or a few candidate genes or proteins at a time, the astonishing advances in omics technologies and computational bioinformatics have made it possible to determine changes in genes, proteins and metabolites at a genome-wide, epigenome-wide, proteome-wide and metabolome-wide scale, often at a single-cell resolution, and using a small amount of sample. The application of these approaches using in vitro and in vivo models has generated a plethora of datasets of flow-dependent transcriptomic, epigenomic, proteomic and metabolomic profiles in endothelial cells and blood vessels under healthy and disease conditions[15,135–140].

Early transcriptomic studies used bulk RNA and miRNA samples from pooled cultured endothelial cells and animal tissues to conduct microarray and RNA sequencing analyses. These studies identified numerous unexpected flow-sensitive genes, miRNAs and long non-coding RNAs (lncRNAs), generating wide-ranging novel hypotheses regarding their various roles in endothelial cell function and atherosclerosis[14,17,73]. Numerous flow-sensitive genes (including *BMP4*, *DNMT1*, *KDM4B*, *KLF2*, *KLK10*, *PLPP3*, *SEMA7A*, *THBS1*, *TMX1* and *ZBTB46*), miRNAs (including miR-95a and miR-712) and lncRNAs (including

MALAT1 and *MANTIS*) were identified from these bulk-RNA studies and subsequently validated, as reviewed elsewhere[14,17,73,141,142]. The roles of flow-sensitive miRNAs and lncRNAs in endothelial function have been reviewed previously[143,144]. *KLK10* (which encodes kallikrein-related peptidase 10) has been identified as one of the most flow-sensitive genes from a gene-array study using the mouse PCL model[135]. *KLK10* expression is increased by stable flow, but nearly lost in response to disturbed flow in endothelial cells in vitro, mouse arteries in vivo and human coronary arteries with advanced atherosclerotic plaques[143]. After the KLK10 protein is produced, it is secreted into the circulating blood (or the conditioned medium in in vitro assays) and functions as an anti-inflammatory and permeability-barrier-protective protein[143]. Interestingly, KLK10, which is a member of the KLK serine/threonine protein kinase family, lacks inherent protease activity, and its anti-inflammatory and permeability-barrier-protective functions are mediated by protease-activated receptor 1 (PAR1)-dependent and PAR2-dependent pathways[143]. Administration of recombinant KLK10 via tail vein injection or ultrasound-guided delivery of a KLK10 expression vector to the carotid endothelium prevented endothelial inflammation and atherosclerosis development in mice[143,144], demonstrating the proof of principle that flow-sensitive proteins such as KLK10 could be used as novel anti-atherogenic therapeutics.

Flow induces epigenome-wide changes in endothelial cells, as revealed by a DNA methylome study that used reduced representation bisulfite sequencing of mouse genomic bulk DNA samples combined with microarray analysis of bulk RNA samples of mouse carotid arteries after PCL surgery[136]. This DNA methylome study, together with other studies, showed that disturbed flow regulates DNA methylation patterns in endothelial cells via the DNA methyltransferases DNMT1 and DNMT3 (refs. 136,145,146). Further studies showed that genetic deletion or pharmacological inhibition of DNMT1 prevented endothelial inflammation and atherosclerosis development in *Apoe*$^{-/-}$ mice[145], demonstrating that flow-sensitive epigenomic modifications could be anti-atherogenic therapeutic targets.

