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Editor-in-chief: Markus Hecker

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Vascular Mechanobiology in Physiology and Disease

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Preface

Biomechanical forces play a major role in organ development, shape, and function. When exceeding the physiological range, however, they may become detrimental to organ structure and function. This is probably best exemplified by the cardiovascular system, with both the heart and blood vessels being continuously exposed to the biomechanical forces exerted by the pressure and flow of blood.

Because the heart mainly has to overcome the resistance to (blood) flow in the pulmonary and particularly in the systemic circulation, it is subjected to its own pressure gradient required to eject the blood into the pulmonary artery and aorta, respectively. If resistance rises, e.g., due to arterial hypertension, tension or stretch in the wall of the left ventricle increases, too, triggering a maladaptive response referred to as left ventricular hypertrophy. While pressure hence stretch therefore is the main biomechanical force affecting the heart and its constituent cell types, the mechanobiology of vascular cells is somewhat more complex. Endothelial cells lining the luminal surface of each blood vessel are continuously subjected to the viscous drag of the flowing blood (designated as shear stress). Unidirectional shear stress mainly affects endothelial cells of the small arteries and arterioles, maintaining them in a dormant state. If blood flow is disturbed, e.g. at bifurcations or curvatures of the large conduit arteries, unidirectional shear stress declines or becomes oscillatory and may give rise to a shift in the phenotype of the endothelial cells. This switch from anti-inflammatory to pro-inflammatory gene expression (also referred to as endothelial dysfunction) in combination with the reduced flow at these sites promotes leukocyte recruitment and diapedesis, ultimately resulting in chronic inflammation within the vessel wall and formation of a vulnerable atherosclerotic plaque.

In addition, endothelial cells and in particular vascular smooth muscle cells are subjected to another biomechanical force: blood pressure determining the degree of tension in the vessel wall and hence stretching of these cells. Volume-dependent distention of the vessel wall (which can be achieved through an increase in blood flow) also results in an increase in wall tension, thereby stretching the endothelial and smooth muscle cells. Like the cardiomyocytes of the heart, the medial smooth muscle cells of the small arteries and arterioles seek to normalize wall tension by active constriction, which cannot be maintained for long. These cells subsequently undergo hypertrophy or hyperplasia (depending on the size of the blood vessel) and

remodel the extracellular matrix so that the vessel wall not only becomes thicker but also (much) stiffer. This in turn raises their resistance to flow and may contribute to the increase in blood pressure in either the pulmonary or systemic circulation.

Research into the mechanobiology of the blood vessels aims to unravel the molecular and cellular mechanisms underlying the physiological response of vascular cells to pressure (wall tension) and flow (shear stress). It also aims to uncover what goes wrong (e.g., in atherosclerosis or hypertension) and to eventually therapeutically interfere with these maladaptive remodelling processes.

The aforementioned aspects of vascular mechanobiology along with many more facets of this fascinating, timely, and highly clinically relevant field of research are addressed by the review articles within this volume. They have been written by some of the leading experts on this subject from around the globe and cover a wide range of topics from basic research to disease-relevant mechanisms and therapeutic options. With this said we wish you an interesting and enjoyable read of this eighth volume in the book series. For those interested in cardiac mechanobiology, there will be another volume in *Cardiac and Vascular Biology* to follow soon.

Heidelberg, Germany — Markus Hecker
Rotterdam, The Netherlands — Dirk J. Duncker
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Hemodynamics and Vascular Remodeling

1

Timothy W. Secomb

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Abstract

The flow of blood in the vasculature generates forces that act on vessel walls. Blood pressure generates circumferential stresses within the walls, while the endothelial lining of vessels experiences shear stresses as a consequence of blood's motion over it. Both types of stress can elicit biological responses from cells in the walls, including growth and remodeling that are required for the normal function of the vasculature, and the development of vascular diseases. The main objective of this review is to summarize the dynamical features of blood flow as they affect the stresses that act on vessel walls. A second objective is to

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summarize the relationship between these stresses and the resulting structural responses in normal and disease states.

1.1 Introduction

The human circulatory system includes blood vessels with a total length of about 10^7 m [1]. They form a network of more than 10^9 segments with diameters ranging from a few μm to a few cm. Adequate and efficient distribution of blood flow throughout the body in accordance with tissue requirements depends on the structural characteristics of the vascular system and the flow behavior of blood as it traverses this network.

The structure of the vasculature is not static. The locations of the major arteries and veins are largely predetermined, but all blood vessels are subject to significant structural changes during development, in response to changing functional demands in health, and due to the effects of the disease. It is obvious that the structural characteristics of the vast number of vessel segments cannot be controlled on an individual basis by genetic information. Instead, the structure of the vasculature must emerge as the collective result of vascular growth and remodeling by each segment in response to the conditions and stimuli that it experiences [2]. These stimuli include the forces acting on vessel walls resulting from blood pressure and flow, namely the circumferential wall stress generated by blood pressure and the wall shear stress generated by blood flow.

In the following sections, the concept of stress in continuum mechanics is presented, followed by a review of the fluid dynamics of blood flow, with emphasis on the stresses acting on vessel walls. The effects of these hemodynamic stresses on vessel wall structures in normal and disease conditions are then discussed.

1.2 Mechanical Stress in Materials

In studies of fluid and solid mechanics, a continuum approach is generally used, in which relevant properties of the material, such as density and velocity, are considered as continuous functions in space, without analyzing the positions or motion of the individual molecules or other particles in the material or the forces acting on them. To describe the forces acting in a continuum, a mathematical entity known as the stress tensor is used. Here, the concepts underlying the stress tensor are described without using mathematical notation. More rigorous expositions are provided in textbooks [3, 4]. Despite the importance of biological responses to mechanical stress in the field of mechanobiology, understanding the concept of stress and its expression as a tensor presents a challenge for many biologists. The following discussion is intended to help bridge this gap.

As a starting point for defining mechanical stress, the forces acting across a hypothetical small planar surface lying within a material or on its boundary are considered. The traction (or stress vector) is defined as the force per unit area acting

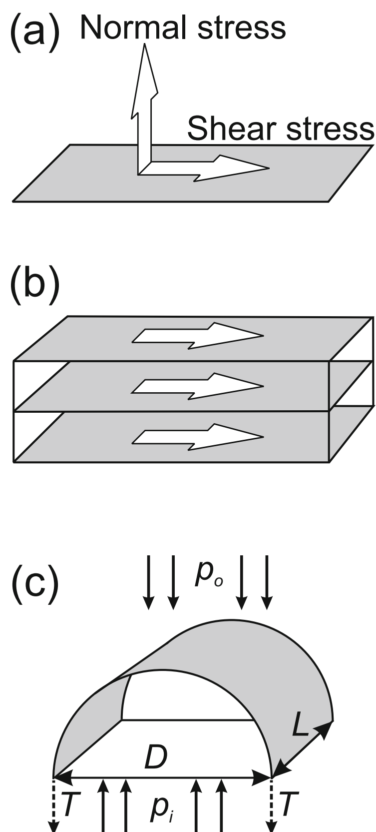

Fig. 1.1 Schematic illustration of basic concepts relating to mechanical stress. (**a**) Normal and shear components of stress acting on a surface (shaded) within or on the boundary of a material. (**b**) When a shear stress acts on the surface of a planar structure of finite thickness, such as a monolayer of endothelial cells, then the shear stress is transmitted uniformly through the thickness of the structure. (**c**) Estimation of the tension in a cylindrical structure subjected to a fluid pressure difference across its wall (shaded). The pressure difference across the wall generates a net force that is balanced by the force generated by tension in the walls

on one side of the surface. More precisely, the traction at a point is defined by considering such a surface containing the given point and taking the limit of force divided by area as the area of the surface approaches zero. Being a vector, traction requires three components to represent its magnitude and direction.

The traction generally depends on the orientation of the surface, represented by the unit vector normal (i.e., perpendicular) to it. For instance, a material may be under tension in one direction and under compression in an orthogonal direction. It can be shown that the dependence of the traction on surface orientation can be fully defined by specifying the tractions acting on three mutually orthogonal coordinate surfaces. The combination of these three traction vectors forms a tensor, which can be expressed as a 3×3 matrix of components in any given coordinate system. In this matrix, the elements on the diagonal are normal stress components, i.e., forces acting normal to a surface (Fig. 1.1a). The off-diagonal elements are shear stress components, i.e., forces acting parallel to a surface (Fig. 1.1a). However, it should be noted that these definitions depend on the orientation of the coordinates. Normal stresses in one coordinate system may appear as shear stresses in a rotated system.

By convention, the outward normal vector to a surface experiencing a traction is used to represent its orientation. It follows that positive diagonal elements in the stress tensor represent tensile forces in the material, while negative diagonal elements represent compressive forces. In the special case of hydrostatic pressure, the only non-zero elements of the stress tensor are on the diagonal, and all are equal to $-p$, where p is the pressure. For any traction acting on a surface with a given normal vector, the force acting on a surface with the opposite normal vector must be equal and opposite, since it represents the reaction force according to Newton's third law of motion.

The stress tensor discussed here is more precisely called the Cauchy stress tensor. In this form of the stress tensor, all quantities are referred to coordinates in the current, possibly deformed, state of the material. For analysis of large-deformation elasticity, other stress tensors (e.g., Piola-Kirchhoff) are often used [5], in which quantities are referred to coordinates in an initial undeformed or reference configuration.

1.3 Conditions for Equilibrium of Mechanical Stress

According to Newton's second law of motion, the force acting on an object equals the product of mass and acceleration. To apply this principle in a continuum, the forces acting on a small volume within the material are considered. For convenience, a cuboidal region is assumed. If the stress in the material is uniform in space, then according to the above discussion, equal and opposite forces act on each pair of parallel surfaces of the cuboid, since their outward normal vectors point in opposite directions. In this case, the zero net force is generated. In general, therefore, the resultant forces generated by a stress field within a continuum must depend on the spatial variations of the stress tensor. The size of the cuboidal region is then considered to approach zero. In this limit, it is found that the resultant force per unit volume of the material is proportional to a particular combination of the spatial derivatives of the stress tensor. If the acceleration of the material is zero or its inertia is negligible, then this combination of derivatives (specifically, the divergence) must be zero to satisfy the mechanical equilibrium of the material.

This result has significant implications for the mechanotransduction of shear stress. Consider a situation in which a multilayer planar structure is subjected to a uniform shear stress on its upper surface, and is immobilized so that its acceleration is zero. According to the condition of mechanical equilibrium, the shear stress (possibly averaged over a surface parallel to the upper surface) must be uniform through the thickness of the structure (Fig. 1.1b). In a curved geometry, such as a blood vessel wall, the same result applies to a good approximation if the structure is thin relative to the vessel radius. This argument can be applied to the endothelial cells lining blood vessels, with the implication that the same (average) shear stress is experienced by the luminal surface, the interior cellular structures such as the cytoskeleton, and the structures connecting the endothelial layer to the basement

membrane. All of these structures are therefore potential sites for mechanotransduction of shear stress by endothelial cells.

The conditions of mechanical equilibrium can also be used to calculate the tension generated in a cylindrical blood vessel wall as a result of the difference between the internal and external pressures acting on the vessel. In this case, the analysis can be simplified by considering the total forces acting on a semicircular part of a vessel segment of length L and diameter D, as shown in Fig. 1.1c. The wall is considered to be thin relative to the radius, and only forces acting in the vertical direction are considered. The pressure difference across the wall, $\Delta p = p_i - p_o$, generates an upward force ΔpLD, which must be balanced by a downward force of $2LT$ generated by tension in the walls, where T is the tension per unit length of the vessel. Equating these forces gives

$$T = r\Delta p \tag{1.1}$$

which is known as the Law of Laplace for a cylinder, where $r = D/2$ is the tube radius. If the wall has thickness h, then the average circumferential stress in the wall is

$$\sigma = \frac{r}{h}\Delta p \tag{1.2}$$

The ratio r/h is generally larger than 1, and it follows that the dominant stress in vessel walls is circumferential stress, not the radial stress generated by blood pressure acting on the walls. Furthermore, r/h varies substantially with vessel type and size [6], being relative small (as low as about 2) in capillaries, and increasing with vessel size up to values of about 10 in arteries and about 50 in veins.

Some caution is needed with regard to the interpretation of Eq. (1.2) in the context of mechanotransduction. Because the circumferential wall stress is a normal component of stress acting in the circumferential direction, it can be variable and even discontinuous with the position in the radial direction (unlike shear stress). The levels of stress carried by the various components of the vessel wall (endothelial cells, basement membrane, smooth muscle cells, elastin, and collagen) may vary widely according to the mechanical stiffness and the degree of stretch (strain) of each component. Also, if r/h is not large, the thin-wall theory presented above may not be a good approximation and a more elaborate theory is needed [7]. The components of the vessel wall that are primarily responsible for responses to changes in intravascular pressure, particularly vascular smooth muscle, may experience levels of stress significantly different from the estimated average given by Eq. (1.2).

1.4 Hemodynamics

For the purposes of this review, we define hemodynamics as "the physical study of flowing blood and of all the solid structures (such as arteries) through which it flows" [8]. This differs from the medical usage of the term, where it generally refers to

parameters such as arterial blood pressure and cardiac output in patients. More detailed discussions of this field are presented in several books [8–12] and articles [7, 13]. In the following presentation, the dependence of wall shear stress on fluid mechanical effects is emphasized, in recognition of the fact that shear stress is an important determinant of vascular remodeling.

1.4.1 Flow in a Cylindrical Tube

The analysis of fluid flow along a cylindrical tube is central to the study of hemodynamics. A first step in this analysis is the determination of the distribution of shear stress in the fluid and at the tube wall, which can be obtained readily under some simplifying assumptions [3, 4]. The tube is assumed to have diameter D and length L, with pressures p_1 at the upstream end and p_0 at the downstream end. The distribution of flow velocity across the tube is assumed to be uniform along the tube and also constant in time. Effects of gravity are neglected. We consider a cylindrical region of fluid with radius $r \leq D/2$, centered on the axis of the tube, and define τ as the shear stress acting in the upstream direction on the curved surface of this region. According to the stated assumptions, τ is uniform over the curved surface. Because there is no acceleration of the fluid, Newton's second law of motion implies that the sum of the forces acting on the cylinder is zero, i.e.,

$$\pi r^2 p_1 - \pi r^2 p_0 - 2\pi r L \tau = 0 \tag{1.3}$$

where each of the stresses, p_1, p_0, and τ, is multiplied by the area of the surface on which it acts. Therefore

$$\tau = \frac{r \Delta p}{2L} \tag{1.4}$$

where $\Delta p = p_1 - p_0$ is the driving pressure. This result shows that the shear stress in the fluid varies in proportion to the distance from the tube axis and that the shear stress acting on the tube wall is

$$\tau_w = \frac{D \Delta p}{4L} \tag{1.5}$$

It is noteworthy that these results do not depend on any assumption about the viscous properties of the fluid.

For the further analysis of flow in tubes, we assume that the fluid is Newtonian, meaning that the shear stress is proportional to the gradient of fluid velocity:

$$\tau = -\mu \frac{du}{dr} \tag{1.6}$$

where μ is a constant, the fluid viscosity, and $u(r)$ describes the variation of fluid velocity with the radial position. (The minus sign appears here because τ was defined with respect to the direction opposite to the flow.) From Eqs. (1.4) and (1.6), it can be shown that

$$u(r) = \frac{\Delta p}{4L\mu}\left(\frac{D^2}{4} - r^2\right) \tag{1.7}$$

i.e., the profile of velocity across the tube has the form of a parabola. Flow in a tube with this velocity profile is known as Poiseuille flow. This solution satisfies the "no-slip" condition for a viscous fluid, which states that the velocity of the fluid adjacent to a solid boundary must match the velocity of the boundary, i.e., $u(D/2) = 0$ in this case. Integration of the velocity over the vessel cross section leads to the equation generally known as Poiseuille's law or the Hagen–Poiseuille equation:

$$Q = \frac{\pi}{128}\frac{\Delta p D^4}{L\mu} \tag{1.8}$$

where Q is the volume flow rate along the tube.

The fourth power dependence of flow rate on diameter, for a given fluid viscosity and for fixed tube length and pressure drop along the tube, was established experimentally in the nineteenth century by J.L.M. Poiseuille [14]. Equation (1.8) was obtained subsequently by theoretical analysis [15].

The derivation of Poiseuille's law depends on a number of assumptions that are generally not satisfied in the vascular system, and its applicability in any given situation has to be carefully evaluated. Even so, it provides a starting point for more detailed analyses and has important physiological implications. In particular, the proportional dependence of Q on D^4 implies that the blood flow rate is very sensitive to changes in vessel diameters. Therefore, blood flow rates can be modulated over a wide range by moderate changes in vessel diameter, and controlled distribution of blood flow according to local tissue needs can be achieved only with relatively precise control of vessel diameters.

Combining Eqs. (1.5) and (1.8) yields the following result:

$$\tau_w = \frac{32}{\pi}\frac{\mu Q}{D^3} \tag{1.9}$$

This relationship is significant with regard to understanding how vascular responses to wall shear stress influence network structure, as discussed below.

1.4.2 Bulk Viscosity of Blood

Blood is a complex fluid, consisting of a suspension of cells in plasma. The mechanical properties of blood are strongly affected by the presence of a high

volume fraction (hematocrit) of red blood cells (erythrocytes), typically in the range of 40–45% for healthy individuals [16].

The bulk viscosity of blood can be measured by placing a sample in a viscometer where it is subjected to a shear flow with a controlled shear rate, defined as $\dot{\gamma} = du/dz$, where u is the fluid velocity and z is a coordinate perpendicular to the flow direction. The shear stress τ on a surface bounding the flow is measured, and the viscosity is computed as $\mu = \tau/\dot{\gamma}$. The viscosity of blood is found to increase as a function of hematocrit [17], as would be expected since the presence of suspended particles tends to oppose the variations of flow velocity within the fluid. Also, blood exhibits shear-thinning behavior and viscosity increases substantially when the shear rate is decreased to low levels [17]. This behavior can be understood as the result of two main effects [16]. Firstly, red blood cells are highly deformable, and this deformation allows them to accommodate to the flow and reduce their contribution to the suspension viscosity. However, this effect is reduced at very low shear rates, because the cells are deformed less at the resulting low levels of shear stress. Secondly, red blood cells of many species, including humans, aggregate at low shear rates, forming larger structures called rouleaux which interfere more strongly with the flow. With increasing shear rates, aggregates are broken up and the viscosity decreases. It is important to note that this dependence on shear rate occurs mainly at shear rates below about 100 s^{-1} [17], whereas shear rates in normally flowing vessels are generally above this range and viscosity shows only slight variations with shear rate. For many purposes, e.g., when analyzing blood flow in arteries, the dependence of viscosity on shear rate can be neglected and blood can be treated as a Newtonian fluid as defined above.

1.4.3 Viscosity of Blood in Microvessels

The preceding discussion is based on the assumption that blood can be represented as a continuum, in the sense that viscosity is considered as an average property over a region containing a large number of red blood cells. This approximation is valid for vessels with diameters of about 300 μm or more. However, in vessels with smaller diameters, the effects of the particulate nature of blood become significant. In particular, red blood cells show a tendency to migrate away from vessel walls, as a result of fluid mechanical interactions between the deformable cells and the imposed flow in the vessel. This phenomenon, which does not involve active motion by the cells, results in the formation of a cell-free or cell-depleted layer near the vessel walls, which causes a significant reduction of resistance to flow relative to what would be expected based on the bulk viscosity of blood [18].

A convenient method to quantify this effect is to define the apparent viscosity, from Eq. (1.8):

$$\mu_{\text{app}} = \frac{\pi}{128} \frac{\Delta p D^4}{LQ} \tag{1.10}$$

where Δp and Q are now measured quantities. Based on an analysis of multiple experimental studies of blood flow in glass tubes, Pries et al. [19] obtained an empirical formula for the dependence of apparent viscosity of blood in vitro on tube diameter and hematocrit, for tube diameters from 3.3 μm up to 2 mm and hematocrits up to 90%. These results show a strong Fåhraeus–Lindqvist effect [20]: apparent viscosity decreases with decreasing diameter down to a minimum at about 7 μm. For hematocrit 45%, the bulk viscosity is $3.2\mu_p$, where μ_p is the viscosity of plasma, whereas the apparent viscosity in a 7-μm tube is $1.25\mu_p$.

Observations of blood flow in microvessel networks of the rat mesentery were, however, found to be inconsistent with model predictions based on the above in vitro estimates of blood viscosity [21]. Based on such observations, a different empirical formula was developed to describe the apparent viscosity of blood in microvessels in vivo, as a function of tube diameter and hematocrit [22]. According to this formula, the apparent viscosity in large vessels matches the in vitro result, but the apparent viscosity is substantially higher than the in vitro estimate in smaller vessels. For example, the estimated apparent viscosity in a 7-μm capillary at hematocrit 45% is about $8.4\mu_p$, almost seven times higher than would be expected based on data from glass tubes.

The shear stress acting on the walls of microvessels can be estimated from the flow rate Q according to Eq. (1.9), setting the viscosity μ equal to μ_{app}. Therefore, the finding of relatively high values of apparent viscosity in microvessels implies that the estimates of wall shear stress in microvessels based on observations of blood flow rate or blood flow velocity are significantly higher than would be obtained if the calculation was based on values of apparent blood viscosity obtained in vitro.

Several potential causes for the high apparent viscosity of blood in microvessels relative to values in glass tubes were identified by Pries et al. [21]. Subsequent studies showed that the main cause for the higher apparent viscosities in vivo is the presence of a layer of macromolecules on the inner wall of blood vessels, termed the endothelial surface layer (ESL) or glycocalyx [23]. This gel-like layer has a very low volume fraction of membrane-bound molecules, but the resistance to fluid motion through the layer is sufficient to reduce the flow velocity of plasma to a much lower value than the velocity in the lumen, outside the ESL.

An important implication of this finding is that the endothelial cell membrane does not experience a significant level of fluid shear stress due to plasma flow over its surface. Instead, shear stress is transmitted to the endothelial cells via the attachment points of the macromolecules that anchor the ESL, including syndecans and glypicans [23, 24]. Being transmembrane proteins, these molecules may in turn transmit the shear stress to the internal cytoskeleton, consistent with an important role for the cytoskeleton in mechanotransduction of shear stress [25, 26].

1.4.4 The Reynolds Number

The flow of fluids is governed by Newton's laws of motion, such that the acceleration of any small part of the fluid, multiplied by its density, is equal to the net force per unit volume acting on it. When the velocity field is viewed in a fixed frame of reference, the fluid acceleration includes a component, known as the advective acceleration, given by a product of the fluid velocity and a spatial gradient of velocity. This represents, for instance, the acceleration experienced by a fluid moving from a region of low velocity to a region of higher velocity, as in a nozzle. The forces acting on the fluid include body forces (i.e., gravity), forces due to gradients in pressure, and forces due to viscosity.

In the study of fluid mechanics, dimensionless parameters are defined that provide indications about the characteristics of the flow under consideration. The most important of these is the Reynolds number [11], defined as the ratio of the inertial effect to the viscous effect. Here, the inertial effect is estimated as the density multiplied by the advective acceleration, while the viscous effect is estimated as the viscous force per unit volume. Suppose that U is a typical fluid velocity and L is a typical length over which the velocity varies. According to the above information, the inertial effect has a typical magnitude $\rho U^2/L$, where ρ is the fluid density. From Eq. (1.6), the shear stress has typical magnitude $\mu U/L$, and the resultant force per volume, which depends on the spatial gradient of stress, has typical magnitude $\mu U/L^2$. The Reynolds number (Re) is then defined as the ratio

$$\mathrm{Re} = \frac{\rho U^2/L}{\mu U/L^2} = \frac{\rho UL}{\mu} \tag{1.11}$$

For flow in tubes, the most relevant spatial dimension is the diameter and the Reynolds number is defined as $\mathrm{Re} = \rho UD/\mu$.

1.4.5 Flow at Low Reynolds Number

Values of the Reynolds number vary widely in the circulatory system, from values below 10^{-3} in the capillaries to values above 10^3 in major arteries. At very low Reynolds numbers, the effects of fluid inertia on the flow are negligible and the flow is dominated by effects of viscosity. This regime is known as Stokes flow. The governing equations of fluid flow are then linear in velocity and pressure, allowing the use of a range of classical mathematical techniques to analyze problems of Stokes flow [27]. Because effects of inertia are negligible, the flow readily adjusts to changes in direction, and vessel curvature or tortuosity does not result in substantial changes in the magnitude of flow or the distribution of wall shear stress. When the pressure gradient driving the flow varies in time, the flow rate varies approximately in proportion to the instantaneous pressure gradient. Irregularities such as bifurcations or changes in vessel diameter produce local perturbations to velocity distributions, which quickly recover their previous characteristics once the

irregularity is passed. The velocity profile of fluid entering a vessel approaches its final form within a short distance. For a Newtonian fluid, this profile is the characteristic parabolic profile of Poiseuille flow. In these respects, fluid flow at low Reynolds number is somewhat simpler than flow at high Reynolds number, as discussed below.

However, when the flow of blood is considered, additional complexity arises because blood is a concentrated suspension of cells, whose dimensions are comparable to microvessel diameters. As mentioned earlier, red blood cells show a tendency to migrate away from microvessel walls. The fluid mechanical mechanisms underlying this behavior depend on the deformability of the cells and on the fluid dynamical interactions of cells with the walls and are only partially understood [18, 28–33]. The distribution of red blood cells across the vessel cross-section affects the distribution of hematocrit in each branch when the flow reaches a diverging microvascular bifurcation [34], and the theoretical understanding of this phenomenon is a challenging problem in the low Reynolds number flow [35–37].

1.4.6 Flow at High Reynolds Number

When the Reynolds number is high, in the range of hundreds or thousands, the flow exhibits very different behavior to that at a low Reynolds number. The phenomena discussed in this section have been considered in more detail in several books [9, 12, 38]. At high Reynolds number, effects of fluid inertia have dominant effects on the fluid motion in most of the flow domain. Even so, viscosity has important effects as a consequence of the no-slip condition, as defined above. This condition is often satisfied through the appearance of a narrow region adjacent to a solid surface in which velocity changes rapidly with the position, known as a boundary layer. A region of rapidly changing velocity can also appear in the interior of the flow, in which case it is referred to as a "shear layer."

An example of a boundary layer is provided by the flow of an (initially quiescent) fluid entering a uniform cylindrical tube (Fig. 1.2a) at high Reynolds number. In the entrance region of the tube, the fluid has a uniform distribution of velocity, except for narrow boundary layers at the tube walls where the velocity drops to zero to satisfy the no-slip condition. This results in a relatively high wall shear stress in this region. As the fluid progresses downstream, this layer gradually thickens until eventually the velocity profile approaches the parabolic shape discussed above. The distance to achieve an almost parabolic profile, known as the "entrance length," is proportional to the Reynolds number. Beyond this point, the flow is considered to be fully developed. For the aorta and major arteries, the entrance length is typically comparable to or longer than the actual length of the artery, and so fully developed flow is never achieved, and the distribution of wall shear stress differs significantly from the estimate based on Poiseuille flow.

The flow of blood through a region of local narrowing of a blood vessel, i.e., a stenosis, provides further examples of phenomena occurring in high-Reynolds flow (Fig. 1.2b). At the stenosis itself, the blood flow velocity must increase as a

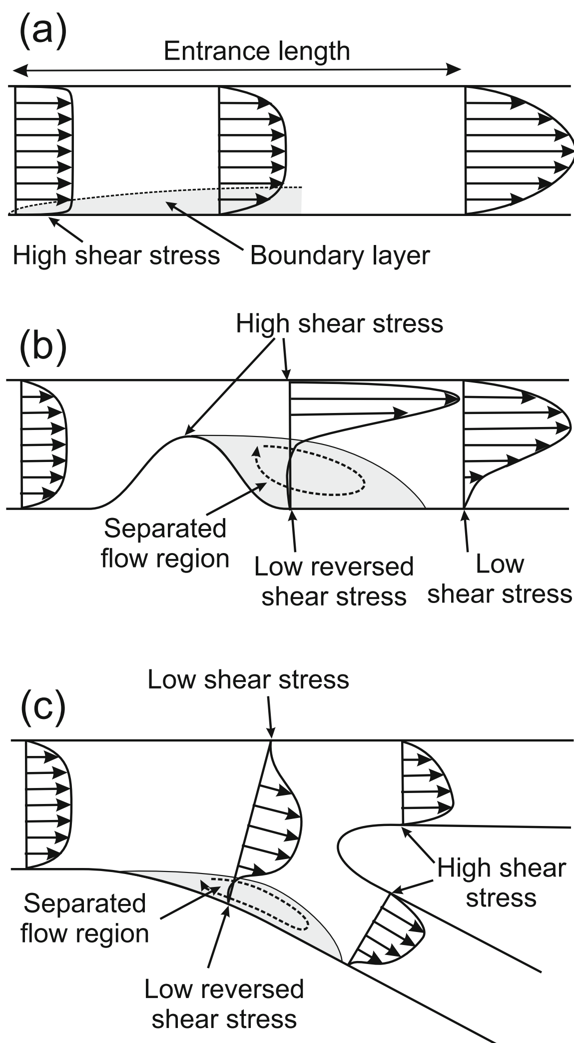

Fig. 1.2 Schematic illustration of phenomena associated with laminar flow in blood vessels at high Reynolds numbers. (**a**) Entrance effects. When steady laminar flow with an initially flat velocity profile enters a tube, a boundary layer is formed in which the flow velocity is progressively retarded. This layer thickens with distance downstream, and eventually the velocity profile approaches the parabolic form of Poiseuille flow. (**b**) Flow past a stenosis (narrowing) in a vessel, exhibiting flow separation. The region between the separating streamline and the wall is shaded and typically exhibits slow recirculating blood flow. Wall shear stresses are increased in some regions and reduced in others, relative to the shear stress in the upstream region. (**c**) Flow through a diverging arterial bifurcation. As in (**b**), the shaded region represents flow separation. Wall shear stress is increased in some regions and decreased in others

consequence of the reduced cross-sectional area of the vessel lumen in the stenosis. The flow exiting the narrow region tends to continue moving parallel to the vessel axis, rather than following the profile of the wall. This sets up a phenomenon known as flow separation, in which fluid streamlines (paths aligned with the flow direction) that are adjacent to the wall in the stenosis separate from the wall, typically reattaching to the wall at some distance downstream, as shown in Fig. 1.2b. In the region between the separating streamline and the wall, shown in gray in Fig. 1.2b, a region of recirculating flow is set up, in which the flow velocities are generally much lower than elsewhere in the vessel.

A similar phenomenon can occur in a diverging arterial bifurcation. In this case, the widening of the parent vessel at the entrance to the bifurcation sets up fluid mechanical conditions analogous to those immediately downstream of a stenosis. The wall shear stress is reduced in the region of widening, and flow separation may occur, leading to the reversal of the wall shear stress in the region of recirculating flow (Fig. 1.2c).

When fluid flows through a curved vessel, the fastest moving fluid, which is near the centerline in a straight tube, is displaced toward the wall at the outside of the curve, and the shear stress is increased there and decreased on the wall at the inside of the curve [9].

Flows at high Reynolds number may be subject to instability, i.e., spontaneous development of time-dependent fluctuations in the flow field. The conditions for instability depend both on the Reynolds number and on the characteristics of the flow. Poiseuille flow, with a parabolic flow profile, is stable up to Reynolds numbers of about 2300 [9]. However, the flow profiles occurring downstream of a stenosis or in the entrance to a bifurcation (Fig. 1.2b, c), which include one or more inflection points, can lead to flow instability at much lower Reynolds numbers, in the range of a few hundred. One result of flow instability is the generation of audible sounds, known as “bruits” when they occur in arteries. The flow in such cases is often referred to in medical literature as “turbulent.” In the fluid mechanics literature, however, turbulence has a stricter definition, including the presence of highly random flow perturbations on a range of length scales. Unstable blood flow at Reynolds numbers in the hundreds is more properly described as “disturbed flow,” with the possibility of true turbulence occurring at higher Reynolds numbers [38]. The term “laminar flow” is used to describe flow that is not turbulent and can include flows that exhibit instability and disturbances.

The pulsatile nature of blood flow in arteries introduces additional fluid mechanical phenomena that influence the stresses experienced by vessel walls, particularly in arteries. Arterial flow has an oscillatory component superimposed on the mean flow rate. The fluctuating flow can cause reversal of the direction of the wall shear stress during part of the cardiac cycle. Flow instabilities may appear and disappear during each cycle, depending on the Reynolds number and the frequency of the cardiac pulsation [12].

In summary, the shear stresses generated by blood flow in the arteries depend in a complex manner on the geometry of the vessels and the characteristics of the flows through them. In general, straight unbranched segments experience a relatively high unidirectional shear stress, with the possibility of reversal during part of the cardiac cycle. However, variations in vessel width, caused, for instance, by stenosis or by bifurcations, can result in regions of low shear stress, with reversals of stress direction associated with flow separation, and fluctuations in stress resulting from flow instability.

1.5 Functional Demands on the Vasculature

The primary function of the circulatory system is the transport of substances from one part of the body to another. This transport is accomplished mainly by two physical processes, convection, and diffusion. Convection refers to the transport of materials carried by the motion of flowing blood, and diffusion refers to random thermal motion of individual molecules which generates net fluxes down gradients of solute concentrations or, more precisely, down gradients of the solutes' thermodynamic potentials. At the cellular level, active transport across cell membranes is also required for many solutes.

Transport of oxygen is a critical function of the circulation. Being a non-polar molecule, it has relatively low solubility in water and in tissue. Its transport from the atmosphere to mitochondria occurs by purely passive mechanisms down gradients in oxygen partial pressure [39]. The necessity for adequate transport of oxygen and other substances places stringent demands on the structure of the circulatory system. As a result of the low concentration of oxygen in tissue, oxygen transport by diffusion is effective only over very short distances, about 100 μm or less in most oxygen-consuming tissue. Therefore, it must be delivered by convective transport within such a distance of all cells that require oxygen. This requires a dense network of tiny vessels throughout the tissue. On the other hand, viscous resistance to blood flow is very high in vessels with very small diameters. For mechanical efficiency, the vascular system must include hierarchical branching structures feeding the microvessels, such that convective transport over larger distances can be achieved by vessels with larger diameters and lower resistance to flow. In effect, the vascular system must solve a complex patterning problem, generating a structure in which a dense meshwork of capillaries is combined with a hierarchical structure of arteries, arterioles, venules, and veins of varying lengths and diameters so that all parts of the tissues are adequately supplied with blood flow [40]. A central challenge in vascular biology is to understand the mechanisms that control the structure of the vasculature in health and disease.

The need to satisfy the metabolic requirements of the tissue suggests that hypoxia is a stimulus for vessel growth and remodeling. The stimulation of angiogenesis (growth of new vessels) mediated by hypoxia-inducible factor 1α (HIF1α) and vascular endothelial growth factor (VEGF) is well established [41]. However, local growth of new vessels in a hypoxic region can have only limited effectiveness in overcoming hypoxia unless it is accompanied by enlargement of the corresponding feeding and draining vessels to allow increased perfusion. This implies the need for mechanisms to coordinate structural adaptation along flow pathways, beyond the region experiencing hypoxia. Structural responses to changes in wall shear stress generated by flowing blood provide such a mechanism, as discussed below. Moreover, the vessel walls must be able to support the forces generated by blood pressure. The dominant stress in the wall is circumferential tension, and the wall must be able to respond to increases in this tension, typically by increasing wall thickness. Evidently, vascular remodeling must involve responses to several different stimuli, including mechanical forces generated by blood flow.

1.6 Role of Hemodynamic Signals in Vascular Remodeling

Evidence for the influence of wall shear stress on vessel diameters can be deduced from a consideration of the relationship between diameter and blood flow rate in the circulation. In the arterial tree, including arteries and capillaries, it has been observed experimentally that flow rate scales approximately as the cube of diameter across a wide range of scales [42, 43]. This scaling is consistent with the optimality theory known as Murray's law [44], which is based on the assumption that vessel diameters are adjusted to minimize a cost function consisting of a linear combination of viscous energy dissipation and blood volume. A potential mechanism by which such a scaling could be achieved is suggested by Eq. (1.9), which states that the wall shear stress is proportional to the ratio of blood flow rate to the cube of diameter (neglecting variations in blood viscosity). If it is assumed that a set point of wall shear stress exists, such that shear stresses above this level stimulate diameter enlargement, and vice versa, then a vessel with a given flow rate will adjust its diameter so as to conform to the cubic dependence of flow on diameter [42, 45]. Indeed, direct experimental evidence supports such a response to changes in wall shear stress [46].

However, some limitations to this theory are apparent. Firstly, veins are consistently larger in diameter than paired arteries carrying the same blood flow rate [42], and levels of wall shear stress are correspondingly lower in veins than in arteries. This implies that other factors besides wall shear stress must affect diameters. Detailed analyses of hemodynamics in microvascular networks of the rat mesentery showed a trend of decreasing wall shear stress with decreasing intravascular blood pressure, which was consistent across arterioles, capillaries, and venules [47]. Such behavior can be accounted for by assuming that intravascular pressure, or more precisely the circumferential stress generated by that pressure, also acts as stimulus for structural remodeling [47, 48]. According to this theory, an increase in intravascular pressure causes inward remodeling with the thickening of the vessel wall. This is consistent with observations of decreased internal diameters of small arteries in hypertension [49].

A second limitation of the theory of diameter adaptation based on shear stress alone becomes apparent when the behavior of vascular networks is simulated [50, 51]. If a flow is divided between two segments connected in parallel, both segments experience the same pressure drop. According to Eq. (1.5), the shear stress in each is proportional to its diameter. Suppose that an initial condition is imposed in which both segments have the same wall shear stress. A small enlargement of one segment would result in an increase in its wall shear stress, in turn stimulating further enlargement, while the other segment would shrink. In other words, such an initial configuration would be unstable.

These considerations imply that other factors in addition to wall shear stress and intravascular pressure must influence the control of vessel luminal diameters through structural remodeling. Pries et al. [52, 53] developed theoretical models for structural adaptation that include a metabolic growth stimulus: if low flow in a given vessel segment leads to reduced oxygen levels, then this stimulates diameter enlargement,

presumably through increased expression of growth factors. Also, these models include effects of convected and conducted responses, such local hypoxia in a given segment generates a growth stimulus in the vessels in the flow pathway upstream and downstream of the given segment. Here, convected responses refer to effects of metabolic signal substances carried downstream from hypoxic regions and simulating growth of downstream vessels [54], and conducted responses refer to signals propagated upstream along the walls of blood vessels by endothelial and smooth muscle cells, which communicate via gap junctions [55, 56]. It was shown that such a model can predict stable network structures with hemodynamic properties consistent with corresponding in vivo observations.

In summary, vascular responses to wall shear stress play an essential role in generating the hierarchical structure of vascular networks, in which the decreases in flow rate in successive branches of the arterial and venular trees are accompanied by corresponding decreases in vessel diameter. Responses to circumferential wall tension generated by intravascular pressure have the effect not only of controlling the thickness of vessel walls but also of generating the asymmetry between the arterial and venular trees, in which venous vessels are systematically larger than corresponding arterial vessels, with lower levels of wall shear stress. These responses, working in concert with responses to metabolites and growth factors, and with upstream and downstream propagated responses, provide a sufficient set of mechanisms to account for the observed structures of vascular networks.

1.7 Conclusions and Translational Perspectives

According to Poiseuille's law, the flows in blood vessels are sensitively dependent on the vessel diameters. At the same time, the structure of the vessels, and in particular their diameters, are capable of continuous adjustment in response to a number of stimuli, including the forces generated by blood flow. These interacting processes are largely responsible for establishing and maintaining the geometric structure of the healthy vasculature so that it can meet the body's requirements. Interactions between hemodynamics and structure also contribute to the development of vascular diseases.

Table 1.1 lists hemodynamic forces acting on vessel walls and some normal and pathological behaviors that are influenced by these forces. The primary structural response of blood vessels to elevated circumferential wall stress is the thickening of the vessel wall, which tends to reduce this stress, according to Eq. (1.2). This response underlies the adaptation of veins used for coronary bypass grafts to arterial blood pressure [57]. It should be noted that circumferential wall stress does not approach a unique set point independent of vessel size; instead, average circumferential stress levels increase with vessel diameter [58]. A second structural response to increased circumferential stress is the reduction of the vessel lumen (inward remodeling). This response may play a role in the development of hypertension [6, 59]. If peripheral resistance is initially increased for some reason, this stimulates an increase in blood pressure in order to maintain flow. The inward modeling of

Table 1.1 Hemodynamic forces acting on vessel walls and biological effects

Hemodynamic property	Role in normal vascular remodeling	Role in disease processes/ responses
Blood pressure, causing circumferential wall stress	Matching of wall thickness to pressure	Adaptation of vein grafts to arterial pressure
Elevated blood pressure		Increased peripheral resistance in hypertension
Wall shear stress	Matching of vessel diameter to flow rate	Enlargement of collateral vessels
Elevated wall shear stress		Initiation of cerebral aneurysms
Low/fluctuating wall shear stress		Initiation of atherosclerotic lesions

arterial vessels in response to this increased pressure has the effect of further increasing peripheral resistance, in a positive feedback loop.

With increased wall shear stress, the primary structural response is increased luminal diameter. This has the important effect of matching vessel diameters to flow rates. When arteries or arterioles become blocked, if collateral flow pathways are available, these vessels may become enlarged and take over the supply of the affected region [60]. This depends on the response to increased shear stress in the collateral vessels. Hemodynamic effects have long been thought to influence the development of atherosclerosis [61]. Normal to high levels of wall shear stress with consistent flow directions promote a stable phenotype in endothelial cells, which inhibits the development of atherosclerosis [62]. However, low and fluctuating levels of shear stress tend to destabilize endothelial cells, and atherosclerosis typically appears initially at regions with such flow conditions, as for example, around arterial bifurcations. It has also been proposed that the complex distributions of pressure-induced wall stresses in such regions may also cause vulnerability to atherosclerosis [63–65], although this hypothesis has not received wide acceptance. Elevated levels of wall shear stress are considered to be a factor leading to the initiation of cerebral aneurysms [66].

In summary, biological responses to stresses acting on vessel walls generated by blood flow are important for the growth and maintenance of functionally adequate and efficient vasculature, and also for the development of diseases of blood vessels. Progress in this area of mechanobiology requires the integration of knowledge of hemodynamics, vessel wall mechanics, mechanotransduction, and growth and remodeling of vessel walls, on scales ranging from molecular and cellular to whole-organ and systemic. Improved understanding of the mechanisms governing vascular structure has the potential to lead to new strategies for preventing or treating diseases of the vasculature.

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2 Contributions of Wall Stretch and Shear Stress to Vascular Regulation: Molecular Mechanisms of Homeostasis and Expansion

Ranganath Maringanti, Elana Meijer, Maarten M. Brandt, Dirk J. Duncker, and Caroline Cheng

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Abstract

Blood vessels are continuously exposed to hemodynamic forces due to the pulsatile nature of the blood flow. In normal physiological settings, these forces are essential in the maintenance of vascular cell function and structure, vascular growth, and in the regulation of vascular tone. However, when exceeding the physiological range these biomechanical forces become detrimental and may initiate pathological pathways. In this chapter, we discuss the types of vascular biomechanical forces, unravel cellular and molecular mechanisms underlying the physiological and pathophysiological response of the vascular cells to these biomechanical stimuli, and describe their role in triggering vascular growth.

2.1 Introduction

The main biomechanical stimuli that affect the vascular system are shear stress and wall stretch [1]. The vascular wall, which is exposed to these forces, is comprised of three main cell types: endothelial cells (ECs), lining the tunica intima, vascular smooth muscle cells (VSMCs) in the tunica media, and fibroblasts within the adventitial layer [2]. The endothelium, which covers the inner lining of blood vessels, mainly responds to shear stress due to its direct contact with the bloodstream. Shear stress is generated by the intraluminal blood flow, which exerts force in the longitudinal direction on the surface of the vessel wall [3]. Circumferential stress or wall stretch, generated by intraluminal pressure of blood flow that exerts forces in perpendicular direction, triggers a direct activation of both ECs and VSMCs [4, 5].

2.1.1 Shear Stress

An important biomechanical stimulus that influences endothelial behavior is shear stress. Shear stress is the force per unit area created when a tangential force (blood flow) acts on a surface (endothelium) (Fig. 2.1). Shear stress is expressed in dynes, where 1 dyne equals 0.1 N/m2. Shear stress ($\boldsymbol{\tau}$) in a circular channel depends on

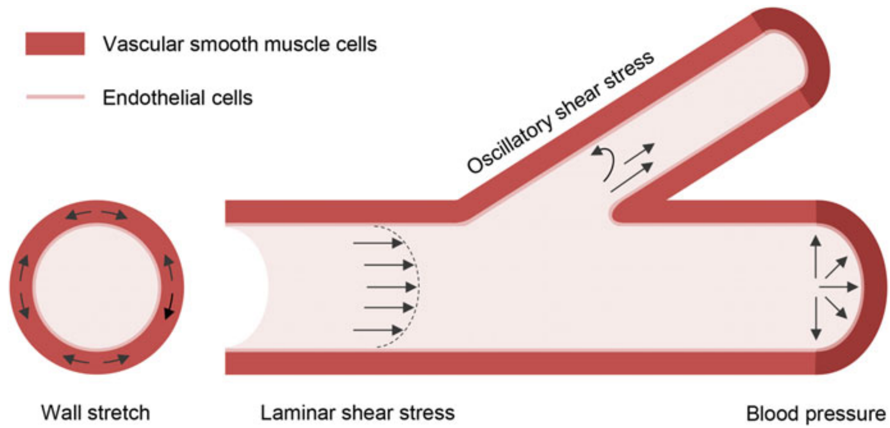

Fig. 2.1 Schematic representation of wall stretch on the left and shear stress on the right. In wall stretch, distension pressure results in the radial forces exerted on the vessel which leads to the extension of the vascular wall and in turn vascular cell elongation in a direction perpendicular to the force applied. Shear stress depends on flow rate, fluid viscosity, and radial distance of the vessel lumen. In straight regions of the vasculature, endothelial cells experience unidirectional shear stress, while at branch points and vascular bifurcations, endothelial cells experience oscillatory or low shear stress

Table 2.1 Physiological ranges of biomechanical forces in the arterial and venous systems

	Arterial system	Venous system	References
Physiological shear stress (dynes/cm^2)	11.4–30.4 (19.0–60.8 for arterioles)	0.76–7.6	Kroll et al. [7]
Pathophysiological shear stress (dynes/cm^2)	< 11.4	< 0.76	Kroll et al. [7]
Physiological wall stretch (%)	5–10%	N.A.	Anwar et al. [4]
Pathophysiological wall stretch (%)	>20%	N.A.	Anwar et al. [4]

volumetric flow (Q), fluid viscosity (μ), and lumen radius (r). The level of shear stress can be calculated according to the relationship: $\boldsymbol{\tau} = (4\mu Q)/(\pi r^3)$, under the additional assumptions that the vessel is a circular tube with a constant diameter, and that the blood flow is fixed [6]. The level of shear stress is actively maintained within a certain range in the circulation as the vasculature responds to shear stress changes by adjusting vascular tone and diameter. The ability of the vasculature to respond is mediated by the conversion of mechanical stress to biochemical responses, the so-called process of mechanotransduction [3]. Throughout the vasculature, ECs experience various flow conditions. Shear stress levels are for instance low (between 0.76 and 7.6 dynes/cm^2) in the venous system, whereas high shear levels are found in arteries and arterioles (between 11.4 and 30.4 dynes/cm^2 for large arteries, and between 19.0 and 60.8 dynes/cm^2 for arterioles) [7] (Table 2.1). Non-uniform

organization of the vascular network based on complex bifurcations, branch points, and curved regions greatly influence shear stress characteristics. In straight sections of the vasculature, both pulsatile and steady laminar flow occurs, resulting in the production of various biological factors by the endothelium for vascular support. In bifurcated regions, the blood flow patterns create oscillating shear stress, characterized by flow reversal, which can include occasional turbulence. Oscillatory shear stress, also referred to as disturbed shear stress, challenges the ECs to respond to the non-laminar changes in hemodynamic forces [8]. Low shear stress is typically associated with the venous system and the inner parts of the arterial curvature. The bigger the angle of the curve, the lower the force on the inner part, which results in differences in shear force between the inner and outer parts of the curvature.

2.1.2 Wall Stretch

The pressure exerted by blood volume produces forces in perpendicular direction to the vessel wall that leads to stretch in circumferential manner, resulting in circumferential stress also termed as ‘‘wall stretch” (Fig. 2.1) [5]. This pressure ‘P’ is the force (F) per unit area (A) exerting in perpendicular direction to the vessel wall, and results in radial forces triggering elongation of the vessel wall, thus causing cellular elongation. This alteration of the vessel wall in response to the amount of stretch applied to it is referred to as ‘‘wall strain” which is a measure of deformation [9, 10]. In the wall of macro- and microvascular structures, VSMCs and ECs are sensitive to wall stretch and undergo structural and functional adaptation in response to it [4]. Wall stretch is mainly studied as a force active in macrovascular vessels. Low magnitude stretches between 5–10% are considered as physiological stretch, which promotes vascular stability, control of vascular tone as well as proliferation, and may also contribute to angiogenesis (Table 2.1). Under these conditions, wall stretch also stimulates a contractile VSMC phenotype, which is characterized by elongated, spindle-shaped cells. On the other hand, high magnitude stretches of 20% and above, are considered as pathological stretch [4] (Table 2.1). Under these conditions, VSMCs switch from the contractile to a “synthetic” phenotype, which shows poor contractility, pro-migratory, and pro-inflammatory behavior on a functional and structural level, and are characterized by a cobblestone morphology with a rhomboid shape and a reduction in length [11, 12].