Proteomics studies using advanced mass spectrometry have identified numerous flow-sensitive proteins that are differentially expressed or post-translationally modified in endothelial cells in response to flow[142,147,148]. Analyses of secreted media (secretome) of endothelial cells show that disturbed flow alters the levels of hundreds of proteins, including ANGPT2 and endothelin 1 (ref. 148). A study to determine proteome-wide *S*-sulfhydration changes of reactive cysteines (*S*-sulfhydrome) in endothelial cells in response to pro-atherogenic conditions in vitro and in vivo identified hundreds of flow-sensitive *S*-sulfhydrated proteins[149], including integrins, which have an important role in the flow-dependent vascular relaxation response. A metabolomics study using plasma samples from *Apoe*$^{-/-}$ mice subjected to PCL surgery showed that disturbed flow induces significant changes in the levels of hundreds of metabolites, including sphingomyelin and the amino acids methionine and phenylalanine[46]. However, the causal effects of flow-dependent changes in metabolites have not been clearly defined in vivo and further investigation is warranted. The targets identified by omics approaches and their roles in endothelial cell dysfunction and in atherosclerosis are summarized in Table 1.

Disturbed-flow-induced reprogramming of endothelial cells

Early transcriptomic and epigenomic studies used mouse bulk RNA and DNA samples obtained from pooled endothelial cells collected by carotid flushing after PCL surgery[135,136,150]. The findings from these studies helped to establish flow-dependent changes in transcriptomic and DNA methylation patterns in endothelial cells in a genome-wide and epigenome-wide manner. However, although these results revealed a definitive list of genes with reduced expression in endothelial cells in response to disturbed flow, identifying genes that are increased under disturbed flow conditions in the PCL model has been difficult. The reason for this dilemma is that disturbed flow induces endothelial cell inflammation and accumulation of other cell types, especially immune cells, in the subendothelial layer, thereby causing substantial contamination of the endothelium-enriched luminal-flushing samples. Therefore, it was difficult to discern whether the increased expression of any gene of interest originated from the endothelial cells or from the contaminating immune cells and VSMCs. To address the difficulty in identifying flow-sensitive genes in endothelial cells, our group carried out scRNA-seq and single-cell assay for transposase-accessible chromatin sequencing (scATAC-seq)[15].

scRNA-seq enables the study of transcriptional changes at a genome-wide scale at single-cell resolution. scRNA-seq quantifies each gene transcript, both unspliced precursor mRNA (pre-mRNA) and mature, spliced mRNA (mRNA), in each cell, providing insights into gene transcript expression profiles and dynamic cellular status. The results show each gene transcript quantity in every cell, while the pre-mRNA and mRNA levels can be used for trajectory inference analysis, such as pseudotime analysis or RNA velocity analysis[151]. scATAC-seq assays reveal genome-wide epigenomic changes in chromatin accessibility at single-cell resolution, providing data on gene *cis*-regulatory elements including enhancers, nucleosome positions and transcription factor binding sites. Integration of scRNA-seq and scATAC-seq analyses provides additional independent validation and comparison of gene transcript levels and epigenomic regulatory profiles for each gene, adding another layer of confidence to the data analysis[152].

Our study of single cells obtained from LCAs (exposed to disturbed flow for 2 days or 2 weeks) and RCAs (exposed to stable flow for 2 days or 2 weeks) in the same mice after PCL surgery enabled the identification of differential transcriptomic and epigenomic changes in endothelial cells and other cell types, in a flow-dependent and time-dependent manner[15]. The scRNA-seq and scATAC-seq results independently showed that disturbed flow induced dynamic changes in cell composition in the mouse carotid arteries in a time-dependent manner. The comparison and integration of scRNA-seq and scATAC-seq data showed remarkable concordance, demonstrating the reproducibility and validity of each dataset[15]. Each individual cell in these datasets was assigned a specific cell type based on the expression of cell type-specific canonical marker genes. The carotid arteries contained eight endothelial cell clusters, four monocyte–macrophage clusters, one cluster of VSMCs, one of fibroblasts, one of dendritic cells and one of T cells, all varying in terms of cell identity and number in a flow-dependent and time-dependent manner[15]. Most interestingly, carotid artery endothelial cells are heterogeneous and dynamic in response to flow. In the RCA exposed to the healthy stable flow condition, four endothelial cell subclusters (E1–E4) were identified and remained unchanged over time. However, in the LCA exposed to the pro-atherogenic disturbed flow condition, most of the healthy endothelial cell subclusters (E2–E4) were nearly lost, whereas new endothelial cell subclusters (E6 and E8) emerged[15]. In addition, few VSMCs were found in the RCA intima, but disturbed flow increased the VSMC numbers in the LCA[15], as expected. Fibroblasts were found in both the LCA and RCA, with the highest number found in the LCA in the 2-day disturbed flow condition. Although monocyte and macrophage

Table 1 | Flow-sensitive genes in endothelial cells

Effect in atherosclerosis	Name	Shear stress type; expression regulation	Target genes	Effect in endothelial cells	Refs.
Protein-coding genes					
Anti-atherogenic	*NOS3*	ULS; ↑	–	Nitric oxide production and maintenance of vascular tone	173
	KLF2	ULS; ↑	–	Antioxidative, antithrombotic, maintenance of vascular integrity and endothelial cell identity	113,174,175
	KLF4	ULS; ↑		Antioxidative, antithrombotic, maintenance of vascular integrity and endothelial cell identity	135,165,176
	SOD2	ULS; ↑		Antioxidative	177
	SOD3	ULS; ↑	–	Antioxidative	177
	TIMP3	ULS; ↑	–	Decreased activities of metalloproteinases and extracellular matrix degradation	178,179
	PLPP3	ULS; ↑		Expression regulated by KLF2 and miR-92; anti-inflammatory	112
	ZBTB46	OSS; ↓	–	Promotes endothelial cell quiescence	180
	BMPR2	ULS; ↑	–	Inhibits oxidative stress and NF-κB activation in endothelial cells	181
	NFE2L2	ULS; ↑	–	Antioxidant responsive element	182
	SOX13	ULS; ↑	–	Anti-inflammatory	134
Pro-atherogenic	*CCL2*	ULS; ↓ OSS; ↑		Promotes immune cell adhesion to endothelial cells	183
	BMP4	OSS; ↑	–	Promotes oxidative stress and inflammatory responses	51,57,184–191
	VCAM1	OSS; ↑	–	Promotes immune cell adhesion to endothelial cells	192
	ICAM1	OSS; ↑	–	Promotes immune cell adhesion to endothelial cells	192
	NFKB	OSS; ↑	–	Increases pro-inflammatory and pro-atherogenic gene expression	193–196
	NOX1, NOX2	OSS; ↑		Increases superoxide generation; pro-atherogenic effects	49–51,55,57, 197–200
	NOX4	OSS; ↑ or ↓		Increases H_2O_2 production leading to pro-atherogenic or anti-atherogenic effects	51,199,201–203
	MMPs	OSS; ↑	–	Increased extracellular matrix degradation	204–207
	TP53	OSS; ↑	–	Promotes cell cycle	39,208
	GADD45	OSS; ↑		Promotes cell growth and proliferation	39
	CDKN1A	OSS; ↑	–	Promotes cell growth and proliferation	39,209
	MAPK1, MAPK3	OSS; ↑	–	Promotes cell growth and proliferation	210–212
	THBS1	OSS; ↑	–	Arterial stiffening	23,213
	SEMA7A	OSS; ↑	–	Increased expression of cell adhesion molecules and monocyte adhesion	214
	HIF1A	OSS; ↑		Promotes glycolysis and angiogenesis	127–129,215
	P2RX7	OSS; ↑	–	Induces ATP-dependent p38 signalling	216
	KDM4B	ULS; ↓	–	Induces EndMT	217
	YAP1, TAZ	OSS; ↑		Increases pro-inflammatory gene expression and atherogenesis by activating JNK	100,218
	HAND2	OSS; ↑	–	Low shear-induced transcription factor, increasing matrix degradation	219
	TXNDC5	OSS; ↑	–	Destabilizes endothelial nitric oxide synthase	220