2.2 Mechanosensing

Mechanical forces affect ECs and VSMCs via mechanotransduction, which refers to the process through which cells sense and respond to biomechanical stimuli and translate these into the biochemical signals that elicit specific cellular responses [4, 13]. Any cellular structure that can detect these mechanical forces is called a mechanosensor. The receptors that are involved in sensing these mechanical forces are termed mechanoreceptors. Activation of mechanosensors by mechanical factors

leads to the initiation of multiple complex intracellular signaling pathways, which affect cellular function and drive the expression of particular genes [2, 4]. Due to the direct contact of ECs with blood flow, shear stress predominantly activates ECs [3]. To date, many mechanosensors have been described in ECs and do not appear to be limited to adhesion molecules, integrins, and ion channels, but for instance also include the membrane lipid bilayer itself and the glycocalyx [13]. These structures enable the vascular wall to adapt to differences in shear forces, by, e.g., stimulating local cell proliferation, or by modulating the vessel diameter. Many of these endothelial mechanosensors are also involved in sensing wall stretch. In addition to ECs, wall stretch also directly activates VSMCs. Accumulating evidence indicates that various aspects of mechanosensing act in parallel and interact rather than operate individually. The mechanosensors and mechanosensing mechanisms of shear stress and wall stretch will be discussed in the following section in more detail.

2.2.1 Receptor Tyrosine Kinases

Receptor tyrosine kinases (RTKs) are high-affinity cell surface receptors that are activated either by mechanical or chemical factors. They undergo dimerization, which allows tyrosine residues of the receptor in the cytoplasmic portion to undergo trans-phosphorylation by its partner receptor, followed by signal transmission through the plasma membrane [14]. This phosphorylation of tyrosine residues creates binding sites for intracellular downstream factors, such as tyrosine protein kinase ‘‘Src” and phospholipase-C γ (PLC-γ). A well-described RTK, vascular endothelial growth factor receptor-2 (VEGFR2), acts as a mechanosensor in association with platelet endothelial cell adhesion molecule 1 (PECAM-1) and vascular endothelial (VE)-cadherin, in response to both shear stress and wall stretch. Previous studies have shown that unidirectional shear stress induces increased tension on PECAM-1, which triggers recruitment and activation of Src family kinases. VE-cadherin links PECAM-1 to VEGFR2, which enables Src-dependent transactivation of VEGFR2 and subsequent activation of phosphoinositide 3-kinase (PI3-K) (Fig. 2.2). The Src-induced activation of VEGFRs does not necessarily require vascular endothelial growth factor (VEGF) stimulation, although it has previously been demonstrated that knockdown of VEGF results in attenuation of the protective effect of shear stress, implicating that VEGF signaling, via an alternative route, may be involved in maintaining a healthy vascular environment [15].

In the early adaptive response to flow, PI3-K activation by VEGFR2 stimulates the serine/threonine kinase protein kinase-B (PKB), also known as Akt (Fig. 2.2). Stimulation of Akt not only stimulates endothelial survival, but also favors endothelial nitric oxide synthase-(eNOS) induced production of nitric oxide (NO) [16]. In this mechanosensor complex, PECAM-1 is responsible for direct transmission of shear forces and Src activation; VEGFR2 is required for the activation of PI3-K, whereas VE-cadherin serves as an adaptor molecule. Disruption of any of these molecules leads to impaired activation and signaling [17]. Furthermore, this tri-molecular complex-mediated PI3-K signaling leads to upstream integrin

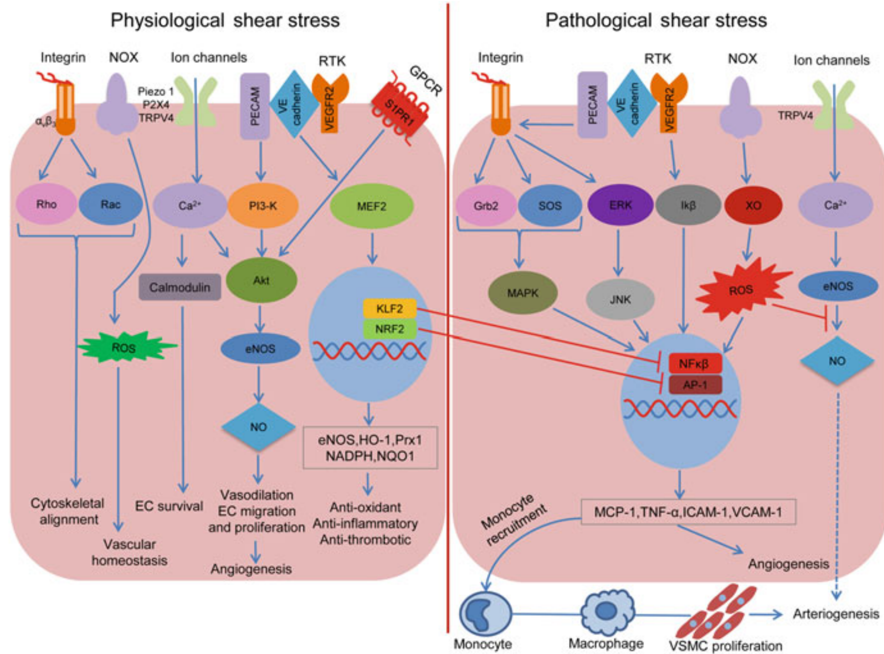

Fig. 2.2 Schematic representation of the mechanosensing and signaling in physiological (left) and pathological (right) shear stress. Shear stress is sensed by various mechanoreceptors or mechanosensory complexes. These mechanosensors initiate various downstream signaling pathways, which at their turn activate transcription factors and co-factors to modulate endothelial cell (EC) function and phenotype. In physiological shear stress shown on the left, these pathways result in vascular homeostasis, vasodilation, EC migration and proliferation, cytoskeletal alignment, and angiogenesis. Physiological shear stress has also antioxidant, anti-inflammatory, and anti-thrombotic functions. Pathological shear stress, in contrast, triggers different pathways, resulting in monocyte recruitment and thereby inflammation, but also eventually in angiogenesis and arteriogenesis. *Akt* protein kinase B, *AP-1* activator protein-1, *eNOS* endothelial nitric oxide synthase, *ERK* extracellular signal-regulated kinases, *GPCR* G-protein coupled receptor, *GRB2* growth factor receptor-bound protein 2 *HO-1* heme oxygenase 1, *ICAM-1* intercellular adhesion molecule 1, *JNK* Jun N-terminal kinase, *MAPK* mitogen-activated protein kinases, *MCP-1* monocyte chemoattractant protein-1, *MEF2* myocyte enhancer factor-2, *NF-κB* nuclear factor kappa beta, *NO* nitric oxide, *NOX* NADPH oxidases, *NQO1* NAD(P)H dehydrogenase quinone 1, *PECAM-1* platelet endothelial cell adhesion molecule-1, *Piezo 1* piezo protein 1, *PI3K* phosphoinositide-3 kinases, *PRX1* paired related homeobox 1, *P2X4* purinoceptor 4, *ROS* reactive oxygen species, *RTK* receptor tyrosine kinases, *SOS* son of seven less complex, *SRPR1* sphingosine-1-phosphate receptor 1,*TNF-α* tumor necrosis factor-alpha *TRPV4* transient receptor potential cation channel subfamily V member 4, *VCAM-1* vascular cell adhesion molecule-1, *VE-cadherin* vascular endothelial cadherin, *VEGF* vascular endothelial growth factor, *VEGFR2* vascular endothelial growth factor receptor-2, *XO* xanthine oxidase

activation, which exerts distinct cellular responses. In response to disturbed shear stress, PI3-K-induced activation of integrins stimulates focal adhesion kinase (FAK) followed by recruitment of a complex consisting of growth factor receptor-bound protein 2 (GRB2)/son of sevenless (SOS), stimulating activation of mitogen-

activated protein kinases (MAPKs) and subsequent nuclear factor κB (NF-κβ) and activating protein-1 (AP-1) mediated expression of pro-inflammatory genes (Fig. 2.2) [18].

In addition to fluid shear stress, PECAM-1 has also been shown to be activated by a wide variety of additional mechanostimuli, including osmotic shock and wall stretch [19]. In response to physiological wall stretch, endothelial PECAM-1 undergoes phosphorylation and initiates protein tyrosine phosphatase-2 (SHP-2) binding, subsequently activating MAPK and extracellular regulated kinase (ERK) (Fig. 2.3). The activation of MAPK and ERK promotes endothelial elongation and reorientation, ensuring that ECs are aligned in parallel to the direction of applied stretch [20, 21]. Under pathological stretch levels, PECAM-1 phosphorylation and subsequently ERK activation instead activate Ras and Raf [22], stimulating the expression of pro-inflammatory genes, such as monocyte chemoattractant protein-1 (MCP-1) [2] (Fig. 2.3). RTKs have also been reported to play a role in VSMCs exposed to wall stretch. In VSMCs, the stretch is involved in activating platelet-derived growth factor-β (PDGFRβ). PDGFRs form a wide subclass of the RTKs family that regulate various cellular responses, such as cell proliferation, differentiation, growth, and development in response to different stimuli. It has been reported that upon the stretch, PDGFRβ undergoes ligand-independent phosphorylation, thereby inducing VSMC proliferation [23].

2.2.2 Integrins

Integrins are transmembrane receptors that attach the cytoskeleton to the extracellular matrix (ECM) and are considered important mechanosensors [24]. They are heterodimeric in structure, meaning that they contain two subunits (α and β), of which in mammals, 18 α- and 8 β-subunits are known [25]. Integrins interact with focal adhesion (FA) proteins across the plasma membrane and contribute to adhesion between the cytoskeleton and ECM via these FA-complexes [13]. In response to physiological shear stress, subsequent conformational activation of integrin $\alpha_v\beta_3$, facilitates an increase in EC binding to ECM. This shear-induced $\alpha_v\beta_3$ integrin binding to ECM leads to transient inactivation of Rho (Fig. 2.2). The Rho family of GTPases is a family of small signaling G-proteins involved in organelle development and cell movement. Short-term inactivation of Rho via $\alpha_v\beta_3$ aids in cytoskeletal alignment to the direction of flow [26] during the late adaptive response. In this process, the previously described tri-molecular complex (PECAM-1, VEGFR2 and VE-cadherin) activates integrins, forming a new integrin-matrix interaction which drives shear-induced adaptive responses in physiological conditions (cell alignment) via transient Rho inactivation [26], and pathological conditions (inflammation) via GRB2/SOS-MAPK- NF-κβ signaling [18] (Fig. 2.2). Regulation of Rho in response to shear stress is complex. Rho-activation occurs quickly and spontaneously under shear stress and immediately induces cell contraction via Rho-kinase. Once Rho-kinase reaches basal levels, ECs elongate in the direction of flow, coinciding with the upregulation of Rac-1 and Cdc42. Rho and Rac activation are both involved

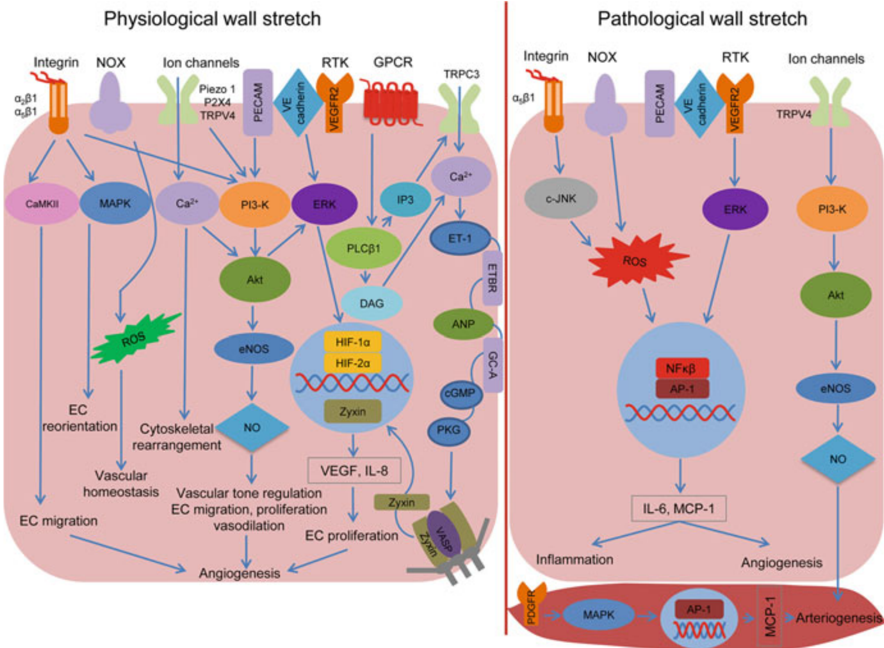

Fig. 2.3 Summary of the mechanosensing mechanisms induced by wall stretch. Wall stretch stimuli are sensed by mechanosensors of both endothelial cells and vascular smooth muscle cells that transduce downstream signals. This results in the activation of transcription factors that regulates gene expression followed by increased protein synthesis which alters cell morphology and function. However, different mechanisms are activated upon different intensities, magnitude, and duration of stretch applied. Physiological wall stretch (left) is beneficial in maintaining healthy phenotype of endothelial and vascular smooth muscle cells and thus regulating vascular growth and function; Whereas pathological wall stretch (right), could activate pathways leading to disease progression or may induce arteriogenesis as a compensatory mechanism that could limit disease development. Thus, it is important to understand the signaling mechanisms that are triggered in response to wall stretch as this could aid in the identification of novel therapeutic targets that could stimulate vascular regeneration and restore vascular function aimed at treating vascular-related diseases. *ANP* atrial natriuretic peptide, *AP-1* activator protein-1, *CaMKII* calmodulin-dependent protein kinase-II, *cGMP* cyclic guanosine monophosphate, *c-JNK* c-Jun N-terminal kinase, *DAG* diacyl glycerol, *eNOS* endothelial nitric oxide synthase, *ERK* extracellular signal-regulated kinases, *ET-1* endothelin 1, *ETBR* endothelin-B receptor, *GC-A* guanylyl cyclase-A, *GPCR* G-protein coupled receptor, *HIF-1α* hypoxia-inducible factor-1α, *HIF-2α* hypoxia-inducible factor-2α, *IL-6* interleukin-6, *IL-8* interleukin-8, *IP3* inositol triphosphate, *MAPK* mitogen-activated protein kinases, *MCP-1* monocyte chemoattractant protein-1, *NF-κ* nuclear factor kappa beta, *NO* nitric oxide, *NOX* NADPH oxidases, *PDGFRβ* platelet cell-derived growth factor receptor-beta, *PECAM-1* platelet endothelial cell adhesion molecule-1, *PI3K* phosphoinositide-3 kinases, *PKG* protein kinase-G, *PLCβ1* phospholipase-C β1, *P2X4* purinoceptor 4, *ROS* reactive oxygen species, *RTK* receptor tyrosine kinases, *TRPC3* transient receptor potential channel 3, *TRPV4* transient receptor potential cation channel subfamily V member 4, *VASP* vasodilator stimulated phosphoprotein, *VCAM-1* vascular cell adhesion molecule-1, *VE-cadherin* vascular endothelial cadherin, *VEGF* vascular endothelial growth factor, *VEGFR2* vascular endothelial growth factor receptor-2

in the regulation of the directionality of cell movement. Inhibition of Rho/Rho-kinase leads to increased cell displacement, indicating that EC reorientation occurs in two-step process which involves Rho-induced depolarization followed by Rho/Rac-mediated polarization and subsequent migration in the direction of flow. In general, transient inactivation of Rho via integrin and Rho/Rac regulation is required for EC reorientation, migration, and flow alignment in response to shear stress [27].

Remarkably, responding to physiological stretch, integrin α2 and β1 subunits are known to activate p38 MAPK, which has also been shown to contribute to endothelial reorientation [28] (Fig. 2.3). In addition, integrin signaling via Rho induces adaptation of cell morphology and orientation and Rho-induced PI3-K activation leads to NO production via the Akt/eNOS pathway that facilitates vascular tone regulation [2]. In contrast, under pathological stretch, integrins induce reactive oxygen species (ROS) production via the c-Jun-N-terminal Kinase (JNK) pathway, which results in the expression of pro-inflammatory genes (such as MCP-1) (Fig. 2.3) [2].

2.2.3 Ion Channels

Ion channels also represent important sensors for shear stress and wall stretch. Shear stress induces the activation of several non-specific cation channels, including Piezo Type Mechanosensitive Ion Channel Component 1 (Piezo1), Transient Receptor Potential Cation Channel Subfamily V Member 4 (TRPV4), and P2X Purinoceptor 4 (P2X4), all causing cellular depolarization and elevated cytoplasmic Ca^{2+} levels [29] (Fig. 2.2). Elevated Ca^{2+} levels stimulate the opening of Ca^{2+}-dependent K^+ channels, leading to K^+ influx and hyperpolarization. In response to shear stress-activated ion channels on ECs and via Calmodulin/Ca^{2+}/eNOS/NO signaling, VSMCs neighboring ECs also hyperpolarize and (partially) through direct electrical coupling via gap junctions, cause vessel relaxation and vasodilation (Fig. 2.2) [30]. The elevated cytoplasmic Ca^{2+} levels furthermore contribute to Akt-mediated activation of eNOS, as Ca^{2+}-activated calmodulin releases eNOS from its bound state in caveolae, which renders the enzyme inactive [31]. Similarly, stretch also causes a rise in cytoplasmic Ca^{2+} levels, as for instance observed in capillary ECs via TRPV4, which facilitated cytoskeletal rearrangements and capillary cell orientation [32]. This stretch-induced rise of Ca^{2+} levels similarly led to Akt-induced activation of eNOS [33] (Fig. 2.3).

2.2.4 G-Proteins and G-Protein-Coupled Receptors

Heterotrimeric G-protein complexes act as molecular switches capable of transmitting a variety of external stimuli into the cell. Their activity is regulated by their ability to bind and hydrolyze guanosine triphosphate (GTP) to guanosine diphosphate (GDP). It has previously been demonstrated that physiological levels of fluid shear stress cause dose-dependent activation of GTP hydrolysis by G-proteins that

were reconstituted into phospholipid vesicles. Increasing or decreasing membrane fluidity by incorporation of lysophosphatidylcholine or cholesterol in the membrane, respectively, affected the activation of G-proteins by shear, indicating that physical properties of the phospholipid bilayer, rather than protein receptor-induced signaling, mediate this activation of G-proteins by shear [34]. Nonetheless, G-protein-coupled receptors do in fact respond to shear stress and may play an important role in mechanosensing. It has for instance been demonstrated that applying shear, as well as a membrane-fluidizing agent, causes rapid and ligand-independent activation of the endothelial bradykinin B2 receptor.

Moreover, the G-protein-coupled receptor (GPCR) sphingosine-1 phosphate receptor 1 (S1P1) has also been demonstrated to be required for shear-induced activation of Akt and eNOS. Similar to bradykinin B2 receptor, activation of S1P1 was ligand-independent, as experiments with ligand binding-deficient mutants of S1P1 led to the functional rescue of the S1P1 knockout phenotype. Using pertussis toxin (PTX), a G_i-protein inhibitor, it has also been demonstrated that applying physiological stretch for 24 h stimulates endothelial migration via activation of G_i-α subunits and GTPase activity [35]. Applying stretch on ECs causes the activation of secondary messengers inositol trisphosphate (IP_3) and Diacylglycerol (DAG) through phospholipase-C-β1 (PLCβ1). These secondary messengers in turn activate Ca^{2+} influx via transient receptor potential channel-3 (TRPC3), eventually leading to secretion of endothelin-1 (ET-1) and thus activating endothelin B-1 receptor (ETB1R). Activation of ET-1 receptor subsequently results in the secretion of pro atrial natriuretic peptide (pro-ANP) that binds to guanylyl cyclase-A (GC-A) receptor, followed by downstream effects such as cyclic guanosine monophosphate (cGMP)-induced protein kinase-G (PKG) activation. This complex stretch-induced autocrine-signaling loop in ECs leads to translocation of zyxin into the nucleus where it controls the expression of stretch-sensitive genes involved in cell cycle regulation and endothelial function [36]. Compared with static ECs, stretch-induced translocation of zyxin also induced the expression of several pro-inflammatory genes, illustrating the various adaptive responses this stretch-responsive GPCR-mediated signaling axis may be involved in [36]. In addition to ECs, stretch-mediated activation of GPCRs has also been demonstrated in VSMCs. Exposing primary renal rat VSMCs to osmotic stretch led to increased Ca^{2+} levels which could be inhibited with the angiotensin 1 receptor (AT_1R) blocker losartan [37, 38].

2.2.5 NADPH Oxidases

The intensity and type of shear stress also directly affects the activity of NADPH oxidases (NOXs). NOXs belong to a family of membrane-bound protein complexes, of which NOX 1, 2, 4, and 5 isoforms are expressed in the vasculature. They facilitate the production of O_2^-, a free radical that is the precursor of most ROS. It has been observed that steady laminar flow leads to limited and transient induction of NOX, which in concert with mitochondrial oxidation contributes to low amounts of ROS (Fig. 2.2) [39]. ROS and shear-induced NO could interact, forming reactive

nitrogen species (RNS) that cause nitrosative damage [39]. During physiological conditions, limited ROS production ensures that this interaction hardly occurs, and as a consequence, the availability of both factors suffices vascular homeostasis. In response to oscillatory shear or flow reversal, however, sustained NOX activity causes xanthine oxidase- (XO) dependent elevation of ROS production [39]. The augmented production of ROS favors an oxidative state and not only causes an increase in RNS, but also lowers NO availability. This lowering of NO bioavailability blunts the vasodilatory capacities, and since NO also provides S-nitrosation of regulatory proteins involved in the suppression of AP-1 and NF-κβ activity, its lowering also results in elevated expression of pro-inflammatory cytokines and adhesion molecules (Fig. 2.2).

In addition to shear stress, wall stretch also affects the NOX activity in ECs. Several studies revealed that upon long-term physiological stretch in ECs, NOX4 was significantly downregulated, leading to reduced ROS formation (Fig. 2.3). This stretch-dependent downregulation of NOX4 expression and ROS formation was found to be abolished when eNOS was inhibited by N omega-nitro-L-arginine methyl ester hydrochloride (L-NAME), implicating that physiological stretch on ECs induces its vasoprotective activity by suppressing NOX4 and ROS via eNOS (Goettsch, [40]). On the other hand, pathological stretch leads to pro-inflammatory conditions in ECs. Pathological stretch causes excessive production of free radicals that can either react alone, or via superoxide dismutase, to generate hydrogen peroxide, which stimulates NF-κβ activity [41, 42]. In addition, phosphorylation of $P66^{Shc}$ in response to pathological stretch leads to elevated superoxide anions and reduced NO levels, indicating that pathological stretch is associated with increased ROS production, promoting oxidative stress and endothelial dysfunction [43].

2.2.6 Glycocalyx

The glycocalyx is a thin apical layer (approximately 500 nm) composed of glycoproteins, glycolipids, and proteoglycans, such as Syndecan-1 and- 4. The cytoplasmic domain of these Syndecans directly interacts with the cytoskeleton, providing a potential route of force transfer from shear stress towards the endothelium. Genetic ablation of syndecans results in dysregulation of Rho-induced structural adaptations required to align ECs to the direction of flow and causes elevated expression of inflammatory genes. Moreover, enzymatic removal of heparin sulfate and hyaluronic acid, both glycosaminoglycans bound to proteoglycans, was shown to attenuate shear-induced production of NO and vasodilation. Suppression of syndecan-4 expression in ECs did not affect shear-induced phosphorylation of VEGFR2, suggesting that Syndecans might signal independent from the PECAM-1/VE-Cadherin/VEGFR2 complex, although since Syndecans also act as Integrin co-receptor during adhesion, it could be argued as well that it signals downstream of this junctional complex.

In addition to shear stress, endothelial glycocalyx was also found to respond to wall stretch. Recent studies have demonstrated that in response to physiological stretch, activated glycocalyx mediates NO production. Using atomic force and fluorescence microscopy techniques, the rapid production of NO was observed in the presence of heparin sulfate and hyaluronic acid which are present on the surface of glycocalyx indicating that glycocalyx mechanotransduction contributes to endothelial function in response to physiological stretch [44].

2.3 Transcriptional and Functional Response

Many aspects of vascular cell function are affected by mechanical stimuli. Endothelial growth, for instance, is highly dependent on the type of shear stress the endothelium is exposed to [45, 46]. In response to laminar flow, only limited numbers of ECs enter the cell cycle, rendering the majority of the cells arrested in G_0 or G_1 phase [47]. This shear-induced growth arrest is mediated by a mechanism involving tumor suppressor protein 53 (p53). Upon p53 activation, cyclin-dependent kinase 4 (CDK4) activity is decreased and retinoblastoma protein (Rb) is not phosphorylated [47]. Rb phosphorylation is required to release E2F transcription factors from the pRb/E2F complex to induce the expression of genes required for DNA replication. Consequently, this shear-induced inhibition of CDK4 limits proliferation [48]. Simultaneously, laminar flow also serves a protective role by reducing EC turnover, preventing the occurrence of lesions [49, 50]. Besides this proliferative response, the inflammatory- and thrombotic state of vascular cells is highly related to shear stress and wall stretch. Depending on type and intensity of mechanical stimuli, particularly transcription factors, including Krüppel-like factors (KLFs) 2 and 4, nuclear factor erythroid 2-like (Nrf2), AP-1, hypoxia-inducible factor 1α (HIF1α), and zyxin are either activated or inactivated (Fig. 2.2).

2.3.1 Krüppel-Like Factors 2 and 4

KLF-2 and 4 are identified as laminar flow inducible factors that play an important role in the regulation of endothelial function. With respect to the vasculature, KLF2 is exclusively present in the endothelium, robustly expressed in straight parts of the vasculature, and notably lower expressed in branch points and bifurcated regions [51]. KLF2, together with Nrf2, modulates the expression of genes critical in regulating vascular tone, hemostasis/thrombosis, and inflammation, and regulates endothelial barrier function and its antioxidative capacity (Fig. 2.2) [52]. Shear stress-induced expression of KLF2 depends on myocyte enhancer-binding factor 2 (MEF2). Under static conditions, histone deacetylase 5 (HDAC5) is bound to MEF2 on the KLF2 promotor and inhibits its transcriptional activity. High unidirectional shear stress results in HDAC5 dissociation from MEF2, allowing increased flow-dependent KLF2 transcription. KLF2 directly binds to the promotor of eNOS, inducing its transcription. At the same time, KLF2 also downregulates Caveolin-1, a negative

regulator of eNOS. In vitro studies also demonstrated that overexpression of KLF2, by inhibiting NF-κβ, lowers the production of inflammatory cytokines and adhesion molecules vascular cell adhesion molecule-1 (VCAM1) and E-selectin [52].

Like KLF2, KLF4 increases the expression of eNOS and thrombomodulin, while reducing the expression of VCAM1 and pro-coagulant factors. Although similarities to the actions and effects of KLF2 exist, KLF4 differs in its expression in response to inflammatory stimuli. In response to inflammatory stimuli, such as tumor necrosis factor-α (TNFα), expression of KLF4 is heavily increased. In the context of inflammation, KLF4 may serve as a back-up to modulate endothelial inflammation when KLF2 levels are strongly attenuated [53]. Compared to shear stress, the effect of wall stretch on KLF2 regulation is less well understood. However, recent evidence has shown that wall stretch also has an impact on KLF2 regulation. In microvascular ECs, pathological stretch halved the expression of KLF2 compared to cells under physiological stretch. This indicates that pathological wall stretch suppresses the functions of KLF2, which may lead to vasoconstriction, inflammation, coagulation, or thrombosis [54].

2.3.2 Nuclear Factor Erythroid 2-Like

Another important laminar flow-responsive transcription factor is Nrf2, which plays an important role in the protection of ECs from oxidative stress [55]. Like KLF2, Nrf2 is activated by shear-induced activation of MAPK signaling which stimulates its release from Kelch-like ECH-associated protein 1 (keap1), resulting in Nrf2 translocation to the nucleus. In addition, the activity of Nrf2 is enhanced by KLF2 and PI3K/Akt signaling, which also enhances the nuclear localization of Nrf2 and stimulates expression of the majority of shear stress-regulated vasoprotective genes [56]. Transcriptional activation of Nrf2 leads to induction of antioxidant response element- (ARE) related genes. These genes, including hemo oxygenase-1 (HO-1), peroxiredoxin 1 (Prx-1), nicotinamide adenine dinucleotide phosphate (NADPH), and quinone oxidoreductase-1 (NQO1) play key roles in the protection of vascular endothelium from oxidative stress during the development of vascular diseases.

2.3.3 Nuclear Factor Kappa Beta

Opposing many of the laminar flow-induced effects, disturbed flow is related to pro-oxidant and pro-inflammatory conditions in ECs, of which chronic activation is associated with the development of vascular disease. Important transcriptional events that reflect the pro-oxidant and pro-inflammatory condition of ECs in response to disturbed flow include the activation of NF-κβ (Fig. 2.2). NF-κβ is a transcription factor involved in both inflammation- and oxidative stress-mediated signaling [57]. In the absence of inflammatory stimuli, the non-activated form of NF-κβ is maintained in the cytoplasm together with its inhibitory subunit IκB, but

when activated, proteolysis of IκB results in the exposition of a nuclear recognition site in NF-κβ, which stimulates it to translocate into the nucleus. Disturbed flow both attenuates KLF2-mediated inhibition of NF-κβ and simultaneously induces IκB degradation, resulting in a pronounced increase of NF-κβ activity [58]. There has been accumulating evidence that wall stretch also induces activation of NF-κβ in ECs by various signaling pathways. Depending on the magnitude of wall stretch, the adaptation of ECs through NF-κβ activation varies. For example, studies have shown that a 12% stretch in ECs leads to NF-κβ activation that results in increased MCP-1 expression, which is chemotactic to monocytes and macrophages [59]. Based on the known role of MCP-1 in ECs, this increased MCP-1 expression via NF-κβ activation may promote capillary tube formation. Other studies demonstrated that in response to 25% stretch, NF-κβ was activated and was shown to significantly elevate levels of interleukin-6 (IL-6), which is involved in inflammatory processes. Mechanistically, NF-κβ activation requires outside-in signaling via integrin activation followed by PI3-K/PLC/PKC signaling, which is also observed in VSMCs upon exposure to stretch [60].

2.3.4 Activator Protein 1

Like NF-κβ, AP-1 is also activated in response to disturbed flow. AP-1 is a heterodimer composed of members of the c-Jun, c-Fos, activating transcription factor (ATF), and Jun dimerization protein (JDP) families. In response to disturbed, or low oscillatory flow, impaired Nrf2 activation causes a reduced inhibition of JNK and p38 MAPK, whereas nuclear translocation of AP-1 subunits is promoted by downregulation of KLF2, eventually both contributing to a rapid activation of AP-1 (Fig. 2.2) [56, 61]. Like NF-κβ, activation of AP-1 eventually results in enhanced expression of numerous adhesion molecules, such as VCAM-1, intercellular adhesion protein-1 (ICAM-1) and E-selectin, thus promoting mononuclear cell recruitment and extravasation, and enhances the transcription of a variety of cytokines, including TNF-α, MCP-1, and interleukins to stimulate an inflammatory response [57]. Oscillatory flow-mediated activation of pro-inflammatory NF-κB and AP-1 and counterbalancing activation of vasculoprotective KLF2 and Nrf2 thus helps to define the shear stress-dependent endothelial phenotype in atheroprone/atheroprotective arterial sites [8]. AP-1 activation has also been reported in response to pathological elevation of wall stretch in both ECs and VSMCs where it regulates many functions such as proliferation, inflammation, and vascular growth. In ECs, wall stretch-induced activation of AP-1 leads to transcription of MCP-1, which stimulates monocyte recruitment and inflammation [59, 62]. Similarly, stretch-induced activation of AP-1 in VSMCs results in MCP-1 synthesis, which via its monocyte recruitment function also stimulates VSMC proliferation [63]. Notably, despite its pro-inflammatory role, deletion of AP-1 family member Jun-D leads to oxidative stress, suggesting that AP-1 also plays a role in protecting ECs from dysfunction [64].

2.3.5 Hypoxia-Inducible Factor 1α

It has previously been demonstrated that HIF1α also is a prominent disturbed shear stress-responsive factor. HIFs are heterodimeric nuclear transcriptional factors consisting of two subunits that regulate the transcription of genes mediating cellular homeostatic responses to altered oxygenation. The expression of HIF1α in many tissues increases exponentially as oxygen concentration declines [65]. HIF1α was found to be upregulated in atheroprone regions of arteries, and HIF1α expression is increased in response to low shear stress in cultured ECs [65]. It has also been demonstrated that disturbed shear activates HIF1α via induction of NOX4 and ROS production. Activation of HIF1α by disturbed shear results in metabolic reprogramming of the endothelium by promoting endothelial glycolytic metabolism and reduction in mitochondrial oxidative phosphorylation. In response to wall stretch, HIF1α activation has not only been reported in ECs [66] but also in VSMCs [67]. Transcriptional activation of HIF1α was observed in skeletal muscle capillary beds and inferior vena cava [68] under prolonged exposure to stretch. In vitro studies of arterial cells have also demonstrated that wall stretch-induced activation of HIF1α promotes VSMC proliferation [67, 69]. Furthermore, physiological stretch on skeletal muscle and capillary ECs promote transcription and DNA binding activity of both HIF1α and HIF2α, leading to transcription of vascular growth- and ECM modulation-associated factors (Fig. 2.3) [66] and matrix metalloproteinases (MMPs) [68].

2.3.6 Zyxin

Zyxin is yet another important transcription factor, recently described for its stretch-dependent activity. Zyxin is a LIM-domain containing protein that appears to be located at focal adhesion regions of ECs and VSMCs, in association with vasodilator adhesion protein (VASP). In ECs, zyxin was found to be activated and translocated directly into the nucleus in response to stretch, where it controls many stretch-sensitive genes, such as interleukin-8 (IL-8), Hairy/enhancer-of-split related with YRPW motif protein 1 (Hey-1) and VCAM. This translocation of zyxin occurs through a complex series of events, where initially GPCRs respond to wall stretch, thereby activating PLCβ that lead to the release of secondary messengers IP_3 and DAG. These secondary messengers activate TRPC3 thereby enhancing Ca^{2+} influx, which leads to activation of ETBR via ET-1 binding. These events further lead to the release of pro-ANP that binds to GC-A leading to downstream cGMP-PKG signaling, finally inducing zyxin phosphorylation at Ser142. Pathway analysis revealed that the genes regulated by zyxin in ECs upon stretch mainly control cell cycle and inflammatory factor release, suggesting that zyxin-induced transcriptional regulation contributes to the maintenance of endothelial cell function [36]. On the other hand, zyxin plays a different role in VSMCs upon stretch induction. Instead of inducing direct translocation to the nucleus, upon its activation, zyxin activates RhoA and increases actin polymerization, transforming G-actin to F-actin, which leads to the

expression of myocardin-related transcription factor-A (MRTF-A) and translocation of MRTF-A to the nucleus. Upon binding of MRTF-A to serum response factor (SRF) in the nucleus, it controls the expression of mechanosensitive genes that mediate the maintenance of the contractile phenotype of VSMCs. Combined, these data suggest that zyxin plays an important role in cellular adaptations in response to stretch [70].

2.4 Mechanical Stimuli in Vascular Growth

The development of the human vascular system starts already in the third week of embryonic development by a process called vasculogenesis. At this point in development, the mechanical factors described in previous sections are still absent. At later stages, however, vascular growth predominantly occurs via angiogenesis and arteriogenesis, two distinctly different processes, in which biomechanical stimuli are of critical importance.

2.4.1 Arteriogenesis

Arteriogenesis, or collateralization, refers to the process of developing pre-existing arterioles into functional muscular collateral arteries, thereby increasing the diameter of these vessels. Physiologically, this is of relevance, for instance, in microvascular remodeling upon exercise training, in which an increase in arteriole diameter leads to an elevation of the total flow a vascular bed can withstand. However, arteriogenesis is mostly known for its vitally important role in overcoming vascular stenosis, which is referred to as abnormal narrowing of blood vessels with subsequent reduction of the lumen. Growth and expansion of pre-existing arterioles to by-pass an occluded vessel provides the vascular system with the ability to compensate for stenosis-induced attenuation of tissue perfusion. Both under these physiological and pathophysiological circumstances, arteriogenesis involves proliferation and migration of ECs and VSMCs, as well as degradation and reorganization of extracellular structures. To facilitate these features, activated ECs express high levels of ICAM1, VCAM1, E-selectin, and MCP-1 to attract monocytes, which in turn secrete a local cocktail of growth factors, proteases, and chemokines, including fibroblast growth factors (FGFs) and MMPs (Fig. 2.2).

Remarkably, in zebrafish embryos, in which all cells can be supplied with oxygen via diffusion, it has been demonstrated that arteriogenesis does not depend on hypoxia, yet predominantly develops in response to altered biomechanical stimuli [71]. During progressive stenosis of an artery, altered hemodynamic patterns emerge. The pressure differs between the proximal and distal portions from the occlusion site, creating a pressure gradient in the artery. The pressure drop distal to the occlusion drives the increase in blood flow and subsequently leads to increased collateral fluid shear stress [72]. This increased shear stress results, among others, in endothelial NO release causing vasodilation [73], subsequently causing an inevitable

rise in wall stretch too. Via the combined activation of various mechanosensors, this rise in biomechanical stimuli activates vascular growth-associated signaling in both ECs and VSMCs recruiting pre-existing arterioles spanning two neighboring arteries. Immunoprecipitation analysis illustrated that in response to pathological shear stress, integrin $\alpha_v\beta_3$, β_1, and β_5 associate with Shc protein (contains Src homology-2 domain). These integrins are known to stimulate arteriogenesis by activation of ERK and JNK signaling, thereby activating the transcription complex AP-1 [74], or via GRB2/SOS signaling, leading to the activation of NF-κβ (Fig. 2.2) [18]. Aided by the disturbed shear-induced loss of Nrf2 activity, which leads to attenuated inhibition of JNK-mediated AP-1 activation, this elevated shear stress-induced activation of integrins initiates the expression of inflammatory factors, such as ICAM1, VCAM1, and MCP-1 [57]. These factors are strongly involved in adhering and attracting monocytes which, upon binding to the vascular wall, transmigrate and differentiate into macrophages, stimulating VSMC proliferation and collateral maturation, mostly in a paracrine manner [75].

Likewise, wall stretch also plays a fundamental role in collateral vessel growth through the activation of integrins. In response to pathological stretch, integrin activation, such as $\alpha_5\beta_1$ in ECs, via JNK signaling, phosphorylates $p66^{Shc}$, and in parallel activates NADPH-oxidase that leads to ROS production [43]. This ROS production leads to the expression of MCP-1 through transcriptional activation of NF-κβ or AP-1 and results in the development of collateral vessel growth [2, 74] (Fig. 2.2). Furthermore, elevated wall stretch leads to integrin β_3 and $\alpha_v\beta_5$ activation in VSMCs, which results in the secretion of platelet-derived growth factor-A (PDGFA), stimulating VSMC proliferation via an autocrine-signaling loop [76]. Although the exact mechanism leading to stretch-induced secretion of PDGFA via integrins activation in VSMCs still needs to be elucidated, it was reported that upon induction of stretch, VSMCs undergo this mitogenic response only when grown on collagen type 1, vitronectin, or fibronectin, but not on elastin or laminin, indicating this response is matrix type-dependent [76].

In addition to integrins, signaling via the mechanosensor PECAM-1 also contributes to collateralization [77]. PECAM-1 is a widely described vascular cell junction molecule, known for its role in endothelial function. In previous studies, it has been demonstrated that shear stress-induced PECAM-1 activation enhances PI3-K/Akt signaling, which in turn may stimulate integrin-mediated inflammatory signaling (Fig. 2.2) [16]. In mice, loss of PECAM-1 causes poor perfusion of collaterals [77] due to impaired complex formation with VE-cadherin and VEGFR2, suggesting that PECAM-1 is essential for arteriogenic signaling [16]. Similar to shear stress, wall stretch also leads to PECAM-1-mediated signaling, stimulating Ras- and Raf-induced activation of ERK signaling, which in turn stimulates the production of MCP-1 and arteriogenesis [19, 77]. In VSMCs, wall stretch activates the RTKs PDGFRα and PDGFRβ [23]. Wall stretch stimulates rapid phosphorylation of PDGFRα and PDGFRβ, which in turn leads to MAPK activation and enhanced DNA binding of AP-1 [23, 78]. Like in ECs, activation of AP-1 in VSMCs enhances the transcription of MCP-1, which via attraction of monocytes

indirectly results in VSMC proliferation, but at the same time also directly stimulates VSMC proliferation and transcriptional activation of NF-κβ [79] (Fig. 2.2).

Although ion channels also serve an important role in sensing shear stress and wall stretch, it is poorly understood how these sensors respond and participate in vascular function and tone under pathophysiological levels of shear stress and stretch. Femoral artery ligation studies have shown that TRPV4 expression is increased in growing collaterals at both RNA and protein levels. The notion that expression was upregulated secondary to femoral ligation suggests that TRPV4 is not directly involved as a primary sensor, though in experiments with a specific TRPV4 activator (4αPDD), it has been demonstrated that elevated TRPV4 levels could stimulate proliferation of both ECs and VSMCs, and thus be an essential factor in collateral vessel growth (Troidl, [80]) (Fig. 2.2). Stretch activated TRPV4 in ECs participates in eNOS and NO production via activation of PI3K and Akt. Blocking of TRPV4 was shown to attenuate eNOS expression, and since several studies have illustrated that lack of NO impairs collateral growth [33, 81], this suggests that stretch-induced activation of TRPV4 may utilize this signaling route to stimulate arteriogenesis. In addition, previous studies have also shown that NO inhibits VE-cadherin, causing increased vascular permeability and allowing infiltration of monocytes, which may contribute to the pro-inflammatory environment required for arteriogenesis [82].

2.4.2 Angiogenesis

Angiogenesis is defined as the formation of new vessels from pre-existing vessels, which either occurs via sprouting of ECs or via intussusception. In sprouting angiogenesis, ECs are activated upon exposure to angiogenic stimuli, such as FGFs and VEGFA. These factors are produced during hypoxia and nutritional stress, and initiate various signaling cascades in ECs. In addition to activating ECs, hypoxia has also been demonstrated to stimulate pre-capillary sphincters [83], a band of contractile mural cells known to regulate blood flow in capillaries [84]. During exercise, for instance, hypoxia in muscle tissue stimulates these pre-capillary sphincters to relax and induces vessel dilation. The subsequent rise in the flow not only provides the surrounding tissue with its oxygen demands, but also stimulates a variety of mechanosensors that may drive angiogenesis [85]. In contrast to sprouting angiogenesis, intussusceptive angiogenesis contributes to vascular growth by the division of existing vessels into multiple smaller vessels. It is initiated by the formation of so-called pillar core structures between opposing ECs. Pericytes and myofibroblasts invade these pillar cores and stabilize them by deposition of extracellular matrix components, followed by expansion of the tissue pillars until eventually the capillary is split in two. Although the exact mechanisms driving intussusceptive angiogenesis are still not fully understood, mechanical forces are suspected to play a vital role [86].

In response to unidirectional shear stress, PECAM-1-induced activation of PI3K and Akt signaling results in eNOS-mediated NO production [16] (Fig. 2.2). Besides its role in vasomotion, endothelial NO is involved in various responses,

predominantly in endothelial survival, but also in EC migration and proliferation [87]. These aspects facilitate normal vascular homeostasis of mature capillaries, but are also indispensable for angiogenesis. In addition, NO has also been shown to suppress Angiostatin, an endogenous antagonist of angiogenesis [88]. Wall stretch-induced activation of PECAM-1 signaling, leading to MCP-1 expression has also been shown to promote endothelial migration and proliferation [19, 77]. Previous studies showed that MCP-1 via MCP-1-induced protein (MCP1-IP) stimulates capillary new growth, whereas silencing of MCP1-IP abrogated this angiogenic response [89]. RTKs, facilitating the above-described production of NO and MCP-1 initiated by PECAM-1, were also reported to stimulate angiogenesis in alternative ways. For instance, in response to shear stress, enhanced Iκβ kinase activity was observed, while this activity was abolished upon inhibition of VEGFR2 [90]. Although, this study has not demonstrated that this pathway promotes angiogenesis, enhanced Iκβ kinase activity could mediate angiogenesis in ECs via NF-κB activation which was supported by other reports, showing that NF-κβ activation promotes capillary tube formation [91]. Physiological stretch has been reported to stimulate angiogenesis via VEGFR2 (Fig. 2.2). Stretch-induced VEGFR2 activation is ligand-independent, though this activation of VEGFR2 stimulates Akt and ERK, which in turn remarkably increases endothelial VEGF sensitivity, resulting in endothelial proliferation [92]. This increased VEGF sensitivity may occur through the activation of HIF-1α or HIF-2α [66].

Shear stress and wall stretch have also been found to regulate angiogenesis via integrins. Integrins such as $\alpha_v\beta_3$ and $\alpha_v\beta_5$ were shown to regulate cell survival and angiogenesis but via distinct ways. Antagonists of $\alpha_v\beta_3$ were shown to disrupt FGF-mediated angiogenesis, while $\alpha_v\beta_5$ antagonists on the other hand disrupted VEGF-mediated signaling [93]. In response to physiological shear stress, studies have demonstrated $\alpha_v\beta_3$ activation with sustained Shc association [94]. In ECs, activated $\alpha_v\beta_3$ along with FGF, leads to Ras-induced protection from apoptosis, whereas $\alpha_v\beta_5$ activation under shear stress, along with VEGF, utilizes Ras- and Raf-induced activation of ERK, promoting both endothelial survival and angiogenesis [93]. In response to physiological stretch, a complex is formed by integrin $\alpha_v\beta_3$ and $\alpha_5\beta_1$, which eventually stimulates angiogenesis. α5β1 mediates EC migration through calmodulin kinase-II (CaMKII) signaling, whereas inhibition of αvβ3 leads to the blockage of this signaling leading to attenuation of EC migration, indicating that both of these integrins are closely involved in this functional response [95].

Ion channels were also reported in the regulation of angiogenesis in response to shear stress and wall stretch. For instance, physiological shear activates various ion channels, including Piezo 1 and TRPV4, which causes cellular depolarization and elevated cytoplasmic Ca^{2+} levels [29] (Fig. 2.2). These elevated Ca^{2+} levels in turn activate K^+ channels leading to K^+ influx and membrane hyperpolarization. The elevated Ca^{2+} levels contribute to eNOS activation and NO production, the role of which in vascular growth had been mentioned before [33]. Similarly, GPCRs such as SP1R1 also activate this eNOS and NO signaling route in response to shear stress and thereby participate in the formation of new blood vessels [96]. As described earlier, physiological stretch also induces GPCR activation which finally leads to

transcription of stretch-sensitive genes, such as IL-8, VCAM-1, and Hey-8 through zyxin translocation into the nucleus [70]. Transcription of these genes may contribute to endothelial migration, survival, and angiogenesis (Li 2003). Moreover, G-proteins have been reported to contribute to stretch-mediated angiogenesis. In response to physiological stretch at 1 Hz frequency for 24 hours on ECs, the G_i-α subunit, which belongs to the heterotrimeric G-alpha subunit family, is activated. This G_i-α subunit is bound to GDP during the inactivated state, whereas in response to stretch, G_i-α releases GDP and binds to GTP, inducing downstream signaling. Despite of the unknown signaling pathway, the enhanced GTPase activity in response to physiological stretch is intimately involved in the regulation of the angiogenic signal to maintain an adequate vascular capacity, as the G_i-protein inhibitor pertussis toxin-induced attenuation of endothelial migration and tube formation in response to wall stretch [35].

Shear stress is also involved in the last phases of angiogenesis; vessel maturation upon onset of flow in the newly formed capillary. The development of a vascular network or vascular plexus is mostly not pre-patterned, leading to highly complex branched vascular networks that require the elimination of poorly flowed branches (pruning) and maintenance of well-flowed blood vessels (maturation) [97]. It has for instance been shown that shear stress-induced downregulation of the pro-angiogenic C-X-C motif receptor 4a results in maturation and stabilization of newly formed vascular connections in the zebrafish trunk- and brain vasculature [98]. Moreover, flow-induced modulation of S1PR1 expression stabilizes the primary vascular network by enhanced cell-to-cell adhesion and by inhibiting angiogenic sprout formation [99]. Another example of shear stress-induced stabilization involves a particular member of the tissue inhibitors of matrix metalloproteinases (TIMPs) family. TIMPs have been shown to coregulate capillary tube stabilization by inhibition of MMPs responsible for ECM breakdown. In particular, TIMP3 has been shown to play a crucial role in vessel stabilization and maturation upon flow [100]. TIMP3 is expressed by pericytes and both regulated by cell-cell contact and shear stress. Increased expression of TIMP3 results in downregulation of A disintegrin and metalloproteinase with thrombospondin motif 1 (ADAMTS-1) [101]. The microvascular stabilizing capacities of pericytes exposed to shear stress are also mediated by miR-27. Unidirectional shear stress in the vasculature of a murine uterus caused the enhanced expression of miR-27, resulting in increased pericyte coverage on the vessel, whereas inhibition of miR-27 resulted in reduced pericyte coverage and therefore reduced vessel stability [102].