Table 1 (continued) | Flow-sensitive genes in endothelial cells

Effect in atherosclerosis	Name	Shear stress type; expression regulation	Target genes	Effect in endothelial cells	Refs.
MicroRNAs					
Anti-atheroprotective	miR-10a	ULS; ↑	*BTRC, MAP3K7*	Anti-inflammatory	221
	miR-19a	ULS; ↑	*CCND1, HBP1, HMGB1*	Inhibits endothelial cell proliferation	222,223
	miR-23b	ULS; ↑	*E2F1, FOXO4*	Inhibits endothelial cell proliferation and EndMT	224,225
	miR-27b	ULS; ↑	*DLL4, FLT1, SEMA6A, SEMA6D, SPRY2, TGFB*	Inhibits angiogenesis, endothelial cell differentiation and vessel integrity	226–229
	miR-101	ULS; ↑	*ABCA1, CUL3, MTOR*	Inhibits endothelial cell proliferation; promotes angiogenesis	230–232
	miR-143–miR-145	ULS; ↑	*CAMK2D, CFL1, ELK1, KLF4, PHACTR4, SSH2*	Inhibits inflammation and promotes anti-atherogenic phenotypes in vascular smooth muscle cells	233–236
	miR-126	NA	*BCL2, CCL2, DLK1, FOXO3, HMGB1, IRS1, LRP6, VCAM1*	Promotes endothelial cell proliferation and vascular protection, and inhibits apoptosis, inflammation and atherosclerosis	237–243
Pro-atherogenic	miR-92a	OSS; ↑	*CXCL12, ITGA5, KLF2, KLF4, PLPP3, SIRT1*	Promotes endothelial inflammation and angiogenesis	170, 244–246
	miR-205	OSS; ↑	*TIMP3*	Increases endothelial inflammation and permeability	181
	miR-663	OSS; ↑	*ATF4, ELK1, KLF2, KLF4, MYOCD, SOCS5, VEGF*	Promotes endothelial inflammation and pro-atherogenic vascular smooth muscle cell phenotype switching	247,248
	miR-712	OSS; ↑	*TIMP3*	Increases endothelial inflammation and permeability	150,249
	miR-21	OSS; ↑	*BCL2, PPARA, PTEN*	Increases endothelial inflammation and apoptosis	250–255
	miR-155	ULS; ↑	*MYLK, NOS3, SOCS1*	Inhibits endothelial inflammation, migration, and proliferation, leading to atheroprotection	256–259
		↑ in atherosclerotic plaque macrophages	*BCL6*	Pro-atherogenic	260
Long non-coding RNAs					
Unknown	MALAT1	ULS; ↑	miR-22-3p	Inhibits endothelial cell proliferation, angiogenesis and migration	261,262
	MANTIS	ULS; ↑	*BRG1*	Promotes angiogenesis and endothelial cell alignment	263
	LINC00341	ULS; ↑	*VCAM1*	Anti-inflammatory	264
	LISPR1	ULS; ↑	*S1PR1*	Promotes endothelial cell migration and angiogenesis	141,265
	STEEL	ULS; ↓	*NOS3, KLF2*	Promotes intact vessel formation	141,266

EndMT, endothelial-to-mesenchymal transition; KLF2, Krüppel-like factor 2; NA, not available; NF-κB, nuclear factor-κB; OSS, oscillatory shear stress; ULS, unidirectional laminar shear stress. Data are from ref. 73.

clusters were rare in the RCA, the LCA had dramatically increased numbers of monocytes and macrophages, reflecting substantial pro-inflammatory and pro-atherogenic vascular changes in response to disturbed flow. Like macrophages, few dendritic cells and T cells were present in the RCA but their numbers were increased by disturbed flow in the LCA[15].

Differential gene expression and gene ontology analyses showed that disturbed flow induces numerous changes that favour pro-atherogenic responses in endothelial cells[15]. To understand the potential underlying mechanisms by which disturbed flow induces endothelial cell phenotype changes, eight different endothelial cell clusters were analysed for their differential gene expression and gene ontology[15]. As expected, the prototypical healthy endothelial cell clusters (E2) expressed the highest levels of the two best known mechanosensitive genes, *Klf2* and *Klf4*, and the expression of these genes was significantly lower in endothelial cell clusters exposed to disturbed flow, such as the prototypical E8 in the LCA. Disturbed flow induced the expression of genes in the E8 cluster of endothelial cells that were also found to be highly expressed in VSMCs (*Acta2* and *Tagln*), fibroblasts (*Dcn1*) and immune cells (*Cd74*, *H2-Eb1*, *H2-Aa* and *H2-Ab1*), indicating potential EndMT and acquisition of immune cell-like features by endothelial cells under the disturbed flow condition. Comparison of the differentially expressed genes between the stable flow-exposed E2 and disturbed flow-exposed E8 clusters by Panther gene ontology analysis showed that disturbed flow induces many well-known biological processes associated with pro-atherogenic pathways, including inflammation, EndMT, apoptosis, angiogenesis and endothelial permeability[15]. A pseudotime trajectory analysis and additional differential gene expression and chromatin accessibility analyses confirmed that E8 cells express higher levels of marker genes for EndMT (*Acta2*, *Cnn1*, *Snai1* and *Tagln*) and EndIT (*C1qa*, *C1qb*, *C5ar1* and *Tnf*) than E2 cells. The evidence for EndMT and EndIT was further validated by immunofluorescence

staining of the key immune cell marker proteins C1QA and LYZ in CDH5^{+} endothelial cells in mice[15]. In addition, chronic exposure of human aortic endothelial cells to disturbed flow in vitro induced the expression of EndMT markers (*SNAI1* and *TAGLN*) and EndIT markers (*C1QC* and *C5AR1*)[15], demonstrating that disturbed flow can induce endothelial cell reprogramming in cultured aortic endothelial cells in the absence of any other cell type, such as immune cells. These results demonstrate that disturbed flow induces the transition of endothelial cells to pro-atherogenic phenotypes, characterized by inflammation, EndMT and EndIT (that is, FIRE)[15] (Fig. 4).

EndMT has a crucial role in endothelial cell dysfunction and atherosclerosis, whereas the role of EndIT has not been defined. Endothelial cells undergoing EndMT exhibit traits of mesenchymal cells, such as fibroblasts and VSMCs, while losing typical endothelial cell characteristics, including the elongated cell morphology and cell–cell junctional integrity[60,153,154]. Mechanistically, the flow-sensitive transforming growth factor-β1 (TGFβ1) is a well-known regulator of the expression of EndMT-related genes[155,156]. Furthermore, endothelial cells without primary cilia have been shown to prime flow-induced EndMT via the TGFβ–ALK5–SMAD2/SMAD3 axis[157]. By contrast, AMP-activated protein kinase is activated by stable flow and suppresses inflammation via nitric oxide-mediated inhibition of NF-κB signalling[158–160]. Cells undergoing EndMT have an important role in atherogenesis by contributing to neointimal thickening, vascular remodelling, and plaque progression and stability[153,161]. A meta-analysis using 28 microarray datasets obtained from endothelial cells exposed to various stimuli (including shear stress, different coronaviruses, hyperlipidaemia and lipopolysaccharide) supports the EndIT concept[162]. Nevertheless, the EndIT concept requires further validation by endothelial cell lineage-tracing studies[163]. Although the pathophysiological importance of EndIT in atherogenesis remains to be tested, the role of disturbed flow-induced endothelial inflammation in atherogenesis is clearly defined.