2.5 Conclusion

Vascular growth occurs through various processes such as vasculogenesis, angiogenesis, and arteriogenesis, which are essential for vessel homeostasis and vascular integrity. Receptors, chemokines, cytokines, and growth factors that regulate these processes have attracted much attention in the past decades due to their roles in vessel growth. However, the underlying complex mechanisms of vessel growth and

the lack of effectiveness of pro-angiogenic or pro-arteriogenic factors, for instance in regenerative medicine, are still not completely unraveled. Recently, much attention has been drawn to studying the role of biomechanical factors in stimulating vessel growth. It has become more evident that these factors stimulate vessel growth by activating mechanosensors, which transduce mechanical stimuli into a vascular growth regulatory response. Shear stress levels are maintained in the vasculature by diameter adaptations via vasoregulation that adjusts the vascular tone. Similarly, wall stretch induces a physiological response and predominantly provides a stimuli to maintain a contractile VSMC phenotype. However, increased magnitude of these forces leads to pathological deviations in vascular wall adaptation and vascular remodeling. Vascular regenerative processes such as arteriogenesis and angiogenesis were initiated in response to deviations in biomechanical stimuli and serve as parallel compensatory mechanism to limit disease progression. As mentioned, there are still considerable gaps in understanding the mechanisms that transduce signals from vascular cell-mechanosensors to regulation of the factors involved in vascular growth. For instance, PDGF a known proliferative marker for VSMCs is upregulated via integrin activation in response to stretch. The signaling mechanism is not clearly understood but may be important for providing targets to combat undesired inflammatory responses or to promote desired arteriogenesis in occlusive artery diseases. Despite many of these mechanosensors have been reported to contribute to vascular diseases, stimulation of vascular growth in response to pathological levels of these forces may serve as an important functional aspect and there is a great need for further research to identify therapeutic targets that could stimulate vascular regeneration and restoration of blood flow thereby limiting disease progression.

Conflict of Interest Authors declare that they have no conflict of interest.

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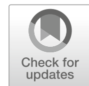

Biomechanics in Small Artery Remodeling

3

Erik N. T. P. Bakker and Ed van Bavel

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Abstract

In this chapter, we discuss how biomechanical forces influence vascular design. We will focus on the small arteries and arterioles, i.e., those vessels that together cause the majority of resistance for perfusion. We will do so because pathological alteration in the caliber of these resistance vessels, "remodeling," is related to both hypertension and impaired local perfusion reserve and tissue ischemia. We will discuss the definitions of remodeling, its role as part of normal homeostasis, and its involvement in a range of pathologies. Subsequently, we address the

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evidence for the involvement of wall shear stress and wall stress under normal and pathological conditions, the need for an integrative, "systems level" understanding, and the translational perspectives of interfering with the regulation of resistance vessel structure.

3.1 Introduction

The human arterial system contains ~10^8 segments covering a ~3000-fold span in diameter. All these segments together achieve the task of bringing the right amount of blood to every corner of our body while limiting the pressure head to some 100 mmHg. This impressive accomplishment has inspired researchers over centuries to study the design of the arterial bed. Pries and Secomb [1] provide an overview of this work, which started by formulating "design principles" by the nineteenth and early twentieth-century pioneers such as Thomas Young and Cecil D. Murray [2]. In more recent times such work included mechanistic studies on the control of vascular structure and function. So how does an individual segment in this huge arterial network come into existence and how does it "know" which diameter to obtain? How tightly is this diameter regulated and what goes wrong in hypertension and other cardiovascular pathologies? We have no full answer. Yet, it has become quite clear that local forces are essential elements in the control of the vascular structure. Thus, wall shear stress (WSS) caused by the local blood flow and wall stress related to local blood pressure is considered to be the major mechanical determinants of the vascular structure.

In this chapter, we discuss how such local forces influence vascular design. We will focus on the small arteries and arterioles, i.e., those vessels that together cause the majority of resistance for perfusion. We will do so because pathological alteration in the caliber of these resistance vessels, "remodeling," is related to hypertension, impaired local perfusion, and tissue ischemia. This discussion requires a clear definition of remodeling, which is less trivial than one might think at first glance. While remodeling may suggest a pathological process, the continuous adaptation of vascular structure is part of normal vascular homeostasis. Understanding this homeostasis requires a detailed comprehension of the involved mechanisms, including sensing of WSS and wall stress. In addition, the physical relations describing forces and flows in the arterial network (e.g., Poiseuille, Laplace) lead to complex network behavior, where local responses have global effects. Therefore, there is a need for a "systems level" analysis of arterial networks in order to really understand their behavior under normal and pathological conditions.

The described work addresses mostly the studies of relevance for such a systems level approach, guided by our ideas on this over the years. We zoom in on particular molecular mechanisms without the intention to provide a comprehensive overview of all described mechanosensors and downstream signaling pathways.

There are clear effects of flow patterns and pressure on the large vessels too, including those on the development of atherosclerosis, plaque stability, and media remodeling. We briefly cover these effects if relevant for understanding the small arteries. Many excellent reviews on these topics are available elsewhere [3, 4].

3.2 Small Artery Remodeling: An Ongoing and Ubiquitous Process

3.2.1 A Biomechanical Definition of Remodeling

In 1994, Gibbons and Dzau defined vascular remodeling as an "active process of structural alteration" that involves the vascular cells (growth, death, migration) as well as the matrix (production and degradation) [5]. Mulvany et al. [6] specifically addressed small artery remodeling, discriminating between changes in wall cross-sectional area (hypertrophic, eutrophic, and hypotrophic remodeling) and changes in inner diameter (inward and outward remodeling) as two consequences of the remodeling response. Mulvany later elaborated on this definition, taking into account the concerns and remarks from others in this field [7]. This included the notion that a eutrophic axis can be identified, of high relevance for the small artery remodeling seen in hypertensive disorders [8]. Figure 3.1 depicts the scheme that is frequently used to characterize the possible remodeling responses. In our vision, remodeling should be seen as an integrated process rather than a combination of independent growth and rearrangement responses. One of the other issues that were raised in [7] is that remodeling suggests a change of a given vessel while the majority of studies are cross-sectional in nature, i.e., comparing different patient groups or, e.g., hypertensive versus normotensive animals. While arguably "differential development" may better explain the process, the term remodeling is generally also used for such inter-individual comparison.

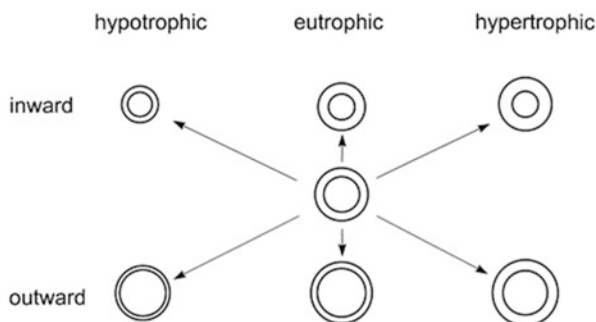

Fig. 3.1 Schematic representation how remodeling can modify the cross-sections of blood vessels. Changes in lumen diameter result in either inward or outward remodeling. Hypotrophic, eutrophic, and hypertrophic remodeling refers to a reduction, unchanged, or increase in wall cross-sectional area respectively. Reprinted with permission from Mulvany [7]

Changes in the content and organization of the wall material will affect the biomechanical properties. This is fundamental: the amount but more so the arrangement of cells and matrix determines the unstressed diameter, the distensibility of the vessel (as measured during full vasorelaxation), and the diameter-dependent ability for the generation of active SMC stress. Conversely, any change in these biomechanical properties reflects remodeling. Indeed, stress-strain relations and pressure-diameter relations, in addition to anatomical measurements, are common instruments for assessing remodeling. The biomechanical properties are crucial for vascular function: these determine the maximal diameter and the ability to constrict and maintain vascular tone under pressure. Considering the intricate relation between wall organization and biomechanical properties, changes in the latter ought to be included in any definition of remodeling. Taken the above together, one could define small artery remodeling as any active change or difference in vascular wall composition or organization, adaptive or not, that affects the geometry and/or biomechanical properties. A gray zone is the rapid reorganization of the SMC cytoskeleton following prolonged activation [9], which could be seen as an intermediate process between vascular tone and remodeling.

3.2.2 Remodeling as Part of Normal Homeostasis

It appears that remodeling is frequently seen as a switch from a static control condition without any relevant biology to an activated state that involves a range of processes involving the cells and matrix. One might thus argue that small arteries are remodeling in hypertension and not in normotension, and look for the involved expression patterns and cellular pathways by a comparison. While such comparison has had its success [10], we believe that the concept of a quiescent vessel in the absence of a stimulus such as elevated blood pressure is illusive. In our view, blood vessels are in a continuous dynamic equilibrium of turnover and notably rearrangement processes in response to their mechanical and chemical environment, and such remodeling is part of normal homeostasis. Sure enough, turnover of elastin is believed to be extremely slow [11], but cytoskeletal reorganization is quick and continuous [9]. The processes in this dynamic equilibrium may be accelerated if conditions change, but this would mostly relate to very sudden changes such as the experienced WSS of collateral vessels in myocardial infarction or ischemic stroke [12]. In the development of hypertension, the environment of the vessels changes very slowly and the continuous process of remodeling allows adaptation to the increasing pressure without a clear switch from "resting" to "activated."

A case for this view comes from our work on the relation between vascular tone and remodeling. We demonstrated in a range of in vivo and in vitro conditions that maintained deep vasoconstriction is a drive for inward remodeling of resistance vessels, while maintained vasodilation inhibits such remodeling or converts it to outward remodeling, depending on the organ, species, and experimental conditions [13–18]. Based on these data, we postulated a continuous turnover and rearrangement of matrix elements, such that they translate the constricted or dilated state into

the anatomy and biomechanics of the vessel wall [19]. We demonstrated the involvement of transglutaminases in this process [20–22]. The actions of these enzymes include the cross-linking of extracellular matrix proteins [23] and their activity may form part of the dynamic equilibrium of blood vessel anatomy and biomechanics. Paradoxically, transglutaminase expression and activity are decreased in the aorta of hypertensive rats [24], making this a case where a better understanding of remodeling cannot possibly be based on molecular data only. Rather, this requires a systems approach that includes biomechanics. Below, after discussing the roles of WSS and wall stress, we will discuss such a systems approach.

3.2.3 Assessment of Small Artery Remodeling

As mentioned above, remodeling is usually determined in a cross-sectional manner, describing a difference in vascular properties between vessels from different groups. In some of these cases, one could argue whether such differences reflect remodeling or should be considered as a difference in vascular development. A fundamental concern in such cross-sectional studies is the need to compare the diameter and wall thickness of small arteries from "identical" anatomical locations and branching order. Due to the stochastic nature of the small artery networks, the diameter of a particular vessel may not be very informative here. Moreover, in some cases vessels are dissected randomly from samples of tissue, such as human gluteal biopsies, introducing possible selection bias. In such cases, the wall-to-lumen ratio (sometimes media-to-lumen ratio) has been used to determine remodeling. This then leaves the question whether an increased ratio is due to eutrophic inward remodeling or wall hypertrophy.

Ideally, one should follow-up on a single artery, studying its structure and function over time in response to a change in hemodynamic conditions or other stimuli. This is possible using an organ culture approach, where the effect of various stimuli can determined ex vivo. With this approach, we and others demonstrated that remodeling can take place in the course of hours and days, by comparing vessel properties at the start and end of the experiment [9, 25–28]. This approach is based on the pressure myograph setup, which enables the recording of lumen diameter and wall thickness at a given pressure. From these data, additional parameters can be calculated, such as the wall cross-sectional area, distensibility, and stiffness. These parameters provide more insight into the underlying mechanisms, such as growth and extracellular matrix properties. Remodeling is then defined as a change in one or more of these parameters. In any case, the measurements should be made under fully relaxed conditions, usually achieved through the addition of calcium-free medium and/or vasodilators.

3.2.4 Involvement of Remodeling in Pathology

Vascular remodeling occurs under many pathological conditions, including obesity, diabetes, peripheral ischemia, and hypertension. Herein it may play an adaptive role; e.g., at the single vessel level wall stress may be normalized by remodeling in the case of hypertension. At the tissue level, remodeling of small pre-existing collaterals can alleviate ischemia due to arterial stenosis. However, it may also play a maladaptive role, when inward remodeling limits vascular reserve [29]. Here we highlight remodeling in some of these conditions, to illustrate the diversity in remodeling responses and the involvement of different elements of the vascular network.

3.2.4.1 Hypertension

Hypertension is a highly prevalent condition, nowadays defined as a systolic pressure >130 mmHg or a diastolic pressure >80 mmHg [30]. Hypertension is associated with vascular remodeling along the entire vascular bed, from the aorta down to the capillaries. The remodeling responses depend on the organ and the experimental model studied [31]. Human abdominal resistance arteries have been studied using the pressure myograph approach [32, 33]. This revealed that the wall-to-lumen ratio is clearly increased [8]. This increased ratio proved to be an independent predictor for cardiovascular events in later studies. Thus, Rizzoni et al. [33] showed that hypertensive subjects with a high wall-to-lumen ratio of gluteal arteries had more fatal and non-fatal cardiovascular events after an average follow-up period of more than 5 years. Pre-clinical work showed that resistance arteries from spontaneously hypertensive rats also display inward remodeling, associated with stiffening of wall components [34]. This finding was however not consistently reported in other studies. Thus, we found that remodeling in spontaneously hypertensive rats differed among sublines, indicating that genetic differences exist that determine the type of remodeling associated with hypertension [35]. Within tissues, remodeling may also depend on the branching order, as stiffening of large cerebral arteries and reduced stiffness in more distal branches has been reported in stroke-prone spontaneously hypertensive rats [36]. Further downstream in the vascular tree, a recurrent finding is a reduction in the number of capillaries, referred to as capillary rarefaction, as reviewed previously by Struijker Boudier [37]. This notion was recently reinforced by observations in the retina of hypertensive subjects [38] as well as in skeletal muscle of elderly hypertensive subjects [39]. However, capillary rarefaction is not a universal finding in hypertension. In our hands, the number of capillaries in the brain of spontaneously hypertensive rats was similar compared to normotensive Wistar Kyoto rats [40].

In hypertension, the notion that subjects with strong vascular remodeling are predisposed to cardiovascular events [33] suggests that remodeling should be prevented or corrected. In this respect, it is very interesting that both experimental studies and comparison of vessels from patients treated with different classes of antihypertensive drugs showed that vasodilatory drugs are able to reverse remodeling, whereas other classes of medication do not [41–43]. However, whether vasodilators also provide better protection against end organ damage in hypertension

is not entirely clear. Systematic reviews on this topic that focus on the brain indicate that studies are not unanimous, but overall favor the notion that antihypertensive medication has beneficial effects on cognitive decline and prevention of dementia [44, 45], with calcium channel blockers and inhibitors of the renin–angiotensin system turning out as most effective.

3.2.4.2 Atherosclerosis

Atherosclerosis is frequently associated with remodeling of the affected vessel wall, a process referred to as the Glagov phenomenon [46]. Herein the vessel wall remodels outward such that encroachment of the vessel lumen by lesion formation is counteracted. Less is known regarding the vascular bed downstream of an occluding stenosis. Experimental work in pigs demonstrated that small arteries downstream of an occlusion show inward remodeling, with reduced distensibility and altered reactivity [47]. Clinical data also suggest increased microvascular resistance downstream of a flow-limiting stenosis [48]. Here, one might argue that the microvasculature distal to a stenosis would remain relatively vasodilated, leading to compensatory outward remodeling. We can only speculate on the discordance with the tone—remodeling hypothesis. Possibly, periods of high WSS (e.g., during exercise) are needed to suppress inward remodeling. In the presence of a significant proximal stenosis, peak WSS during episodes of coronary vasodilation and hyperemia is strongly reduced. In any case, coronary angioplasty may not always be sufficient to fully restore blood flow to an ischemic area, and additional measures to restore perfusion are needed. At present, such treatment options are limited.

3.2.4.3 Obesity, Insulin Resistance and Diabetes Type 2

Arterial remodeling in the context of obesity, insulin resistance, and type 2 diabetes is characterized by large artery stiffening [49]. In isolated resistance arteries from obese patients, an increase in media-lumen ratio was found, which was attributed to hypertrophy [50]. A similar finding was reported by the same group in type 2 diabetic patients [51]. In a mouse model of obesity, large artery stiffening is recapitulated in femoral arteries [52]. In this study such stiffening was not observed in coronary arteries, indicating heterogeneity among vascular beds. In another animal model, the obese Zucker rat, penile arteries show inward remodeling, while coronary arteries again were not affected [53]. In the same model hypotrophic inward remodeling was found in skeletal muscle arteries, associated with low blood flow. On the other hand, the cerebral vascular bed was spared [54].

Taken together, vascular remodeling is a key feature in several pathological conditions. Large heterogeneity is observed in the type and degree of remodeling, depending on the organ, level within the vascular tree, and genetic background. Remodeling may have adverse consequences, as inward remodeling increases peripheral resistance in hypertension [8], which limits maximal blood flow, and potentially further increases blood pressure. The beneficial effects of remodeling include outward remodeling of collaterals, which alleviates tissue ischemia. Conditions such as diabetes [55] and hypertension [56] may however impair the capacity for beneficial remodeling, as shown in animal studies on arteriogenesis and

flow-induced remodeling. We speculate that endothelial dysfunction, either at the level of blood flow sensing or signaling, might be a crucial step herein.

3.3 Current Evidence for Involvement of Wall Shear Stress Sensing

3.3.1 Role of WSS in Normal Homeostasis

3.3.1.1 Regulation of WSS

WSS (WSS) associated with the flowing blood is considered to be a major factor driving vascular caliber. As worked out in this book by Tim Secomb, in Poiseuille flow WSS is proportional to flow divided by the third power of the diameter. It follows that regulation of WSS to a specific value throughout the network would lead to a third power relation between local flow and diameter ($Q = kd^3$, with Q the flow, d the diameter, and k a constant including viscosity), and between diameters in vascular nodes (i.e., ${d_0}^3 = {d_1}^3 + {d_2}^3$, with d_0 diameter of the mother segment and d_1 and d_2 the diameters of the daughter segments). Such relations, referred to as Murray's law, conveniently provide a mechanism for the principles of cost optimization in vascular transport systems based on such third power relations [2]. Yet, life is more complicated, with square rather than cubic relations for large vessel branching [57], clear differences between arterial and venous WSSes [58], non-Newtonian fluid [59], and effects of oscillatory versus steady WSS [60]. Despite these complications, it seems commonly accepted that WSS is the primary drive for the development of blood vessels to a certain caliber, and for changes in caliber in cases where the flow is altered.

While Murray's law and related observations on the relation between flow and caliber make a strong case for WSS as a fundamental driving force for remodeling, it is far from trivial how one should test this and unravel the possible underlying mechanisms. As useful as endothelial cell cultures in flow chambers with molecular read-outs are for identifying shear sensing mechanisms, such studies at best provide clues for further in vivo testing. Yet, in vivo, one cannot change local WSS without influencing pressures, flows, wall stress, and oxygenation throughout the network and tissue. This renders the differentiation between direct WSS effects and indirect effects extremely difficult. In an attempt to overcome these complications, we kept isolated small porcine coronary arteries cannulated in organ culture [61]. In these experiments, a fixed pressure gradient was applied that was dissipated over the resistances of both pipettes and the cannulated vessel. Flow and WSS in this setting are determined by the inner diameter of the vessel. We found that in the course of several days the vessels controlled their WSS to $\approx$10 dyn/cm^2, by a combination of vascular tone and remodeling. Inhibiting nitric oxide synthesis left such control intact albeit at a higher set point of $\approx$14 dyn/cm^2 (Fig. 3.2). While the involvement of nitric oxide may not have been too surprising, a similar approach could have been used for other putative shear sensors. Unfortunately, the experiments were technically demanding and as far as we know, this approach has not been followed up. Yet,

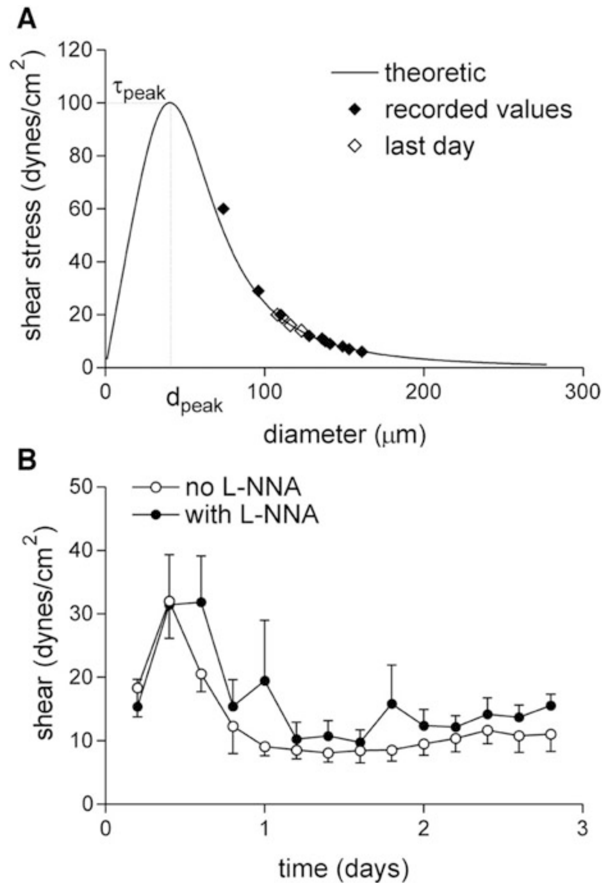

Fig. 3.2 WSS regulation by isolated porcine coronary arterioles in organ culture. Vessels were kept in the presence or absence of the nitric oxide inhibitor L-NNA. (**a**) WSS and diameter values recorded in a typical experiment. Recorded values are plotted with theoretical values of WSS as a function of diameter. At this constant pressure gradient and cannula resistance, the vessel can achieve any value of WSS between 0 and maximum WSS by active constriction and dilation. WSS at the end of the experiment (*day 3*) varied slightly at ~15.5 dyn/cm^2. (**b**) WSS values were highly irregular on *day 1* and then settled between 10 and 20 dyn/cm^2. Nitric oxide inhibition did not induce a significant difference in the level of WSS attained. Reprinted with permission from Pistea et al. [61]

such work suggests that also in vivo, higher WSS is associated with an elevated set point for shear stress regulation, which may be due to impaired sensing (such as damage to the glycocalyx) or interference of modulating factors (such as oxygen-free radicals quenching nitric oxide).

3.3.1.2 Mechanisms of WSS Sensing

As covered by several chapters in this book, WSS has a range of effects on vascular biology, function, and structure. Many WSS sensors have been suggested [62]. Several of these are associated with the endothelial glycocalyx, a vulnerable layer of proteoglycans that covers the lumen of blood vessels [63, 64]. Here we limit the discussion to recent evidence for WSS sensors in the context of remodeling.

First, Piezo proteins have been implicated in WSS sensing. These proteins are subunits of calcium-permeable nonselective cation channels [65]. Li et al. demonstrated Piezo1 involvement in WSS-dependent endothelial cell calcium signaling, alignment of cultured cells underflow, alignment of endothelial cells in vivo,

and vascular development [66]. Endothelium-specific *Piezo1*-modified mice are embryonically lethal, with lack of development of larger vessels from the capillary plexus around embryonic day 10. Wang et al. revealed their role in shear-dependent regulation of NO formation, vascular tone, and blood pressure [67]. Their role in tone control is complicated by their inhibition of endothelium-derived hyperpolarizing factor, resulting in constriction. Regional differences seem to exist, possibly related to myo-endothelial gap junctions [68] and with a possible role in flow redistribution in exercise [69]. Piezo1 is also involved in flow pattern-specific endothelial inflammation [70]. In addition to these shear-mediated effects, Piezo1 expression in smooth muscle cells is involved in small artery remodeling [71], as will be discussed below.

Second, in a recent study in *Cell*, Xu et al identified a G protein-coupled receptor, the proton-sensitive GPR68, as an important sensor for flow-mediated dilation and remodeling in mesenteric arterioles [72]. Their work is based among others on extensive screening of cultured cells under WSS, followed by in vivo validation. Here, high flow in third order mesenteric arterioles, which perfuse the intestinal wall, causes flow-mediated dilation and induces outward remodeling within 14 days in GPR68$^{+/+}$ but not GPR68$^{-/-}$ mice. Yet, the passive diameter under normal flow was not different between both genotypes and the knock-out animals were normotensive in this study, which in our view suggests that GPR68 may not be the primary sensor for the regulation of caliber during development.

3.3.2 Role of WSS in Pathological Remodeling

3.3.2.1 Intracranial Aneurysms

As far as we know, some form of WSS sensing occurs in all arteries, and the role of WSS in vascular pathology has been studied for decades. In almost all studies, WSS is not directly measured but rather estimated from the recorded geometry and velocity field or pressure gradients. A large artery example demonstrates the challenges of studying WSS involvement in pathological remodeling: intracranial aneurysms are saccular cerebral artery evaginations of mostly a few mm in diameter. Development and rupture of these aneurysms have been associated with WSS for decades. Yet it remains unclear to what extent high or low WSS is a trigger for outgrowth, or whether spatial gradients [73] or temporal fluctuations are involved [74]. There are many reasons for this slow progress. This includes the difficulty of estimating WSS, leading to a "computational fluid dynamics challenge" for the prediction of WSS [75]. Moreover, since WSS is not directly measured, it is difficult to dissociate between a direct WSS effect and involvement of other flow-dependent processes (breakdown of stagnant boundary layers, gradients of endothelium-derives factors, local availability and residence time of leukocytes, and so on). Similar concerns hold for evaluation of the role of WSS in small artery pathological remodeling, where the advantage of microscopic resolution is offset by the complex rheology and presence of an endothelial surface layer.

3.3.2.2 Altered Shear Sensitivity in Small Arteries

The mesenteric vascular bed of rats and mice is a frequently used model for studying shear-induced remodeling. In this model, ligation of one or more arteries increases flow in the adjacent vessels to restore blood flow to the intestine via small collaterals. In this model, impaired outward remodeling of the vessel exposed to increased WSS has been reported with normal aging [76], hypertension [77], and diabetes [78]. The impaired outward remodeling that is observed in these conditions, is associated with altered acute responses to WSS in isolated small arteries. Thus, in small arteries from various organs and species, including mesenteric arteries, WSS typically induces the release of vasodilatory factors such as nitric oxide and prostaglandins. In aging, diabetes, and exposure to high pressure, impaired vasodilation is frequently observed, due to an excessive amount of reactive oxygen species. Oxidative stress may quench nitric oxide and thereby interfere with downstream signaling pathways [78–81]. It is therefore tempting to speculate that impaired remodeling as observed in various pathological conditions is due to an altered balance in factors that modulate tone, or at least shares common pathways.

3.3.2.3 Arteriogenesis

An innate mechanism to restore blood flow in the case of arterial stenosis is arteriogenesis. This process refers to the outgrowth of small pre-existing collateral arteries that bypass an occlusion [82]. These vessels experience a substantial increase in WSS upon blockade of a main feeding artery. Particularly flow reversal is critical for amplified arteriogenesis [83]. In animal models, such as the femoral artery ligation model, arteriogenesis leads to a dramatic enlargement of small arterioles in the leg muscles. This process is highly dependent on a local inflammatory response, creating an environment of tissue degradation, growth, and reorganization. In laboratory animals, arteriogenesis takes days to weeks to develop. As such, arteriogenesis may be particularly relevant in conditions where blood flow to vital organs gradually decreases due to large artery lesion formation. The end result of arteriogenesis is the formation of a number of tortuous collaterals that partially restore blood flow to the affected organ [84]. Unfortunately, studies in humans aimed at stimulation of arteriogenesis have reported little or no success [85]. Further research on this topic is therefore needed to develop novel therapeutic approaches. For instance, an approach based on microRNAs appears to be very promising, as a single microRNA may affect several targets involved in remodeling [86].

3.4 Current Evidence for Involvement of Pressure and Wall Stress

3.4.1 Role of Wall Stress in Normal Homeostasis

3.4.1.1 Acute Regulation of Wall Stress

Wall stress is the force per unit of surface area that is elicited by the transmural pressure difference. The circumferential component equals (in the thin-walled approximation) $P \cdot r/h$, where P = pressure, r = lumen radius, and h = wall thickness. This relation holds in equilibrium, where the distending effect of pressure is in balance with the elastic stresses in the matrix and active smooth muscle cell stress. It is considered to be one of the fundamental parameters that determine vascular design [87]. In principle, regulation of wall stress elicited by a change in pressure can be achieved by a change in lumen diameter, an adaptation in wall thickness, or a combination of these. The role of wall stress in remodeling cannot be discussed without a consideration of smooth muscle cell contraction (vascular tone), and notably the myogenic response to changes in pressure. The myogenic response is a mechanism intrinsic to the smooth muscle layer within the vessel wall. It involves increased contractile activation and vasoconstriction following a rise in pressure, and the reverse response to a fall in pressure. The myogenic response, in concert with modulation of vascular tone by WSS and metabolic factors, forms the cornerstone of autoregulatory mechanisms that regulate tissue perfusion and capillary pressure. The signal transduction pathways of the myogenic response have been reviewed previously [88–90] but remain an area of active research.

The contractile response to a rise in pressure that is induced by the myogenic response lowers wall stress through a reduction in diameter and increase in wall thickness. To function as a regulatory feedback loop, some wall stress-sensitive mechanism is required that affects the contractile response. Otherwise, the vessel would tend to constrict until closure when stimulated. The presence of such an intrinsic property was tested experimentally by preventing vessels to reduce their diameter upon contractile activation [91]. These isometric versus pressure-controlled (isobaric) experiments on small arteries showed fundamental differences in concentration-response relations to various agonists, consistent with the presence of a stress sensor regulating that affects contractile activation and regulates wall stress under normal, pressure-controlled conditions.

In the presence of vascular tone, the smooth muscle cells are considered the main load-bearing elements in the vessel wall. However, under conditions of full dilation, the extracellular matrix elements are assumed to bear the load. Herein elastin dominates at lower pressure levels, while much stiffer collagens take up most of the load upon recruitment at higher pressure levels. This concept is based on the assumption that smooth muscle cells and extracellular matrix elements are in parallel arrangement [92]. An intriguing question is whether this is indeed the case. Partial digestion of collagens leads to a shift in the length-tension relationship of the contractile machinery suggesting that the organization of cells and matrix is more complicated [93].

3.4.1.2 Structural Regulation of Wall Stress

While in the short term wall stress is regulated by vasoconstriction and dilation, in the course of days and weeks remodeling and growth processes contribute [92]. Remodeling responses tend to follow the habitual level of tone, with inward remodeling following prolonged periods of constriction, and outward remodeling upon long-term vasodilation. As noted earlier, the correlation between tone and remodeling is a concept that we strengthened in several studies using an organ culture approach. Herein we showed that chronic vasoconstriction, irrespective of the nature of the stimulus, was sufficient to induce inward remodeling (for review see [94]). When vessels were kept dilated, either outward remodeling was observed, or vessel diameter was maintained. Exposure to a vasoactive substance alone was not sufficient, as in the absence of tone, achieved by maintaining vessels at low pressure, remodeling responses were lacking. Typically, remodeling in these experiments was characterized by a change in distensibility, with diameter changes at higher pressures, but not at low pressure levels in the passive pressure-diameter relationship.

Searching for the mechanisms that couple vascular tone to eutrophic inward remodeling we identified transglutaminases as crucial mediators (for review see [95]). Among the pleiotropic actions of these enzymes is their ability to cross-link extracellular matrix proteins. Exposure to exogenous transglutaminases, or activation of endogenous transglutaminases, induces a reduction in distensibility similar as observed after chronic vasoconstriction. In addition, constriction-induced remodeling can be inhibited by transglutaminase blockade, supporting a causal relationship between inward remodeling and transglutaminase activity. An intriguing question herein is how transglutaminases are activated by vasoconstriction. We speculated that conformational changes in response to mechanical stimulation could activate type 2 transglutaminase [96]. Others more recently showed that the nonselective cation channel Piezo-1 is able to stimulate transglutaminase activity via an increase in intracellular calcium upon stretch [71].

There is a clear relationship between the mechanical load that a vessel experiences in vivo and its extracellular matrix properties. The forces acting on the vessel wall are not restricted to the effects of pressure and flow. Thus, also other mechanical forces may play a role. These include forces that result from cardiac- and skeletal muscle contraction. Thus, there is a marked difference in extracellular matrix architecture between human resistance arteries obtained from pericard as compared to frequently used rat mesenteric arteries [97]. This is evident also from a study where elastin degradation caused marked lengthening of skeletal muscle arteries, but not cerebral arteries [98]. The latter may be explained by the large differences in length a blood vessel is exposed to during skeletal muscle contraction, whereas the cerebral vessels lie protected within the skull.

Based on their orientation, smooth muscle cells are an obvious candidate to translate wall stress into a biological response, including cell growth and extracellular matrix turnover. Examples of these are the induction of fibronectin synthesis [99] and activation of MMP-9 by pressure [100]. The role of other cell types in the response to wall stress is beginning to emerge. Thus, the adventitia of large arteries

contains fibroblasts, nerves, progenitor cells, and immune cells that contribute to remodeling after injury [101]. Particularly in the pulmonary circulation, evidence has been collected that substantiates a major role for adventitial cells in remodeling [102]. Whether the adventitia and its resident cells play a similar active role in resistance artery remodeling in other organs remains established.

3.5 Need for an Integrative Understanding of Remodeling

Above we have discussed the complex interaction between mechanical stimuli (wall stress and WSS) and the responses of the vascular wall, ranging from acute changes in tone to long-lasting structural alterations. It leaves no doubt that these interactions form a tightly regulated control loop that ensures proper control of vascular tone and structure. Consequently, a systems approach will be needed to truly understand such control. As a first step, we considered a single resistance vessel subjected to given levels of pressure and flow, as well as vasoconstrictors and dilators [103]. Figure 3.3

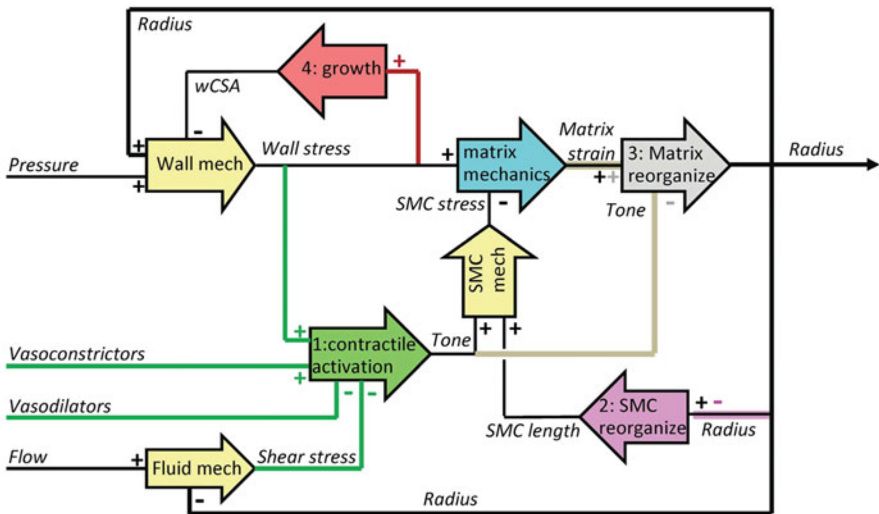

Fig. 3.3 "Schematic representation of the model, with color-coded biomechanics and vascular adaptation. The arrow blocks reflect the processes, with input and output signals. For each input, the plus or minus sign indicates a positive or negative effect on the output. Note that a plus sign also indicates a reduction in output upon a reduction in input. Double signs indicate two separate effects of the same input. Yellow blocks are static mechanical relations, other blocks are causal with first-order dynamics; outputs of the five causal blocks define the state of this system. Mechanical loading is represented by the static relations (yellow blocks) and by strain following stress with first-order kinetics (blue). The four adaptation processes are Adaptation 1: smooth muscle cell contractile activity, tone (green lines and block). Adaptation 2: SMC reorganization (plasticity, pink). Adaptation 3: eutrophic matrix remodeling (gray). Adaptation 4: wall cross-sectional area growth (red)." (text reproduced from VanBavel and Guvenc Tuna [103]). Figure reproduced with permission from VanBavel and Guvenc Tuna [103]

shows this model, which includes the key relations discussed above. These are mechanical in nature (vascular wall mechanics based on the Laplace relation, fluid mechanics linking WSS to flow and radius, the concept of matrix and cells carrying wall stress), with additional biological control (regulation of tone, organization of smooth muscle cells and matrix, and hypertrophy). There is little or no doubt about the existence of these relations but only by considering them together it becomes clear how they together establish long-term vasoregulation. In particular, in this model, long-term tone (defined as relative smooth muscle cell active stress), smooth muscle cell length, wall stress, and vascular reserve are fully independent of pressure, flow, and vasoactive agents. In that sense, tone acts as an "error" signal between changes in stimuli and final anatomical adaptation. Endothelial dysfunction in this model had only limited effect on the vascular caliber, while plasticity of the smooth muscle cell length-active stress relation was one of the critical processes. This uncovers the ill-studied process of cytoskeletal adaptation as a key mechanism in vascular dysregulation.

The above model only considers a single segment subjected to well-controlled pressure and flow, i.e., an isolated, cannulated segment. In vivo, the next levels of complexity are formed by the vascular network and balance of oxygen supply and demand. Seminal work here has been done over the years by Secomb and Pries [104]. A full discussion of the developments is beyond the scope of this chapter, but such studies clearly demonstrate how integrative approaches can help understanding adaptation of arteries and arterial networks. This involves a continuous interplay between experiments and modeling, leading to insight into needed data (e.g., arterial network data in the heart and brain, mechanisms that control the smooth muscle cell (SMC) active stress curve) and needed modeling (e.g., sensitivity analyses of pathophysiological changes on local perfusion, central blood pressure), an exciting challenge indeed!

3.6 Conclusion

Small artery remodeling is a continuous process that forms an essential part of vascular regulation, in close interplay with the regulation of tone. Pressure and flow, and the associated wall stress and WSS, form critical stimuli. Remodeling is affected in a range of pathologies, as cause or consequence. The importance of physical stimuli for both the local vessel wall and the functioning of the whole cardiovascular system (cardiac output, oxygenation, blood pressure) is one of the main reasons why a systems approach is needed for a proper understanding of cardiovascular control.

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New Kids on the Block: The Emerging Role of YAP/TAZ in Vascular Cell Mechanotransduction

4

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Abstract

YAP and TAZ are transcriptional coactivators controlled by the Hippo signaling pathway and by actin dynamics, and that are critical for cell growth and organ size control. YAP and TAZ are exquisitely sensitive to mechanical cues, such as substrate stiffness and shear stress, and the realization is growing that they play roles in arterial remodeling and aneurysm formation. This chapter discusses how YAP and TAZ are activated by mechanical cues, their potential arterial target genes, and plausible roles of YAP/TAZ in arterial health and disease. It is

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concluded that local and specific strategies are needed for YAP/TAZ targeting in cardiovascular disease treatment.

Abbreviations

ACTA2	actin alpha 2, smooth muscle
AMOT	angiomotin
AP-1	activator protein 1, a heterodimer of FOS and JUN
ARHGEF17	Rho guanine nucleotide exchange factor 17
ARIH1	ariadne RBR E3 ubiquitin protein ligase
ARID1A	AT-rich interaction domain 1A
AXL	AXL receptor tyrosine kinase
BAPN	β-aminopropionitrile, lysyl oxidase inhibitor
BMP	bone morphogenetic proteins are a group of growth factors and morphogenetic signals
cAMP	cyclic adenosine monophosphate, second messenger causing, e.g., vasodilatation
CArG box	DNA sequence that binds SRF $CC(A/T)_6GG$
CDC42	cell division cycle 42, monomeric GTPase of the Rho subfamily
Cpd22	cell-permeable inhibitor of integrin-linked kinase
CRIM1	cysteine-rich transmembrane BMP regulator 1
CTGF	from connective tissue growth factor, but official gene symbol is CCN2
CYR61	from cysteine rich angiogenic inducer 61, but official gene symbol is CCN1
DEK	DEK proto-oncogene
DLL4	delta-like canonical Notch ligand 4
DNA	deoxyribonucleic acid
EC	endothelial cell
EMT	epithelial-to-mesenchymal transition
F3	coagulation factor III, tissue factor
FN1	fibronectin 1
F-actin	filamentous actin (formed by polymerization of globular (G-) actin)
GLS	glutaminase
GPCR	G protein-coupled receptor
iBOP	thromboxane A_2 mimetic
IL6	interleukin 6
IL6ST	interleukin 6 signal transducer, also known as GP130
IL8	interleukin 8
ILK	integrin-linked kinase
JNK	c-Jun NH2-terminal kinase, official gene symbol is MAPK8
LATS	large tumor suppressor kinases 1 and 2, also known as WARTS

LDHA	lactate dehydrogenase
LOX	lysyl oxidase, crosslinks collagens and elastin
MAP4K	mitogen-activated protein kinase kinase kinase kinase (MAP 4 K1/2/3/5)
MBNL1	muscleblind-like splicing regulator 1
miR	microRNA
MOB	MOB kinase activator 1A, activator of LATS1/2 in the Hippo signaling pathway
mRNA	messenger ribonucleic acid
MRTF-A	myocardin-related transcription factor A, also known as MAL, BSAC, and MKL1
MRTF-B	myocardin-related transcription factor B, also known as MKL2
MST	mammalian sterile 20-like kinase or STK4 (serine/threonine kinase 4)
MYH11	myosin heavy chain 11, smooth muscle myosin heavy chain (SMMHC)
MYOCD	myocardin
MYC	MYC (from myelocytomatosis) proto-oncogene, bHLH transcription factor
MYOF	myoferlin
NCOA3	nuclear receptor coactivator 3
NEXN	gene encoding the F-actin-binding protein nexilin
NF2	neurofibromin 2
NOTCH	notch receptors (NOTCH1–4) named after a wing defect in drosophila
NT5E	5′-nucleotidase ecto
PAH	pulmonary arterial hypertension
PC	pyruvate carboxylase
PDGF	platelet-derived growth factor, A and B subunits homo- or heterodimerize
PDLIM5	PDZ and LIM domain 5
PDLIM7	PDZ and LIM domain 7
PKD2	from polycystic kidney disease, polycystin 2, cation channel
PTPN14	protein tyrosine phosphatase non-receptor type 14
RBMS3	RNA binding motif single-stranded interacting protein 3
RHOA	ras homolog family member A, monomeric GTPase that controls actin dynamics
RNA-Seq	RNA sequencing, method for analyzing the cellular transcriptome
SAV1	salvador family WW domain containing protein 1
SMC	smooth muscle cell
SRC	SRC (after a Rous sarcoma virus gene), non-receptor tyrosine kinase
SRF	serum response factor

STAT3	signal transducer and activator of transcription 3
SWI/SNF complex	chromatin remodeling complexes (SWItch/Sucrose Non-Fermentable)
TAOK	TAO kinase 1 (and 2), originally called TAO1 for its one thousand and one amino acids
TAZ	WW domain containing transcription regulator 1 or WWTR1
TEAD	TEA (named after TEF-1 and abaA) domain transcription factor
TGFB2	transforming growth factor beta 2
THBS1	thrombospondin 1, mediates cell–cell and cell–matrix interactions
TPM1	tropomyosin 1, actin-binding protein
USS	unidirectional shear stress
VEGF-A	vascular endothelial growth factor A
VEGFR2	VEGF receptor 2 or KDR (kinase insert domain receptor)
YAP	Yes1-associated transcriptional regulator also known as COB1, YAP, YAP65, and YKI

4.1 Introduction

Work on the mechanosensitive coactivators YAP (*YAP1*) and TAZ (*WWTR1*), best known for their ability to regulate the balance between proliferation and apoptosis, has virtually exploded in the last decade [1]. This is not surprising given their roles in various facets of development, homeostasis, and disease [2], and is, perhaps, a testimony to the profuse influences of mechanical cues in numerous processes critical to life [3, 4]. YAP and TAZ have also, not surprisingly, been shown to influence vascular biology [5, 6] and disease [7–12]. As a matter of fact, the vascular system is exposed to forces exerted by flowing blood and the pressure surges generated by the heart throughout life [13]. These forces (shear stress and wall tension) are acting on the vascular wall causing structural and pathological changes in the aging human population. Hypertension is therefore the leading risk factor for the burden of disease across the globe [14], vouching for a deleterious yet insidious impact of exaggerated mechanical inputs on blood vessels. Here, we summarize what has been learnt of the mechanosensitive coactivators YAP and TAZ in the normal and diseased vascular wall.

4.2 An Overview of the Hippo Signaling Pathway

YAP and its paralog TAZ are targets of a signaling cascade called the Hippo pathway. An overview of this pathway is shown in Fig. 4.1. The coactivators YAP and TAZ depend on one of four TEA domain (TEAD) transcription factors for DNA binding and transcriptional activation [15]. YAP and TAZ are depicted inside the

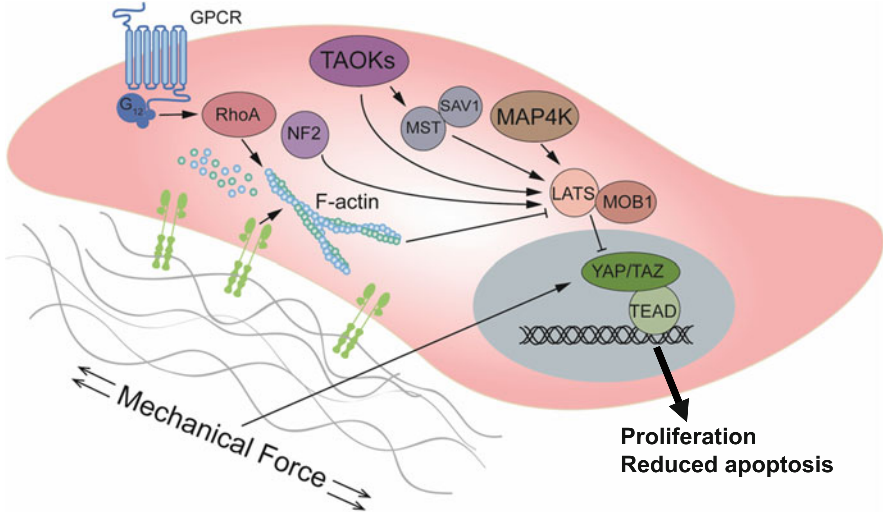

Fig. 4.1 Overview of the Hippo signaling pathway and the mechanical forces acting on the blood vessel wall. Many of the regulators of YAP and TAZ activity are cytosolic, but YAP and TAZ shuttle between the cytosol and the nucleus depending on their activation state. When active, YAP and TAZ are nuclear driving gene activation through TEAD proteins. Other interaction partners include AP-1, NOTCH, and MRTFs

nucleus in Fig. 4.1, but the nucleocytoplasmic distribution is a key determinant of activation. Upstream of YAP and TAZ are two inhibitory kinase cassettes: LATS/ MOB1 and MST/SAV [16]. The LATS kinase(s) phosphorylates YAP on five residues (one of which is S127). This causes nuclear export and reduced activity [17]. SRC kinases, a family of eight non-receptor tyrosine kinases that includes YES1, phosphorylate YAP on three residues (one is Y357), causing activation [18]. Several of the components of the Hippo pathway, which refers to the inhibitory kinases upstream of YAP/TAZ, were identified by genetic screens for negative regulators of cell growth in the fruit fly. In 2005, Pan and colleagues [19] identified YAP as a LATS-binding protein and showed that overexpression of YAP promotes cell proliferation and reduces apoptosis. Selective loss of YAP on the other hand caused severe growth defects. During in vitro culture YAP plays an important role in contact inhibition of cell proliferation; in sparsely seeded cells YAP is nuclear, whereas in dense cell cultures YAP is cytosolic. Additional inputs on this pathway have been described, including MAP4K [20], TAO kinases 1 and 2 [21], and NF2 (merlin) [22]. Another important, if not critical, input on YAP/TAZ is through G protein-coupled receptors and RhoA-dependent changes in actin dynamics [21, 23]. A part of the RhoA effect is funneled via changes in LATS activity and YAP phosphorylation [17, 21], but RhoA dependence is often maintained following LATS silencing [3, 24, 25], arguing that additional mechanisms must be involved. One focus of this chapter is on the possible role of YAP and TAZ in the vascular changes that occur in response to altered mechanical load on blood vessels.

Activation of YAP and TAZ by high substrate stiffness, cytoskeletal tension, and cell geometry was described in seminal work by Dupont et al. [25], and molecular underpinnings of these processes, considered here broadly to reflect cellular mechanoactivation, are discussed herein.

4.3 Why Study YAP and TAZ in Mechanically Challenged Arteries: Arguments from Public DATA Repositories

When inspected in the GTEx Portal, a repository for RNA expression across human organs [26, 27], it is clear that both YAP (Fig. 4.2a) and TAZ (Fig. 4.2b) are highly expressed in arteries compared to other tissues. The sum of YAP and TAZ is highest in the aorta (rank #1), followed closely by the tibial artery (rank #2) and shortly thereafter by the coronary artery (rank #5, Fig. 4.2c). Skeletal muscle, highlighted in blue for comparison, ranks much lower (rank #32). Immunohistochemical staining for YAP and TAZ in the Human Protein Atlas [28] similarly reveals positive (brown) staining of ECs and SMCs, the major cellular constituents of blood vessels (Fig. 4.2d–j). YAP staining (HPA038885) is particularly striking, with intense staining of small and medium-sized arteries in essentially all organs (Fig. 4.2d–g), and SMCs stain somewhat more intensely than ECs. This is most easily seen in areas of oblique sectioning where both SMCs and ECs are present with perpendicular orientations (boxed areas in Fig. 4.2d). TAZ staining (CAB068248) is more intense in ECs than in SMCs (Fig. 4.2h–j), but several SMC nuclei are also positive, both in arteries and in bundles of non-vascular SMCs (white stars in Fig. 4.2e and j highlight non-vascular SMCs). In all, this shows that the expression pattern of YAP and TAZ favors a function in blood vessels of various calibers, and argues that scientists interested in vascular mechanobiology need to make an inroad into a field currently dominated by cell biologists and cancer scientists. The relevance of the apparent difference in staining pattern for YAP (SMCs>ECs) and TAZ (ECs > SMCs) in human blood vessels is not known, but these coactivators are highly redundant [3]. Our work on the YAP/TAZ target gene NEXN in SMCs, for example, showed that silencing of YAP caused sizeable upregulation of TAZ [29].

4.4 The Vascular Wall and Its Mechanical Forces

Shear stress is the force of friction exerted by flowing blood on the vascular wall in the direction of flow. Blood pressure creates an additional outward force on the vessel that increases wall tension (Fig. 4.2k). In the latter case, SMCs, which wrap around arteries perpendicular to the direction of flow, are longitudinally stretched. Arteries and arterioles respond to both of these forces in acute and chronic fashions. We start here by describing the acute responses of arteries to changes in flow and pressure, and this is followed by a description of the structural impact of such forces.

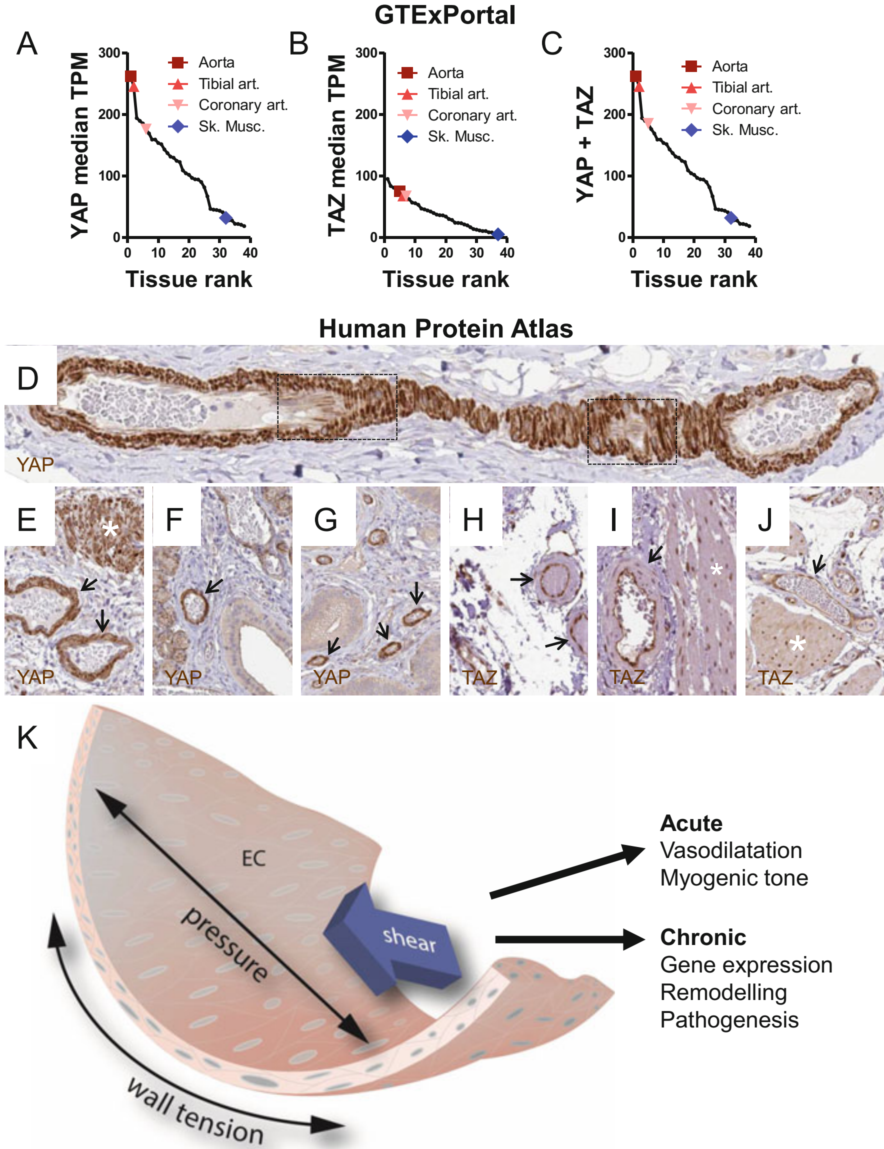

Fig. 4.2 YAP and TAZ are highly expressed in the human arterial wall as shown using RNA-Seq and immunohistochemistry. Panel **a** through **c** show mRNA data from the GTExPortal.org for YAP (**a**), TAZ (**b**), and the sum of YAP and TAZ (**c**) [26, 27]. Panels **d** through **j** show staining for YAP (**d–g**) and TAZ (**h–j**) in arteries. Arrowheads highlight arteries in **e** through **j** and white stars highlight non-vascular SMCs. Images are from the Human Protein Atlas [28]. YAP and TAZ are activated by mechanical forces acting on cells and tissue, and panel **k** depicts the forces acting on the vascular wall along with the acute and chronic consequences of such forces

Flow-mediated dilation [30] is the acute response to an increase in shear stress that is registered by the vascular endothelial cell, producing vasodilatation [31]. How this shear stress is sensed by the endothelial cell and transmitted to the smooth muscle cells remains debated. It has become clear that the glycocalyx on the endothelial cell may bend in response to shear stress and transmit a signal that activates receptors, ion channels, and cytoskeletal elements [32]. This results in endothelial cell production of nitric oxide [33] as well as other vasodilators which relax the underlying smooth muscle cells, increase blood flow, and restore shear stress. Shear stress modulates the morphology of the ECs making them elongated and aligning them in the direction of flow. Unidirectional shear stress within the physiological range typically protects against pathological changes in the vessel wall, including atherosclerosis [32, 34]. Low or disturbed flow, on the other hand, can induce endothelial cell proliferation and transforms cell morphology toward a cobblestone shape. This promotes a vascular inflammatory response and plaque development [32, 34]. Thus, areas in the vessel with disturbed flow patterns, such as branching points in the aorta, are particularly prone to atherosclerosis.

The myogenic response is defined as the intrinsic ability of vascular smooth muscle to contract in response to acute stretch or increased transmural pressure [35]. The sustained contraction at elevated pressure is referred to as myogenic tone. This phenomenon was originally described by the British physiologist William Bayliss in 1902 [36], and was later shown to be crucial for the autoregulation of blood flow and capillary pressure in a variety of tissues including the brain, kidney, skeletal muscle, and mesenteric circulation [37]. The myogenic response is primarily observed in small arteries and arterioles termed resistance arteries due to their role in vascular resistance. Accordingly, these vessels collectively also play an important role for pressure regulation in larger systemic vessels.

The myogenic response is most likely initiated by a variety of stretch sensors in the smooth muscle cell membrane including receptors, and ion channels [38], such as the polycystines 1 and 2 (PKD1 and PKD2) [39]. In addition, the actin cytoskeleton and actin anchoring sites such as focal adhesions and dense plaques are involved in mediating mechanotransduction in vascular SMCs. Activation of these stretch sensors results in depolarization of the smooth muscle cell and calcium influx via voltage-gated calcium channels. This, combined with calcium sensitization via activation of the RhoA/Rho-kinase signaling pathway [40], results in a sustained contraction in response to increased pressure levels in small arteries.

The importance of actin cytoskeletal dynamics for myogenic constriction and stretch-dependent signaling events has been demonstrated in a number of vascular beds including cerebral arteries [41], portal vein [42], and mesenteric arteries [43]. The portal vein has been used as a model of stretch-dependent responses as its longitudinally oriented smooth muscle cells are highly sensitive to mechanical forces and exhibit myogenically activated contractions. Stretch of the portal vein promotes actin polymerization via activation of the Rho/Rho-kinase pathway [42]. Stretch-induced activation of actin polymerization has also been demonstrated in pressurized arteries and is crucial for the development of myogenic tone [44, 45].

The chronic responses to changes in flow and pressure include so-called remodeling, which entails a structural change of the vascular elements. Generally speaking, remodeling of arteries and arterioles can be defined along two axes: inward to outward, depending on the change in lumen diameter, and hypotrophic, eutrophic, or hypertrophic, depending on the accompanying change of wall material [46]. In hypertension, for example, remodeling typically involves an increase in the relationship between the media thickness and the lumen diameter [47]. This structural change, especially if it involves a reduction of lumen diameter, may fortify hypertension. Several studies have shown that antihypertensive treatment can normalize resistance artery structure, arguing that pressure as such contributes to the alteration of arterial design [47]. Hypertrophic remodeling implies an increase of the amount of wall material. Hypertension causes hypertrophic remodeling in larger vascular segments, and this blends with eutrophic inward remodeling in the resistance arterioles. Chronic changes in shear stress similarly associate with altered vascular structure: reduced flow causes inward remodeling [48], whereas increased shear stress causes outward remodeling [49], in the latter case involving a clear hyperplastic response of both SMCs and ECs. The molecular mechanisms of remodeling differ depending on the physiological situation or vascular disease considered, and can arise simply by rearrangement of the existing wall material, or may involve recruitment of inflammatory cells, stem cells, or even transdifferentiation and growth of resident cells depending on the disease model. Whatever the case, it is clear that mechanical forces acting on the vascular wall have major and dynamic influences on the structure of blood vessels (Fig. 4.2k).

4.5 Mechanoactivation of YAP and TAZ

As outlined above, it was not until 2011 that it was unambiguously demonstrated that YAP and TAZ are activated by mechanical cues [25], and this has since been corroborated in several studies in various model systems [3]. The exact molecular details of the mechanosensitivity of YAP and TAZ have however remained unclear [3]. It involves movement of the coactivators from the cytoplasm to the nucleus, and there are examples where this occurs independently of the Hippo pathway [3, 50]. There are, however, also examples where mechanical forces and the actin cytoskeleton act through Hippo, and one such mechanism involves inhibition of LATS kinase activity by filamentous (F-) actin [51]. Most likely, mechanosensitivity of YAP and TAZ therefore arises due to summation of several layers of regulation and some of these are discussed below.

One mechanism that may be relevant in SMCs involves the Enigma proteins PDLIM5 and PDLIM7 [52]. These proteins bind to YAP and bring it to focal adhesions and thus in close proximity to integrins (Fig. 4.3a). Integrin activation by mechanical forces [54] subsequently leads to the localized activation of SRC family kinases that inhibit LATS and phosphorylate YAP (Fig. 4.3a), promoting YAP nuclear translocation. The reasons that this model is attractive are twofold. First, and most importantly, this model fits the fine-grained staining pattern for YAP

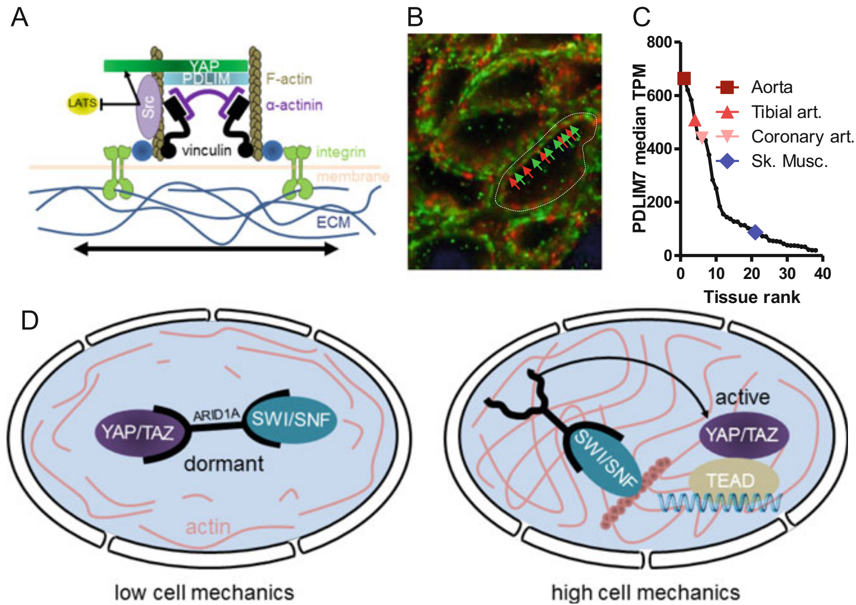

Fig. 4.3 Multilayered mechanical control of YAP and TAZ involves interactions in different cellular compartments. Panel **a** depicts the Enigma model of YAP/TAZ activation. Here, YAP is anchored at the membrane via binding to the Enigma proteins PDLIM5 and PDLIM7. Integrin-dependent local activation of SRC family kinases inhibits the LATS kinases and activates YAP. This then allows for YAP release from the adhesion structure and nuclear translocation. The figure was adapted from Elbediwy et al. [52]. In contractile SMCs, YAP (green) is located at the membrane as indicated by double labeling with the membrane protein caveolin-1 (red, panel **b**). Panel **c** shows that PDLIM7 expression is particularly high in arteries (from the GTExPortal.org). Panel **d** shows a model for nuclear activation of YAP/TAZ, occurring through polymerization of nuclear actin and release of YAP/TAZ from ARID1A [53]

in SMCs in situ. In SMCs, focal adhesions have a peculiar form, coalescing into large longitudinal membrane ribbons [55] that are referred to as “dense bands” or “dense plaques.” These ribbons may be straight (venous and gastrointestinal SMCs) or they may have a spiral form (arterial SMCs). Dense bands and membrane caveolae create two complementary and non-overlapping domains in SMCs [56]. YAP localizes to the membrane in between caveolae domains [29], arguing that it is situated in dense bands. This is depicted in Fig. 4.3b, where YAP staining is shown in green and caveolin staining in red in cross-sectioned SMCs. The subcellular distribution of YAP in SMCs thus implies that, for translocation to nuclei, YAP must first dislodge from its membrane anchorage site. The second reason that the Enigma model is attractive is that PDLIM5 and PDLIM7 are highly expressed in the arterial wall compared to other tissues. This is illustrated for PDLIM7 in Fig. 4.3c (data from GTExPortal.org). Of note, cytoplasmic anchorage of YAP may occur via different binding partners depending on the cell type, and ECs express high levels of angiomotin [57], a well-known regulator of YAP/TAZ activity [58]. Mechanical

activation of YAP/TAZ in ECs and SMCs may thus involve different binding partners.

Another layer of mechanical regulation likely occurs inside the nucleus. A recent study [53] demonstrated that ARID1A and the SWI/SNF complex retain YAP and TAZ in partly dormant form in nuclei (Fig. 4.3d shows YAP/TAZ activation in nuclei). Upon mechanical challenge, nuclear actin polymerizes to form filaments that anchor the SWI/SNF complex. This in turn releases YAP/TAZ, presumably through a conformational change in the SWI/SNF family member ARID1A, allowing for productive interaction of YAP/TAZ with TEAD transcription factors on DNA and transcriptional activation. The validity of this activation mechanism was confirmed in numerous model systems and in many species. Thus, interactions in different cellular compartments (nuclei, focal adhesions) as well as changes in phosphorylation (SRC, LATS) confer multilayer mechanosensitivity on YAP and TAZ.

4.6 Role of YAP and TAZ in the Formation of Blood Vessels

The vascular system arises in a process called vasculogenesis that gives rise to a primitive vascular plexus. This primitive network of blood vessels is then expanded and remodeled in a process called angiogenesis. YAP and TAZ play roles in both of these processes. The earliest indication of a role in vasculogenesis was the finding that global YAP knockout results in embryonic lethality with failure of blood vessel formation in the yolk sac [59]. YAP and TAZ similarly play a role in blood vessel formation in the heart. Here, the coronary arteries arise from epithelial cells in the epicardium via epithelial-to-mesenchymal transition (EMT). Using two distinct epicardial Cre-deleter lines, it was demonstrated that the lack of epicardial YAP/TAZ results in embryonic lethality and impaired coronary vasculature development [6]. These effects were attributed to reduced epicardial cell proliferation and impaired EMT. As a result, a reduced number of ECs and SMCs were available for formation of new blood vessels.

Developmental angiogenesis involves the development of new blood vessels from the existing vascular plexus. This requires EC proliferation and a phenomenon called sprouting. Morphologically, the angiogenic process is initiated by the formation of tip cells from existing ECs. Tip cells use a network of filopodia to sense the environment and they lead the angiogenic front (see Fig. 4.4 where filopodia resemble see weed). Below tip cells in the sprouts are stalk cells. Stalk cells proliferate to support growth. Phalanx cells are found proximal to the stalk cells, and there is a continuum of lumen formation and barrier maturation from phalanx to stalk cells. Angiogenesis is an exquisitely complex process involving numerous signaling pathways [60], and a handful of recent studies have underscored a role of YAP and TAZ in both developmental and pathological angiogenesis [61, 62].

Using EC-specific and inducible knockout (ECKO) of YAP and TAZ postnatally it was shown that developmental angiogenesis in the retina and brain, via control of microvessel length, density, and branching, was severely impaired [63]. ECKO of LATS1/2, on the other hand, increased branching and EC proliferation.

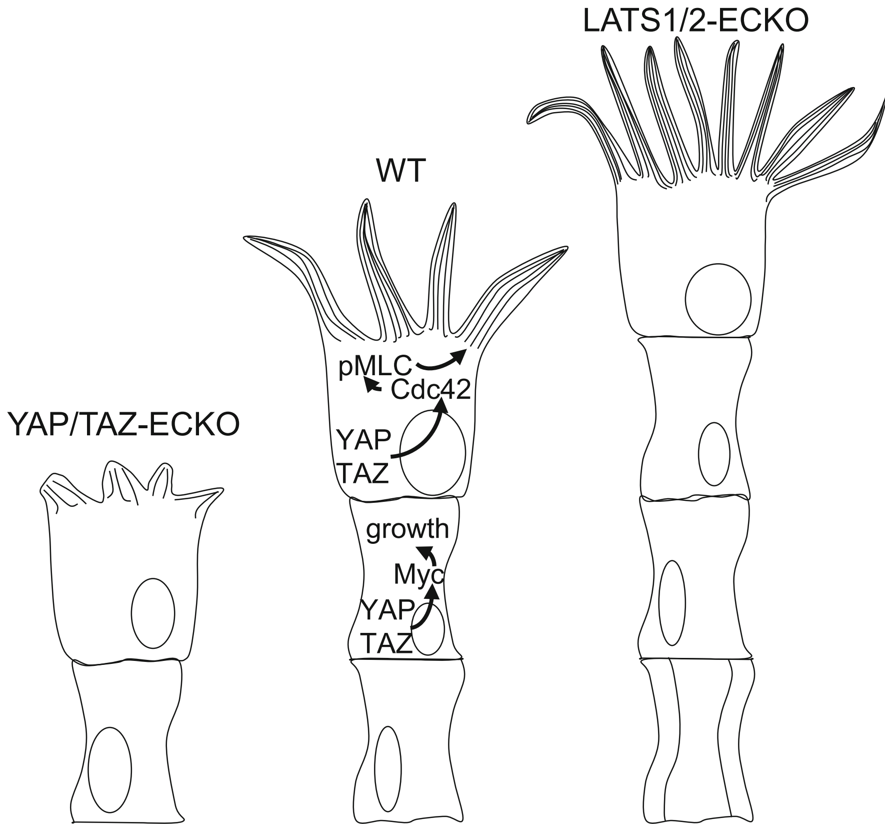

Fig. 4.4 Angiogenesis occurs through growth of endothelial sprouts from existing vessels, and this depends on YAP and TAZ. Three sprouts are shown: to the left from an animal that lacks YAP and TAZ specifically in endothelial cells (ECKO), in the middle from a wild-type (WT) mouse, and to the right from a mouse that lacks LATS1 and LATS2 in ECs. YAP and TAZ control sprouting via Cdc42 activity and myosin phosphorylation in tip cells. This in turn influences filopodia which are actin-dependent structures protruding from the tip cell. YAP and TAZ also control growth of stalk cells via Myc. Consequently, sprouts are smaller with less developed filopodia when YAP and TAZ are knocked out and larger with more extensive filopodia when LATS1 and LATS2 are knocked out

Mechanistically, it was found that YAP and TAZ regulate filopodia in tip cells via Cdc42 activity and myosin phosphorylation (Fig. 4.4, middle). Stalk cell growth was moreover controlled by YAP/TAZ through Myc to allow for sprout elongation (Fig. 4.4, middle). Impaired angiogenesis via Cdc42 was also highlighted in another study [64]. This argues that changes in Cdc42 activity are likely an important mechanism controlled by YAP/TAZ, but the two studies disagree on how Cdc42 is activated. Of further interest is the prior demonstration that the myosin phosphatase, which controls myosin phosphorylation, is a direct target of LATS kinase activity [65]. Two additional studies on YAP and TAZ in angiogenesis focused on the fact that YAP and TAZ are activated by VEGF via SRC family kinases and actin

dynamics [51, 66]. Embryonic and EC-specific YAP/TAZ depletion resulted in severe vascular defects and growth arrest, and microvessels were shorter with fewer branches and poorer EC coverage [51]. Reduced angiopoietin-2 levels and retention of VEGF receptor 2 (VEGFR2) in the Golgi apparatus were among the mechanisms proposed. Cross talk of YAP/TAZ with BMP signaling and possibly Dll4/Notch was moreover demonstrated [67]. Collectively, these studies argue in favor of an important role of YAP and TAZ in angiogenesis and indicate dependence of major angiogenesis-relevant signaling pathways on YAP/TAZ in ECs.

4.7 Shear Stress-Dependent Control of YAP and TAZ in Endothelial Cells

Several recent studies have established that mechanoactivation of YAP and TAZ plays an essential role in ECs. Atherosclerosis is a major contributor to vascular disease and atherosclerotic lesions generally develop in areas with disturbed flow and relatively low shear stress, for example where arteries branch or bend (Fig. 4.5a). Disturbed flow patterns are associated with vascular inflammation, EC proliferation and apoptosis, as well as production of reactive oxygen species. In experimental models where cultured endothelial cells were exposed to unidirectional shear stress (USS), inactivation of YAP (increased S127 phosphorylation) was observed,

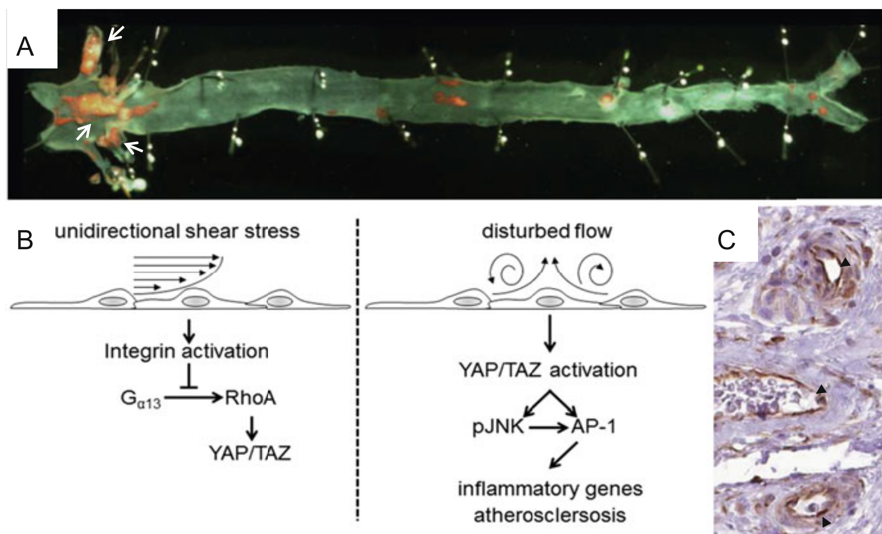

Fig. 4.5 Flow-mediated changes in YAP and TAZ activity control atherogenesis. Panel **a** shows atherosclerosis the aorta from an ApoE knockout mouse. The aorta was cut open along the greater curvature of the arch. Oil red O staining was performed to show lipid inclusions in red. Predilection sites for lipid inclusion include the center of the lesser curvature and at branch sites (white arrows) where flow is disturbed. Panel **b** shows YAP/TAZ activation by disturbed flow. Panel **c** shows AMOT staining in ECs (black arrowheads) of human arteries (from the Human Protein Atlas [28])

whereas YAP activation instead occurred with disturbed or oscillatory flow [7, 8]. This is illustrated in Fig. 4.5b (right vs. left). Accordingly, decreased YAP activity (cytoplasmic localization) was found in the outer curvature of the aortic arch, where flow is laminar, and nuclear YAP was found in the inner curvature of the aortic arch where flow is disturbed [7, 8]. The mechanism for YAP/TAZ inhibition by unidirectional shear stress was proposed to involve integrin β_3 activation and scavenging of $G_{\alpha 13}$, which in turn abrogates normal RhoA-GTP loading and thus YAP/TAZ activation (Fig. 4.5b, left). Accordingly, silencing of $G\alpha_{13}$ abolished shear stress-induced inactivation of YAP. Disturbed flow and dyslipidemia, on the other hand, activated YAP (Fig. 4.5b, right). Silencing and overexpression of YAP moreover decreased and increased atherosclerosis, respectively [8]. The proatherogenic effect of YAP was shown to involve JNK activation as well as synergy with AP-1 on its target genes. Several inflammatory gene targets were also identified, including IL6 and IL8. YAP/TAZ activation in ECs also promoted proliferation. Thus, to conclude, YAP is activated in ECs by disturbed flow, and this is a proatherogenic event that depends on AP-1-driven transcription of inflammatory cytokines.

The impact of unidirectional shear stress on YAP/TAZ may be time-dependent. Experiments in developing zebrafish elegantly show that when blood starts to flow through a blood vessel, YAP is rapidly translocated to the nucleus [68] and thus activated. However, after 6–24 h of continuous unidirectional shear stress, YAP is excluded from the nucleus, suggesting differences in the acute and chronic responses to flow. The nuclear accumulation in response to flow in zebrafish occurs without changes in S127 phosphorylation and LATS activity. Instead, the sudden increase in flow induces reorganization of the cortical cytoskeleton allowing for binding and neutralization of angiomotins (AMOTs), molecules that otherwise inhibit YAP translocation to the nucleus. Interestingly, AMOT mRNA levels are high in vascular tissue and immunohistochemistry reveals rather specific AMOT staining of the endothelium compared to the surrounding SMCs (Fig. 4.5c, arrowheads).

4.8 YAP and TAZ in Growth and Differentiation of Arterial SMCs

SMCs exhibit remarkable phenotypic plasticity. In the healthy arterial wall SMCs are “contractile” and are characterized by proliferative quiescence and high expression of SMC differentiation markers such as myosin and actin [69–72]. In arterial lesions, on the other hand, the expression of differentiation markers is reduced as is contractility, and proliferation and matrix synthesis are increased (Fig. 4.6a). The latter state is referred to as the “synthetic” phenotype, and the switching between phenotypes is called phenotypic modulation. Phenotypic modulation depends on a family of coactivators called myocardin-related transcription factors (MRTFs: MRTF-A, MRTF-B, MYOCD), and in particular myocardin [71, 73]. The majority of SMC differentiation markers, including smooth muscle myosin (MYH11) and actin (ACTA2), are direct transcriptional targets of myocardin [74].

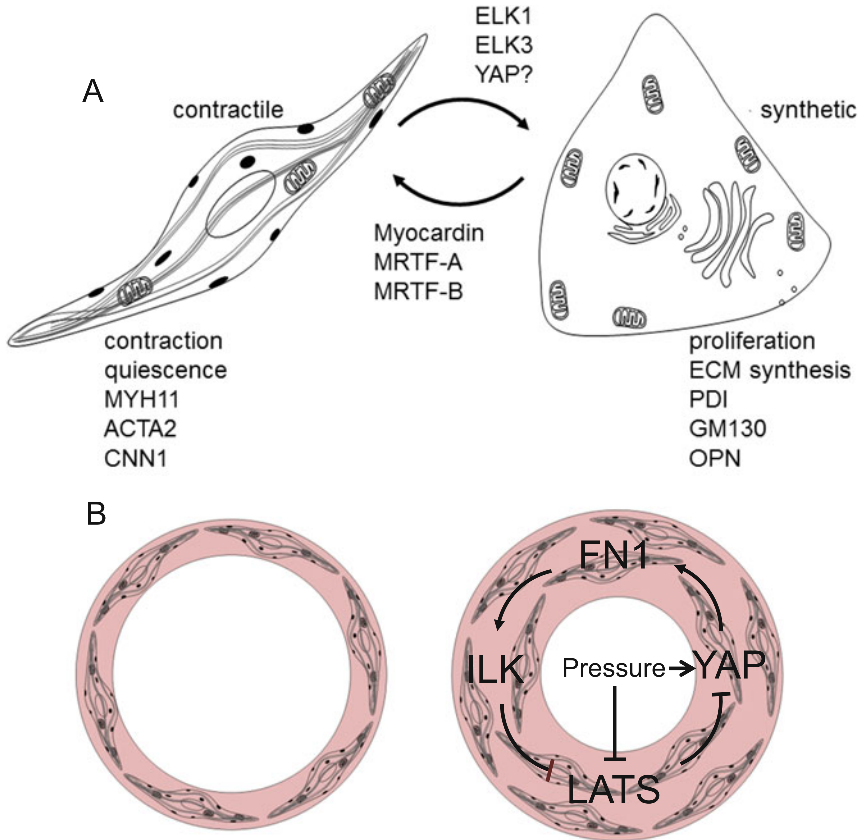

Fig. 4.6 Phenotypic modulation of vascular SMCs and inward hypertrophic remodeling in pulmonary arterial hypertension (PAH) may depend on YAP/TAZ. Panel **a** shows that SMCs toggle between distinct phenotypes characterized by contractility and quiescence (left) and growth and matrix synthesis (right). YAP may favor transition to the "synthetic" phenotype whereas myocardin-related transcription factors (MRTFs) favor transition to the contractile phenotype. Panel **b** shows vicious circle in PAH causing hypertrophic inward remodeling (right) compared to normotensive animals (left). This vicious circle involves activation of YAP, causing increased expression of fibronectin (FN1) and integrin-linked kinase (ILK) activation which further promotes YAP activation via LATS inhibition

One of the first studies of YAP in SMCs [75] focused on phenotypic modulation. It was demonstrated that the mitogen PDGF induces YAP and reduces myocardin. Silencing of YAP, on the other hand, reduced proliferation and increased myocardin. It was moreover shown that YAP binds to myocardin, and the authors argued that such sequestration of myocardin may preclude myocardin-driven transcription. In all, this indicated that YAP contributes to growth and phenotypic modulation of SMCs. Another study published the same year [9] used an arterial injury model and reached similar conclusions: (1) YAP increased SMC proliferation following arterial injury, (2) overexpression of YAP reduced a comprehensive panel of SMC markers

and increased the proliferative marker cyclin D1, and (3) silencing of YAP increased SMC markers and reduced cyclin D1. Two recent studies echo similar findings [76, 77]. Given that all of these studies unanimously favor an important role of YAP in modulation of SMCs toward the synthetic phenotype, it is rather surprising that one study using SM22α promoter-driven deletion of YAP [78] in vivo reported unchanged SMC differentiation marker expression. SMC proliferation, on the other hand, was perturbed, causing arterial walls to become thinner. So, to conclude, while the role of YAP in SMC proliferation holds up in vivo, its role in phenotypic modulation is less clear.

There are several possible explanations for the discrepant findings regarding YAP in phenotypic modulation. One possibility is that YAP may drive proliferation of SMCs without loss of differentiation in their native environment in vivo. Another is that transient overexpression and knockdown in vitro does not involve the same degree of compensation, via TAZ, which occurs in vivo. It is also important to underscore that a caveat of the conditional knockout strategy used [78] is that YAP was depleted both in SMCs and in the embryonic heart, causing cardiac hypoplasia and septal defects. This may cause structural changes in arteries due to reduced blood flow independently of YAP and TAZ. Inducible deletion of both YAP and TAZ in SMCs using more specific Cre-deleter lines [79] would allow for a more precise delineation of the role of these coactivators in phenotypic modulation and in remodeling and disease of systemic arteries.

Several studies indicate that vasoactive and mitogenic mediators beyond PDGF influence the activation of YAP and TAZ in SMCs. These include the thromboxane A2 receptor agonists iBOP and U-46619, and the lipid mediator sphingosine-1-phosphate, all of which activate YAP [29, 80]. Agents that elevate cAMP, on the other hand, inhibit YAP in SMCs [11]. YAP regulation by these mediators typically involves RhoA, changes in the actin cytoskeleton, and changes in LATS-dependent phosphorylation on S127. SRC-dependent changes in YAP phosphorylation (e.g., Y357) remain comparatively poorly studied in SMCs. Such studies are needed, because important aspects of YAP activation depend on tyrosine phosphorylation.

4.9 YAP and TAZ Contribute to Pulmonary Arterial Hypertension

The blood pressure in the lung is much lower than that in the systemic circulation. Pulmonary arterial hypertension (PAH) is defined as a pressure at rest exceeding 25 mm Hg [81]. Over time, PAH almost invariably leads to inward hypertrophic remodeling irrespective of initial arterial diameter. In extreme situations, this can completely obliterate small arterioles. Such remarkable changes depend on an altered balance of proliferation and apoptosis in the media, along with remodeling of the extracellular matrix [82]. A number of recent studies have underscored a role of YAP and TAZ in these changes. In a string of papers [83–85], Bertero et al. used systems biology to first define a role of the microRNA miR-130/301 in PAH. They

then went on to show that YAP contributes to induction of miR-130 and to matrix remodeling. The stiffened matrix then further promoted YAP activity in a feedforward manner. One of the downstream effectors of the proposed pathway was the collagen and elastin cross-linking enzyme LOX (lysyl oxidase). It was shown that β-aminopropionitrile (BAPN), an inhibitor of the LOX family of enzymes, normalized pulmonary blood pressure and vascular architecture. In the last paper in this series, the authors demonstrated that YAP and TAZ also target the metabolic enzymes glutaminase (GLS), lactate dehydrogenase (LDHA), and pyruvate carboxylase (PC) to cause metabolic changes that contribute to remodeling and PAH. A GLS inhibitor normalized arterial architecture and pressure similar to the LOX inhibitor BAPN. As a whole, these papers provide remarkable system level insight into PAH, and strongly incriminate YAP/TAZ in this rare disease, but they also raise a number of questions, regarding for example the specificity of the pharmacological interventions.

Strong supportive evidence for a role of YAP in remodeling of lung arterioles has also been generated by other research groups working on PAH [86]. It was demonstrated that LATS1 phosphorylation (and thus activity) was reduced in SMCs from PAH patients both in vivo and in vitro. This is associated with increased total YAP/TAZ levels and increased DNA synthesis. An increase of total YAP was also seen in the PAH work cited above, and in the arterial injury model [9], arguing that YAP induction is a shared property of hyperplastic arterial lesions irrespective of anatomical location. It was further found that silencing of YAP and TAZ reduced DNA synthesis and increased apoptosis in pulmonary SMCs from PAH patients. Interestingly, YAP/TAZ induction is associated with increased levels of fibronectin (FN1), a major integrin ligand, and integrin-linked kinase (ILK). FN1-ILK signaling was found to further repress LATS1 activity. The authors also reported attenuation of remodeling with the ILK inhibitor Cpd22. It was proposed that a rise in pulmonary arterial pressure activates YAP/TAZ to initiate a self-sustaining feedforward loop involving matrix remodeling and pursuant FN1/ILK1 signaling. This is illustrated in Fig. 4.6b.

4.10 YAP and TAZ in Aneurysmal Disease

So far, only one study has focused on YAP in aortic aneurysm development and dissection (Stanford type A). Elastin fragmentation and apoptosis was demonstrated in patient aneurysm samples and this is associated with reduced YAP levels in SMCs. It should be noted here that inhibition of YAP activity as a consequence of aneurysm formation is counterintuitive from a biomechanical point of view. This is because an increase in lumen diameter (>50% to classify as an aneurysm) at the same pressure will increase the wall tension according to the law of Laplace. Repression of YAP may thus be the cause rather than the consequence of aneurysm formation. Whatever the case, the authors then used the LOX inhibitor BAPN, i.e., the same substance used above to treat PAH, to induce aneurysms with dissections in mice. BAPN reduced the aortic YAP levels and increased apoptosis. Taken together,

this study suggested that repression of YAP may be a pathogenic mechanism in aneurysm development and strongly cautions against the use of BAPN in PAH treatment. The pro-aneurysmal effect of the LOX inhibitor BAPN is expected, because knockout of LOX causes neonatal aneurysms [87], as do various matrix disorders, including Marfan syndrome, which is caused by mutations in fibrillin.

4.11 YAP and TAZ Target Genes in the Mature Vascular Wall

Numerous RNA-Seq analyses have identified YAP/TAZ target genes in different cell types, including ECs [51, 63], but to the best of our knowledge, no study has focused on human SMCs. A recent study defined a list of signature YAP/TAZ target genes in human cancer cell lines [88]. CTGF (CCN2) and CYR61 (CCN1), which are among the classical targets, were present on this list as expected (Fig. 4.7a). This list of signature targets gives food for thought. For instance, one may speculate that TGFB2 contributes to the reported profibrotic effect of YAP/TAZ in PAH. Similarly, MYOF (Myoferlin), which has been shown to control trafficking of VEGFR2 [90], could contribute to the VEGFR2 trafficking defect that was documented in YAP/TAZ KO mice in angiogenesis [51]. ARHGEF17 could contribute to activation of small GTPases and thus perhaps to specialized actin-dependent structures such as filopodia. F3 (tissue factor), finally, may provide a connection between mechanical forces (e.g., shear stress) and coagulation.

Because it is unclear to what extent target genes of YAP and TAZ are cell type- and species-specific, we have examined if the mRNAs from the signature gene list correlate with YAP and TAZ in human arteries (Fig. 4.7a, data from GTExPortal). Surprisingly, none of the classical target genes CTGF and CYR61 correlate in a consistent manner with YAP or TAZ at the mRNA level in the three arteries represented in this database. This does not imply that CTGF and CYR61 are not regulated by YAP/TAZ in arteries. Rather other transcriptional inputs, such as the MRTFs [91], may dominate the transcriptional drive on these genes in the arterial wall. Four mRNAs correlate consistently with YAP and TAZ across arteries (highlighted in red), and others correlate with either YAP or TAZ in each of the arteries, and satisfy the additional criterion that they are highly expressed (bold face).

The signature gene list (Fig. 4.7a) is rather small considering that several hundred genes are activated when YAP/TAZ are overexpressed in vitro. One may want to cast a wider net to define additional targets in the arterial wall. We therefore cross-referenced a recent RNA-Seq study [89] with genes that are highly expressed (top 600) in the human coronary artery [26] and those that correlate positively with YAP (Fig. 4.7b, top 600). Among genes that are both highly expressed and regulated by YAP (blue-yellow overlap in 7B) are THBS1, FN1, and IL6ST. THBS1 (thrombospondin-1) plays a role in angiogenesis [92], and is conspicuously upregulated in the hypertensive compared to the normotensive arterial wall [93]. FN1 (fibronectin) is regarded by many as a master regulator of extracellular matrix assembly, and FN1 can bind both integrins and matrix constituents, including collagens [94]. FN1 was highlighted as target gene in one of the PAH studies [86],

A

signature targets cancer	coronary YAP	coronary TAZ	tibial YAP	tibial TAZ	aorta YAP	aorta TAZ
CYR61	R=0.21**	R=0.14	R=-0.27***	R=-0.22***	R=0.21***	R=-0.05
CTGF	R=0.42***	R=0.25**	R=-0.04	R=0.04	R=0.20***	R=-0.03
AMOTL2	R=0.27***	R=-0.07	R=-0.04	R=-0.01	R=0.13*	R=-0.21***
ANKRD1	R=0.31***	R=0.03	R=0.23***	R=0.01	R=0.07	R=-0.18**
IGFBP3	R=0.01	R=-0.07	R=0.13**	R=0.12**	R=-0.07	R=0.10
F3	R=-0.36***	R=-0.34***	R=-0.03	R=-0.04	R=-0.01	R=-0.14*
FJX1	R=-0.14	R=-0.01	R=-0.02	R=0.01	R=-0.20***	R=0.01
NUAK2	R=-0.21**	R=-0.34***	R=-0.24***	R=-0.09	R=-0.26***	R=-0.23***
LATS2	R=0.69***	R=0.34***	R=0.57***	R=0.28***	R=0.62***	R=0.03
CRIM1	R=0.62***	R=0.32***	R=0.24***	R=0.31***	R=0.53***	R=-0.07
GADD45A	R=0.38***	R=0.29***	R=0.11*	R=-0.05	R=0.33***	R=-0.03
TGFB2	R=0.67***	R=0.56***	R=0.34***	R=0.43***	R=0.43***	R=0.17**
PTPN14	R=0.56***	R=0.23**	R=0.52***	R=0.29***	R=0.32***	R=0.14*
NT5E	R=0.42***	R=0.51***	R=0.29***	R=0.48***	R=0.12*	R=0.22***
FOXF2	R=-0.31***	R=-0.19*	R=-0.21***	R=-0.10*	R=-0.15**	R=-0.12
AXL	R=0.45***	R=0.41***	R=0.28***	R=26***	R=0.04	R=0.29***
DOCK5	R=0.23**	R=0.16*	R=0.25***	R=0.17***	R=0.26***	R=-0.04
ASAP1	R=-0.06	R=-0.21**	R=0.14**	R=0.14**	R=-0.08	R=-0.06
RBMS3	R=0.18*	R=0.45***	R=0.27***	R=0.16***	R=0.41***	R=0.25***
MYOF	R=0.63***	R=0.34***	R=0.29***	R=0.23***	R=0.47***	R=-0.04
ARHGEF17	R=0.46***	R=0.33***	R=0.09	R=0.06	R=0.30***	R=-0.02
CCDC80	R=-0.16*	R=0.04	R=-0.08	R=0.18***	R=-0.16**	R=-0.09

B

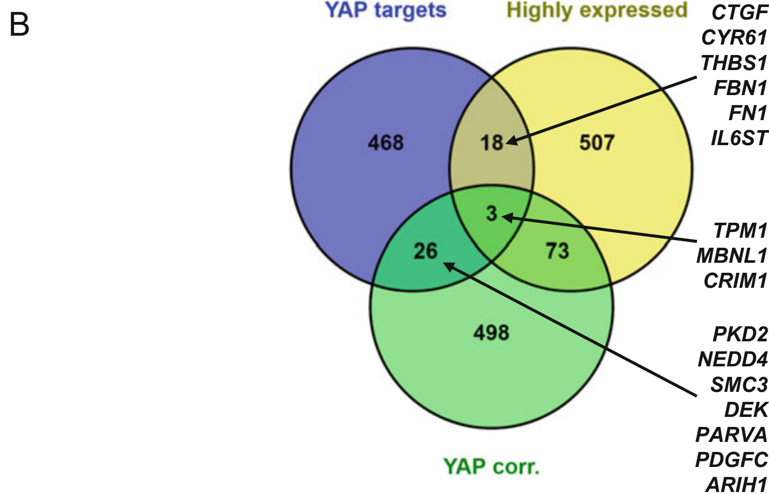

Fig. 4.7 Prediction of YAP/TAZ target genes in human arteries. Panel **a** shows signature target genes of YAP and TAZ in human cancer cell lines (left column) and how these correlate with YAP and TAZ at the mRNA level in human arteries (right columns). Positive correlation coefficients are depicted in red and negative correlation coefficients in blue ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$). Genes that correlate in a positive manner with both YAP and TAZ in all arteries are highlighted in red in the left column. Genes that correlate with either YAP OR TAZ in all arteries and that are highly expressed are shown in bold. Correlation analyses were made using data from the GTEx

and FN1 is indeed regulated by mechanical cues, including flow [95]. IL6ST (a.k.a. GP130) is part of the IL6 receptor complex, and acts through the STAT3 pathway. IL6ST plays a critical role in epithelial regeneration and can itself activate YAP via SRC family kinases [96]. IL6ST moreover plays a role in cardiac hypertrophy following pressure overload [97], and contributes to atherosclerosis in mice [98]. Thus, among highly expressed genes in the arterial wall, YAP and TAZ potentially target several beyond the core signature that are essential for vascular homeostasis and disease.

Among the potential YAP targets in the overlay of all three datasets (Fig. 4.7b, center) are TPM1, MBNL1, and CRIM1. TPM1 (tropomyosin-1) is an actin-binding protein and a component of the contractile apparatus in SMCs. It is also regarded as one of the classical smooth muscle differentiation markers. MBNL1 regulates pre-mRNA processing on multiple levels [99], and knockout of MBNL1 causes dystrophy-like histological changes in skeletal muscle [100]. MBNL1 has yet to be studied in the arterial wall. This may be worthwhile given its sizeable expression in arteries [26]. CRIM1, finally, plays a role in capillary tube formation [101], in part by presenting growth factors such as VEGF-A to ECs [102]. As discussed above, CRIM1 also belongs to the signature target gene list.

A decent number of genes that are regulated by YAP and that correlate with YAP in the arterial wall also exist (blue-green overlap, Fig. 4.7b), and some of these are notable. PKD2, for example, encodes a mechanosensitive Ca^{2+}-permeable cation channel that may play a role in myogenic tone [103]. DEK, on the other hand, is a proto-oncogene potentially involved in growth. ARIH1, finally, was shown in recent work to link myonuclei to the cytoskeleton, and mutations in this gene gave rise to aortic aneurysms with dissections [104]. ARIH1 may thus be a critical target gene for YAP/TAZ-dependent protection from this catastrophic disease. Thus, to conclude this section, there are many genes that are highly relevant for cardiovascular function and disease among the predictions of arterial YAP/TAZ targets. Importantly however, with a few exceptions, direct experimental validation of these predictions has yet to be provided.

4.12 Cross Talk Between YAP/TAZ and MRTFs

MRTFs are essential for SMC differentiation and act through the Serum Response Factor and so-called CArG boxes in DNA (Fig. 4.8a). The YAP/TAZ and MRTF coactivator families are similar in many ways. Both are inhibited by drugs that depolymerize F-actin such as Latrunculins and Y27632 [3, 73, 105, 106]. YAP/TAZ and the MRTFs are moreover activated by substrate stiffness [25, 107]. From the

Fig. 4.7 (continued) Portal [26]. Panel **b** shows YAP target genes from a recent RNA-Seq study (blue circle [89]), cross-referenced here with genes that are highly expressed in the human coronary artery (yellow circle), and those that correlate with YAP across arteries (green circle). Examples of genes from the overlays are given to the right

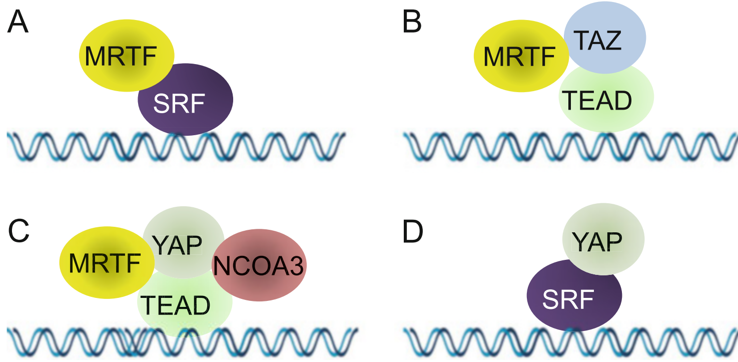

Fig. 4.8 Cross talk between mechanosensitive coactivators from the MRTF and YAP/TAZ families. Panel **a** shows prototypical interaction between MRTFs and SRF, driving transcription via CArG boxes in the genome. Panels **b** and **c** show that MRTFs may also partner with TAZ/TEAD and YAP/TEAD complexes to drive transcription via MCAT elements. YAP may also bind to SRF as depicted in **d**. **b** through **d** depict direct coupling mechanisms, but coupling may also be indirect and involve F-actin assembly

discussion above, it is evident that the coactivator families share gene targets, and CTGF and CYR61 are good examples [88, 91]. Genomic TEAD binding motifs are significantly enriched in the vicinity of SRF binding sites [108], and well over 100 distinct target genes are shared [89]. Overall resemblance between these coactivator families is therefore conspicuous. In fact, a number of studies argue that the MRTF and YAP/TAZ signaling pathways are coupled.

Recent work by Foster et al. first defined target genes that are unique for MRTF/SRF signaling (MRTF-only genes) and for YAP/TEAD signaling (YAP-only genes) [107]. The authors then went on to silence MRTF and found that both MRTF-only and YAP-only genes were reduced. Conversely, when YAP was silenced, both YAP-only and MRTF-only genes responded. This interdependence was ascribed to downstream effects of both coactivator families on actin assembly. Indeed, activation of YAP by overexpression of MRTF was abolished by simultaneous treatment with Latrunculin. This was taken to support an indirect coupling mechanism, involving downstream target genes and actin dynamics.