Therapeutic implications in atherosclerosis

As discussed in the Introduction, the CANTOS trial[12] demonstrated that targeting a non-lipid pathway, such as an inflammatory pathway, could be an effective anti-atherogenic therapy. We propose that flow-sensitive genes, proteins and pathways in endothelial cells that regulate FIRE, such as endothelial inflammation, EndMT and EndIT, could be promising novel anti-atherogenic targets. In support of this notion, our transcriptomics study conducted in the mouse PCL model of atherosclerosis showed that both statins and blood flow regulate the expression of hundreds of genes, and the transcriptional profile changes are remarkably distinct from each other[164]. This result suggests that flow-dependent and cholesterol pathways have different roles in atherosclerosis, highlighting the rationale for targeting flow-sensitive molecules (genes, proteins and signalling molecules) as a complementary therapeutic approach. Two therapeutic strategies are conceivable: stimulating or increasing stable-flow-induced atheroprotective molecules, or inhibiting disturbed flow-induced pro-atherogenic molecules with the use of small molecules, recombinant proteins or gene therapies delivered in a systemic or targeted manner.

Several stable-flow-induced molecules are promising anti-atherosclerotic targets. *KLF2* and *KLF4* account for >50% of all stable-flow-induced gene transcription and the encoded proteins affect nearly all facets of atheroprotective responses in endothelial cells[165]. Given their dominant importance, numerous strategies to stimulate *KLF2* and *KLF4* expression have been proposed. Statins are a well-known inducer of *KLF2* expression in cultured endothelial cells[123]. However, whether statins also induce *KLF2* and *KLF4* expression in vivo under flow-conditions has been disputed given the potent effect of flow on the expression of these genes[110,164]. Betulinic acid has also been shown to induce *KLF2* expression, as well as expression of its target gene *NOS3* (which encodes eNOS), via the upstream ERK5–MEF2C pathway[166]. PIEZO1 agonists (Yoda1, Jedi1 or Jedi2) or antagonists (salvianolic acid B) have been shown to modify *KLF2* and *KLF4* expression[167–169]. However,