Direct coupling mechanisms have also been proposed. Speight et al. [109] demonstrated that MRTF-A and TAZ coprecipitate in cells where RhoA is active. It was also shown that MRTF-A and TAZ are synergistic on isolated TEAD elements (Fig. 4.8b). Another study showed that MRTFs may bind to YAP [89], allowing for recruitment of the coactivator NCOA3, and activation of isolated TEAD elements (Fig. 4.8c). Remarkably, the latter study demonstrated that mutations in MRTFs that disrupt YAP binding, but that are otherwise benign, completely ablate the pro-metastatic activity of the MRTFs. Yet another study showed that YAP may interact with SRF (Fig. 4.8d) to promote stem-like properties in epithelia [110]. Thus, to conclude, both indirect and direct coupling mechanisms between YAP/TAZ and MRTFs have been proposed.

4.13 Clinical Translation

Work summarized here argues that YAP/TAZ play important roles in hyperplastic arterial disease and in angiogenesis. YAP and TAZ also seem to play a role in aneurysm development, but the detailed mechanism is not understood. Both activation and inhibition of the transcriptional impact of YAP and TAZ may be desirable to ameliorate vascular disease. In fact, as clearly illustrated by the LOX inhibitor BAPN, YAP/TAZ inhibition is beneficial in pulmonary arterial hypertension, but deleterious in aneurysm development. This argues that local and highly controlled pharmacologic targeting is required if real patient benefit is to be achieved. There are ongoing efforts to develop small molecule modulators to control Hippo signaling [111]. This raises hopes that more specific targeting strategies will become available. Beyond local delivery, specificity may be achieved by focusing on cell type-specific activation mechanisms. For example, the Enigma proteins and AMOTs may allow for differential targeting of YAP/TAZ in SMCs and ECs. All in all, there is thus hope that we may be able to control these coactivators for therapeutic benefit in vascular disease caused by deleterious mechanical inputs.

Note Added in Proof While this chapter was processed for publication two papers appeared (Daoud et al. Cell Mol Gastroenterol Hepatol. 2020 Sep 28:S2352-345X (20)30157-0 and Wang et al. iScience 23, 101860, December 18, 2020) unanimously showing that dual deletion of YAP and TAZ in adult smooth muscle causes loss of contractile differentiation and death due to loss of colonic contractility and colonic obstruction. The latter paper demonstrated similar loss of arterial contractility. This is consistent with the cross talk discussed in Sect. 4.12, but additional mechanisms for loss of contractile differentiation likely also play a role.

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Ethical approval: This article does not contain any studies with human participants performed by any of the authors. This article does not contain any studies with animals performed by any of the authors with the exception of Fig. 4.5a which is from ongoing work where the ethical permit was granted by Malmö/Lund Ethics Committee (M433–12 and M46–13) at Lund University.

Informed Consent: Not applicable.

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GPCRs Under Flow and Pressure

5

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Abstract

G protein-coupled receptors (GPCRs) are commonly known as membrane-bound cellular sensors for chemical stimuli like small molecules and hormones that activate intracellular signal transduction pathways resulting in cellular responses. However, GPCRs are not only sensitive to agonist stimulation but also to mechanical forces. There is growing evidence that mechanosensitive GPCRs are involved in several physiological and pathophysiological states, such as vascular regulation, cardiac hypertrophy, and preeclampsia. In blood vessels, two different mechanical forces occur that contribute to autoregulation of the vessel tone: shear stress induced by blood flow and wall stretch induced by blood pressure. Recent findings suggest that blood flow activates mechanosensitive GPCRs in endothelial cells resulting in flow-induced vasodilation, and blood pressure activates mechanosensitive GPCRs in vascular smooth muscle cells mediating myogenic vasoconstriction. Seven GPCRs are currently discussed as potential mechanosensors in blood vessels. However, a principal molecular mechanism underlying mechanically induced GPCR activation remained largely elusive. Recent findings suggest that at the molecular level, GPCRs undergo stimulus-specific patterns of conformational changes. Moreover, GPCRs that possess a C-terminal helix 8 (H8), a common feature of many GPCRs, are mechanosensitive, while GPCRs that lack H8 are not mechanosensitive. In addition, transfer of H8 to non-mechanosensitive GPCRs confers whereas elimination of the H8 precludes mechanosensitivity. Thus, there is evidence pinpointing H8 as an essential structural motif that endows GPCRs with mechanosensitivity. Deeper insights into the mechanistic basis of mechanosensation of GPCRs may lay the foundation for a better understanding of the roles of mechanosensitive GPCRs in health and disease.

5.1 Introduction

G protein-coupled receptors (GPCRs) are heptahelical receptors that are essential for many physiological and pathophysiological processes, thus representing important drug targets for approximately 34% of all approved drugs [1]. The most frequently targeted receptors are the histamine H_1 receptors (H_1Rs), the dopamine D_2 receptors, the muscarinic acetylcholine M_1 receptors (M_1Rs), and the adrenergic α_{1A} receptors (α_{1A}Rs) [2]. GPCRs are commonly regarded as cellular sensors for neurotransmitters and hormones. However, they are also sensitive to physical and chemical stimuli like voltage [3–7], ions [8], and mechanical forces [9, 10].

So far, several GPCRs have been characterized as mechanosensors (see Table 5.1) that contribute to various physiological and pathophysiological

Table 5.1 Mechanosensitive GPCRs. Overview of mechanosensitive GPCRs that are described so far

Receptor	Coupling	Expression system	Stimulation	Potential function	References
Adenosine A_{2A} receptor ($A_{2A}R$)	G_s	Overexpression in HEK293 cells	Hypotonicity	?	[11]
Apelin receptor	$G_{i/o}$	Endogenous expression in human umbilical vein endothelial cells (HUVEC), human umbilical artery endothelial cells (HUAEC), and zebrafish endothelial cells	Shear stress	Endothelial cell polarization	[12]
Angiotensin II AT_1 receptor (AT_1R)	$G_{q/11}$	Overexpression in HEK293, COS-7, and rat A7r5 cells; endogenous expression in murine and rat cerebral, murine renal, and murine mesenteric vascular smooth muscle cells and in murine and rat cardiomyocytes	Hypotonicity, direct membrane stretch, pressure overload, increased intravascular pressure	Sensor of blood pressure mediating myogenic vasoconstriction	[13–16]
				Pathophysiological role as mechanosensor inducing cardiac hypertrophy	[17, 18]
		Overexpression of mutant receptors in HEK293 cells	Direct membrane stretch	?	[19]
Bradykinin B_2 receptor (B_2R)	$G_{q/11}$	Overexpression in bovine aortic endothelial cells	Shear stress, hypotonicity	Sensors of shear stress in endothelial cells	[20]
B_2R/AT_1R dimer	$G_{q/11}$	Overexpression in aortic smooth muscle cells of transgenic mice	Pressure overload	Pathophysiological role as mechanosensor mediating in preeclampsia	[21]
Cysteinyl leukotriene 1 receptor ($CysLT_1R$)	$G_{q/11}$	Overexpression in HEK293 cells; endogenous expression in murine renal and mesenteric vascular smooth muscle cells	Hypotonicity; increased intravascular pressure	Sensor of blood pressure mediating myogenic vasoconstriction	[16]
Dopamine D_2 receptor (D_2R)	$G_{i/o}$	Overexpression in CHO-K1 cells	Hypotonicity	?	[11]
Dopamine D_5 receptor (D_5R)	G_s	Endogenous expression in murine vascular endothelial cells	Shear stress	Sensor of shear stress in endothelial cells	[22]

(continued)

Table 5.1 (continued)

Receptor	Coupling	Expression system	Stimulation	Potential function	References
Endothelin ET_A receptor (ET_AR)	$G_{q/11}/G_s$	Overexpression in HEK293 cells	Hypotonicity	?	[14]
Formyl peptide receptor 1 (FPR_1)	$G_{i/o}$	Endogenous expression in human leukaemia cell line HL60	Shear stress	Sensor of shear stress in neutrophils that reduces retraction of pseudopods	[23]
GPR68	$G_{q/11}/G_s$	Overexpression in HEK293 cells; endogenous expression in murine endothelial cells of small resistance arteries and in human breast cancer cell line MDA-MB-231	Shear stress	Sensor of shear stress in the endothelium mediating flow-induced vasodilation	[24]
Histamine H_1 receptor (H_1R)	$G_{q/11}$	Overexpression in HEK293 and HeLa cells; endogenous expression in HUVEC and murine mesenteric artery endothelial cells	Hypotonicity, direct membrane stretch, shear stress, intravascular flow	Sensor of shear stress in the endothelium mediating flow-induced vasodilation	[11, 14, 25]
Muscarinic M_5 receptor (M_5R)	$G_{q/11}$	Overexpression in HEK293 cells	Hypotonicity, direct membrane stretch	?	[14]
Parathyroid hormone 1 receptor (PTH_1R)	$G_s/G_{q/11}$	Overexpression in HEK293 and in murine preosteoblastic cells	Shear stress	Bone growth	[26]
Sphingosine 1-phosphate receptor (S_1PR)	$G_{i/o}$	Endogenous expression in endothelial cells of the murine retinal vasculature; endogenous expression and overexpression in HUVEC	Shear stress	Vascular sprouting during development	[27]
Vasopressin V_{1A} receptor ($V_{1A}R$)	$G_{q/11}$	Overexpression in HEK293 cells	Hypotonicity	?	[14]

mechanisms such as regulation of vessel tone or cardiac hypertrophy. Among them are the following GPCRs: the apelin receptor [12, 28], the sphingosine 1-phosphate receptor (S_1PR) [27], the parathyroid hormone 1 receptor (PTH_1R) [26], the dopamine D_5 receptor [29], the angiotensin II AT_1 receptor (AT_1R) [13–16, 18, 19, 30], the GPR68 [24], the cysteinyl leukotriene 1 receptor ($CysLT_1R$) [16], the bradykinin B_2 receptor (B_2R) [20], the formyl peptide receptor 1 [23], the H_1R [11, 14, 25], and a mechanosensitive B_2R/AT_1R dimer [21]. For some mechanosensitive GPCRs like the endothelin ET_A receptor, the muscarinic M_5 receptor, the vasopressin V_{1A} receptor [14], the dopamine D_2 receptor (D_2R), and the adenosine A_{2A} receptor ($A_{2A}R$) [11], a physiological or pathophysiological role has not been identified yet. Furthermore, another class of receptors, the adhesion GPCRs (aGPCRs), are also discussed as potential mechanosensors that are involved in proprioception of the fruit fly *Drosophila melanogaster* [31–33], in mechanically induced skeletal muscle hypertrophy [34], and in mechanically induced myelination [35].

Although there is growing evidence that GPCRs are mechanosensitive, the exact mechanism of mechanically induced GPCR activation remains largely obscure until now. Up to now, it is not even clear how mechanical forces cause activation of mechanosensitive transmembrane proteins. Currently, three models for mechanosensation of transmembrane proteins originally developed to explain the activation mechanism of mechanosensitive ion channel are discussed [36, 37]: First, the membrane model, which is based on the assumption that mechanical stimulation causes changes in the lateral pressure profile of the phospholipid bilayer [38, 39] resulting in conformational changes of integral membrane proteins without the contribution of the cytoskeleton or the extracellular matrix. Hereby, mechanically induced changes of the membrane tension might affect the flexible structures of the integral membrane proteins, thereby resulting in stabilization of active protein conformations [39, 40]. The membrane model is probably valid for intrinsically mechanosensitive bacterial ion channels that are mechanosensitive even after reconstitution in artificial bilayers [41]. Further studies will have to show whether this model is also applicable to intrinsically mechanosensitive GPCRs.

Second, the tethered model applies to the mechanosensitive degenerin/epithelial sodium channel (DEG/ENaC) multiprotein ion channel complex identified in the worm *Caenorhabditis elegans* [42–45]. The ion channel complex is tethered to the extracellular matrix as well as to the cytoskeleton [46] such that mechanically induced membrane stretch decompresses the protein complex resulting in opening of the channel pore. This model is suitable to characterize intrinsically mechanosensitive proteins, too. Eventually, such aGPCRs might also represent candidates for the tethered model of mechanosensation since their long N-terminus is tethered to the extracellular matrix and the C-terminus is tethered to the cytoskeleton [33]. Notably, aGPCRs possess an extraordinarily long extracellular domain and an additional autoproteolysis inducing domain, which allows for cleavage of an N-terminal receptor fragment.

The third model highlights indirect mechanosensitivity based on the concept that the integral membrane protein itself is not intrinsically mechanosensitive. Instead, the membrane protein is activated via a signalling pathway that is induced by

mechanical stimulation (mechano-biochemical conversion). Because of the additional interconnected signalling pathway, the kinetics of this indirect activation is expected to be slower than the kinetics of the direct activation [9, 10]. The indirect model might apply e.g. for TRPC6 channels in the vasculature [9, 14, 47] and P_2X_4 channels in podocytes [48]. These channels rather represent mechanotransducers than mechanosensors.

The structural basis for mechanosensation by GPCRs is still largely elusive. Interestingly, applying a cysteine accessibility assay on AT_1R, the first evidence was obtained that mechanical forces might elicit active receptor conformations that are distinct from agonist-induced receptor conformations [19]. By applying the method of dynamic fluorescence resonance energy transfer (FRET), we were recently able to monitor conformational changes of mechanosensitive H_1Rs in real time confirming distinct mechanically induced receptor conformations.

Altogether, mechanosensitive GPCRs play essential roles in physiological settings like vascular regulation [13–16, 49], and they critically contribute to pathophysiological processes [18, 21]. Thus, elucidation of the structure–function relationship of mechanosensitive GPCRs might represent a first significant step towards a deeper understanding aiming at improved pharmacotherapy.

5.2 Mechanosensitive Proteins Discussed as Mechanosensors in Blood Vessels

Blood vessels can perceive mechanical forces and convert them into biological responses. Mainly two different mechanical stimuli occur in blood vessels: intravascular blood pressure and blood flow [50]. Increased intravascular blood pressure results in stretching of the vessel wall, which has an impact both on endothelial and on vascular smooth muscle cells. In vascular smooth muscle cells, the cell membrane, the cytoskeleton, and the contractile apparatus that are structurally and functionally associated are all distorted by vessel stretch [51]. Membrane stretch leads to activation of mechanosensitive vascular smooth muscle cells followed by massive calcium influx resulting in smooth muscle cell contraction and subsequent vasoconstriction [52]. This autoregulatory mechanism of small resistance arteries and arterioles is also known as myogenic vasoconstriction or Bayliss effect named after its discoverer Sir William Maddock Bayliss [53]. This mechanism is essential for keeping the capillary perfusion of organs and tissues constant. Notably, myogenic vasoconstriction is independent of the endothelium and agonist stimulation. Besides blood pressure, blood flow can also exert mechanical forces on blood vessels. Blood flow causes fluid shear stress that solely activates endothelial cells which finally results in flow-induced vasodilation [54–57]. This second autoregulatory mechanism is essential for an adequate increase of vessel perfusion, e.g. during physical exercise, and it is frequently found disturbed in cases of endothelial damage like in the context of diabetes mellitus and arteriosclerosis (endothelial dysfunction) [58]. Until now, at the protein level, the mechanosensors in vascular smooth muscle and endothelial cells have not been fully identified.

In vascular smooth muscle cells, various proteins were suggested as potential mechanosensors. Among them are intracellular membrane-associated proteins like dense bands [59] composed of vinculin and α-actinin [60] that can stabilize smooth muscle cells and the intracellular structural protein desmin that influences the rigidity of smooth muscle cells, thereby stabilizing the vascular wall [61]. Furthermore, the actin cytoskeleton itself can affect myogenic vasoconstriction [62–64] by regulating the plasticity of small resistance arteries in response to different mechanical stimuli [65]. Integrins, which are heterodimeric transmembrane receptors important for cell-extracellular matrix adhesion and mediating cellular signals like the organization of the cytoskeleton, were also discussed as direct mechanosensors [66] that contribute to myogenic tone [67–70]. However, until now, it is not clear whether integrins represent mechanosensors rather than mechanotransducers. In addition, tyrosine kinases of the Src family were suggested as mechanosensors [71] or as mechanotransducers [72, 73]. Furthermore, membrane-associated enzymes like phospholipase A2 [74, 75] and phospholipase C [76–80] contribute to mechanosensation. However, while phospholipase C should rather be classified as a mechanotransducer [14], the role of phospholipase A2 is not well defined yet [81].

Moreover, various ion channels have been suggested as mechanosensors or mechanotransducers in the vascular system. Among them are chloride channels [82–86] as well as cation channels of the epithelial sodium channel (ENaC) family [87–92], TREK-1, a member of the two pore-domain background potassium channels [93–95], and inwardly rectifying potassium (Kir) channels [96]. In addition, Piezo1 and Piezo2 channels that were characterized as intrinsically mechanosensitive cation channels [97, 98], which are activated by membrane stretch, by cell deformation through poking, and by fluid shear stress [99, 100] might serve as direct mechanosensors in the vasculature. Piezo1 is involved in vascular development [101, 102] and in hypertension-dependent arterial remodelling [103]. However, there is no experimental evidence yet to support the concept that Piezo1 is intrinsically involved in myogenic vasoconstriction [103]. Three members of the TRP channel family, namely the smooth muscle *transient receptor potential classical* channel TRPC6 [104], the smooth muscle *transient receptor potential melastatin* channel TRPM4 [105, 106], and the endothelial *transient receptor potential vanilloid* channel TRPV4 [107–111], are also involved in mechanosensing and/or mechanotransduction in blood vessels. It is still a matter of debate whether TRPC6 channels should be regarded as direct [81, 104, 112–114] or indirect mechanosensors [14, 115]. Mederos y Schnitzler et al. [14] applied a classical approach by performing single-channel measurements on membrane patches using suction as a mechanical stimulus. Physiological membrane stretch did not result in TRPC6 channel activation, suggesting that TRPC6 is not directly mechanosensitive. These findings were recently confirmed by a study demonstrating that after reconstitution in artificial bilayers TRPC6 channels are not mechanosensitive [47]. These findings strongly point to an indirect mechanosensitivity of TRPC6. Moreover, TRPC6 channels could restore mechanoresponse of a touch-insensitive *Caenorhabditis elegans* mutant, but this effect depended on the second messenger diacylglycerol (DAG) additionally pointing to indirect mechanosensitivity [47]. In

vascular smooth muscle cells, TRPC6 channels represent mechanotransducers that are activated downstream of $G_{q/11}$ protein-coupled receptors [14]. In these cells, stretch-induced GPCR activation causes activation of phospholipase C resulting in cleavage of phosphoinositide-4,5-bisphosphate into the two second messengers inositol trisphosphate and DAG. DAG in turn activates TRPC6 channels which leads to membrane depolarization, activation of voltage-gated calcium channels, and massive calcium influx which causes smooth muscle cell contraction and results in myogenic vasoconstriction. Hereby, TRPC6 channels play an important role as mechanotransducers, while the $G_{q/11}$ protein-coupled AT_1R and $CysLT_1R$ were identified as potential mechanosensors [13, 14, 16, 49].

Flow-induced vasodilation is another essential autoregulatory mechanism of blood vessels [54–57] that enables increased vessel perfusion. Fluid shear stress elicited by blood flow results in activation of the endothelial nitric oxide synthase (eNOS) which causes nitric oxide (NO) release and leads to flow-induced vasodilation. Notably, eNOS activation can be calcium-dependent [116, 117] or calcium-independent [118, 119]. It is commonly accepted that an increase in the intracellular free calcium concentration in endothelial cells leads to a Ca^{2+}/calmodulin-dependent activation of the eNOS [120] resulting in NO release and subsequent vasodilation [121–123]. However, shear stress can also directly activate shear stress-sensitive protein kinases, which results in phosphorylation of eNOS at specific amino acids leading to activation or inactivation of eNOS according to the respective phosphorylation site [124, 125]. Moreover, shear stress can even influence the eNOS expression levels by activation of different transcription factors [126]. Although it is commonly accepted that endothelial cells are sensitive to shear stress, the mechanisms underlying endothelial mechanosensation have remained largely elusive.

Several proteins have been discussed as potential mechanosensors or mechanotransducers in endothelial cells [127]. Among them are junctional proteins like platelet endothelial cell adhesion molecule-1 (PECAM-1), vascular endothelial cadherin (VE-cadherin), and VEGF receptors. It is well documented that shear stress causes phosphorylation of PECAM-1 [128], suggesting that endothelial PECAM-1 might rather serve as a mechanotransducer. Moreover, a mechanosensory complex comprising PECAM-1, VE-cadherin, and VEGF2 receptor that contributes to shear stress-induced endothelial signalling was described [129]. In addition, basal sensors such as integrins were discussed as potential endothelial mechanosensors [130, 131]. However, integrins may rather act as integrators of the shear stress-induced signalling pathway than as direct mechanosensors.

Other mechanosensitive endothelial proteins appear to be localized in primary cilia [22, 132–135], which may sense blood flow thereby amplifying the shear stress signal, and the glycocalyx [136–138], which dampens the force of shear stress reaching the plasma membrane [139]. Furthermore, receptor tyrosine kinases might be involved in endothelial mechanosensation [140] as well as caveolae [131, 141, 142] that might reduce the effect of fluid shear stress acting on transmembrane receptors [143].

Ion channels like the potassium inwardly rectifying channel Kir2.1 [96], which is sensitive to shear stress when expressed in *Xenopus laevis* oocytes [144], and the TRPV4 cation channel that is sensitive to hypoosmotically induced membrane stretch [110] as well as to fluid shear stress [107, 108, 145–147] were also suggested as endothelial mechanosensors. However, since mechanically induced TRPV4 activation requires phospholipase A2 activity, TRPV4 channels might rather serve as mechanotransducers than as direct mechanosensors. The mechanosensitive Piezo1 and Piezo2 channels are also involved in mechanosensation of endothelial cells. The role of Piezo2 for endothelial mechanosensation has remained largely elusive until now. However, there is first evidence that Piezo1 in combination with Piezo2 might mediate aortic baroreceptor pressure sensing [148]. Nevertheless, this hypothesis was recently questioned [149]. Still, Piezo2 might be involved in the sensation of endothelium-dependent pain [150]. Especially for Piezo1 channels, there is a substantial body of data suggesting that this channel is involved in endothelial flow sensing [101, 102, 151–154]. Piezo1 channels are known to essentially contribute to blood vessel maturation during development [101, 102], angiogenesis [101, 155], and blood flow recovery after hind limb ischemia [155]. In addition, Piezo1 channels were recently identified as endothelial sensors of shear stress that cause ATP and adrenomedullin release which results in GPCR and subsequent eNOS activation leading to NO production thus resulting in flow-induced vasodilation [154, 156]. In this case, Piezo1 might serve as a mechanosensor and the GPCR as a mechanotransducer. Moreover, Piezo1 channels were still mechanosensitive after reconstitution in artificial bilayers [98] strongly pointing to inherent mechanosensitivity. However, the role of Piezo1 is still a matter of debate. It was recently reported that Piezo1 is activated downstream of a mechanosensitive multiprotein complex consisting of GPCRs, the $G\alpha_{q/11}$ protein, and PECAM-1 [157]. Hereby, fluid shear stress might cause activation of the GPCRs/$G\alpha_{q/11}$/PECAM-1 complex resulting in G protein activation and dissociation of the Gβγ subunit to Piezo1 channels which in the end causes channel opening [157]. This signalling pathway downstream of GPCR activation would be analogous to the signalling pathway leading to G protein-mediated potassium inwardly rectifying (Kir) channel activation, which requires $G_{i/o}$ protein activation and subsequent Gβγ interaction with the channel resulting in channel opening.

Altogether, several proteins contribute to mechanosensation or -transduction of endothelial and smooth muscle cells, and there is growing evidence that endothelial Piezo channels represent key components that are involved in flow-induced vasodilation.

5.3 Mechanosensitive GPCRs Essentially Contribute to Vascular Mechanosensing

Until now, several GPCRs were characterized as mechanosensors mediating vascular responses towards mechanical forces. The first GPCR identified as a direct mechanosensor is the AT_1R [18]. The intrinsic mechanosensitivity of AT_1R is

well established and was identified in the heterologous overexpression system [14, 17–19], in the heart, where AT_1R is involved in the development of load-induced cardiac hypertrophy [17, 18], and in the vasculature, where AT_1R contributes to myogenic vasoconstriction [13–15]. Interestingly, one isoform of the AT_1R, the AT_{1B} receptor, which is highly expressed in small resistance arteries of rodents, is a key component for mechanosensing by vascular smooth muscle cells and is essentially involved in myogenic vasoconstriction [13, 15]. In mesenteric resistance arteries of adult mice, pharmacological blockade of the mechanosensitive $CysLT_1R$ resulted in a reduction of the myogenic tone of about 39%, while blockade of the AT_1R reduced myogenic tone by about 27%. Blockade of both receptors together even had an additive effect and resulted in a reduction of the myogenic tone of about 64%, which was similar to the blockade of the complete $G_{q/11}$ protein signalling pathway applying the selective $G_{q/11}$ protein inhibitor YM-254890 [16, 49]. These findings suggest that $G_{q/11}$ protein-coupled receptors contribute to myogenic vasoconstriction by about 64%. Consequently, other mechanosensitive signalling pathways still contribute by about 36% to myogenic tone. Notably, mechanically induced AT_1R and $CysLT_1R$ activations were agonist-independent [13, 14, 16, 18]. However, during mechanical stimulation AT_1Rs showed sensitization towards agonist stimulation [14], indicating that under physiological conditions in the presence of the endogenous agonists, mechanosensitive GPCRs might show increased activity. This kind of sensitization towards agonist stimulation may be a new mechanism of fine-tuning of vascular reactivity.

In addition, GPCRs represent mechanosensors in the endothelium. In particular, endothelial S_1PRs [27] were suggested as mechanosensors that suppress shear stress-induced vascular hypersprouting. Notably, shear stress-induced receptor activation was agonist-independent pointing to a direct mechanosensitivity of S_1PR. Moreover, the B_2R [20] was identified as an endothelial sensor of shear stress applying the method of intramolecular dynamic FRET. B_2R-FRET constructs transiently expressed in endothelial cells responded to mechanical stimulation with conformational changes. Another candidate for a direct endothelial mechanosensor is GPR68 [24] which was identified as a shear stress sensor mediating flow-induced vasodilation in small resistance arteries. Recently, we identified the highly mechanosensitive H_1R [14] as an endothelial mechanosensor. H_1R that is highly expressed in endothelial cells is sensitive to fluid shear stress, which leads to G protein activation, an increase of the intracellular free Ca^{2+} concentration, NO production, $G_{q/11}$ protein activation, and subsequent vasodilation [11]. Pharmacological tools like an application of inverse agonists as well as genetic tools like global knockout of the H_1R and smooth muscle-specific, inducible knockdown of the $G_{q/11}$ protein largely abolished flow-induced vasodilation [11]. However, H_1Rs endogenously expressed in endothelial cells were not only sensitive to shear stress but also to hypoosmotically induced membrane stretch.

As stated above, conflicting data suggest that GPCRs might serve as mechanotransducers activated downstream of Piezo1 [154, 156]. However, opposing findings propose that Piezo1 is activated downstream of a mechanically induced GPCR/$G_{q/11}$ protein signalling pathway [157]. Furthermore, in endothelial cells, a

$G\alpha_{q/11}$ protein/PECAM-1 complex was identified that dissociates after application of shear stress [157, 158]. In this complex, the G protein itself might serve as a direct mechanosensor since $G\alpha_{q/11}$ proteins were still sensitive to fluid shear stress after reconstitution in phospholipid vesicles in the absence of GPCRs [159]. Notably, the role of GPCRs for mechanosensation critically depends on their expression levels, and in some tissues e.g. in podocytes, GPCRs are not critically involved in mechanosensing or mechanotransduction [48].

Altogether, autoregulation of blood vessels is of such importance that several different signalling pathways may have evolved that work together in sensing blood flow and blood pressure and converting mechanical forces into cellular signals, thereby enabling adequate vessel perfusion [127]. Endothelial and smooth muscle cells mediate opposing responses to mechanical forces promoting vasodilation in response to blood flow and vasoconstriction in response to blood pressure. During the last few decades, a sizable portfolio of different proteins was identified that contribute to mechanosensing or mechanotransduction in smooth muscle and endothelial cells [127]. However, it should be taken into account that in different vessel beds different mechanosensitive signalling pathways have developed and that the expression levels of mechanosensitive proteins differ among cells, tissues, and organs, thereby fine-tuning mechanosensing. To summarize, there is growing evidence that mechanosensitive GPCRs represent promising candidates for direct mechanosensors in blood vessels that are activated by mechanical forces independently of their endogenous agonists, thereby contributing to autoregulation of the vessel tone.

5.4 Agonist and Mechanical Stimulation Induce Distinct Active Receptor Conformations

It is well known that GPCRs possess seven transmembrane domains and that many but not all GPCRs exhibit an additional C-terminal eighth α-helical structure (helix 8; H8) that is situated parallel to the cell membrane. Recent advances in the structural analysis of GPCRs showed that GPCRs adopt distinct active and inactive receptor conformations. In particular, agonist stimulation mainly causes a movement of the transmembrane domain (TM) 6, which results in G protein activation and subsequent cellular signalling [160]. To characterize the conformational changes of GPCRs in response to mechanical stress, a substituted cysteinyl accessibility mapping of the AT_1R was performed suggesting that mechanical stimulation mainly causes a movement of TM7 [19]. However, structural analysis and substituted cysteinyl accessibility mapping only allow for static information, and data regarding the kinetics of conformational changes are missing.

To monitor the dynamics of conformational changes of GPCRs, the technique of intramolecular FRET is well suitable [161–166]. Employing that method, we recently analysed whether agonist and mechanical stimulations foster distinct receptor conformations using the highly mechanosensitive H_1R [14]. For this, three H_1R-FRET constructs were deployed with a C-terminally attached cyan

fluorescence protein (cerulean) serving as a FRET donor. Moreover, small tetracysteine binding motifs for selective labelling with the fluorescein arsenical hairpin binder FlAsH which served as a FRET acceptor were inserted at different positions into the receptor: at the beginning of the third intracellular loop, at the end of the third intracellular loop, and at a proximal position of the C-terminus after TM7 and directly in front of the H8 [11] (see Fig. 5.1). Agonist stimulations with histamine of the H_1R-FRET constructs with the binding sites for FlAsH at the beginning and end of the third intracellular loop and mechanical stimulations of all three FRET constructs resulted in FRET signal decreases, suggesting that receptor activation results in conformational change corresponding to a movement of the fluorophores away from each other. The agonist-induced FRET signal decreases were substantially smaller than the mechanically induced FRET signals, strongly supporting the notion that agonist and mechanical stimulations foster distinct receptor conformations. Interestingly, the H_1R-FRET construct with the binding site for FlAsH at the beginning of the C-terminus just upstream of H8 showed the most pronounced FRET signal decreases in response to mechanical stimulation, but no agonist-induced FRET signal changes confirming the occurrence of stimulus-dependent active receptor conformations (see Fig. 5.1a). Similar results were observed by analysing the G_s protein-coupled adenosine A_{2A} receptor (A_{2A}R). Using an A_{2A}R-FRET construct with a FlAsH binding site at the end of the third intracellular loop [162], higher mechanically than agonist-induced FRET signal decreases were monitored [11] in line with the findings analysing the H_1R-FRET construct with the binding site for FlAsH at the corresponding position in the intracellular loop. These findings suggest that A_{2A}Rs are also mechanosensitive.

The highest mechanically induced FRET signal decreases were detected employing the FRET construct that possesses two fluorophores encompassing the H8. Thus, it can be speculated that mechanical stimulation might cause elongation of H8 (see Fig. 5.1b). However, a movement of TM7 cannot fully be excluded. An elongation of H8 would be compatible with the notion that α-helices can act as constant force springs that can unfold as the length of the helix increases and refold progressively as the length decreases [167]. Nevertheless, one should keep in mind that FRET signal changes can be elicited by fluorophore rotations as well. Therefore, further studies are needed to investigate mechanically induced elongations of H8 in more detail.

The kinetics of agonist-induced FRET signal changes of the H_1R was slightly slower than that of the muscarinic M3 receptor [164]. Notably, hypoosmotically induced FRET signal changes were slower ($\tau_{1/2} = 381 \pm 32$ ms) compared to agonist-induced FRET signal changes ($\tau_{1/2} = 251 \pm 19$ ms in isotonic bath solution with reduced NaCl concentration and $\tau_{1/2} = 148 \pm 7$ ms in physiological bath solution). However, hypoosmotically induced cell swelling cannot be regarded as a physiological stimulus since rapid decreases of tonicity are not common in the vascular system. Performing patch-clamp experiments in the whole-cell configuration, hypoosmotically induced cell swelling elicited similar effects as direct membrane stretch either induced by application of positive pressure through the patch pipette or by vertical movement of the patch pipette [14, 48]. Thus, hypoosmotically induced

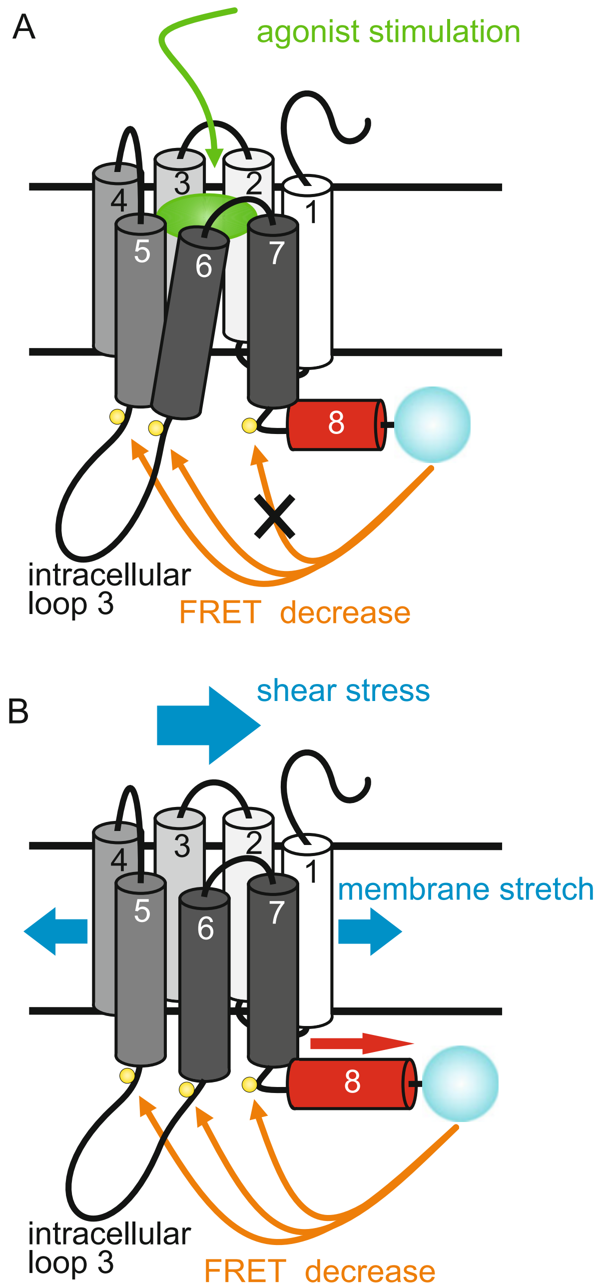

Fig. 5.1 Agonist and mechanical stimulation elicit distinct active receptor conformations. Schematic representation of H_1R-FRET constructs in their agonist-bound (**a**) or mechanically activated conformations (**b**). Transmembrane domains (TM) are numbered. The C-terminal helix 8 (H8) is displayed in red and labelled as 8. Black horizontal lines indicate the plasma membrane. The cyan ball indicates the C-terminally fused cyan fluorescent protein Cerulean. Yellow dots indicate the three binding sites for the yellow fluorescent dye FlAsH: at the beginning and the end of the third

membrane stretch was used as a fast screening method. Nevertheless, the use of hypotonicity has its limitations and the sugar alcohol mannitol that is often used to supplement the hypotonic solution to isotonic values might even have additional antioxidative side effects. Therefore, fluid shear stress was used as a more physiological stimulus. The applied shear stress in the range of 3–60 dyn cm^{-2} resulted in FRET signal decreases similar to the effects observed after applying hypoosmotically induced membrane stretch [11] (see Fig. 5.1b). There was even a correlation between the strength of the mechanical stimulus that was either induced by increased hypotonicity or by fluid shear stress and of the FRET signal, which is an important criterion for the characterization of directly mechanosensitive proteins [10, 43]. Under physiological conditions, veins and venules are usually exposed to fluid shear stress in the range of 1–29 dyn cm^{-2} [168, 169]. While conduit arteries receive shear stress of about 10 dyn cm^{-2}, arterioles are usually exposed to higher shear stress up to 70 dyn cm^{-2} [169]. Thus, shear stress of 1–70 dyn cm^{-2} can be regarded as a physiological stimulus.

Until now, the exact mechanism of mechanically induced GPCR activation is not fully understood, and it is still elusive how different mechanical stimuli can influence integral membrane proteins. Interestingly, there is first evidence that membrane stretch and shear stress can both affect the lipid order of the plasma membrane [170, 171]. Yamamoto and Ando showed that shear stress increases membrane fluidity [170]. Moreover, it was reported that membrane stretch changes the lateral pressure profile of the plasma membrane [38]. Thus, it is likely that alterations of the phospholipid bilayer by mechanical stress might allow integral membrane proteins like GPCRs to adopt an active conformation resulting in G protein activation and subsequent cellular signalling. These considerations are suggestive of the membrane model to be valid for explaining the mechanosensitivity of GPCRs.

Interestingly, in the overexpression system the mechanically induced FRET signal changes were significantly but not fully suppressed by application of selective inverse agonists, while intracellular calcium increases were nearly fully abolished [11]. These findings suggest that FRET signals monitored in the presence of inverse agonists reflect inactive receptor conformations. Moreover, mechanically induced H_1R activation was agonist-independent [11] since H_1R mutants with a disrupted histamine binding site by select amino acid exchanges were still mechanosensitive which is in line with what was previously reported for the AT_1R [13, 14] and the $CysLT_1R$ [16].

Fig. 5.1 (continued) intracellular loop and the beginning of the C-terminus just before H8. (**a**) The agonist is depicted in green. Agonist binding causes a movement of the TM 6 which was monitored as FRET signal decreases analysing the FRET constructs with the binding sites for FlAsH located in the third intracellular loop. No FRET signal changes were detected upon agonist stimulation using the FRET construct with the binding site for FlAsH at the beginning of the C-terminus. (**b**) Mechanical stimulations by membrane stretch or shear stress are displayed as blue arrows. Mechanical stimulation causes FRET signal decreases analysing all three FRET constructs. Mechanical stimulation mainly causes elongation of H8

Altogether, there is compelling evidence suggesting that GPCRs are intrinsically mechanosensitive and that mechanical and agonist stimulations evoke distinct active receptor conformations. Mechanical stimulation of GPCRs leads to conformational changes that might result in an elongation of H8, while agonist stimulation mainly results in movement of TM6 (summarized in Fig. 5.1). However, both stimuli cause G protein activation and result in subsequent signalling.

5.5 Helix 8 Is the Essential Structural Element Conferring GPCRs with Mechanosensitivity

Until now, the role of H8 for receptor function is not fully understood. There is first evidence that H8 might be involved in protein/lipid interaction, receptor internalization, receptor dimerization, or coupling to G proteins (summarized in [172]). However, as mentioned above, mechanical GPCR stimulation might involve conformational changes of H8. Nevertheless, the role of H8 for mechanosensing remained largely elusive. Interestingly, structural analysis of mechanosensitive GPCRs like the H_1R [173], the AT_1R [174–176], the M_5R, the $A_{2A}R$ [177], the D_2R [178], or the $CysLT_1R$ [179] revealed that these receptors possess a H8 as a unifying structural element. Receptors like the human neurotensin 1 receptor (NTS1R) [180, 181] and the human gonadotropin-releasing hormone receptor (GnRHR), which lack a H8, were insensitive to mechanical stimulation. In the case of the GnRHR, the C-terminus is extremely short and only comprises three amino acids. Structural analysis of the human CXCR4 [182] showed that this receptor does not possess a complete H8 but exhibits only one α-helical loop. There are two different porcine GnRHR isoforms: a short isoform, which lacks H8, and a long isoform that was predicted to possess a H8. While the short isoform was not mechanosensitive similar to the human GnRHR, the long isoform turned out to be mechanosensitive [11]. These findings suggest that GPCRs containing a H8 are mechanosensitive, while GPCRs lacking a H8 are insensitive to mechanical forces. Moreover, the transfer of H8 conferred mechanosensitivity, while truncation of H8 rendered the receptor non-mechanosensitive [11]. Besides, disruption of the α-helical structure of H8 by select amino acid exchanges from phenylalanine or tyrosine to proline likewise impaired mechanosensitivity of GPCRs which was shown for the H_1R, the AT_1R, and the $A_{2A}R$ [11].

To summarize, H8 emerged as the essential structural motif that endows GPCRs with mechanosensitivity. The presence of an intact H8 is a pivotal prerequisite for mechanosensation of $G_{q/11}$, $G_{i/o}$, and G_s protein-coupled receptors.

5.6 Conclusion

There is growing evidence that GPCRs represent mechanosensors in the vascular system essentially contributing to the autoregulation of vessel tone. An overview of the potential mechanosensitive GPCRs that are involved in the regulation of vessel

tone is given in Table 5.2. Recent findings suggest that the endothelial H_1R [11] and the GPR68 [24] are activated agonist independently by fluid shear stress that is induced by the blood flow, thereby contributing to flow-induced vasodilation. It can be speculated that mechanical stimulations cause changes of the lipid order in the phospholipid bilayer resulting in stabilization of a distinct active receptor conformation that comprises elongation of the C-terminal H8, which in the end allows for G protein activation, an increase of the intracellular free calcium concentration, NO production, and subsequent vasodilation. In addition, smooth muscle AT_1R and $CysLT_1R$ [13–16] emerged as sensors of blood pressure. Increased intravascular blood pressure increases the wall tension of the blood vessel resulting in membrane stretch, which might change the lateral pressure profile of the phospholipid bilayer, thereby allowing for stabilization of an active receptor conformation characterized by H8 elongation, G protein activation, increase of the intracellular free calcium concentration, and subsequent myogenic vasoconstriction. Altogether, agonist-independent GPCR activation by different mechanical stimuli significantly contributes to the autoregulation of vessel tone. This concept is depicted in Fig. 5.2.

As already mentioned, the application of inverse agonists precluded the mechanically induced GPCR activation. Among the mechanosensitive GPCRs, AT_1Rs are the best-examined receptors. There is compelling evidence that AT_1Rs are involved in several physiological and pathophysiological states such as myogenic vasoconstriction [13–16], load-induced cardiac hypertrophy [18], and preeclampsia [21]. Notably, AT_1R that also requires an intact H8 for mechanosensation already represents a target for commonly used drugs against hypertension, diabetic nephropathy, and congestive heart failure. Our findings suggest that AT_1R blockers do not only impair agonist- but also mechanically induced signalling. However, in our hands, higher drug concentrations were needed to block the mechanically induced compared to the agonist-induced responses [11, 13, 14, 16].

H_1R antagonists or inverse agonists that are frequently used as antihistaminic drugs abolished mechanically induced receptor activation and hence flow-induced vasodilation [11]. Thus, it can be speculated that antihistaminic drugs might cause blood pressure dysregulation. However, while first-generation antihistaminic drugs caused dizziness and orthostatic hypotension due to their anti-α-adrenergic adverse side effects, an impact of the more selective second-generation antihistaminic drugs on blood pressure is not documented yet. A reason for this might be the fact that antihistaminic drugs cannot initiate active vasoconstrictions. Another reason might be the variety of other mechanosensitive signalling pathways that act together to keep vessel tone and blood pressure constant so that dysregulation of the vessel tone by antihistaminic drugs might be masked. Moreover, signalling pathways might vary between the vessel beds depending on variable protein expression levels. However, antihistaminic drugs that are taken prior to sportive activities significantly reduced post-exercise hypotension [183] and blood flow [184], suggesting that in this particular case, flow-induced vasodilation is indeed impaired.

Altogether, a better understanding of the molecular mechanism of mechanosensing by GPCRs and the characterization of H8 as the essential structural motif affording GPCRs with mechanosensitivity sheds new light on the field of

Table 5.2 Mechanosensitive GPCRs that contribute to regulation of vessel tone

Potential function for regulation of vessel tone	Stimulus	Receptor	Coupling	Cell type	Vascular bed	References
Myogenic vasoconstriction	Blood pressure/ membrane stretch	AT_1R	$G_{q/11}$	Murine and rat cerebral, murine renal, and murine mesenteric artery vascular smooth muscle cell	Third- and fourth-order branches of murine resistance arteries of the brain, kidney, mesentery	[13–16]
		$CysLT_1R$	$G_{q/11}$	Murine renal and mesenteric artery vascular smooth muscle cells	Third- and fourth-order branches of murine resistance arteries of the mesentery	[16]
Flow-induced vasodilation	Blood flow/ shear stress	GPR68	$G_{q/11}/G_s$	Murine mesenteric artery endothelial cells	First-, second-, and third-order branches of murine mesenteric arteries	[24]
		H_1R	$G_{q/11}$	Murine mesenteric artery endothelial cells	First- and second-order branches of murine mesenteric arteries	[11]

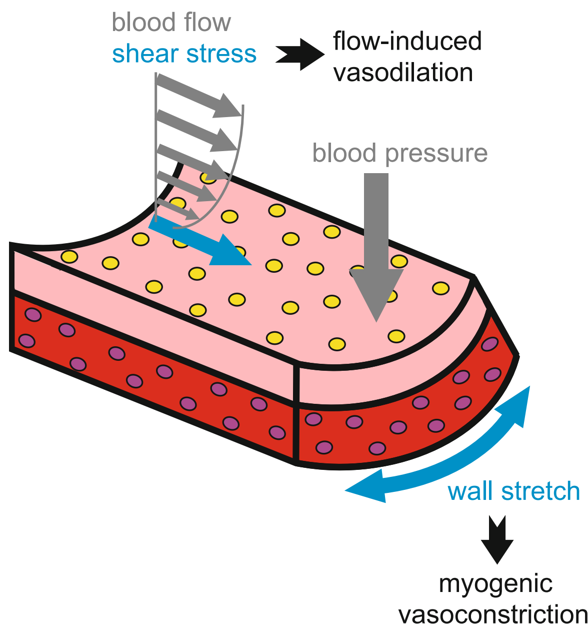

Fig. 5.2 Mechanosensitive GPCRs are involved in mechanosensing by endothelial and smooth muscle cells. The schematic represents two different mechanical forces (shear stress induced by the blood flow and wall stretch induced by blood pressure) impacting on an arterial wall section. The endothelium is displayed in pink and the vascular smooth muscle in red. Endothelial GPCRs that are sensitive to shear stress such as H_1R, B_2R, S_1PR, D_5R, GPR68, and apelin receptor are depicted as yellow dots. Vascular smooth muscle GPCRs that are sensitive to increased intravascular blood pressure such as AT_1R and $CysLT_1R$ are displayed as violet dots. Intravascular pressure causes wall stretch leading to membrane stretch of smooth muscle cells. While activation of the endothelial GPCRs by shear stress results in flow-induced vasodilation, the activation of the smooth muscle GPCRs by increased blood pressure leads to myogenic vasoconstriction (Bayliss effect)

GPCR research and offers novel insights into the structure–function relationship of GPCRs, which might help further improve medical treatment of mechanically induced diseases such as hypertensive nephropathy, hypertensive cardiomyopathy, hypertensive retinopathy, hypertensive encephalopathy, pulmonary hypertension, or preeclampsia.

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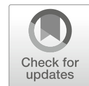

Hemodynamic Control of Endothelial Cell Fates in Development

6

Hanna M. Peacock, Margo Daems, and Elizabeth A. V. Jones

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Abstract

The vascular system initially forms as a random network of capillaries, which remodels under the influence of flow into efficient, hierarchical vasculature. Blood flow determines the final expression pattern of arteriovenous markers and guides the pruning of excess vasculature, including the restructuring of the six pairs of aortic arches. Changes in blood flow can overrule arterial, venous, and lymphatic fates, causing both morphological and genetic switching between identities. Flow patterns are essential for determining locations of sprouting, regression, fusion, and intussusception, as well as for guiding the formation of both vascular and cardiac valves. The force of blood flow is detected by various mechanosensors on the luminal cell surface, in cell–cell junctions, and in contact with the ECM. These mechanosensors activate multiple interacting signaling cascades which are understood to play important roles in vascular development and endothelial cell fate specification.

6.1 Introduction

The cardiovascular system is the first organ system to become fully functional during development. The proper working and organization of the heart and blood vessels is critical for growth of the embryo beyond the distance that oxygen can diffuse. Endothelial cells—which line the inner surface of all blood and lymphatic vessels in the body—are exposed to the physical forces of blood flow from early in development. On top of genetically programmed cues, these physical forces sculpt the chaotic array of growing vessels into an efficient, hierarchically organized transport system, perfusing every tissue in the body. During this process, endothelial cells take on roles as arterial, venous, capillary, or lymphatic endothelium. While each subtype of endothelial cells has its own morphological properties and genetic signature, there is a spectacular amount of plasticity in endothelial cell fate, which allows the vasculature to adapt dynamically to maintain optimal functioning.

6.2 Hemodynamics in Vascular Development

6.2.1 The Vasculature at the Onset of Flow

Vasculogenesis, the de novo differentiation of endothelial cells, is a largely genetically driven process which lays down a “first draft” of the vascular system. In mice, vasculogenesis is initiated around embryonic day (E)7, beginning in the blood

islands in the distal aspect of the yolk sac [1]. The blood islands are populated by VEGFR2+ mesodermal cells originating from the posterior primitive streak [2] (Fig. 6.1). These cells give rise to both primitive endothelial cells (angioblasts) and primitive red blood cells (erythroblasts) [3]. Following their differentiation, angioblasts migrate from the blood islands along the surface of the yolk sac to form a randomly organized, honeycomb-shaped network of vessels [4]. Angioblasts also coalesce along the embryonic midline, forming paired dorsal aortae that connect with the yolk sac vasculature [5].

The onset of blood flow acts as a profound signal to remodel the primitive vascular plexus into a functional and hierarchical vasculature. Blood flow has many roles, including delivery of oxygen and nutrients, distribution of growth factors, and removal of waste products, as well as imparting physical forces—shear stress and cyclic stretch—on the vasculature. Initially, the erythroblasts are

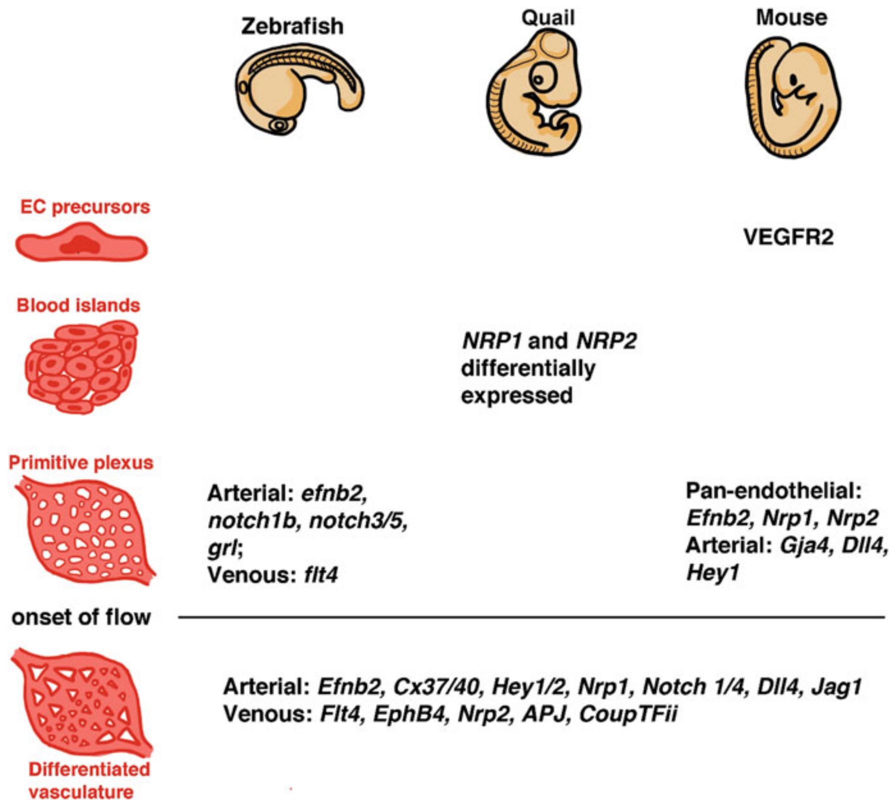

Fig. 6.1 Summary of arteriovenous gene expression during vascular development in zebrafish, avian, and mouse embryos before and after the onset of flow

confined to the blood islands and only low-viscosity blood plasma is flowing [6]. As the heart matures and becomes a more efficient pump, flow increases in velocity and the patterns of the flow changes [6]. Beginning at E8.5 (5–8 somites) in mice or 11 somites in chicks, the erythroblasts are swept up by the flowing plasma and leave the blood islands [6]. This causes a rapid change in the hematocrit level (percent red blood cells), significantly increasing the viscosity of the blood [7, 8]. Over the following day, the primary capillary plexus remodels into a hierarchal structure of arteries, veins, and capillaries [6, 9].

Before the onset of blood flow, distinct populations of endothelial cells have already begun to express specific arterial and venous markers (Fig. 6.1). Here, however, important differences exist between species. Zebrafish express a large range of arterial-specific genes in the presumptive arteries before the onset of flow (around 28 hpf, 35 somites), including EphrinB2 (*efnb2*), *notch1b* [10], *notch3/5* [11], and the zebrafish homologue of the Notch target gene *Hey2*, *gridlock* (*grl*) [12]. Furthermore venous marker, *flt4*, is already restricted to the posterior cardinal vein [11]. In the chick, blood island cells express either *NRP1* or *NRP2*, but not both [13, 14]. Similarly, in mouse, arterial-specific gene expression can be found before the onset of flow (at 5–6 somites); however, the number of genes identifying arteries is much more limited compared to other species [5]. EphrinB2 (*Efnb2*), *Nrp1*, and *Nrp2* are expressed before the onset of flow in a pan-vascular manner and only become restricted to the arterial vascular bed after flow. Only Cx37 (*Gja4*), *Dll4*, and *Hey1* are arterial specific before 5–6 somites [5]. No venous-specific markers are expressed before the onset of flow, as assessed by *in situ* hybridization, though faint expression of *EphB4*, *Nrp2*, and *Flt4* can be observed in the inflow tracts of the sinus venosus [5]. Despite the apparent genetic specification of arterial and venous endothelial cells during early differentiation, the vasculature retains a remarkable plasticity, allowing it to functionally adapt as required. This plasticity is critical for the development of organized vasculature consisting of arteries, capillaries, veins, and lymphatic vessels, as well as for the proper formation of lymphatic, venous, and cardiac valves.

6.2.2 Flow Induces Differentiation of Hierarchical Vasculature

In the absence of blood flow, the vasculature fails to remodel from a primary capillary plexus into morphologically differentiated arteries and veins. As such, mutations in cardiac-specific genes, causing specific heart development defects, also present with secondary vascular defects. For example, mutations in *Titin* result in defects in cardiomyocyte alignment, causing poor contractility of the heart and consequently a delayed onset of blood flow [15]. Secondary to this, the primary capillary plexus is retained, and the aorta does not become lumenized, thereby causing lethality by E11.5 [15]. Similarly, loss of the *Mlc2a* gene causes diminished

arterial contraction with a secondary impairment of vascular remodeling and is lethal at E10.5–11.5 [6, 16]. More bluntly, excision of the heart before the onset of flow halts remodeling of capillaries into arteries and veins in the yolk sac vasculature [17].

The mechanical forces created by blood flow are the key to its effects on vascular remodeling. Shear stress exerted on the vessel wall depends strongly on the blood viscosity, which is determined by the hematocrit [7, 18]. In mice with mutations in *RapGEF2* or *cMYC*, hematopoiesis is impaired such that red blood cells do not differentiate in the blood islands [19, 20]. Although the primary capillary plexus develops normally, in the absence of erythroblasts, remodeling fails to occur [19, 20]. The increase in blood viscosity, and thereby shear stress, is the crucial component of circulation for guiding remodeling. If erythroblasts are trapped in the blood islands of the yolk sac, so that only plasma is allowed to circulate, the yolk sac vasculature does not remodel [6] (Fig. 6.2). Restoring the blood viscosity by injecting hetastarch intravascularly is sufficient to rescue vascular remodeling even though erythroblasts are absent [6], demonstrating the profound role of the physical force of blood flow on the remodeling process.

6.2.3 Endothelial Cell Orientation and Active Vessel Regression

Vessels are generally formed in excess during vasculogenic processes. Unneeded vessels are subsequently pruned, where the selection of specific vessel segments that will regress is a flow-driven process [21]. Obstructing flow or reducing cardiac output is sufficient to induce ectopic pruning of a vessel segment, while increasing flow velocity maintains vessel segments which otherwise would have regressed [22]. Flow is such a powerful influencer of this process that computational analysis of hemodynamics in an area of vasculature can be used to predict locations of vessel regression: vessel segments exposed to low or variable shear stresses compared to the surrounding vasculature are likely to regress [22]. Thus, how many vessels regress is controlled by the need of the tissue, but which ones will regress is determined by flow.

Endothelial cells experiencing high flow are strongly polarized against the direction of flow (i.e., the Golgi apparatus is located upstream of the nucleus), while the axial polarity misaligns in vessels experiencing low flow, which are predicted to regress [23]. Endothelial cells of regressing segments extend filopodia and their Golgi apparatuses are polarized toward the base of the segment, indicating active migration out of the retracting vessel segment [23]. These endothelial cells migrate out of the regressing vessel toward the feeding vessel and are redeployed into higher flow segments of the vascular bed [21–23].

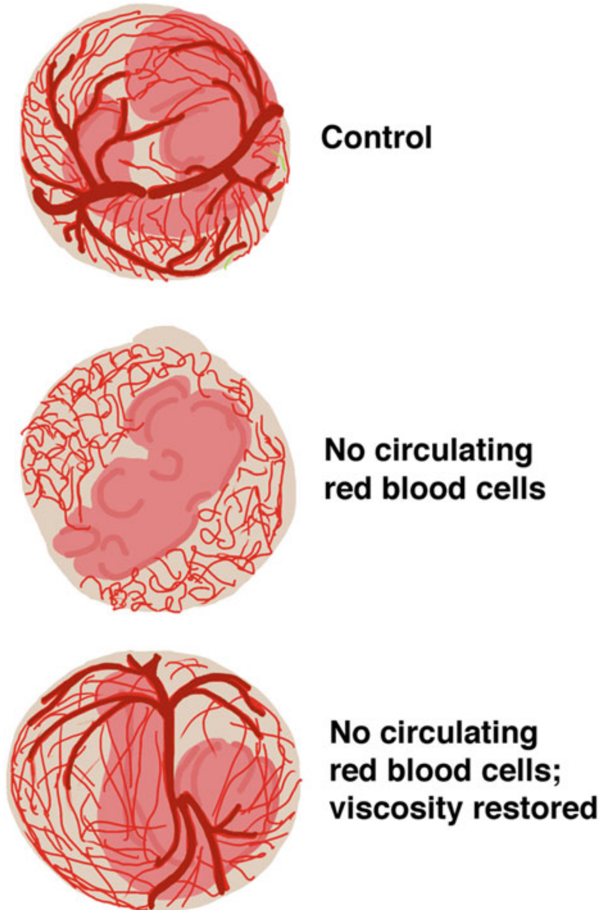

Fig. 6.2 Vascular remodeling in the mouse embryonic yolk sac depends on blood viscosity. In control embryos, the vascular plexus remodeled into hierarchically organized vasculature. When red blood cells were trapped in the blood islands, thereby lowering the blood viscosity, vascular remodeling did not occur. When red blood cells were trapped in the blood islands, but the blood viscosity was restored by injecting hetastarch, vascular remodeling was rescued. Based on [6]

6.2.4 Flow Shapes the Aortic Arch

The aortic arch and its initial branches are formed from the asymmetric reorganization of the six matching pairs of aortic arches (also known as pharyngeal or brachial arch arteries) [24, 25]. The decision of which aortic arches grow in diameter and which regress is a flow-driven process. Disruption of blood flow patterns in the heart, for example by ligation of the vitelline vein or left atrium, disrupts both cardiac morphology and aortic arch selection [26, 27]. In the chick embryo, Hamburger Hamilton stage 21 (HH21, 43–44 somites) is a moment of great individual variability in aortic arch architecture, which coincides with a moment of strong changes in cardiac output, due to the active looping of the heart [28, 29]. At HH21, the aortic arches experience high levels of shear stress, which are correlated to the changes in diameter of the different aortic arch branches [28]. Branches that experience high shear stress levels grow in diameter, and those under lower shear stress

shrink [28]. Further supporting this observation, decreasing the heart rate of mouse embryos is sufficient to induce inappropriate regression of aortic arches [30].

Cardiac looping breaks the symmetry in the cardiac output and is thus crucial for proper aortic arch selection. As the angle of the outflow tract changes with heart looping, flow is directed differently through the aortic arch arteries and the relative arch diameters shift accordingly [29, 31]. PITX2 is expressed in cells of the secondary heart field, which contribute to the left ventricle and outflow tract [30]. PITX2 is necessary for the rotation of the outflow tract, which directs flow to the left sixth aortic arch in mice [30]. In the absence of PITX2, the outflow tract fails to rotate and both sixth aortic arches receive equal blood flow. As a result, rather than forming a single aortic arch, both arches remain patent during development [30] (Fig. 6.3). PITX2 itself is not expressed in the vessels but only in the heart, indicating that changes in blood flow to the aortic arches, rather than gene expression in the vessels themselves, are the determining factor in this process.

6.3 Mechanosensors: Structures for Detecting Shear Stress

It is clear that blood flow, as mechanical force, has a profound influence on the structure of the developing vasculature. But for this mechanical force to be "understood" by endothelial cells, it needs to be translated—mechanotransduced—into molecular signals, which ultimately affect gene expression and direct cell fate. There have been many mechanosensors identified to date, some of which respond to flow within seconds, while others respond to long-term changes in flow intensity or direction [32]. Below, we detail a selection of mechanosensors that are active during embryonic development and which are speculated or shown to define early endothelial cell fates (Fig. 6.4).

6.3.1 Ion Channels

Ion channels are some of the first recognized mechanotransducers identified in endothelial cells [33]. They function by hyperpolarizing the endothelial cell membrane in response to mechanical stimuli, followed by rapid depolarization [34]. The velocity of this hyperpolarization in response to flow matches with the opening of an ion channel, supporting their role in mechanotransduction. Hyperpolarization is quickly followed by depolarization [34]. The early phase is driven by potassium (K^+) currents, whereas the latter is driven by chloride (Cl^-) currents [34]. Certain ions such as calcium (Ca^{2+}) activate cofactors for enzymes, thus driving downstream pathways. Additionally, Ca^{2+} opens Ca^{2+}-gated K^+ channels and Cl^- channels [35, 36] and therefore can be located upstream of hyperpolarization and depolarization events. Intracellular increases in Ca^{2+} occur rapidly after the onset of flow, but are transient and largely subside within 5 min [37].

The earliest identified endothelial mechanosensitive ion channel is the inward-rectifying K^+ channel Kir2.1, which is activated by shear stresses as low as 0.1 dyne/

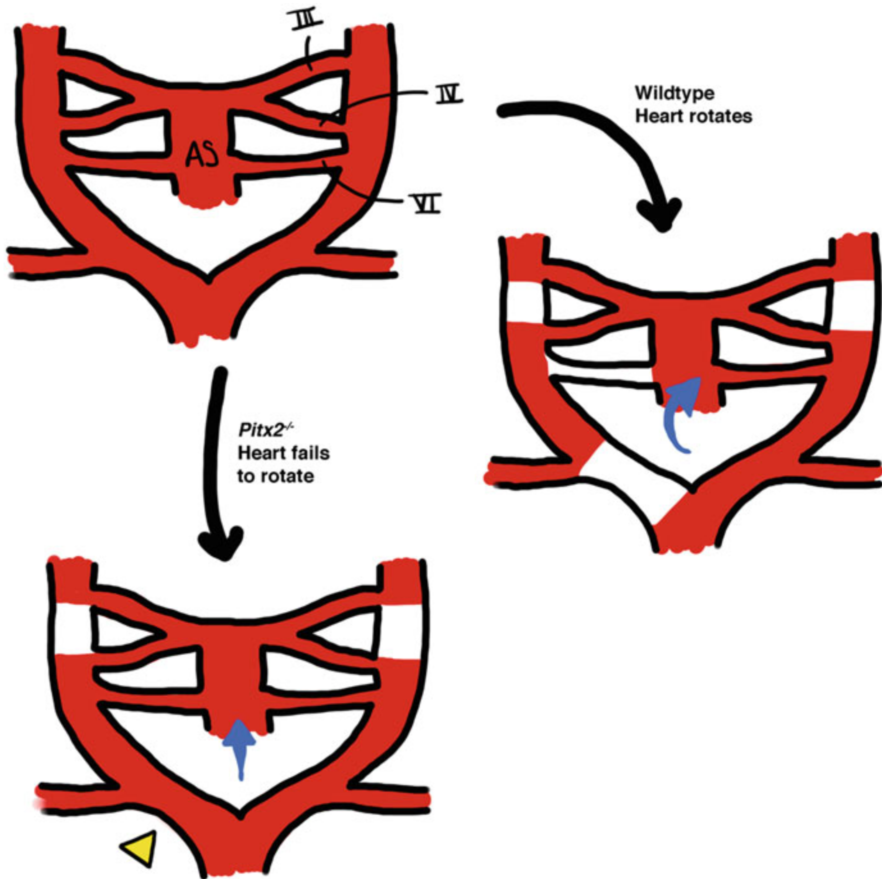

Fig. 6.3 Aortic arch selection depends on flow. In wild-type mice, the left sixth aortic arch regresses secondary to rotation of the heart directing flow through the right sixth arch. In Pitx2-knockout mice, when the heart fails to rotate, flow is directed through both sixth aortic arches equally and both remain patent. AS = aortic sac, III, IV, and VI indicate the third, fourth, and sixth aortic arches, respectively. Red indicates perfused vessels. White indicates regressed vessels. Based on [30]

cm^2 [33]. Mechanical activation of Kir2.1 induces a hyperpolarization of the cell membrane of which both duration and magnitude are dependent on shear stress exposure [33, 38]. Additionally, the Kir2.1 channel recovers fully after exposure to shear stress, without desensitization [33, 38]. Studies in mice demonstrate that the full knockout of *Kir2.1* dies perinatally due to a severe cleft palate phenotype. K^+ channel is important during retinal angiogenesis [39]. *Kir2.1*$^{-/+}$ retinas show an increased vessel density, vessel length, and number of branching points, accompanied by a decreased αSMA coverage [39]. Moreover, heterozygous loss of *Kir2.1* downregulates pruning of excess vessels, reduces alignment of endothelial

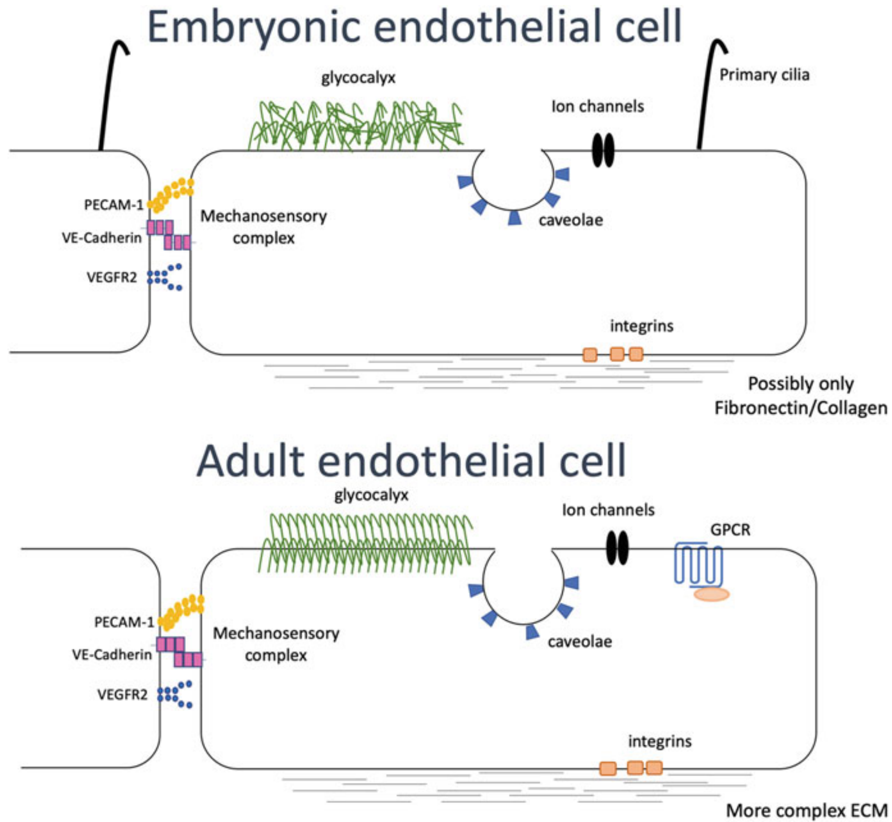

Fig. 6.4 Important mechanosensors in embryonic and adult endothelial cells. Both adult and embryonic endothelial cells share the junctional mechanosensory complex (VEGFR2, PECAM-1, VE-cadherin), caveolae, integrins, and ion channels. The glycocalyx on adult endothelial cells is more structurally organized than on embryonic endothelial cells. The basement membrane is more complex in adult than in embryo. The primary cilia play a prominent role sensing low shear stress but would be absent from larger vessels in the adult. *GPCR* G protein-coupled receptor, *PECAM-1* platelet and endothelial cell adhesion molecule 1, *VEGFR2* vascular endothelial growth factor receptor 2

cells in arteries, and delays vascular remodeling [39], all processes known to involve mechanotransduction.

The transient receptor potential vanilloid subtype 4 (TRPV4) Ca^{2+} channel is important for vessel dilation, as well as heart valve formation. TRPV4 opens in response to flow, resulting in an intracellular Ca^{2+} elevation that indirectly stimulates hyperpolarization of the cell membrane [40, 41]. TRPV4-null mice completely lose the ability to induce vasodilation in response to shear stress, indicating that the Ca^{2+} channel is crucial for shear stress-mediated regulation of the vascular tone [40, 41]. Transient receptor potential 1 (TRPC1) is demonstrated to pair up with TRPV4, which prolongs the shear stress-induced Ca^{2+} influx and delays intracellular

Ca^{2+} decay [42]. Importantly, specifically blocking TRPV4 activity within the complex is sufficient to abolish the shear stress-induced Ca^{2+} influx, indicating that TRPC1 merely enforces the mechanosensing capacity of TRPV4 [42]. Nonetheless, the mechanosensitivity of the TRPV4/TRPC1 complex is diminished by cGMP-dependent protein kinase (PKG) activity by specifically inhibiting TRPC1 [42]. Besides TRPV4, members of the TRPP2 family, such as Polycystin-2, induce a Ca^{2+} influx in response to shear stress [43].

PIEZO1 was recently identified as a highly conserved mechanosensitive Ca^{2+} channel, abundantly expressed in the endothelium [44, 45]. PIEZO1 is necessary and sufficient to induce mechanosensitive currents in endothelial cells [46]. PIEZO1 opens in response to unidirectional shear stress, causing rapidly adapting Ca^{2+} currents. Moreover, knockdown of PIEZO1 in endothelial cells causes a reduced although not completely abolished Ca^{2+} flux in response to shear stress [46]. Additionally, endothelial cells lacking PIEZO1 lose their ability to align with flow [46]. Impressively, transfection of *PIEZO1* in normally unresponsive HEK293 cells is sufficient to induce Ca^{2+} spikes in response to shear stress [46]. Murine PIEZO1 is crucial during early vascular development. Homozygous deletion of *Piezo1* in mice, either globally or endothelial-specific, is embryonically lethal between E9.5 and E11.5 [46, 47]. At E9.5, the yolk sac vasculature of *Piezo1*$^{-/-}$ embryos is disorganized and exhibits a delay in the formation of large vessels, suggesting that sensing shear stress through PIEZO1 is crucial for vascular remodeling [47].

6.3.2 GPCRs

G protein-coupled receptors (GPCRs) constitute a large family of receptors that are involved in numerous signal transduction pathways and cellular responses [48]. GPCRs ultimately activate two principal signal transduction pathways: the cAMP signal pathway and the phosphatidylinositol signal pathway [48]. Shear stress affects the conformational dynamics of several GPCRs, such as the B_2 bradykinin GPCR and the AT1 angiotensin receptor, similarly to stimulation by their respective ligands [49–51]. Mechanical activation of GPCRs induces several pathways, including ERK/Akt phosphorylation, through $G\alpha_q$ activation and intracellular Ca^{2+} release [51]. However, the specific mechanism of activation is not yet understood. $S1P_3$/$G\alpha_{q/11}$ is directly activated by shear stress within seconds, independently of GPCR activation [52]. Although GPCRs are important mechanosensors in the adult, no developmental phenotypes have yet been reported.

6.3.3 Integrins

Focal adhesions are multiprotein complexes at the apical cell surface where membrane components connect to extracellular matrix (ECM) proteins, including vitronectin, fibronectin, laminin, and collagen [53]. Focal adhesion-associated integrins, such as $\alpha_v\beta_3$, are able to sense flow through conformational changes

caused by mechanical stimuli [53]. However, this mechanosensitivity requires specific and dynamic integrin–ECM interactions; shear stress specifically induces $\alpha_v\beta_3$-fibronectin or vitronectin interaction [53, 54]. Integrins activated by shear stress activate a variety of protein kinases and adaptor molecules, including focal adhesion kinase (FAK), c-Src tyrosine kinase, and Shc [55–57]. These kinases and adaptor molecules activate the downstream ERK/MAPK pathway, eventually stimulating MAPK-driven transcription of target genes [58].

Given that integrins have numerous functions that exceed mechanotransduction, it is difficult to dissect the role of flow sensing by integrins from their other functions during vascular development. Null mutations in five of the integrins result in embryonic lethality (α4, α5, αV, β1, and β8), although the β1 mutant dies before the vasculature has even formed [59–63]. As noted, the ECM itself is an important determinant of integrin mechanotransduction. The ECM is not fixed during vascular development, where the earliest vessels express mostly fibronectin with a virtual absence of laminin [64]. In the chick, laminin expression is introduced from 8 to 10 days of development, which is approximately equivalent to a stage of E15.5 in mouse [64]. However, in the mouse, collagen IV and laminin can be detected at least from E10.5 onward [65]. These results indicate that mechanotransduction through integrins will vary depending on the stage of development.

6.3.4 The Glycocalyx

The glycocalyx is a structure on the luminal surface of endothelial cells and is responsible for maintaining blood vessel integrity [66–68]. The glycocalyx extends hair-like structures into the vascular lumen that consist of glycosaminoglycans including chondroitin sulfate, hyaluronan, sialic acids, and heparan sulfate. These glycosaminoglycans connect to the cytoskeleton through syndecans [66, 69]. The glycocalyx transmits shear stress directly to the cytoskeleton and mediates NO production [70–72]. The transmission of shear stress is thought to occur through a bending motion of the structure [73]. Specific degradation of either heparin sulfate or sialic acid reduces shear stress-induced NO production in endothelial cells, indicating that only specific components of the glycocalyx are necessary for mechanosensation [70–72].

In the developing embryo, the glycocalyx is already present when blood flow is initiated; however, it changes in organization and structure with the onset of flow [74]. During embryonic development, luminal hyaluronan degradation prevents vascular remodeling, while heparan sulfate degradation avoids the upregulation of shear stress-sensitive genes, without specifically inhibiting the vascular remodeling process [74]. Together, these results suggest that mechanosensation by specific components in the glycocalyx is necessary during vascular remodeling.

6.3.5 Primary Cilia

Endothelial cells present with a non-motile cilium on their surface, known as the primary cilium. The primary cilium consists of an axoneme, the stabilizing microtubule scaffold, which is surrounded by the ciliary membrane that contains several receptors, ion channels, and transporter protein complexes, such as the intraflagellar transport (IFT) complex [75]. The mechanosensory properties of the primary cilium depend on several proteins, including IFT88, KIF3a, and a mechanosensory complex formed by polycystin1 and polycystin2 [76–78]. Cells lacking functional polycystin1 fail to induce a shear stress-provoked Ca^{2+} influx [76, 79, 80]. Additionally, *Ift88*$^{-/-}$ endothelial cells lack a primary cilium due to instability and exhibit a reduced alignment to flow, but not a complete loss of mechanosensation [80]. Primary cilia respond to low shear stress levels and disassemble by exposure to high shear stress levels both *in vitro* and *in vivo* [81, 82].

Primary cilia are important regulators during embryonic development, as evidenced by the presence of developmental defects in diseases caused by ciliary dysfunction [79]. Mutations in IFT-linked genes, such as *Ift88* and *Kif3a*, result in embryonic defects that closely resemble those induced by loss of Sonic Hedgehog [83–85]. In the developing retina, the primary cilia maintain the collective polarization of endothelial cells in low shear stress regions, ultimately preventing vascular regression [80]. The primary cilia are necessary during vascular morphogenesis of zebrafish, when flow forces are low; however, it is dispensable during later development [80, 82]. Primary cilia disruption in mice, however, does not cause embryonic lethality, as occurs in mutants where vascular remodeling is completely inhibited [78]. Therefore, the primary cilium is suggested to enforce and fine-tune mechanotransduction in endothelial cells but is not critical for vascular function.

6.3.6 The Junctional Mechanosensory Complex

The junctional mechanosensory complex consists of vascular endothelial growth factor 2 (VEGFR2), platelet and endothelial cell adhesion molecule 1 (PECAM1), and VE-cadherin and is located within adherens junctions [86]. The mechanosensory complex is critical for endothelial cell alignment to flow in response to shear stress; deletion of either PECAM1 or VE-Cadherin impairs this ability [86]. Furthermore, transfection of these 3 proteins enables normally non-responsive fibroblasts to align to flow, demonstrating the importance of the complex for mechanotransduction [86]. Molecularly, shear stress phosphorylates PECAM1 through Src family kinases, activating VE-cadherin [87, 88]. VE-cadherin functions as an adaptor molecule, recruiting VEGFR2 to the complex by binding β-catenin and activating it independent of ligand [86, 89, 90]. Activated VEGFR2 binds PI3K directly and induces its activation by phosphorylation, subsequently activating further downstream pathways [86].

The junctional mechanosensory complex is likely necessary during early embryonic development. *VE-Cadherin*$^{-/-}$ mice go through vasculogenesis but die at E9.5

due to impaired vascularization and vascular remodeling, demonstrating its importance in early embryonic development and mechanosensation [91, 92]. *Vegfr2*$^{-/-}$ mice experience embryonic death between E8.5 and E9.5, before the onset of blood flow, due to disrupted blood-island formation and vasculogenesis [93]. Surprisingly, *Pecam1*$^{-/-}$ mice develop normally and do not exhibit any major vascular defects, suggesting that PECAM1 is not critical for vascular development [94]. However, PECAM1 is important during postnatal collateral remodeling and angiogenesis [95, 96]. Postnatal development of the retinal vasculature and retinal neovascularization are impaired in *Pecam1*$^{-/-}$ mice; *Pecam1*$^{-/-}$ mice show a disorganization of the retinal vasculature, associated with an increased and irregular expression of arteriovenous markers EphB4 and EphrinB2 [97]. Additionally, *Pecam1*$^{-/-}$ arterioles show an impaired dilation in response to rapid changes in shear stress levels, suggesting that PECAM1 senses sudden changes in shear stress levels [97]. Taken together, these findings indicate that PECAM1 is dispensable for vascular remodeling; however, it is required during postnatal alterations. Both VEGFR2 and VE-Cadherin are necessary for embryonic development, suggesting that a functional mechanosensory complex is required, although separate components appear to be dispensable.

6.3.7 Caveolae

Caveolae are invaginations of the plasma membrane present on the apical surface of endothelial cells linked to actin filaments and are rich in signal molecules [98–101]. Caveolins CAV1, CAV2, and CAV3 are necessary for caveolae formation [102]. Caveolae flatten in response to shear stress, enabling the activation of multiple signaling pathways and long-term shear stress exposure increases caveolae density, where CAV1 relocalizes from the Golgi apparatus to the luminal cell surface [103–105]. Shear stress phosphorylates CAV1, which activates several downstream pathways such as Ras homolog gene family, member A Rac1 (1RhoA) and Ras-related C3 botulinum toxin substrate (Rac1), ERK/Akt phosphorylation, as well as the eNOS system and KLF2 signaling [104–106]. Caveolins are expressed as early as E7.0 in mouse embryos [107]. Surprisingly, *Cav1*$^{-/-}$ mice develop normally, despite a decreased Cav2 expression and the absence of caveolae [101, 108]. However, *Cav1*$^{-/-}$ mice present with an uncontrolled endothelial cell proliferation, resulting in thickened alveolar septa [101, 108]. Although *Cav1*$^{-/-}$ mice show no fetal defects caused by impaired mechanosensation, caveolae are considered important mechanosensitive structures consolidating several mechanosensing structures, such as TRPV4 channels and the mechanosensory complex, inducing colocalization of its components and enforcement of mechanotransduction [109].

6.3.8 Shear Stress-Induced Gene Regulation for Arteriovenous and Lymphatic Differentiation

As mentioned, endothelial cells express highly conserved arterial and venous markers early in embryonic development, suggesting that genetic predetermination occurs [110]. Endothelial identity can be manipulated by environmental cues, such as local blood pressure, hemodynamic forces, oxygenation, or pH [111]. Arterial identity is initiated by the Sonic Hedgehog (Shh)–VEGF–Notch axis, which induces arterial-specific genes, including Neuropilin-1 (*Nrp1*), EphrinB2 (*EFNB2*), Uncoordinated-5b (*Unc5b*), and members of the Notch pathway [11, 13, 112]. On the other hand, venous specification is dependent on active repression of arterial identity by chicken ovalbumin upstream promoter transcription factor II (*Coup-TFII*), which results in expression of venous-specific genes, such as *EphB4* [113].

The lymphatic vasculature drains protein-rich and fat-rich interstitial fluid into the venous circulation and functions as a highway for the immune system [114, 115]. Lymphatic development is initiated at the cardinal vein, where specific venous endothelial cells differentiate into lymphatic endothelial cells that start budding to form lymph sacs [115]. These lymph sacs then remodel to lymphatic capillaries and collecting lymphatics. Additionally, non-venous derived cells also contribute to lymph vessel development [116]. Lymph flow and fluid shear forces are the essential drivers of these late lymphatic developmental processes, which include remodeling of the primitive mesenteric lymphatic plexus, development of lymphatic valves, and smooth muscle coverage on the collecting lymphatic vessels [117].

6.3.9 Notch Signaling

The Notch signaling pathway is a crucial regulator of arterial endothelial identity. Interrupting components of the Notch signaling pathway *in vivo* induces embryonic lethality around E9–E10, and often presents with impaired vascular remodeling [118–121]. Shear stress stimulates the Notch signaling pathway by stimulating Notch cleavage and subsequent notch intracellular domain (NICD) translocation [122–125]. Endothelial cells subjected to unidirectional shear stress induce the expression of arterial markers, such as *EFNB2*, *NOTCH1/3*, *HEY1*, and *HEY2*, while decreasing the expression of venous endothelial markers *EPHB4* and *NRP2* [123]. Moreover, blocking Notch signaling downregulates the expression of *EFNB2*, suggesting that shear stress-activated Notch signaling preserves the arterial phenotype [126]. Molecularly, mechanical Notch activation drives arterial specification by arresting the cell cycle [127]. Notch activation induces the expression of *GJA4* (commonly Cx37), which phosphorylates downstream cell cycle inhibitor CDKN1B (p27) through ERK/MAPK signaling. Stabilization of CDKN1B protein arrests the cell cycle, which subsequently enables endothelial cells to express arterial genes [127].

Besides its indispensable role in arteriovenous differentiation, shear stress-modulated Notch signaling also affects lymphatic differentiation, the maturation of

collecting lymphatic vessels, luminal valve formation, and sprouting lymphangiogenesis [117, 128, 129]. Low oscillatory shear stress levels open the ORAI1 Ca^{2+} channel in lymphatic endothelial cells [130]. Extracellular Ca^{2+} entering the cell binds calmodulin, which interacts with PROX1 and facilitates an interaction between PROX1 and KLF2 [130, 131]. The PROX1/KLF2 complex induces the expression of DTX1 and DTX3L, which aggregate and subsequently downregulate Notch signaling to permit lymphatic sprouting [130].

6.3.10 ALK1/Endoglin and ALK5

The ALK1/BMP/Endoglin complex is activated in response to ligand binding by BMP9 or BMP10 and phosphorylates SMAD1/5/8. Phosphorylated SMAD1/5/8 binds to SMAD4, which dimerizes and translocates to the nucleus to affect downstream signaling pathways. In zebrafish, ALK1 expression is observed in arteries upon onset of flow, demonstrating that high shear stress levels induce ALK1 expression [132]. Additionally, both SMAD1/5 phosphorylation and TGF-β activation are induced by shear stress [133, 134]. Shear stress potentiates the response of the receptor complex to BMP9/10, therefore stimulating ALK1 signaling. Disrupted ALK1/Endoglin signaling results in improper endothelial cell behavior in response to shear stress, including migrating downstream, increasing proliferation, and abnormally increased endothelial cell surface area [135]. Moreover, ALK5 is exclusively activated by shear stress in embryonic endothelial cells, suggesting a role for mechanically activated TGF-β signaling during development [134].

NOTCH and ALK1 signaling converge on many common targets, including binding of SMAD1/5/8 to the regulatory sites of Notch target genes [136–138]. Both NOTCH and ALK1 signaling affect recombining binding protein suppressor of hairless (RBPJ) and bind GC-rich palindromic sites present in the *HEY1* promoter, synergistically inducing HEY1 expression [136]. Additionally, ALK1–NICD interaction is stabilized through p300/CBP-associated-factor (P/CAF), which improves its ability to bind DNA [136, 137]. Interestingly, inhibiting both ALK1 and NOTCH signaling exacerbates the hypervascularization observed in separate ALK1 or NOTCH blockade [138]. Moreover, hypersprouting induced by NOTCH blockade is rescued by BMP9-mediated ALK1 activation, demonstrating that ALK1 and NOTCH signaling are intertwined during development [138].

6.3.11 PROX1/FOXC2/GATA2

PROX1 and FOXC2 are key regulators of lymphatic development. Prox1 drives lymphangiogenesis, as well as lymphatic endothelial specification [139]. Both *FOXC2* and *PROX1* expression are induced in lymphatic endothelial cells by shear stress [117, 140]. Lymphatic budding from veins by a subpopulation of endothelial cells is arrested in *Prox1*$^{-/-}$ mice, so they develop without a lymphatic

vasculature [139, 141]. On the other hand, FOXC2 is essential for the initiation and development of collecting lymphatic vessels [142, 143]. *FoxC2*$^{-/-}$ mice fail to mature their primary lymphatic plexus, due to persistent expression of lymphatic capillary markers and abnormal capillary sprouting [140]. Mechanical PROX1 and FOXC2 activation induces Cx37 and calcineurin/NFAT signaling, pathways necessary for lymphatic valve development [128]. Additionally, introducing *PROX1* in blood endothelial cells reprograms their transcription profile toward lymphatic identity [144].

Although PROX1 and FOXC2 are key regulators of lymphatic development, GATA2 is their upstream regulator; GATA2 recognizes a putative enhancer element upstream of *PROX1* and *FOXC2* and regulates its gene expression [117, 145]. *GATA2* expression is increased in lymphatic endothelial cells *in vitro* exposed to oscillatory shear stress [117]. Furthermore, *GATA2* expression is upregulated by reduction in matrix stiffness, indicating that it is a very mechanically sensitive gene [146]. Neither *PROX1* nor *FOXC2* knockdown completely prevents shear stress-induced expression of lymphatic valve genes; however, *GATA2* knockdown is sufficient to completely prevent the shear stress-induced expression of lymphatic valve genes [117, 147]. Therefore, shear stress probably induces *GATA2* expression, which induces PROX1 and FOXC2 and regulates lymphatic valve development. Endothelial-specific loss of *GATA2* results in late-embryonic lethality caused by anemia, hemorrhage, and edema, demonstrating that GATA2 is essential for proper lymphatic development [147, 148].

6.3.12 YAP and TAZ

YAP/TAZ are mechanotransducers that translate a broad range of mechanical cues into biological responses, as evidenced both *in vitro* and *in vivo* [149–151]. Molecularly, Yap/Taz activity is regulated by ECM stiffness and cell spreading, as well as shear stress [149, 151]. YAP/TAZ activation is dependent on both small GTPase Rho and the actin cytoskeleton [149, 152, 153]. YAP/TAZ signaling is a key regulator of cell–cell contacts and cell growth. Low density or loss of cell–cell contacts stimulates *Yap*/*Taz* expression, which induces downstream effectors, including SMADs, ErbB4, and tumor protein p73, to activate the cell cycle and thus regulates proliferation and differentiation [154–157].

Endothelial cell-specific deletion of both YAP/TAZ during embryonic development results in severe vascular defects followed by embryonic death due to impaired growth and sprouting of endothelial cells [158, 159]. When YAP/TAZ are ablated in endothelial cells postnatally, vascular remodeling still occurs, and large arteries and veins form normally in the neonatal retina [159]. However, these retinas display impaired sprouting and excessive pruning, suggesting that YAP/TAZ are still necessary to fine-tune the remodeling process [159].

In lymphatic endothelial cells, YAP/TAZ signaling is induced by oscillatory shear stress, which is present in lymphatic valves [128]. However, *FoxC2* expression efficiently attenuates this expression and the subsequent pro-proliferative effect

exerted by Yap/Taz signaling [140]. Thus, FOXC2 maintains quiescence and survival of lymphatic endothelial cells in regions of disturbed flow by blocking YAP/TAZ activation [140]. These results suggest that certain regions, such as lymphatic valves, require high expression levels of *FoxC2* to maintain their barrier function and prevent endothelial cell overgrowth [140].

6.3.13 Wnt/β-Catenin Signaling

The Wnt/β-catenin signaling pathway is heavily involved in cardiac embryonic development, more specifically in arteriovenous differentiation, heart valve morphogenesis, and angiogenesis [160–163]. Oscillatory shear stress activates the Wnt signaling pathway and known downstream Wnt target genes in cultured endothelial cells [164, 165]. Furthermore, oscillatory shear stress drives the nuclear relocalization of β-catenin, which is strictly dependent on the presence of PECAM1, suggesting that oscillatory shear stress is sensed by the junctional mechanosensory complex [165]. Oscillatory shear stress induces the expression of angiopoietin-2 (Ang-2) by activating the Wnt/β-catenin signaling pathway *in vitro* and *in vivo* [164]. Inhibition of *Ang-2* during development impairs de novo vessel formation [164]. In amputation experiments in zebrafish embryos, targeting Wnt/β-catenin signaling delays vascular repair [164]. Both phenomena are rescued by *Ang-2* mRNA, illustrating the crucial role of Wnt/β-catenin in vascular development and repair [164].

The Wnt/β-catenin signaling pathway is implicated in the lymphatic development as well. Wnt signaling is activated by oscillatory shear stress in lymphatic endothelial cells [166, 167]. Lymphatic endothelial cell-specific *β-catenin* deletion in mice induces severe embryonic lymphedema due to the absence of lymphatic valves, closely resembling the *FoxC2*$^{-/-}$ and *Gata2*$^{-/-}$ phenotype, demonstrating that Wnt/β-catenin signaling is crucial for lymphatic valve development [147, 148, 168]. Furthermore, *FoxC2* expression is impaired in β-catenin-deficient mice and ectopic *FoxC2* expression partially rescues this β-catenin$^{-/-}$ phenotype [168]. Therefore, oscillatory shear stress is proposed to activate β-catenin, which uses *Prox1* to induce *Gata2* and subsequently *FoxC2* expression, driving lymphatic development [166, 167]. Importantly, PROX1 is also expressed downstream of GATA2, resulting in a positive feedback loop that amplifies the signaling pathway [167].

6.3.14 TIE and ANG

TIE1 and TIE2 comprise a family of receptor tyrosine kinases exclusively expressed in endothelial cells that bind ANG-2 and ANG-1, respectively, and exert distinct roles during embryogenesis [169]. TIE2 and its ligand ANG-1 regulate developmental angiogenesis [170, 171]. *Tie2*-deficient mice die at E10.5, while *Ang-1* deficiency results in embryonic death between E11 and E12.5 [170, 172]. Both *Tie2*- and *Ang-1*-deficient mice present with a reduced complexity in their immature vasculature,

suggesting that vascular development is not completed [170, 172]. On the other hand, TIE1 is crucial for lymphatic development; loss of *Tie1* in mice leads to edema and hemorrhage, which causes pups to die immediately after birth [170].

ANG-2 has an inhibitory role, where it binds TIE2 exclusively and prevents binding of ANG-1 [173]. *Ang-2* overexpression *in vivo* induces a similar phenotype as observed in *Tie2*$^{-/-}$ or *Ang-1*$^{-/-}$ mice, although *Ang-2* overexpression results in more severe vascular defects [173]. Mice lacking *Ang-2* die postnatally within a few weeks and exhibit retinal vascular defects [174]. *Ang-2* deletion results in chylous ascites, demonstrating that *Ang-2* is required for lymphatic function [174]. Unexpectedly, gene replacement with *Ang-1* in mice lacking *Ang-2* rescues the lymphatic defects only. ANG-2 is therefore thought to inhibit TIE2-mediated angiogenesis, but acts as a TIE2 agonist in the lymphatic vasculature [174].

Ang-2 expression is induced by oscillatory shear stress and inhibited by unidirectional shear stress both *in vitro* and *in vivo*, while *Ang-1* expression is unaffected [175]. Furthermore, inhibiting *Ang-2* expression *in vitro* inhibits oscillatory shear stress-mediated migration of blood endothelial cells [175]. These results suggest that unidirectional shear stress downregulates ANG-2 expression, allowing ANG-1 to stimulate TIE2-mediated angiogenesis. On the other hand, oscillatory shear stress, such as present in lymphatics, induces Ang-2 expression in endothelial cells, which is necessary for lymphangiogenesis [164, 176]. Thus, TIE/ANG-driven signaling consists of a careful balance between stimulation and inhibition of angiogenesis, which is crucial during early vascular development.

6.3.15 KLF2

Krüppel-like transcription factor 2 (KLF2) is a global transcription factor regulating blood vessel development, vascular tone, thrombosis/hemostasis, and inflammation [177]. KLF2 upregulates expression of eNOS [178, 179]. Furthermore, KLF2 induces the pro-inflammatory VCAM-1 and E-selectin through regulating IL-1β and inhibits TNF-α-mediated expression of tissue factor (TF) [178, 180, 181].

KLF2 is a wide-acting transcription factor and, as such, is an important mediator of shear stress. KLF2 is upregulated by laminar flow in endothelial cells through the MEK5/ERK5/MEF2 signaling pathway [177, 178]. In this pathway, ERK5 senses shear stress and subsequently targets the myocyte enhancing factor (MEF2)-binding site present in the KLF2 promoter region [177, 182]. *Klf2*-deficient mice develop normally through vasculogenesis, angiogenesis, and vascular remodeling; however, they die embryonically at E13.5 due to internal hemorrhages [183–185]. *Klf2*$^{-/-}$ mice exhibit a poorly developed vascular smooth muscle cell layer, accompanied by the absence of vascular smooth muscle cells along the dorsal aortic wall [185]. During vascular remodeling, vessels are stabilized by the recruitment of vascular smooth muscle cells. Differentiation of smooth muscle cells is initiated at the ventral side of the aortic wall, after which they migrate around the aorta [186, 187]. Thus, KLF2 signaling is crucial during vascular remodeling to ensure vessel stability by regulating vascular smooth muscle cell recruitment and migration. Additionally,

Klf2 expression suppresses *Ang-2* and induces both *Tie2* and *NFATc*, which is critical for the formation of the arterial wall, possibly explaining the observed phenotype [177, 188].

KLF2 signaling also plays an important role in lymphatic development. Endothelial-specific loss of *Klf2* in mice results in the formation of a defective lymphatic network, characterized by reduced lymphatic branching, irregular vessel thickness, and round-end sprouts [131]. Molecularly, mechanical activation of the ORAI1 Ca^{2+} channel upregulates *KLF2* expression in lymphatic endothelial cells [131]. Extracellular Ca^{2+} influx facilitates an interaction between PROX1 and KLF2, which downregulates Notch signaling to enhance lymphatic sprouting [130, 131].

6.3.16 Coup-TFII

COUP-TFs are members of the steroid/thyroid hormone receptor superfamily that act as transcription factors to regulate a wide variety of genes [189]. Murine *Coup-TFII* is widely expressed during the early stages of development; however, expression levels stabilize once organogenesis is completed [190]. Furthermore, Coup-TFII is an important marker of venous identity by actively blocking Notch signaling and is required for the maintenance of venous identity [113, 191].

Coup-TFII-deficient mice die embryonically around E10 due to hemorrhage and edema of the brain and heart, accompanied by extensive growth retardation [190]. *Coup-TFII*$^{-/-}$ mice exhibit severe vascular defects, including sinus venosus, atrial, and cardinal vein malformations, abnormal angiogenesis, and defective vascular remodeling of the primary capillary plexus [190]. The observed impaired vascular remodeling was suggested to be secondary to the disrupted flow caused by severe heart defects, rather than due to the loss of Coup-TFII. However, COUP-TFII expression is also mechanosensitive. Laminar steady flow, but not pulsatile flow, induces *COUP-TFII* expression in HUVECs *in vitro* [192]. Additionally, in chicken embryos, *COUP-TFII* expression is upregulated if the vitelline artery is ligated, such that it becomes exposed to venous flow patterns [192]. Together these results suggest that flow changes as caused by ligation induce *COUP-TFII* expression and drive remodeling of arteries into veins.

6.4 Specific Examples of Endothelial Differentiation Based on Flow Mechanics

Vascular remodeling involves multiple processes, including sprouting angiogenesis into avascular areas, regression of poorly perfused vessels, fusion of smaller vessels, intussusception or splitting of large vessels, and the differentiation of localized endothelial populations, as occurs during valve formation. The specific locations of these events are governed by the mechanical forces exerted by blood flow.