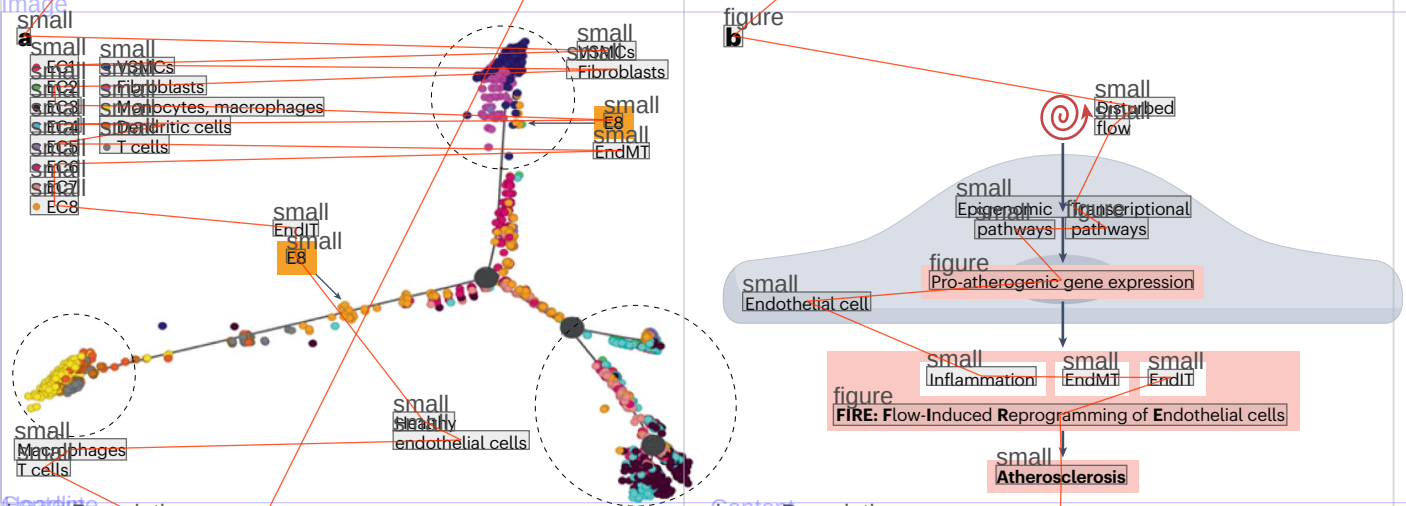

Fig. 4 | Single-cell RNA sequencing reveals disturbed-flow-induced reprogramming of endothelial cells. a, Disturbed flow stimulates the transition of healthy endothelial cells (ECs) to mesenchymal cells (EndMT; E8) and to an immune cell-like state (EndIT; E8), as determined by a pseudotime trajectory analysis of single-cell RNA sequencing datasets obtained from a mouse model of partial carotid artery ligation. The dots along the trajectory lines represent the status of the cells transitioning towards differentiated cell types. **b**, Disturbed flow induces epigenomic changes, such as chromatin remodelling, and transcriptomic changes that lead to pro-atherogenic gene expression patterns, which in turn induce flow-induced reprogramming of ECs (which we term as FIRE, an emerging concept that collectively refers to EndMT, EndIT and EC inflammation) and, eventually, atherosclerosis development. VSMC, vascular smooth muscle cell. Panel **a** adapted with permission from ref. 15, Elsevier.

given the dual atheroprotective and pro-atherogenic roles of PIEZO1, drugs targeting this receptor would require safety and specificity studies in order to be used as atherosclerosis therapies. The use of recombinant KLK10 or targeted overexpression of *KLK10* as an anti-atherogenic therapy is discussed above.

Inhibition of disturbed-flow-induced molecules is a promising anti-atherosclerosis strategy. Pharmacological inhibition of disturbed-flow-induced HIF1α using the small-molecule inhibitor PX-478 was shown to reduce atherosclerosis in mice[129]. Inhibition of disturbed-flow-induced miRNAs, including the antagomiRs of miR-92a, miR-205 or miR-712, effectively reduced atherosclerosis development in mice[150,170–172]. The agent 5-aza-2′-deoxycytidine inhibits the disturbed-flow-induced DNMT activity and prevented atherosclerosis in a mouse model[136]. Numerous flow-sensitive genes, proteins and pathways, including NF-κB, YAP, TAZ and BMP4, as well as specific inhibitors, drugs and RNA therapeutics, are suitable for further investigation, but research on therapeutic strategies targeting disturbed-flow-induced atherogenesis is scarce. Developing approaches to overcome this limitation is a major research area to be developed.

Conclusions

In conclusion, shear stress from blood flow potently regulates phenotypic and functional changes in endothelial cells that either prevent or promote atherogenesis. Endothelial cells transduce these biomechanical cues through mechanosensors that mediate various mechanosignal transduction pathways, which in turn regulate transcriptomic and epigenomic changes and cellular functions. The advent of high-throughput omics combined with in vivo and in vitro experimental models have revealed numerous flow-sensitive genes, proteins and pathways that regulate endothelial cell dysfunction and atherosclerosis development. Whereas stable flow induces an atheroprotective endothelial cell phenotype, disturbed flow induces an atherogenic phenotype characterized by alteration of endothelial morphology and barrier function, impairment of endothelial metabolism, redox regulation, proliferation and apoptosis, induction of inflammatory pathways, and transdifferentiation to other cell types, such as EndMT. Additionally, scRNA-seq and scATAC-seq studies in vivo have revealed that disturbed flow not only induces endothelial inflammation and EndMT, but also EndIT, which we define as FIRE (flow-induced reprogramming of endothelial cells). The mechanisms and roles of FIRE in endothelial dysfunction and atherosclerosis are major unanswered questions that could reveal important novel mechanisms underlying atherosclerosis. Moreover, the flow-sensitive molecules regulating FIRE could be novel therapeutic targets.

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Competing interests

H.J. is the founder of Flokines Pharma. The other authors declare no competing interests.

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