6.4.1 Shear Stress and Endothelial Cell Sprouting

In addition to more direct effects of hemodynamics on endothelial cell biology, blood flow modulates the distribution of chemical signals by controlling interstitial flow. Sprouting angiogenesis involves the selection of a tip cell and designation of stalk cells based on VEGF gradients [193]. Interestingly, high shear stresses inhibit vascular sprouting [9, 194]. Simultaneously imaging blood flow and vessel geometry showed that sprouts form where shear stress reaches a minimum (Fig. 6.5). Thus, though factors such as VEGF control when a sprout will form, the flow dynamics control where that sprout will be located [195]. Additionally, sprouts grow toward a

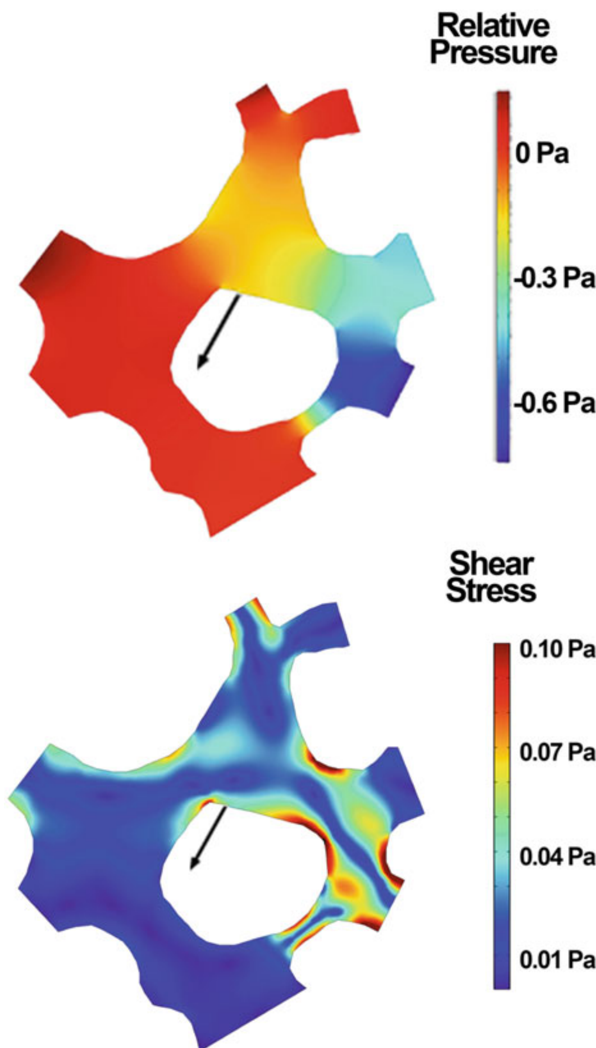

Fig. 6.5 Sprouts form at shear stress minimum and are directed toward areas of high pressure. Computational assessment of relative pressure and shear stress in vessels of the yolk sac capillary plexus of quail embryos. Arrow indicates direction of sprout elongation. Sprout is predicted to form from low pressure to high, and at the location of a shear stress minimum. Based on [195]

vessel of higher pressure, and will dynamically change direction as pressure differences change [195]. This information was enough to predict the location of sprout formation in a given vascular area [195], indicating the power of hemodynamic forces in sculpting the vessel geometry. When interstitial flow was calculated to predict VEGF distribution, interstitial flow rather than diffusion was the dominant factor in VEGF distribution, and the pressure gradient modulating sprouting direction correlated to the areas of high VEGF concentration [196].

6.4.2 Shear Stress Governs Intussusception

New vessels can be formed by splitting up of an existing vessel through a process called intussusceptive angiogenesis. Intussusception involves the formation, elongation, and connection of pillars through the vascular lumen. Blood flow is the main driving force for intussusception. Pillars form more readily when blood flow is higher, with new pillars visible as early as 15 min after an increase in blood flow [197, 198]. However, within areas of higher flow, pillars form and fuse at regions of the vasculature with low shear stress and are particularly prominent at bifurcations in the vasculature [197–199]. Computationally, the pillar was observed to be confined to an island of low shear stress within the bifurcating vessel [199]. Additionally, the length and direction of the pillar depend on the angle of the bifurcation and the direction of the "dominant" vessel branch, suggesting that the geometry of the flow, and thereby shear stress, sculpts the ultimate shape and growth of the pillar [199].

6.4.3 Vascular Fusion is a Flow-Driven Process

During vascular development, the major vessels form from a plexus through a process of vascular fusion, which has also been called reverse intussusception [200, 201] (Fig. 6.6). Vascular fusion is reported to occur in regions of the vascular plexus experiencing the highest flow, namely the forming vitelline artery [21]. However, the process is actually driven by low shear stress levels. Preventing the entry of erythroblast into circulation, which creates a low shear stress environment in the yolk sac vascular plexus, leads to an excessively fused vascular plexus [9]. Fluid dynamic analysis of vessels undergoing fusion shows that in high-flow regions, low shear stress regions form downstream of tissue "pillars" that drive the fusion process, similar to how eddies form behind rocks in a high-flowing stream (our unpublished results). Phosphorylation of the junctional protein VE-Cadherin increased in situations of low shear stress, and inhibition of this phosphorylation prevents fusion from occurring [202]. The proposed molecular mechanism is that under low shear stress VE-Cadherin phosphorylation initiates rearrangement of the junctional proteins [202].

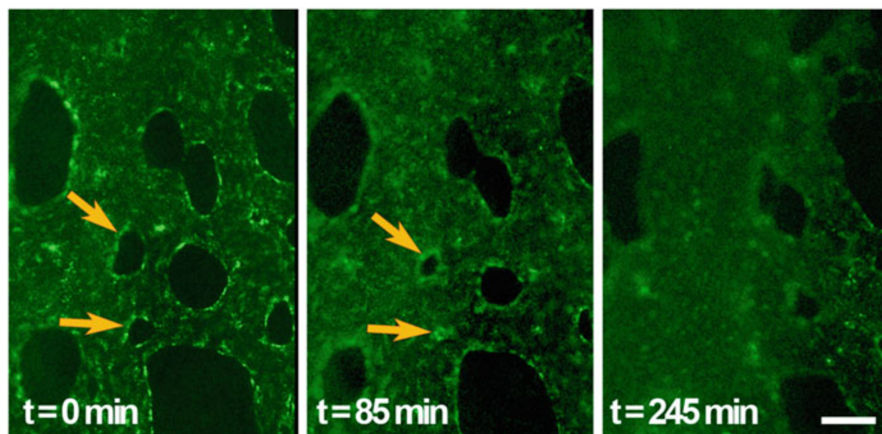

Fig. 6.6 Vascular fusion in the remodeling yolk sac. Vascular fusion occurs when two vessels merge. Time lapse images of chick yolk sac (green = endothelial cells) show a pillar of avascular space shrinking and finally disappearing (yellow arrow). Scale bar = 100 μm

6.4.4 Plasticity in Arteriovenous Identity

Endothelial cells show extensive plasticity with regard to arteriovenous fate during development, although this plasticity becomes more limited as development proceeds. This was demonstrated by quail-to-chick grafting experiments where segments of quail embryonic vessels were grafted onto the coelom of chick hosts and the endothelial cells were allowed to incorporate into the chick vasculature. Immunostaining for QH1, an antigen specific to quail but not chick endothelial cells, allowed for observation of where grafted endothelial cells had migrated. Interestingly, grafts of dorsal aorta from quails colonized both arteries and veins in the chick host equally [203]. Quail aortic endothelial cells incorporated into chick veins lost expression of *EphrinB2* and *NRP1* [203], indicating individual endothelial cell plasticity. However, after 7 days of development, quail aortic endothelial cells preferentially migrated to the chick arteries [203]. Likewise, grafts of quail veins incorporated equally into chick arteries and veins until 7 days, but after that migrated preferentially into veins [203]. Quail venous endothelial cells incorporated into arteries upregulated *NRP1* and *EphrinB2*, indicating arterial differentiation [203]. Therefore, during early development, endothelial cells are able to contribute to arteries or veins, regardless of their vessel of origin.

Not only can arterial or venous-specified endothelial cells integrate into the complementary vessels, but entire vessel segments can shift between arterial and venous identity based on the flow they experience. In avian embryos, initial flow within the yolk sac vascular plexus is a loop, where arterial vessels are located in the caudal half of the yolk sac and venous vessels in the rostral half of the yolk sac. Through the process of vascular remodeling, this loop circulation becomes restructured into an out-and-back circulation, with the vitelline artery and vein

running in parallel [17]. During formation of the vitelline vein, areas of the arterial capillary plexus expressing *EphrinB2* and *NRP1* become exposed to venous flow, causing them to rapidly downregulate arterial markers [17]. Arterial segments also disconnect from the forming vitelline artery and sprout out to reconnect into the venous system [17]. As such, formation of the vitelline venous system depends strongly on the plasticity of arterial vessel segments.

Manipulations of flow in arterial and venous vessel segments of the forming yolk sac further display the astonishing plasticity in vessel identity. Ligation of the right vitelline artery after it has differentiated results in a complete switch to venous identity in the right side of the yolk sac, including downregulation of *GJA5*, *NRP1*, and *EphrinB2* and upregulation of *Coup-TFII*, *NRP2*, and *TIE2* [17, 192]. Removal of ligation restored arterial identity, including *NRP1* expression, within 4 hours [17]. Following ligation, the anterior vitelline vein carried arterial flow, causing it to rapidly remodel into an artery, including downregulating the venous markers *NRP2* and *TIE2* and upregulating the arterial marker *GJA5* [17, 192].

6.4.5 Altered Flow Reprograms Lymphatic Vessels to Blood Vessels

Lymphatic vessels can be prompted to take on a blood vessel phenotype in the presence of blood flow. Mice with mutations in *Slp76* develop direct connections between blood and lymphatic vessels, after which mesenteric lymphatic vessels become exposed to blood flow [204, 205]. Ultimately, the mesenteric lymphatic vessel remodels into a second vein, which mimics the original vein both anatomically and molecularly [204]. The formerly lymphatic vessel loses expression of lymphatic markers such as *Lyve1* and instead starts expressing blood vasculature markers, such as *vWF* [204]. More specifically, the lymphatic vessel is replaced by an *EphrinB2*$^{-}$; *EphB4*$^{+}$ vein [204]. Tracing of formerly lymphatic endothelial cells indicates that these extra veins are populated by lymphatic endothelial cells that had undergone a change in identity to become blood (specifically venous) endothelial cells [204].

Hemodynamics, rather than the presence of blood, drives the transition from lymphatic to blood endothelial cell identity. Wall shear stress levels were inferred from Doppler ultrasound in the mesenteric vessels of *Slp76* knockout and wild-type mice. In wild-type mice, lymphatic vessels could not be visualized due to such low flow levels, while in *Slp76* knockouts, lymphatic vessels and mesenteric veins had comparable flow profiles [204], supporting the role of blood flow in the reprogramming of the lymphatic vessels. To better understand whether this apparent transition of the lymphatic vessel into a vein was secondary to exposure to blood components or to blood vascular hemodynamics, *Slp76* knockout mice were compared with wild-type mice which were lethally irradiated, after which their bone marrow was reconstituted with *Slp76*-negative cells. The *Slp76*-chimera mice still develop blood-filled lymphatics, but the vessels have very little flow, compared to *Slp76* knockout mice [204, 205]. In the *Slp76*-chimera mice, lymphatic vessels

retained their identity, despite the presence of blood [205], indicating that blood vascular hemodynamics drives the observed transition. The important role of hemodynamics in lymphatic versus blood endothelial cell identity was confirmed *in vitro*. Human lymphatic endothelial cells exposed to arterial levels of shear stress (20 dyne/ cm^2) lost expression of *PROX1* and upregulated the arterial markers *HEY1*, *HEY2*, and *EphrinB2* [204]. Curiously, when flow was stopped, *PROX1* expression was restored [204], demonstrating the lasting plasticity of endothelial cells under influence of hemodynamic forces.

6.4.6 Oscillatory Flow in Valve Formation

Atrioventricular valve formation begins with the convergence of endocardial cells within the atrioventricular canal, a subset of which undergo endocardial-to-mesenchymal transition (endocardial EMT). These cells invade and proliferate within the cardiac jelly, forming the cardiac cushions, which are subsequently remodeled into the valve leaflets (reviewed in [206]). The initial convergence of the endocardial cells in the region of the atrioventricular canal depends on shear stress patterns created by the reversing oscillatory flow [207]. Using flow imaging techniques, it was shown that endocardial EMT initiates and is dependent on the presence of flow recirculation in the heart [208].

Many of the signaling pathways involved in valve formation are hemodynamically regulated pathways and their expression is lost in the absence of a heartbeat (Fig. 6.7). Luminal and abluminal endocardial leaflet cells originate from the atrial and ventricular endocardium, respectively [209, 210]. Luminal cells show Notch activation and abluminal cells show Wnt/β-catenin activation [209]. KLF2 is also crucial for heart valve formation and specifically expressed in the endocardial cells of the forming valve in both zebrafish and mouse models [211, 212]. KLF2 expression is higher in areas of the atrioventricular canal that are predicted to have higher shear stress levels [211]. In this region, Trpv4 and Trpp2 are necessary for oscillatory shear stress-induced *klf2a* expression [213]. Loss of *Klf2* in mice or zebrafish, as well as loss of either *trpv4* or *trpp2,* results in impaired valve formation [210–212].

Klf2a is highly expressed in a subset of ventricular-derived endocardial cells which undergo endocardial EMT and invade into the cardiac jelly, and drives expression of *Wnt9b* and *fibronectin1b*, which facilitates migration and proliferation [210, 211]. This invasion does not occur in the absence of heart beat, and is particularly dependent on the reversing flow, as indicated by impaired invasion in *gata2* morphants [210]. *KLF2a* drives expression of *fibronectin1b*, which facilitates the invasion of endocardial cells into the cardiac jelly [210]. *KLF2a* also regulates expression of endocardial *Wnt9b*, which is expressed specifically in the endocardial cells of the forming valves and is dependent on shear stress [211]. Endocardial loss of *Wnt9b* phenocopied the *Klf2* knockout [211]. Wnt9b is necessary for induction of

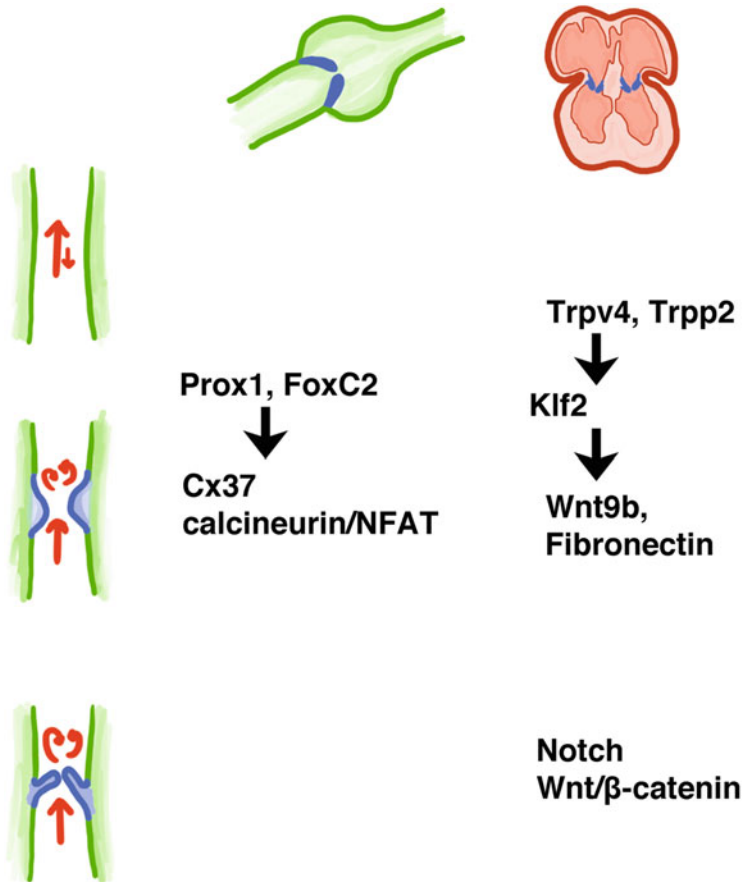

Fig. 6.7 Shear stress-induced gene expression during lymphatic and cardiac valve formation is driven by oscillatory shear stress

Wnt signaling in mesenchymal cells of the cardiac cushion, regulating their proliferation and condensation into valve leaflets [211].

It is not only heart valves but also lymphatic valve formation that is driven by oscillatory flows (Fig. 6.7). Impaired lymphatic flow, such as occurs in *CLEC2* mutants, results in a failure of lymphatic valve formation [117]. In the initial phase of valve development, expression of *GATA2*, *PROX1*, *FOXC2*, and *Cx37* and activation of Wnt/β-catenin and calcineurin signaling are elevated [117, 128, 145, 166]. Disruption of any of these molecules results in the loss of functional valve formation [117, 128, 145, 166]. GATA2 and Wnt/β-catenin signaling is regulated by oscillatory shear stress upstream of *PROX1* and *FOXC2* both in vitro and in vivo [117, 145, 166]. PROX1 and FOXC2 further regulate *Cx37* expression, which itself is upstream of NFAT/calcineurin signaling and necessary for segregation of the valve forming territory [128].

6.5 Conclusion

Shear stress created by blood flowing over endothelial cells is a critical morphogenic force which sculpts the entire cardiovascular system. It has the power to override genetically determined arteriovenous specification and govern the location and formation of lymphatic and cardiac valves. Moreover, among species and even within adult and embryonic systems, there are important differences in the involved mechanosensors and shear stress levels that regulate the functional organization and maintenance of the entire vascular system. This complex role can only occur due to a myriad of mechanosensors that activate signaling through multiple distinct pathways. These various mechanically activated signals converge within the nucleus, ultimately driving cardiovascular development and remodeling.

Compliance with Ethical Standards

Conflict of Interest: The authors declare that they have no conflict of interest.

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The Biomechanics of Venous Remodeling 7

Hanna Kuk, Christina Jeanneret, Thomas Noppeney, and
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Abstract

The remodeling of veins is associated with an alteration of both the architecture as well as the functional properties of the vein wall. On the morphological level, such changes contribute to the development of bulged and dilated varicose or

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spider veins. On the functional level, they may evoke venous insufficiency, which is characterized by reflux of venous blood or disturbed flow and elevated intraluminal pressure levels. While the primary cause of venous remodeling remains obscure in the majority of cases, epidemiological and etiological studies indicate that the development of varicose veins is driven by risk factors, which support the development of venous hypertension and thus chronically augment circumferential stress of the venous wall (e.g., dysfunctional venous valves, pregnancy, or obesity). As such, changes in flow and blood pressure appear to act as both cause and consequence of venous remodeling, thereby delineating pathophysiological biomechanical parameters as critical determinants of impaired vein function.

This review intends to highlight biomechanical forces capable to activate vascular endothelial and smooth muscle cells in the vein wall and discuss the underlying molecular mechanisms. A special emphasis is given to pressure-induced circumferential wall stress as a plethora of experimental and epidemiological evidence underlines the relevance of venous hypertension for venous remodeling processes.

7.1 Introduction

Blood vessels that constitute an integral component of the cardiovascular system are constantly exposed to biomechanical forces such as biomechanical stretch and shear stress. Depending on the magnitude and duration of such hemodynamic stimuli, this may lead to the pathophysiological structural and functional changes of the vessel wall through altered cell proliferation, cell death, cell migration, and production or degradation of the extracellular matrix (ECM) [1]. The term vascular remodeling may thus be applied to any long-lasting structural changes to the vessel, measured by the reduction or an increase in its luminal diameter, or as a result of the thickening of the vessel wall [2, 3].

The pathologic remodeling of veins often results in significant morbidity and discomfort for the patient as well as causing a cosmetic discontent. Some of the chronic pathophysiological changes associated with pathologic venous remodeling processes may include, among others, ulcerations, chronic venous insufficiency (CVI) as well as varicose veins [4]. The latter are classified as enlarged, tortuous corkscrew-like veins in the subcutaneous tissue, 3 mm in diameter or larger [5, 6]. Importantly, chronic conditions that lead to the maladaptive functional and morphological alterations of the venous system may further constitute the development of chronic venous disease (CVD) [7]. Generally, CVD and diseases of the venous system may be classified by the venous blood clot formation (thrombosis), inadequate venous drainage (insufficiency) or complex/congenital venous malformations. The latter may involve a number of hereditary, congenital, and/or other conditions, discussion of which is outside the scope of the current chapter review. Venous malformations however are considered the most common of the vascular congenital anomalies [8]. These conditions may include Maffucci

syndrome, Proteus syndrome, Parkes Weber syndrome, Klippel–Trenaunay syndrome (KTS), and May–Thurner syndrome (MTS), among others [8, 9].

The thromboembolic events in the venous system are most often associated with deep and superficial veins of the lower extremities and include superficial thrombophlebitis involving veins below the skin surface as well as deep vein thrombosis (DVT) involving deep veins [10]. While thrombus formation is usually associated with the veins of the lower limbs, axillo-subclavian vein thrombosis, also known as the Paget–Schroetter syndrome is a relatively uncommon condition associated with the venous circulation of the upper limbs [11]. The condition is known to affect primarily young competitive athletes and is a result of an extrinsic compression of the subclavian vein between the clavicle and first rib, particularly as a result of activities involving arm elevation or exertion [11]. Additional risk factors associated with venous blood clot formation may include inactivity, prolonged immobility, obesity, advanced age, underlying coagulation disorders, surgery, pregnancy, trauma as well as inherited tendency for blood clotting [10, 11]. DVT is an especially serious condition that may ultimately result in pulmonary embolism. In addition, an impaired outflow of blood due to thromboembolic events may lead to dermatitis, increased skin pigmentation and swelling, symptoms characteristic of venous insufficiency [10].

CVI is generally characterized by the pathophysiological changes of the venous vasculature of lower extremities, usually as a result of an obstruction, reflux, or both, over a prolonged period of time (1). Manifestations of the chronic venous disease may be thought to arise as a result of primary venous insufficiency or as a secondary manifestation to other processes, such as DVT or congenital and inherited disorders and malfunctions. Additional symptoms of CVI typically include leg swelling (edema), pain, lipodermatosclerosis, dermatitis, skin pigmentation, and coarsening of the skin texture [12, 13]. Burning, itching, pain, and development of moist, irregular ulcers are also commonly reported in advanced cases [10]. Often presenting in the lower extremities, varicosis is commonly associated with swollen, painful legs which may potentially develop complications including skin ulcers, superficial thrombophlebitis, and bleeding, among others [14]. During the onset of varicose vein development, the activation of the venous smooth muscle (SMC) and endothelial cells (EC) as well as preliminary changes in the composition of the ECM may result in further weakening of the venous wall, culminating in a typical, crock-screw-like morphology associated with varicosis [15]. Although not comparable in the extent of their severity, spider veins are defined as dilated, intradermal venules less than 1 mm in diameter (teleangiectases) or between 1 mm and 3 mm in diameter (reticular veins) and often resemble varicosities albeit on a much smaller scale [6]. It is estimated that close to 25 million Americans are affected with varicose veins and approximately 300,000 yearly varicose vein surgeries are performed in Germany alone [16, 17]. Indeed, while not associated with a significant risk of mortality, varicose veins, and spider veins are nevertheless common, with a reported 20% prevalence of varicose veins in the adult Western population and a 59% prevalence rate associated with spider veins [5, 6].

Depending on the severity and progression of the CVD, the major aim of the currently available therapies may range from an improvement of a cosmetic appearance (i.e., spider veins) to the need for an invasive and/or continuous therapy in order to alleviate pain, reduce edema, reverse skin changes as well as to heal and/or reduce ulcer formation or thrombus development associated with later stages of varicosis [5, 6, 12]. Sclerotherapy (use of a sealing/sclerosis agent) as well as laser ablation are commonly utilized in the clinical practice for the treatment of lower stage varicose veins while phlebotomy (surgical removal) of an advanced-stage varicose veins may be needed in other instances [5, 17]. Varicose vein recurrence rate however remains high, with an estimated 7–70% recurrence rate and with 20% of procedures carried out for recurrent veins [18]. Medical intervention to remove pre-established varicose veins therefore does not address the problem of the underlying cause of varicose vein formations [18]. Compression stockings are also generally prescribed following treatment although the compliance rate among patients may be variable in some cases [5, 19].

In addition, alternative therapies such as topical formulations are also gaining importance, with a noted emphasis on the use of naturally derived, herbal products and plant extracts as have been the consideration in the past [20–23]. Currently available herbal or drug-based topical therapies aim at alleviating the symptoms of varicose veins, especially in the later stages of ulceration. Generally, these are classified as topical steroid-based creams (short term-reduction of swelling and pain), lidocaine cream formulations (short-term alleviation of pain), heparin based creams (improvement of blood circulation as well as keeping thrombosis at bay), and ointments which incorporate vitamins or plant extracts such as horse chestnut as an active ingredient [22, 24–29]. In addition, oral formulations such as Pycnogenol®, a pine bark extract with strong antioxidant, anti-inflammatory, and vasodilator properties, have reported effectiveness in the management of asthma, mental function, venous thrombosis, and CVI-associated edema, among other symptoms [30]. Given the variable composition of such extracts and the further presence of additional active ingredients and excipients, mechanistic insights as to the general function of such products cannot be precisely characterized. Naturally derived, single molecular entities applied in the varicose vein treatment and prevention have yet to be established. Likewise, the precise molecular mechanism(s) of varicose vein and CVD development in humans remain to be fully elucidated. A common hypothesis for the pathologic venous remodeling suggests that an increase in the venous filling pressure may serve as the initial trigger for venous EC and SMC to respond in a maladaptive fashion, thus leading to further structural and functional changes of the venous wall [12, 13, 31]. Consequently, there is a need for a greater understanding and manipulation of biomechanical mechanisms controlling venous remodeling at the molecular level.

7.2 Anatomy and Physiology of the Venous System

7.2.1 Overview of the Cardiovascular System and Characterization of Blood Vessels

The human cardiovascular system is a closed-loop system consisting of organs and tissues that enable the flow of blood throughout the body, namely, the heart and blood vessels. With every contraction of the ventricles, blood is pumped from the heart through the system of blood vessels which are further subdivided into the pulmonary and systemic circulatory loops. The former allows for O_2/CO_2 exchange at the lungs, ensuring the elimination of CO_2, the waste by-product of cellular respiration, and re-oxygenation of the blood. The latter allows for the transport of the oxygen-rich blood to the cells, tissues, and organs of the body.

In a healthy individual at rest, the cardiac output, or the product of heart rate and stroke volume (defined as the amount of blood pumped by the left ventricle of the heart in one contraction) is estimated at approximately 5 L of blood per minute and may increase three–fourfold depending on the metabolic demand. This circulating blood volume further serves as a transport system, permitting the removal of cellular metabolic waste (i.e., CO_2, adenosine, hydrogen ion, lactate) and transport of nutrients (i.e., glucose, amino acids, lipids, electrolytes), hormones, and blood cells, including immune cells, throughout the body [32].

Blood vessels may be classified into large and small arteries and veins that comprise the macrocirculation as well as smaller size arterioles and venules which together with the capillaries make up the microcirculation in vertebrates. In the systemic circulation, oxygenated blood derived from the pulmonary circulation is carried away from the left ventricle of the heart at high pressures via large conduit arteries that subsequently branch out into small arteries and arterioles and feed into the capillary beds. Blood is then returned back to the right ventricle of the heart via venules and small veins that converge into larger, deep veins.

Both arteries and veins comprise a similar three-layer structure: the innermost tunica intima layer consisting of one layer of endothelial cells (ECs) and the basal membrane, the middle or tunica media layer consisting primarily of one to several layers of smooth muscle cells (SMCs) and the outermost, tunica adventitia layer comprised of connective tissue as well as vasa vasorum, a circulatory network of thin-walled blood vessels that provide an adequate oxygen and nutrient exchange in larger veins and conduit arteries [32]. Further, along with the vasa vasorum, both lymphatic and nerve plexi (nervi vasorum) are observed in the adventitia of larger vessels [32]. Capillaries, which essentially consist of one layer of ECs and the basal membrane, link the arteriolar and venular branches of the microcirculation and primarily serve as a highly adjustable interphase for oxygen/nutrient exchange with the cells and tissues of the body. The transition from capillary to a smaller size venule is generally marked by the gradual re-appearance of SMCs in the vessel wall followed by an added complexity of a layer of collagen and other extracellular matrix (ECM) fibers in the adventitia layer as the vessel size increases [32].

7.2.2 The Venous System, Structure and Function

The human venous system is a low pressure system carrying approximately ~65% of the total blood volume at any given time. Known as capacitance vessels, veins serve predominantly as a blood reservoir given their high degree of compliance (especially for the splanchnic and cutaneous venous vasculature), thus accommodating changes in blood volume and maintaining the filling pressure in the right heart [33]. Aside from their role as the main blood reservoir, veins serve two additional, albeit major functions, namely, the return of de-oxygenated blood back to the heart (venous return) as well as temperature regulation [4]. The latter is carried out primarily by the superficial vessels beneath the skin, albeit with the input from the sympathetic nervous system such as during cold-induced vasoconstriction [34]. In addition, higher density of α1- and α2-adrenergic receptors associated with the splanchnic and cutaneous veins makes them highly sensitive to adrenergic stimulation, important in the induction of venoconstriction, mobilization of blood volume, and temperature regulation [33]. Veins are generally classified as deep (located beneath the muscle layer), superficial (located beneath the skin), and perforator/connecting veins that act as a link between the superficial and deep venous plexus [4]. The superficial and deep systems connect at numerous points at various non-junctional perforators. In the lower limbs, the venous blood returns to the right heart against gravity through both the superficial (i.e., the great and small saphenous veins) and deep (i.e., femoral) venous systems connected by numerous non-junctional perforators. Indeed, an estimated 90% of venous return from the legs is transmitted through the deep venous system. Not surprisingly, obstruction, reflux, or other maladaptive changes to the structural integrity of veins of the lower limbs may limit venous return and promotes edema formation, pain, skin pigmentation as well as the development of ulcers at the later stages of venous disease progression, among other symptoms [10, 12, 13].

While veins are similar in structure to the arteries, some major differences are observed reflecting primarily on the differences in their functional roles. The tunica intima layer of the larger-sized veins, deep and superficial veins contain one-way, bicuspid venous valves that are made up of ECM, namely, collagen and elastic fibers, and are covered by the EC monolayer [4]. These valves ensure the orthograde flow of blood from the periphery to the heart and are absent from the arterial system. The tunica media layer of veins is comprised of SMCs and scant elastic fibers with a more pronounced collagen fiber contribution as compared to similarly sized arteries [32]. The outer, tunica adventitia is comprised mainly of collagen fibers and connective tissue. Further, the internal and external elastic membranes that encase the tunica media layer in the arteries are absent from the venous blood vessels [32]. While a more pronounced smooth muscle cell layer is typical for the small- and medium-sized veins, larger veins exhibit an increase in the amount of connective tissue in the tunica media layer and a more pronounced collagenous, as opposed to elastic fiber contribution in the adventitia [32]. Given that the blood travels at relatively low pressures within veins, the vasa vasorum is found to penetrate the venous vessel wall closer towards the lumen, while the higher pressures exerted by the blood passing

within the arteries would collapse this intricate vascular network, which is thus situated on the periphery of arterial vessel walls. The size of the veins typically varies between 1 and 10 mm in diameter, with the largest vein, the inferior vena cava, ranging in size from 20 mm to over 28 mm in diameter while the smaller venues generally range from 8 to 100 μm in size [32, 35].

Aside from the histological and structural differences, differential classification of arteries and veins may be further complemented with the understanding of the early genetic programming in the developing embryo, as well as molecular markers that are thought to be characteristic for the venous and arterial branches of the circulatory system [36]. In fact, a number of findings suggest that additional signaling is required to induce arterial differentiation from the venous state, where the differentiation toward a venous pathway appears to be the default programming state of blood vessels during development [36]. Some of the identified molecular markers specific to the arterial vessels are known to include Dll4, Jag1, Notch1, Notch4, and Hey2 [36]. Similarly, a biomolecular marker characteristic of the arteries includes Ephrin B2 (Efnb2), a transmembrane ligand for the ephrin family. Ephrin B2 is known to be specifically expressed in the EC precursors that lead to the arterial differentiation. In contrast, the expression of Ephb4, a receptor for Efnb2, is reported preferentially in the veins [36, 37]. Additional markers, including chicken ovalbumin upstream promoter transcription factor II (COUP-TFII) as well Phosphatidylinositol-3 kinase (PI3K) signaling pathway have been also associated predominately with the venous molecular marker expression and maintenance of venous differentiation, respectively. COUP-TFII is a member of the orphan nuclear receptor superfamily and a factor implicated in the development of a number of tissues and organs including the heart and blood vessels, among others [38, 39]. Conditional ablation (removal) of COUP-TFII from the endothelium resulted in arterial differentiation of the veins while ectopic expression of COUP-TFII led to the loss of a number of arterial markers [36, 40]. Similarly, the PI3K signaling pathway has been further implicated in the differentiation and maintenance of the venous phenotype in zebrafish, where PI3K was found to promote venous cell fate by blocking arterial p42/44 MAPK activation [36, 41].

7.3 Morphological and Functional Features of Varicose and Insufficient Veins

The functional features of healthy veins meet the necessary criteria of a system that appears to be well designed to store and release blood capacity on request, features which are in turn critically dependent on the architecture, diameter, and distensibility of the vessel. In varicose (diseased) veins, both the vessel diameter and distensibility parameters are known to be increased [42, 43] as assessed by duplex-ultrasound techniques measuring changes in the venous diameter [44] (Fig. 7.1).

Further, the biophysical properties of healthy and varicose veins assessed by ultrasonography correlate with the distribution of the major structural proteins of the vein wall. In addition to the noted “rearrangement “of the smooth muscle cell layers,

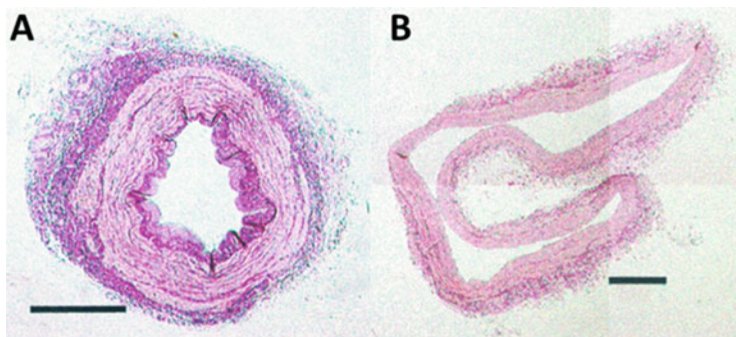

Fig. 7.1 Hematoxylin-eosin staining of normal and varicose saphenous veins. (**a**) Healthy vein with regular media. (**b**) Varicose vein with thinned media

the distribution of the major structural proteins of the vein wall is known to be altered from their physiologic, baseline levels. These include collagens, elastin, and smooth muscle cell actin, bearing further effect on the venous diameter and distensibility [45–47]. For instance, vascular smooth muscle actin is preferentially expressed in the intima and media, and to a lesser degree in the adventitia, in both healthy and varicose veins. Elastin, however, is abundantly detectable in the adventitia of healthy veins but significantly diminished in the adventitia of varicose veins. Loss of elastin in the adventitia may contribute to the functional features of varicose veins which are more distensible but show less elasticity. These histologic alterations were observed in the proximal as well as the distal superficial varicose veins (great saphenous vein, GSV). On the other hand, selective reduction of type III collagen in the intima and media is seen in the proximal varicose veins. This might explain the increased distensibility of the varicose veins. Interestingly, these findings are less prominent in the distal varicose veins although mRNA expression of MMP-2, MMP-9, TIMP-1, and TIMP-2—transcripts encoding collagenases and regulators of the proteolytic activity of cells—appears to be more pronounced in the distal as compared to proximal vein segments [48].

Indeed, elastin and type III collagen have an impact on the biophysical properties of varicose veins. Tissue array analysis has been carried out in the past, where the assessment of numerous tissue samples assembled on a single histologic slide allowed for a high throughput analysis of multiple specimens at the same time. The tissue array analysis examined the correlation of the biophysical and histological variables with the expression level of the structural proteins analyzed. Only elastin and collagen III, both reduced in varicose veins, were significantly and sufficiently correlated with duplex ultrasonographic measurements in vivo. Elastin, which was found exclusively in the adventitia of the vein, was negatively correlated with the vein diameter at rest. Type III collagen in the intima was negatively correlated with distensibility as measured by the absolute increase in venous diameter. Elastin in the adventitia and type III collagen in the intima did not correlate with each other at the same sector of the vein ring, suggesting that both pathological events may occur

independently. The finding of elastin loss in the whole length of the GSV yet a reduction in type III collagen expression in only the proximal part of the varicose vein supported this further. Elastin loss in the adventitia coupled with the generalized remodeling of the vein wall was associated with the generalized signs of varicose vein development and progression as these were conserved across both proximal and distal vein segments [42, 43].

This tissue microarray analysis linking dilatation and increased distensibility in varicose veins to the structural protein content in the vein wall identified two proteins that were specifically affected: adventitial elastin and intimal type III collagen. Similar to elastin, type III collagen has elastic properties, unlike type I collagen, which is a more rigid fibrillar protein. Both are preferentially located in different layers of the vein wall; elastin is found exclusively in the adventitia, whereas type III collagen is present in all three vein wall layers. The sites of deficiency, the intima for type III collagen and the adventitia for elastin, were separated by the circular media layer of vascular smooth muscle cells. Within the same sector of the vein, elastin, and type III collagen are not affected to the same degree. A selective decrease in type III collagen compared with type I collagen has been observed in varicose veins [46] which was attributed to the diminished synthesis of type III collagen by venous smooth muscle cells [49]. Type III collagen synthesis is not inhibited at the transcriptional but at a post-translational level [50].

Elastin loss may be a key factor in the development of varicose veins, preceding and precipitating dilatation. The concomitant reduction in type III collagen is involved in abnormal distensibility. The assessment of vein diameter and distensibility is a non-invasive diagnostic tool to quantify the elastin and type III collagen content of diseased veins. The prevention of elastin and collagen loss might be a possible therapeutic target for varicose veins.

7.4 Risk Factors of Varicose Vein Development and Venous Insufficiency

A number of hereditary or life-style related risk factors have been associated with the development of varicose veins including, among others: female gender, familial history of varicose veins, advancing age, multiple pregnancies, history of blood clots, and injury to the veins as well as conditions that cause increased pressure in the abdomen (i.e., prolonged standing, being overweight, prolonged sitting with legs crossed, among others) [1, 7, 51]. The initiating triggers for venous remodeling processes associated with varicose vein formation however have been a subject of debate. Malfunction of the valves in the larger size, deep and superficial vessels (venous valve reflux) has been previously associated with the onset and development of varicose veins as well as chronic venous insufficiency (CVI) [4, 6]. Other studies however report valve reflux as a secondary phenomenon to venous remodeling occurring at the molecular level due to other underlying conditions such as primary venous hypertension [1, 15, 52–54]. In fact, risk factors including prolonged sitting with legs crossed, wearing tight undergarments or clothes, prolonged standing as

well as conditions that cause increased pressure in the abdomen (e.g., obesity, pregnancy) have been associated with varicose vein development, supporting the hypothesis that venous hypertension may indeed contribute, if not initiate, the maladaptive processes associated with venous remodeling and varicose vein development [1, 51, 55]. Moreover, the simple fact that varicose and insufficient veins usually develop in lower extremities which are exposed to the highest level of hydrostatic pressure levels in upright position underlines the relationship between venous hypertension and venous remodeling [31].

To date, knowledge of the underlying mechanisms triggering the onset of varicose vein formation and CVI nevertheless remains lacking. Functional analysis of the underlying pathological mechanisms of varicose vein initiation and progression is further limited in humans given that the disease is already established at the time patients seek medical advice. Consequently, there is a need for the identification and manipulation of mechanisms controlling CVI and varicose vein development at the molecular level as well as expansion of current strategies to therapeutically interfere with the initiation and progression of CVI. In line with a number of the above-mentioned risk factors, venous hypertension may nevertheless be considered as the primary underlying pathogenic mechanism in varicose vein formation [1, 13, 31, 56, 57].

7.5 Flow and Pressure in Healthy and Insufficient Veins

As mentioned above, a common hypothesis for venous remodeling may suggest that an increase in the venous filling pressure acts as the initial trigger by exerting tension on the venous wall, leading to an increased circumferential wall stress. For example, when standing still, venous vasculature of the lower limbs is subject to high hydrostatic pressures exerted by the effect of gravity and the pooling of blood [58]. At the level of the right atrium, the venous hydrostatic pressure approaches 0 mmHg and then increases by an estimated by +0.8 mmHg with each additional cm beneath that point, reaching as high as 90–100 mmHg in deep and superficial veins of the ankle and the lower leg [58, 59]. Upon active contraction of the calf muscles of the lower leg however (i.e., while walking), this pressure is subsequently reduced due to an enhanced venous return of blood from the lower limbs against the forces of gravity that is supported by (1) the active compression of the deeper veins during muscle contraction, (2) prevention of reflux by competent vein valves (mainly located in the veins of the limbs, rarely in the iliac veins and absent in the inferior caval vein), (3) the relative (diastolic) negative pressure of around −4 mm Hg in the right heart and (4) the “pumping” effect generated by in- and expiration. Indeed, the activation of the so-called “calf muscle pump” alone evokes physiological decrease in the hydrostatic venous pressure to approximately 40 mmHg–25 mmHg in the veins of the lower leg and foot [58]. A corresponding example is shown in Fig. 7.2.

Further, when the muscles relax, the emptied deep veins are now found at a lower pressure which allows for the drainage of blood from the superficial veins, thereby also reducing their pressure. In contrast, the hydrostatic pressure of popliteal and

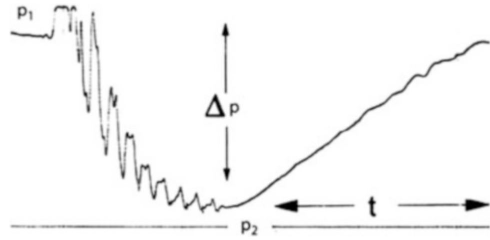

Fig. 7.2 Venous pressure of a healthy volunteer in the upright position, during exercise and rest. p_1: 90 mm Hg upright position, p_2: 20 mmHg after ten times standing on tiptoes, Δp: 70 mm Hg decrease in pressure during exercise

femoral veins of the thigh is not affected to the same extent by the calf muscle activity. This difference in the pressure gradient between the thigh and the venous vasculature of the lower limb is commonly referred to as the "ambulatory pressure," estimated at 37.4 ± 6.4 mm Hg [58, 59].

The ambulatory venous pressure in patients with varicose and/or insufficient veins is chronically increased above normal levels and may rise up to 80–90 mmHg in patients with obstruction of the iliac vein after deep vein thrombosis. In fact, ambulatory venous hypertension may contribute to all clinical manifestations of chronic venous insufficiency, i.e., varicose veins, edema, skin changes, and ulceration. In this context, Nicolaides demonstrated that the incidence of venous ulceration is 100% in patients when the ambulatory venous hypertension is 90 mm Hg [60]. In insufficient veins, muscle activity is not capable to reduce the pressure due to the increase in venous reflux. The failure to reduce the superficial ambulatory venous pressure on exercise may occur as a result of malfunctioning valves of the deep or superficial veins, leading to venous reflux or regurgitation of blood flow and pooling of blood in the lower limb [7]. In patients with CVI, the pressure in the surface veins of the leg remains elevated, leading to venous dilatation and further changes in the structural integrity and function of the venous valves and vessel wall [1].

As indicated by the association of venous reflux and venous hypertension, there is a close relationship between flow and pressure. While being in upright position the venous flow velocity in the extremities is controlled by muscle activity, blood volume, the diameter, and compliance of veins (25-fold higher as compared to arteries), the blood viscosity, and the temperature. In supine position, the flow velocity in venules is 0.03 cm/sec and rises up to ~20 cm/sec in the inferior caval vein. These values change ~2.5-fold if the extremities are lifted by an angle of 20°, ~0.6-fold in upright position, 1.2-fold while walking, and up to 4.4-fold while cycling. Considering a reflux velocity of ~62 cm/sec (as measured by spectral Doppler ultrasound [61] in insufficient saphenous veins and the corresponding volume of recirculating blood, venous reflux chronically elevates the intraluminal pressure in veins of the lower extremities in the upright position.

7.6 Biomechanically Evoked Venous Remodeling in Experimental Models

Veins are constantly exposed to biomechanical forces such as shear stress and biomechanical stretch exerted by the movement of blood inside the vessel which, depending on the magnitude and duration, may stabilize the vessel wall or trigger the maladaptive remodeling processes. Shear stress is borne directly by the endothelial cells (ECs) as a result of friction of the passing blood and is applied in parallel to the vessel wall (Fig. 7.3).

In contrast, the biomechanical stretch is applied perpendicularly to the vessel wall as a result of increased intraluminal pressure inside the vessel, which subsequently distends the vessel wall [62]. According to the Law of Laplace, this promotes an increase in circumferential wall tension that is borne by venous ECs and smooth muscle cells (SMCs) making up the vessel wall (Fig. 7.1) [1]. Unlike biomechanical stretch, mean shear stress levels are rather low in the venous circulation.

Feldner et al., have reported that an increase in the venous filling pressure alone is sufficient to support varicose-like vein formation in mice by stretch-activating venous SMCs and ECs lining the vessel wall [52]. In this study, Feldner et al. have adopted a model where occlusion of one of the larger veins in the mouse auricle allowed for a local increase in intravenous filling pressure (P_{tm}) and an associated

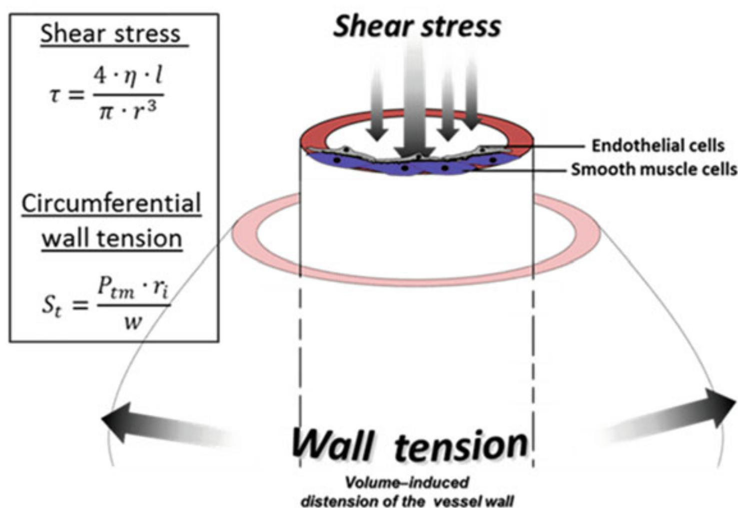

Fig. 7.3 Biomechanical forces in the vasculature. Shear stress is directly proportional to the length of the blood vessel (l) and blood viscosity (η) and correlates inversely with the vessel radius (r). Circumferential wall tension is directly proportional to the transmural pressure difference (Ptm) and the initial, resting vessel radius (ri) and correlates inversely with the vessel wall thickness (w). ECs are exposed to shear stress generated by the movement of blood and to biomechanical stretch as a result of the increase in the volume of the moving blood during systole. SMCs cells are exposed primarily to biomechanical stretch

rise in circumferential wall tension (S_t) in the collateral venules (refer to Fig. 7.1) [52]. This initial venous “stretch activation” served as a driving force for subsequent (up to) 2.5 fold enlargement of the remodeling veins, which were found to be comparable in appearance to the cork-screw like varices observed in humans [52]. Additional in vivo rodent models of acute and chronic venous hypertension have been utilized in the past and are based on a similar principle of venous ligation, namely, an increase in the filling pressure that results in the remodeling of the collateral venules [63–66]. In comparison to Feldner et al., these ligations have been carried out on the inferior vena cava, bilateral common iliac veins or bilateral common femoral veins in the hindlimb of mice and/or rats, involving a rather invasive surgical procedure. Moreover, venous remodeling associated with deep venous occlusion was found to be accompanied by severe venous hypertension, leukocyte infiltration (inflammation), hindlimb edema, increased valve height, and increased valvular reflux [52, 63–66]. While these responses are commonly associated with the later stages of varicose vein disease development, they are generally absent in early varicose lesions in humans or other murine models that report mild venous hypertension and a limited inflammatory response [52, 63–66]. Further, the application of biomechanical stretch in an in vitro culture of venous SMCs or ECs serves as an additional model of stretch-induced venous remodeling and is often used to better characterize stretch-induced molecular pathways on a cellular level [62, 67–70].

7.7 Mechanisms of Biomechanically Induced Venous Remodeling

Shear stress and hypertension-derived circumferential wall stress are well-established determinants of the functional and molecular phenotype of vascular cells which have the capacity to alter the structure and functional properties of blood vessels. Given the relevance of these environmental stimuli for survival and cellular functions, nature has developed a plethora of mechanisms sensing these forces.

Besides many cell type and partially site-specific sensing mechanisms, one of the most relevant and ubiquitously present parts of the mechanosensing system is the cytoskeleton. It is connected to the environment via cellular adherens junctions or focal adhesion sites and thus able to sense any subtle change in the relative position of neighboring cells or components of the extracellular matrix and their rigidity, respectively. Moreover, it is also linked with many signaling complexes located on the surface of vascular cells and thus forms a structural network integrating multiple external physical parameters acting on the cell. Depending on the direction and level, biomechanical forces may evoke a deformation of the cytoskeleton raising or lowering its tension which is subsequently translated into specific signaling events once a distinct threshold has been passed. The corresponding cellular behavior has been elegantly described by the “tensegrity model” [71, 72] which explains how biomechanical forces are transmitted through the cytoskeleton to finally stimulate

clustering of integrins in focal adhesions and induce multifaceted signaling cascades involving focal adhesion kinases, Src-kinase-, Rho-kinase-, or G-protein-dependent pathways. Based on this principle, laminar flow shears the surface of the endothelial cells in relation to their base inducing a discrete tension of the cytoskeleton which translates into conformational activation of integrins avb3, transient inhibition of Rho allowing the realignment of the cytoskeleton in flow direction [73]. More recent work indicates that junctional receptor complexes comprising PECAM-1, VE-cadherin, and VEGFR2/3 may also act as autonomous mechanotransducers [74] and form a signaling hub that orchestrates shear stress-mediated activation of eNOS [75, 76], via PI3K/Akt.

Similar mechanosensing mechanisms exist for the assessment of circumferential wall stress which may increase as a consequence of raising intraluminal pressure and lead to biomechanical stretch of vascular cells. Stretching the membrane of arterial SMCs stimulates the activation of Gq/11-coupled angiotensin II AT(1) receptors, which subsequently induces phospholipase C-dependent signaling to activate type C transient receptor potential (TRPC) ion channels, raise the membrane potential and ultimately increase the myogenic tone [77]. As can be deduced from the tensegrity model, exposure of arteries to elevated pressure levels results in integrin/src-dependent FAK activation [78]. Src-dependent signaling has also been shown to unleash NADPH-dependent superoxide generation [79] which is a prototypic response of ECs and SMCs exposed to hypertension- and/or biomechanical stretch [80, 81].

Despite a substantial amount of studies that have delineated the aforementioned mechanisms, their relevance and validity for the venous system remain unclear as both the pressure and flow profile significantly differ from that observed in arteries. For instance, the mechanosensitive properties of arterial endothelial cells allow distinguishing between laminar and oscillatory flow from which the former promotes the shear stress-induced release of nitric oxide, arterial relaxation, anti-thrombotic, anti-inflammatory, and quiescence-mediating effects [1, 82]. In contrast, oscillatory flow or absence of shear stress is associated with the development of endothelial cell dysfunction driving pro-inflammatory differentiation and lay the ground for the development of arteriosclerosis-prone sites [83–85]. As such, the inhomogenous muscle-pump-dependent and partially oscillatory flow in veins may limit the baseline capacity of the venous endothelium to produce NO even under physiological conditions. In insufficient veins, the reflux of blood and accompanying venous hypertension may further limit the bioavailability of NO due to both the decreased fraction of laminar flow and pressure-induced NADPH-dependent superoxide production which would transform NO to peroxynitrite. Under these conditions, the protective functions of EC may be limited and enable the development of varicosis-prone areas in veins. In fact, lower NO levels have been reported in blood collected from foot veins of patients with primary varicose as compared to the control group [86] and several reports indicated that ROS production is elevated while antioxidative mechanisms (e.g., superoxide dismutase) are impaired in varicose and insufficient veins [87–89].

Although such studies contribute to the identification of cellular responses in established varicose and/or insufficient veins, mechanisms initiating the development of these pathologies and the relevance of biomechanical parameters in this context are still under debate [31]. However, as summarized above, results from different animal models underline the relevance of venous hypertension as a primary cause for the development of varicose and/or insufficient veins [90]. Specifically, creating femoral arteriovenous fistulae and subsequent venous hypertension resulted in edema formation, expression of matrix metalloproteinases 2 and 9 (MMP-2 and MMP-9), increasing valvular reflux, and further morphologic changes comparable to those observed in human insufficient veins [66]. Likewise, increasing the intraluminal pressure in porcine jugular veins has an immediate impact on their tortuosity [91]. Results from experimental models as mentioned above suggested that ligation of a single central auricle vein is sufficient to promote the development of tortuous and bulged veins in the connected venous network. Endothelial as well as smooth muscle cells in remodeling veins show an increase in proliferation and MMP-2 expression thereby mimicking features of stretch-exposed venous smooth muscle cells, mouse veins exposed to supraphysiological pressure levels, and human varicose veins (CEAP-classification: C2-C3) [52]. Under these conditions, blocking the activity of the mechanoresponsive transcription factor activator protein 1 (AP-1) inhibited MMP-2 expression and proliferation which underlines its relevance for the orchestration of biomechanically evoked venous remodeling processes. Moreover, treatment of remodeling or hypertension-exposed veins with statins—pharmacological inhibitors of the 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase which also attenuate AP-1 and NF-kB activity [92]- showed similar effects [67]. Along these lines, smooth muscle cells in varicose veins from patients under cholesterol-lowering statin therapy show a lower degree of proliferation, MMP-2 abundance, and activated (phosphorylated) c-fos—a subunit of AP-1. Considering that multiple mechanosensitive signaling pathways converge on AP-1 and modulate its activity by phosphorylating/dephosphorylating its subunits c-jun and c-fos including mitogen-activated kinases such as JNK and ERK1/2 [93, 94], it is tempting to assume a critical role of AP-1 as integrative signaling hub that translates biomechanical stimuli into an altered transcriptome of ECs and SMCs as a prerequisite for the initiation of venous remodeling. As such, activation of AP-1 in venous cells that are chronically exposed to elevated levels of biomechanical stretch or circumferential wall stress may promote the expression of genes regulating the proteolytic capacity, proliferation, and inflammation [95–98]. Such a mechanism would explain observations suggesting a venous hypertension/wall stress-dependent increase in the abundance and activity of matrix metalloproteinases especially in atrophic regions of human varicose veins [99] which may additionally spurred by inflammatory events in varicose veins complicated by thrombophlebitis [100]. Consequences of locally increased MMP activity and matrix degradation may comprise venous dilation and valve dysfunction, amplify the level of circumferential wall stress (see Fig. 7.1) and initiate a self-promoting vicious cascade of events. These environmental conditions may also promote an altered composition of the extracellular matrix as has been observed for collagen type I and III, aggrecan,

laminins, and proteoglycans [46, 50, 101]. Increasing levels of collagen type I and decreasing levels of the more distensible collagen type III in varicose veins would ultimately affect the rigidity and compliance of the vein wall, modify the way how biomechanical forces are transmitted to the cells, and further impair the venous architecture. In fact, malfunction of vein valves is supposed to be secondary to changes in the elastic properties of the vein wall, the rate of arterial inflow, vein dilation, or [102, 103] distension of the valve "rings" as a consequence of venous hypertension [104].

In addition to biomechanical forces generated by an increase in intraluminal pressure levels, alterations of the physiological venous flow profile may also contribute to the development of varicose and insufficient veins. On the one hand, venous reflux causes ambulatory venous hypertension by impairing the return of venous blood, and thus the physiological pressure drop [105]. On the other hand, non-pulsatile venous blood flow or the disturbed venous return may cause local hypoxemia (e.g., indirectly through deep vein thrombosis) which is thought to injure venous valves and thus promote their incompetence [104]. Along these lines, blood stasis in varicose veins is associated with lower venous oxygen partial pressure and an increase in plasma pro-MMP-9 activity as a consequence of polymorphonuclear leukocyte activation which would in fact contribute to a flow-mediated amplification of the local proteolytic capacity [106]. While further detailed mechanistic studies are missing in this context, reflux velocity and flow also appear to correlate with the diameter of great saphenous veins and the severity of primary varicosis [107]. However, from a mechanistic point of view, such observations may be interpreted as both cause and consequence of venous remodeling processes. Particularly the bidirectional relationship of venous return and intraluminal pressure (i.e., venous hypertension opposes venous return and venous reflux causes hypertension) prohibits a more decisive judgment about the individual relevance of these biomechanical parameters in initiating the remodeling of the vein wall.

7.8 Conclusions

The vein wall is exposed to different types of biomechanical forces from which circumferential wall tension is mainly generated by venous pressure while the flow profile determines the level of shear stress to which endothelial cells are exposed. Due to site-specific features of veins (e.g., position of a vein segment in the lower extremity while standing upright; different flow profiles at the location of the valves) and the bidirectional relationship of flow and pressure in this vascular entity, the individual mechanistic relevance of these parameters for the initiation and promotion of venous remodeling processes remains obscure. Nevertheless, the characteristics of risk factors for varicose/insufficient veins as well as the experimental evidence from animal models strongly suggest venous hypertension and chronically elevated circumferential wall tension as a critical biomechanical determinant which has the capacity to initiate venous remodeling. Genes controlling structural features of both the vein wall and the connective tissue may additionally support or counteract this

process by altering the susceptibility of veins to biomechanical stress. However, precise mechanistic studies delineating the nature of vein-specific cellular responses to specific biomechanical stressors are scarce. Assuming that comparable sensing mechanisms exist in venous and arterial endothelial and smooth muscle cells, the question whether biomechanical stimuli are also translated in a similar way remains unanswered. From a clinical point of view, arterial hypertension in combination with oscillatory flow promotes site-specific pro-inflammatory arteriosclerosis-related responses of endothelial and smooth muscle cells and arterial stiffening. Venous hypertension primarily results in the irregular remodeling of the vein architecture whereby the relevance of inflammation and leukocytes is unclear. Are there compensatory mechanisms protecting vascular cells in veins from a similar fate as in hypertension-exposed arteries, which may include foam cell and plaque formation or do they in fact respond differently to changes in biomechanical forces? Would statins have similar beneficial effects on the pathophysiological remodeling of veins (e.g., indirectly by blocking AP-1 activity) as on neointima formation and sclerosis of arteries?

Answering such questions will be a critical challenge for the future and requires causative mechanistic studies based on state-of-the-art animal models as the analyses of human samples usually evaluates distinct (end) stages of a complex disease allowing for the demonstration of analogies rather than causal relationships to the cellular responses. Although limited by a reductionist design that may only partially mimic the complex and long-lasting mechanisms triggered by biomechanical stimuli in venous cells, animal models may help to identify individual molecular determinants which are rate-limiting for the initial steps or chronic promotion of biomechanically induced venous remodeling processes.

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Compliance with Ethical Standards

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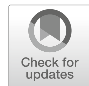

Mechanobiology of Lymphatic Vessels

8

Anish Mukherjee and J. Brandon Dixon

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Abstract

While the cardiovascular system acts as the major circulatory system in the human body, the existence of a parallel, almost equally important, secondary circulatory system is frequently overlooked. This system is called the lymphatic system, which serves to perform three primary functions in the human body: (1) drainage of interstitial fluid, (2) transport of immune cells, and (3) transport of lipid. These three functions are performed with the help of an intricate network of vessels and nodes, called the lymphatic system. The lymphatic system has its own unique anatomy and physiology that interacts with, and is influenced by, its local mechanical microenvironment. In this review, we will highlight studies that have delineated the molecular mechanisms underlying the mechanosensitivity of the lymphatics and the role of mechanomodulation on lymphatic development, physiology and pathophysiology. These topics will be explored in the context of early development, postnatal development and maturation, normal physiology, and disease.

8.1 Introduction

While the cardiovascular system acts as the major circulatory system in the human body, the existence of a parallel, almost equally important, secondary circulatory system is frequently overlooked. This system is called the lymphatic system, which serves to perform three primary functions in the human body: (1) drainage of interstitial fluid, (2) transport of immune cells, and (3) transport of lipid. These three functions are performed with the help of an intricate network of vessels and nodes, called the lymphatic system [1, 2]. The lymphatic system has its own unique anatomy and physiology, and a discussion about both is crucial before delving into the effect of mechanical forces on their function.

The journey of the lymphatic system begins with lymphatic capillaries (also called initial lymphatics), which are closed-ended vessels formed by a single layer of endothelial cells without any basement membrane [3–5]. This is where the uptake of interstitial fluid occurs in the lymphatic vasculature, through specialized junctions between the endothelial cells. The endothelial cells form overlapping flap-like structures that are called primary lymphatic valves, which are held together with button-like junctions [6, 7]. A defining feature of these endothelial cells is their connection to their surrounding extracellular matrix with anchoring filaments. During periods of excess interstitial fluid accumulation, the anchoring filaments open the primary lymphatic valves, which results in the movement of interstitial fluid into the initial lymphatics [8, 9]. The interstitial fluid is referred to as “lymph” once it enters

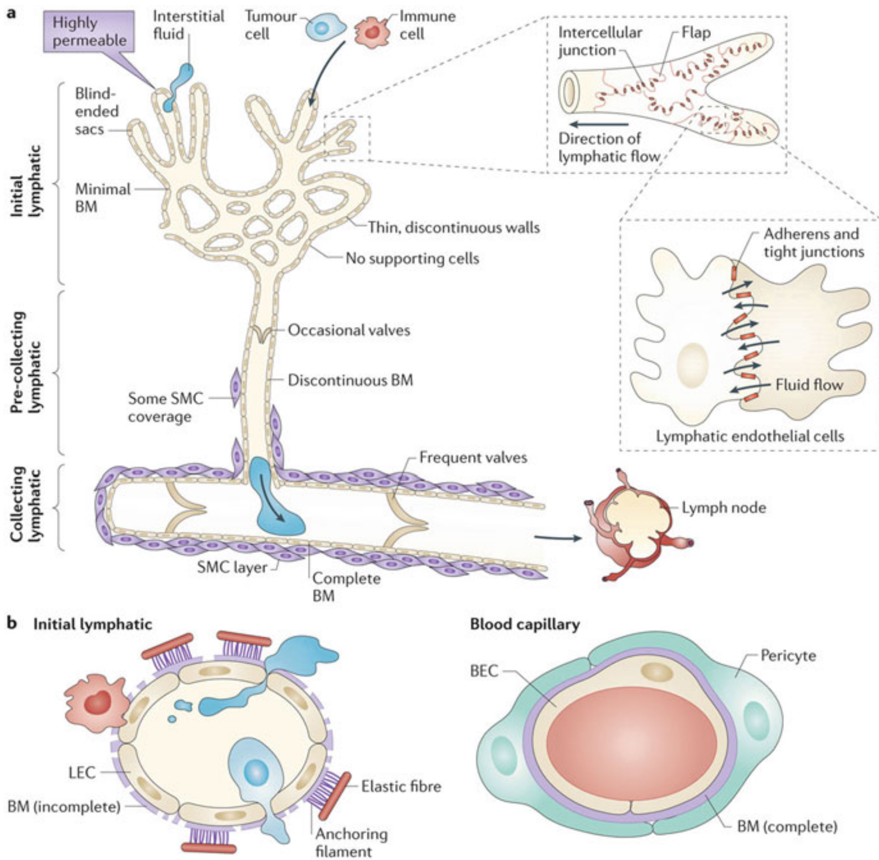

Fig. 8.1 An overview of the various components of the lymphatic system. (**a**) The organization of the lymphatic network is shown, starting from the initial lymphatic capillaries and moving to the pre-collecting and collecting lymphatics. The primary lymphatic valves in the initial lymphatics formed by the “button-like” junctions are highlighted. (**b**) The initial lymphatic structure is compared to a blood capillary, showing a nearly absent basement membrane and the presence of special anchoring filaments that help in the formation of lymph by opening the primary lymphatic valves [10]

the initial lymphatics and flows through the lymphatic vasculature. Figure 8.1 shows the main components of the lymphatic vasculature with an emphasis on the structure of the initial lymphatic vessels. Figure 8.1b includes a comparison with the blood capillaries to show the key structural differences.

The second major structure is the collecting lymphatic vessel (Fig. 8.2). The initial lymphatics connect and form structures called pre-collecting lymphatics which are characterized by a basement membrane surrounding the endothelial cells and sparse muscle cell coverage [12, 13]. These develop downstream into

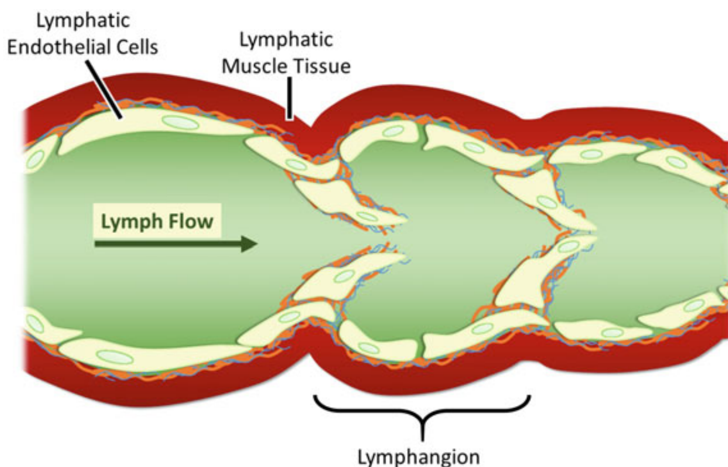

Fig. 8.2 The structure of the collecting lymphatic vessels is shown. The vessels are primarily composed of lymphatic endothelial cells and lymphatic muscle cells. The presence of valves ensures a unidirectional lymph flow [11]

collecting lymphatic vessels, which are characterized by a complete basement membrane, lymphatic muscle cell (LMC) coverage, and well-developed valves (called secondary lymphatic valves) that ensure a unidirectional flow through the vessels [14, 15]. The collecting lymphatic vessel physiology is unique since they can spontaneously perform fast phasic contractions on top of the slow tonic constrictions that are also seen in blood vessels [16, 17]. The segment of a collecting lymphatic vessel between two valves is called a lymphangion, which is the basic spontaneously contractile unit of the lymphatic vasculature. The lymphangions, with the assistance of the secondary lymphatic valves, ensure a unidirectional flow of lymph through the lymphatic vasculature.

The lymphatic vasculature is also interspersed with multiple lymph nodes. The lymph nodes serve as the points of exchange of nutrients and immune cells, while also participating in the activation of the adaptive immune system [2, 18]. Afferent lymphatic vessels carry lymph, along with nutrients, chemokines, and cells, into the lymph nodes [19]. It is estimated that as much as half of all lymph is reabsorbed into the blood circulation by high endothelial venules within lymph nodes as observed by the fact that efferent lymph has a higher protein concentration than afferent lymph [20–22]. The rest of the lymph continues on its journey through the lymphatic circulation through the efferent lymphatic vessels. The physiological differences of the lymphatic system with the blood vasculature are reflected in the differences in mechanical forces experienced by cells within the vessels. Because of the fast tonic contractions of the lymphatic vessels, they are exposed to a highly oscillatory mechanical microenvironment. A typical lymphangion can contract to around 40% of its diastolic diameter with a frequency of up to 15 contractions per minute. The periodic contraction of the lymphangion creates an oscillatory pressure and flow, with periodic flow reversal due to delayed secondary valve closure. The

oscillatory flow can exert a peak shear stress of 4–12 dynes/cm^2 as has been observed in in situ preparations of rat mesenteric lymphatic vessels [23]. The primary forces experienced by the lymphatic vessels can be grouped into five types: (1) forces due to interstitial fluid flow and (2) interstitial pressure which exerts external forces on the lymphatic vessels and are primarily relevant in the context of developing and initial lymphatics, (3) transmural pressure which exerts a radial force on the contracting lymphangions and correlates with the basal stretch of the lymphangions, (4) axial force due to stretch from the surrounding tissue pulling on the lymphangion chains, and (5) the wall shear stress (WSS) exerted by the flow of lymph within the lumen of the lymphangions. These forces act together to control various aspects of lymphatic function, starting from the development of the lymphatic vasculature and lymphatic fate specification, to lymphangiogenesis and lymphatic vessel maturation, to the control of lymphatic vessel contraction and synchronization. Further, exposure to pathological levels of these forces can lead to a maladaptive response which can lead to a reduced sensitivity of the lymphatic vessels to the mechanical microenvironment, remodeling of the vessel, and various other complications that may arise out of these dysfunctions.

The fact that the lymphangions and, in turn, the lymphatic vessels can respond to their mechanical microenvironment necessitates the presence of mechanotransduction machineries that serve to transduce the mechanical forces to biologically relevant actions. The field of vascular physiology has managed to capture a wide range of these mechanotransduction mechanisms which primarily act through the endothelial cells, activating a number of mechanosensory complexes that have some physiological effect [24–29]. The physiological effects of the endothelial mechanotransduction of shear stress and stretch can range from regulation of the tone of the blood vessel through vasorelaxation [30, 31], to angiogenesis [32, 33]. Pathological levels of shear and stretch can cause remodeling of blood vessels [33, 34] and complications like atherosclerosis [35–37]. Lymphatic vessels also show a similar adaptation and reaction to their changing microenvironment, which dictates their growth, maturation, and physiology [38]. Pathological levels of these mechanical forces can also lead to maladaptive lymphatic remodeling and other complications that may directly impact the lymphatic system or have secondary effects on other systems like the cardiovascular system.

This review will attempt to encompass the various mechanotransduction pathways that have currently been investigated in lymphatic vessels, the effect of mechanotransduction on lymphatic development and physiology, the involvement of mechanically mediated adaptations to lymphatic growth and remodeling, and the pathologies related to elevated mechanical forces and/or reduced sensitivity of lymphatic vessels to their mechanical microenvironment. Starting from an analysis of the role of mechanical forces on lymphatic vessel development, the review will go on to elucidate the role of the lymphatic vessel mechanical microenvironment on postnatal lymphangiogenesis, transport of solute across lymphatic vessel walls, control of lymphatic contractility, and finally end with a discussion on pathologies associated with abnormal mechanical forces or impaired response of the lymphatics to the mechanical microenvironment. Each section will dive into the various

molecular mechanisms by which mechanical forces are transduced by lymphatic endothelial and muscle cells, all the while distinguishing between the forces that trigger these pathways and lead to the appropriate physiological response.

8.2 Expansion and Maturation of Lymphatic Vessels

Lymphatic endothelial cells (LECs) share similarities with vascular endothelial cells in terms of their molecular structure but also express lymphatic specific markers [39, 40]. Some of the markers that are specific to the LECs are LYVE-1 [41, 42] (a hyaluronan receptor homologous to CD44), VEGFR-3 [43, 44] (a receptor of VEGF-C and VEGF-D, involved in endothelial cell proliferation), Podoplanin [40, 45] (a small membrane glycoprotein), and Prox-1 [46] (a transcription factor important in lymphatic development). The lineage of lymphatic vessels can be traced back to lymph sacs that have a venous origin in mammalian embryos [47]. The mechanical forces that control the initial lineage commitment, the migration, and organization of LECs, and eventually lymphatic valve formation and maturation of the collecting lymphatic vessel, can be broadly categorized as the interstitial forces (interstitial pressure and flow) and luminal shear stress.

8.2.1 Interstitial Forces

The interstitial forces primarily come into play during the initial stages of lymphatic development, i.e., during the initial commitment to lymphatic lineage and the proliferation and distribution of LECs in a developing lymphatic vasculature [48]. The various physiologically relevant forces acting on the initial lymphatic vasculature are shown in Fig. 8.3a. Interstitial pressure has been found to be correlated to the expansion of LECs in a developing mouse embryo and has been identified as an important factor in the proliferation of LECs during development [49]. Peak periods in LEC elongation, proliferation, and VEGFR-3 signaling were found to coincide with the peaks in interstitial pressure during the development of mouse embryo. The phosphorylation of VEGFR-3 and LEC proliferation were found to increase or decrease with addition or removal, respectively, of interstitial fluid in the embryo. β_1 Integrin, which links the ECM to the actin cytoskeleton of LECs, was significantly upregulated in response to increased interstitial pressure and was co-localized with phosphorylated VEGFR-3. Further, the role of β_1 Integrin in the phosphorylation of VEGFR-3, and LEC proliferation, was confirmed in the presence of stretch and increased interstitial pressure, consolidating the idea that the mechanotransduction of interstitial pressure during development is necessary for the proliferation of LECs and consequently lymphatic vessel expansion (Fig. 8.3b).

Interstitial flow can dictate the formation of the lymphatic vasculature by controlling the morphogenesis of initial lymphatic networks. An in vitro study on LECs and blood endothelial cells (BECs) have shown the differential morphogenesis of these cells, regulated by the interstitial flow [50]. In a 3D cell culture performed in

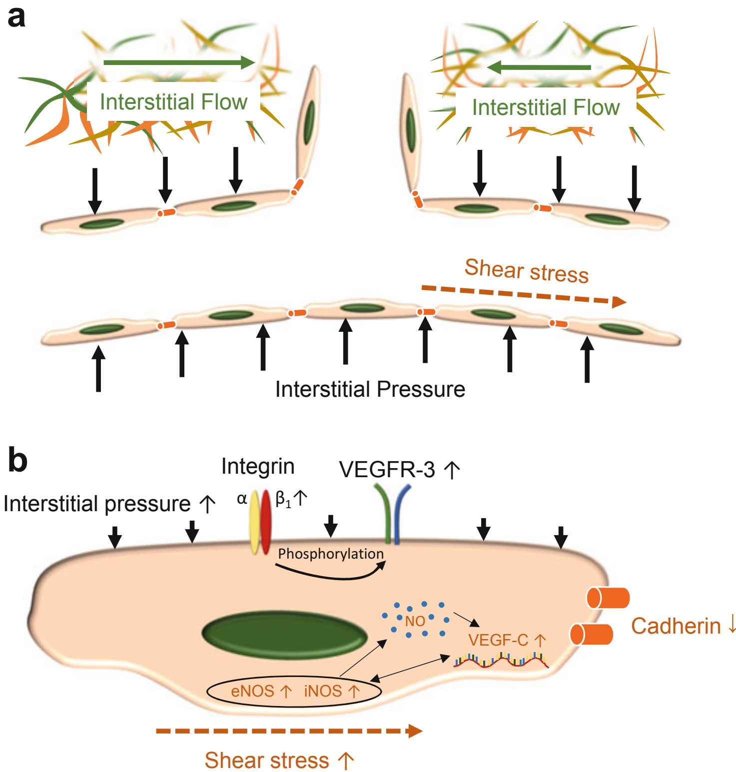

Fig. 8.3 The various physiologically relevant forces which drive the morphogenesis and proliferation of neonatal/postnatal lymphatic capillaries. (**a**) The interstitial pressure drives the development of the initial lymphatic vasculature while interstitial flow directs the formation of growth factor gradients and guides lymphangiogenesis. Luminal shear stress is most relevant during postnatal lymphangiogenesis. (**b**) Interstitial pressure drives the proliferation of LECs by upregulating VEGFR-3, with β_1 integrin being involved in mechanotransduction. Luminal shear stress acts through the eNOS and iNOS pathways, which stimulate VEGF-C mRNA expression. Shear stress can also regulate pan-cadherin expression, which may be important in the morphogenesis of lymphatic capillaries

collagen gel, both LECs and BECs exhibited the formation of multicellular networks and lumen which are characteristic of endothelial cells in culture inside unconfined collagen gels. An application of an interstitial flow with a velocity of 10μm/s increased the network and lumen formation significantly for LECs and BECs. BECs formed complex lumen containing networks while LECs preferentially formed longer actin extensions that interacted with the ECM, which is reminiscent of the ECM interactions that are characteristic of initial lymphatics. Furthermore, pan-cadherin expression was drastically reduced in LECs in response to flow, while the expression was unchanged for BECs. The reduced cadherin expression is suggestive of a lymphatic morphology, where the initial lymphatics are characterized

by the formation of loose cell–cell junctions unlike the tight endothelial junctions present in BECs.

8.2.2 Luminal Shear Stress

Apart from the interstitial flow, the luminal shear stress also plays an important role in the patterning, maturation, and stabilization of lymphatic vessels. One of the most important steps in the maturation of lymphatic vessels is the formation of the secondary lymphatic valves, which enable the unidirectional flow of lymph through the lymphatic system. Dysfunctional valves can lead to a compromised lymphatic system and have been implicated in numerous lymphatic-associated complications such as lymphedema [51–53]. Valve malformation is frequently a hallmark of primary lymphedema that is caused due to genetic defects and leads to a compromised lymphatic system [54, 55]. The expression of transcription factors that regulate valve formation is also heavily correlated to the fluid shear stress that developing lymphatic vessels experience. Thus, mechanotransduction has been implicated as playing a major role in the development of lymphatic valves. It will be prudent to look at some of the molecules involved in the major mechanotransduction pathways leading to lymphatic valve formation.

PROX1 and FOXC2 transcription factors have been shown to be major factors in the formation of lymphovenous valves [46, 56, 57] and their absence is correlated to lymphatic valve failure, as studied using $FOXC2^{-/-}$ mice [51]. Connexin37 (cx37) [58] and calcineurin [57] play important roles in the precise development of lymphatic valves and postnatal maintenance. Calcineurin leads to the activation of NFATc1 which plays a role in the formation of collecting lymphatic vessels, cooperatively with FOXC2 [57]. Sabine et al. [59] showed that PROX1 and FOXC2 expression is dependent on the flow that developing LECs are exposed to, which may act cooperatively with connexin37 and calcineurin/NFAT to promote the development and maturation of lymphatic valves. In vitro application of oscillatory shear stress (OSS) upregulated cx37 and nuclear NFATc1 expression, which was dependent on the presence of PROX1 and FOXC2. The application of OSS was also found to upregulate the expression of endothelial nitric oxide synthase (eNOS) and FOXC2, while a low, steady shear stress (LSS) upregulated KLF2. PROX1 and OSS were found to be required for the expression of cx37. Prox-1 and FOXC2 were also found to affect the cytoskeletal structure of LECs. A follow-up work by the same group showed that the absence of FOXC2 results in dysfunctional lymphatics, characterized by malformed or absent valves, reduced barrier integrity, and collapsed lumen near the valve sites. FOXC2 deletion was also associated with an abnormal response of LECs to OSS, resulting in increased cell proliferation and cell death through YAP1/TAZ signaling [60]. The increased proliferation is probably due to reduced contact inhibition in $FOXC2^{KD}$ mice and shows that FOXC2 is required to maintain the cell–cell junction integrity and hence the quiescence of LECs in response to an OSS.

Given that WSS seems to be an important mechanical cue in lymphatic development, and that WSS is usually much lower in the lymphatics than in the blood vasculature, LECs would presumably need to have a much lower set point for WSS, in order to elicit a response, than BECs. To test this hypothesis, Baeyens and colleagues cultured LECs and human umbilical vein endothelial cells (HUVECs) in gradient flow chambers to determine the magnitude of WSS necessary to induce cell alignment [61]. HUVECs achieved maximum alignment in the shear range of 8–20 dynes/cm^2 whereas LECs aligned in the range of 4–10 dynes/cm^2. Interestingly, silencing of VEGFR3 in LECs shifted their WSS set point up, and the increased expression of VEGFR3 in HUVEC shifted their set point down. This shows that the WSS set point for cell alignment in vitro depends on VEGFR3. However, the extent that VEGFR3 is crucial for determining this WSS threshold in vivo remains to be determined.

An interesting study performed by Sweet et al. [62] attempted to isolate the effect of impaired lymph flow on lymphatic vessel maturation. C-type lectin receptor-2 (CLEC2) is expressed on platelets and bind specifically with podoplanin, a cell-surface protein unique to LECs. This supposedly prevents the flow of blood into the lymphatics at points of contact of the lymphatic system with the venous system. The authors used a CLEC2$^{-/-}$ mouse to impede the lymph flow in vivo, which resulted in impaired lymphatic valve development with fewer PROX1 LEC clusters. Another interesting finding was the abnormal recruitment of lymphatic muscle cells (LMCs) and excess coverage of LMCs in CLEC2$^{-/-}$ mice, very similar to that observed in FOXC2 deficient vessels [51]. Interestingly, CLEC2 deletion did not affect the formation of mesenteric lymphatic vessels or neonatal lymph flow. The results suggest that lymph flow primarily helps in the maturation of valves when the lymphatic network is forming and possibly regulates the LMC coverage on lymphatic vessels to facilitate the development of healthy lymphangions.

Another molecule that has been implicated in the mechanosensitive response of LECs that leads to lymphatic valve maturation is GATA-2 [63], which is required for the lymphatic valve formation driven through PROX1 and FOXC2. The application of OSS in vitro was found to upregulate GATA-2 in LECs and the deletion of GATA-2 was marked by an absence of PROX1 and FOXC2 in sites of lymphatic vessel formation in vivo. OSS was also found to stimulate the Wnt/β-catenin pathway on LECS cultured in vitro, and the Wnt/β-catenin pathway had a demonstrated role on lymphatic vessel maturation in vivo through FOXC2 [64]. Specifically, β-catenin was found to be important in the development of lymphatic valves and patterning of LECs during lymphatic development. OSS induced Wnt/β-catenin pathway activation was found to upregulate the expression of FOXC2, which reinforces the idea that OSS is important in lymphatic vascular patterning and valve development.

More recently, the mechanosensitive cation channel PIEZO1 has been identified as an important player in the development of lymphatic valves [65, 66]. A Tye2Cre: Piezo1^{cKO} mouse model was used to show that the absence of PIEZO1 leads to infrequent and deformed valve formation [65]. However, the absence of PIEZO1 does not affect the expression of FOXC2 or NFATc1, which by themselves are

upregulated in the presence of OSS. The authors suggested that PIEZO1 acts through other transcription factors that might be involved in the development of lymphatic valves. However, another recent publication concerning PIEZO1 has shown that PIEZO1 did upregulate transcription factors like FOXC2 and GATA-2 that are important for lymphatic valve development [66]. There are differences in the mouse models and experimental techniques which might possibly explain these contradictory results, and further research needs to be conducted to elucidate the pathways through which PIEZO1 can stimulate OSS induced lymphatic valve formation.

It is important to note that all of the experiments described in the above paragraphs that directly demonstrate that OSS is an important driver of the respective signaling pathway are done in vitro, which is certainly artificial and removes the cells from the relevant in vivo microenvironment. The extent that OSS is the initiator or primary enhancer of these signaling pathways in vivo is unknown. While significant strides have been made to elucidate the role of various signaling molecules in forming lymphatic valves, and the fact the OSS is an important mechanical cue necessary for their activation in vitro, the underlying mechanobiological principle guiding the frequency and localization of lymphatic valve formation remain less clear. Because of the dynamic contractile nature of the lymphatics and the bias of lymphatic valves to be in an open position, all LECs within the vessel experience OSS, albeit with varying magnitudes due to geometry. A recent work has shown that simulating shear stresses, in vitro, that are more relevant near lymphatic valve sites can orient the LECs perpendicular to the flow and enhanced FOXC2 (required for valve formation) expression [67]. Thus, the local distribution of shear stress can influence the formation of lymphatic valves. However, how the exact initial location of valve specification is determined is unclear. Furthermore, all measures of flow and estimates of WSS in lymphatics have been limited to adult mice and measurements of forces experienced by LEC during embryonic or early postnatal development are lacking. The current knowledge of the role of OSS in lymphatic valve formation has been briefly summarized in Fig. 8.4.

Another hallmark of a developing lymphatic vessel is the tight regulation of lymphatic proliferation and migration through a number of transcriptional factors like GATA-2, NOTCH1 signaling, and YAP/TAZ signaling, which all depend on mechanical cues. GATA-2 was also found to be upregulated in LECs in response to the stiffness of the matrix on which they are cultured [68]. LECs cultured on a softer matrix show an upregulation of GATA-2 with a correlated increase in VEGFR-3 mRNA expression. Further, GATA-2 upregulation due to culture on softer matrices lead to a downregulation of certain markers of proliferation, as well as some targets of YAP/TAZ signaling, while upregulating markers of cell migration and lymphatic vascular development like VEGFR3 and NOTCH1, among others. Interestingly, NOTCH1 activity is decreased in LECs exposed to a steady, laminar flow, increasing lymphatic sprouting [69]. This response is mediated by ORAI1, a subunit of a plasma membrane calcium channel, which increases calcium influx in the LECs in response to flow [70]. Increased cytosolic calcium leads to the activation of

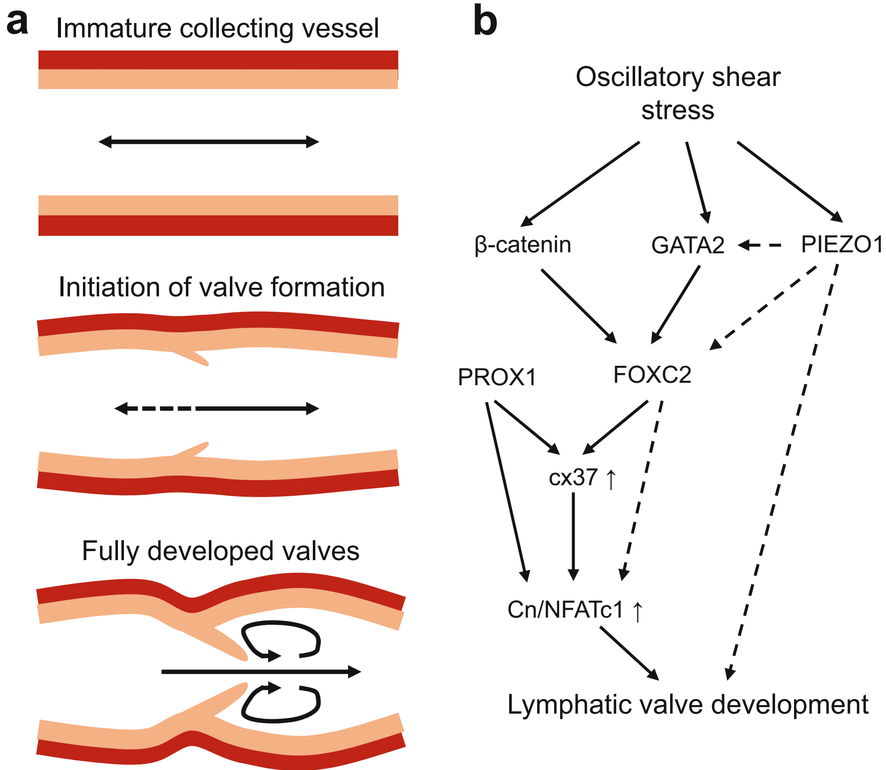

Fig. 8.4 The development of the secondary lymphatic valves, influenced by the oscillatory shear stress environment, along with the key molecular regulators of valve morphogenesis (Figure adapted from Sabine et al. [59]). (**a**) The progression of secondary lymphatic valve formation is shown along with the corresponding changes in the shear environment. (**b**) The major pathways through which oscillatory shear stress is transduced to affect lymphatic valve development are shown. The dashed lines represent pathways which have not been fully delineated as of now, or which need to be investigated further

calmodulin which triggers the formation of a Kruppel-like factor 2(KLF-2)/PROX1 complex that downregulates NOTCH1 and promotes lymphatic sprouting.

Summary The development of the lymphatic vasculature is regulated by a number of mechanical cues, including interstitial flow, luminal shear stress, and even matrix stiffness. Interstitial pressure regulates the proliferation of LECs during embryonic development and interstitial flow regulates the morphogenesis of initial lymphatics. Luminal shear stress, specifically OSS, regulates the development of lymphatic valves through transcription factors like PROX1, FOXC2, and GATA-2. PIEZO1, a mechanosensitive cation channel, has also recently been shown to be important in the development of lymphatic valves, although further studies are needed to establish the pathways through which PIEZO1 acts to influence the development of

lymphatic valves. Finally, matrix stiffness (upregulating GATA-2) and luminal shear stress (upregulating ORAI1 and downregulating NOTCH1) have been established to play a role in the proliferation and migration of LECs.

8.3 Postnatal Lymphangiogenesis

As discussed in the previous section, the proliferation of LECs and maturation of lymphatic valves during the early stages of the formation of lymphatic vasculature are heavily influenced and regulated by the mechanical microenvironment. Although a hallmark of lymphatic maturation is quiescence, lymphatic sprouting and proliferation happen throughout the lifetime of the organism through the process of lymphangiogenesis. In fact, lymphangiogenesis is the primary wound response of lymphatic vessels [71, 72] and is also heavily involved in the process of inflammation [73, 74]. Lymphangiogenesis has been the focus of therapeutic approaches to the treatment of lymphedema and has been found to be important for the resolution of many other diseases such as arthritis [73, 75, 76] and the fibrotic response to myocardial infarction [77]. However, excess lymphangiogenesis has also been found to be involved in the progression of lymphatic diseases like lymphedema [78]. Hence, the process of lymphangiogenesis must be tightly regulated in the context of both healthy lymphatic physiology and dysfunctional lymphatics. Much like neonatal LEC proliferation, postnatal lymphangiogenesis is also regulated by mechanical forces like interstitial flow and shear stress. In this section, the effect of interstitial flow and luminal shear stress on lymphangiogenesis will be investigated, along with a discussion on its implications for therapy.

8.3.1 Interstitial Flow

Interstitial flow has been shown to induce lymphatic proliferation and sprouting, which is important in the context of both neonatal and postnatal lymphangiogenesis. A model of skin lymphatic regeneration has been developed by Swartz et al. [79] that utilizes implanted collagen in the rat tail following lymphatic injury. It was shown that fluid channels form in the collagen gel prior to the proliferation of LECs. Interstitial flow was suggested as a driving factor for the unidirectional degradation of the matrix along with preferential directional proliferation and patterning of LECs as compared to BECs. The localized expression of matrix metalloproteinases (MMP) and VEGF-C in the distal portion of the collagen gel further affirm the role of interstitial flow in the transportation of MMPs that degrade the collagen gel, forming the channels, and VEGF-C which causes directional proliferation of LECs in the direction of the interstitial flow.

A follow-up study with this skin regeneration model showed that the directional migration of LECs by interstitial flow happens by the proliferation of single LECs that later organize into vessels [80]. Interestingly, VEGF-C expression was found to be the highest during the initial migration of the LECs and was reduced during the

reorganization and maturation phase. The pro-lymphangiogenic effect of interstitial flow was further investigated using the skin regeneration model, where it was found that a suppression of interstitial flow impaired lymphangiogenesis and directional LEC migration, leading to irregular patterns of LECs in the collagen gel [81]. The importance of interstitial flow in the directional migration of LECs was further recapitulated by the diffuse distribution of MMPs and VEGF-C in the collagen gel. These findings delineate the process by which interstitial flow may affect LEC proliferation both during development and in adults.

The pro-lymphangiogenic effect of interstitial flow was also shown in LECs cultured in vitro in a microfluidic platform that attempted to mimic the lymphatic microenvironment [82]. The presence of interstitial flow significantly increased the proliferation of the cultured LECs and showed more prominent lumen formation and filopodia projections. Application of interstitial flow also upregulated ERK, which is a well-known mediator of mechanotransduction cascades, thus showing that interstitial flow stimulates mechanotransduction cascades that can lead to LEC proliferation. The LECs also showed an increase in the level of PROX1, consistent with other studies that have identified a shear stress-induced upregulation of PROX1.

8.3.2 Shear Stress

Shear stress is another important stimulator of mechanotransduction that enhances lymphatic proliferation, as has been investigated through in vivo and in vitro experiments. As mentioned in the previous section, ORAI1, a plasma membrane-bound calcium channel protein responds to laminar flow and leads to an increase in the cytosolic calcium, which binds with calmodulin [70]. The calcium/calmodulin complex leads to the formation of a Kruppel-like factor 2 (KLF-2)/PROX1 complex that activates downstream proteins DTX1 and DTX3L, eventually resulting in the activation of NOTCH E3 ligase that deactivates NOTCH1 [69]. Downregulation of NOTCH1 leads to increased LEC proliferation.

Nitric oxide (NO) has been identified as a key player in lymphangiogenesis and metastasis [83–85] (Fig. 8.3b). Inducible nitric oxide synthase (iNOS) was found to be significantly correlated with increased density of lymphatic vessels in sites of tumor metastasis in head and neck squamous cell carcinoma [83] and melanoma [84]. In vitro studies have shown that stimulation of the iNOS pathway or exogenous application of NO leads to the upregulation of VEGF-C mRNA expression, and blocking NO leads to a downregulation of VEGF-C mRNA [83]. The involvement of endothelial nitric oxide synthase (eNOS) has also been confirmed in lymphangiogenesis in the context of tumor metastasis. VEGF-C application has been found to stimulate eNOS expression through the PI3K/Akt pathway and blockage of NOS using the inhibitor L-NMMA blocks lymphangiogenesis in a mouse tail model of dermal lymphatic regeneration. Further, exogenous application of NO has been found to stimulate lymphatic proliferation in vitro [85]. While the effect of flow on lymphatic proliferation has not been studied in the context of eNOS expression or NO production, it is interesting to note that in an acute setting elevated

shear stress can induce eNOS expression and increase NO production in LECs [86, 87]. Thus, further studies are needed to investigate the role of sustained flow-mediated NO production in lymphangiogenesis.

However, the role of fluid shear stress in lymphangiogenesis is not as straightforward as simply increasing lymphatic proliferation. In fact, the level of fluid shear stress may dictate the upregulation or downregulation of lymphangiogenesis. As an example, the obstruction of lymph flow in vivo by ligating sheep popliteal lymphatic vessels resulted in increased sprouting near the point of ligation [88]. An analysis of the lymphatic tissue surrounding the sprout showed an increase in PROX1 expression, and a coincident increase in Tie2 phosphorylation, and MAPK activation. Similar observations have been made in a mouse model of lymphatic ligation, with the lymphangiogenic sprouting response being shown to be dependent on cooperative interactions of macrophages and T cells [78]. However, the extent that the low WSS, as opposed to some secondary response to distal tissue inflammation, is necessary for the phenotype is unclear. It should be noted here that exposing cultured LECs to stretching in vitro has been shown to induce lymphatic proliferation [49]. It is advisable to appreciate the complexity of the mechanical environment which the LECs are exposed to, in order to understand these apparently confounding results.

While KLF-2 has been shown to be involved in the shear-mediated lymphatic sprouting as mentioned above, the role of KLF-2 might also be dependent on the magnitude of the shear stress that the vessel is experiencing. One study has shown that KLF-2 expression is increased in LECs isolated from an experimental model of chronically increased pulmonary lymph flow. This was coincident with a reduction in PPAR-γ expression, which is involved in regulating the production of cellular reactive oxygen species (ROS). Cellular ROS expression was consequently increased, leading to a subsequent reduction in the availability of NO. This chronic increase in cellular ROS might be a mechanism by which pathogenesis might happen in the form of endothelial dysfunction. As mentioned before, NO has been implicated in lymphangiogenesis. Hence, chronically increased ROS might impact the lymphangiogenesis in pathological scenarios.

Summary Postnatal lymphangiogenesis is regulated by the mechanical microenvironment of the lymphatic vessels, primarily depending on the interstitial flow and shear stress in the lumen. Interstitial flow can stimulate lymphangiogenesis and LEC proliferation by upregulating VEGF-C and MMPs. Matrix metalloproteases help with channel formation in the ECM, and VEGF-C leads to the subsequent LEC proliferation and lymphatic vessel maturation. Shear stress can stimulate lymphatic proliferation through the activation of ORAI1, a plasma membrane calcium channel, and downstream factors of which KLF-2 and NOTCH1 are important in the stimulation of LEC proliferation. NO has also been found to stimulate lymphatic proliferation. iNOS and eNOS expression is coincident with lymphatic proliferation around regions of tumor metastasis, and exogenous blockage of NOS can block lymphangiogenesis. The lymphangiogenic response to shear stress may be contradictory, however, since models of lymphatic flow blockage have also been shown to

induce lymphangiogenesis. Chronically increased flow can also lead to the under-production of NO, having implications in endothelial proliferation and dysfunction.

8.4 Trans-Endothelial Transport

The lymphatic vasculature is a specialized network of closed vessels that show a unique morphology compared to blood vessels. Their function as a system complementary to the cardiovascular system requires that they take up the extracellular fluid that has been extravasated from blood vessels into their own lumen. This function is performed by a specialized network of lymphatic capillaries called initial lymphatics. The initial lymphatics are made up of a single layer of endothelial cells that are loosely connected to each other through “button-like” junctions that form the primary lymphatic valves. The LECs are connected to their extracellular matrix through anchoring filaments that help open the primary lymphatic valves during excess interstitial fluid accumulation. This results in the formation of lymph, which travels through the collecting lymphatics and lymph nodes until it reaches the blood circulation again. Hence, a key component of lymphatic function is the regulation of the barrier function of LECs, which controls the formation of lymph and maintenance of the lymph composition through trans-endothelial transport. As with other aspects of lymphatic function, the trans-endothelial transport is also dependent on the mechanical forces experienced by the vessel (Fig. 8.5).

Blood endothelial cells have been found to alter their barrier function in response to fluid shear stress, showing an acute increase in solute transport and permeability [89–92]. LECs were found to have a similar response to fluid shear stress, as shown by studies done on their barrier function in vitro [93]. LECs showed an immediate increase in the trans-endothelial electrical resistance, which is a measure of the endothelial barrier function, in response to a steady shear of about 10 dyn/cm^2, which gradually decreased till the imposed shear stress was removed. In fact, the application of a pulsatile shear stress of the same magnitude (10 dyn/cm^2) produced a similar increase in barrier function. The role of the actin cytoskeleton in the shear stress-mediated increase in barrier function was also confirmed by the blocking of actin dynamics. Blocking Rac1, a GTPase of the Rho family that can enhance barrier function by stabilizing the actin fibers, inhibited the shear stress-induced increase in barrier function. Further, the authors showed that the increase in barrier function was primarily because of an increase in the resistance between the LECs, which in turn produced tighter cell–cell junctions. It is important to understand the impact of shear stress on lymphatic permeability in vivo as well, which can possibly be investigated with partial lymphatic ligation models.

The transmural flow, which is the flow perpendicular to the LEC layer and is reflective of fluid uptake by the initial lymphatics, has also been shown to increase the conductance across the LEC layer [94]. Aquaporin-2, which is a channel that regulates the inflow of water into endothelial cells, showed increased expression in response to an elevated transmural flow. The expression of VE-cadherin (the proteins that form the button-like junctions between LECs) and PECAM-1 (the

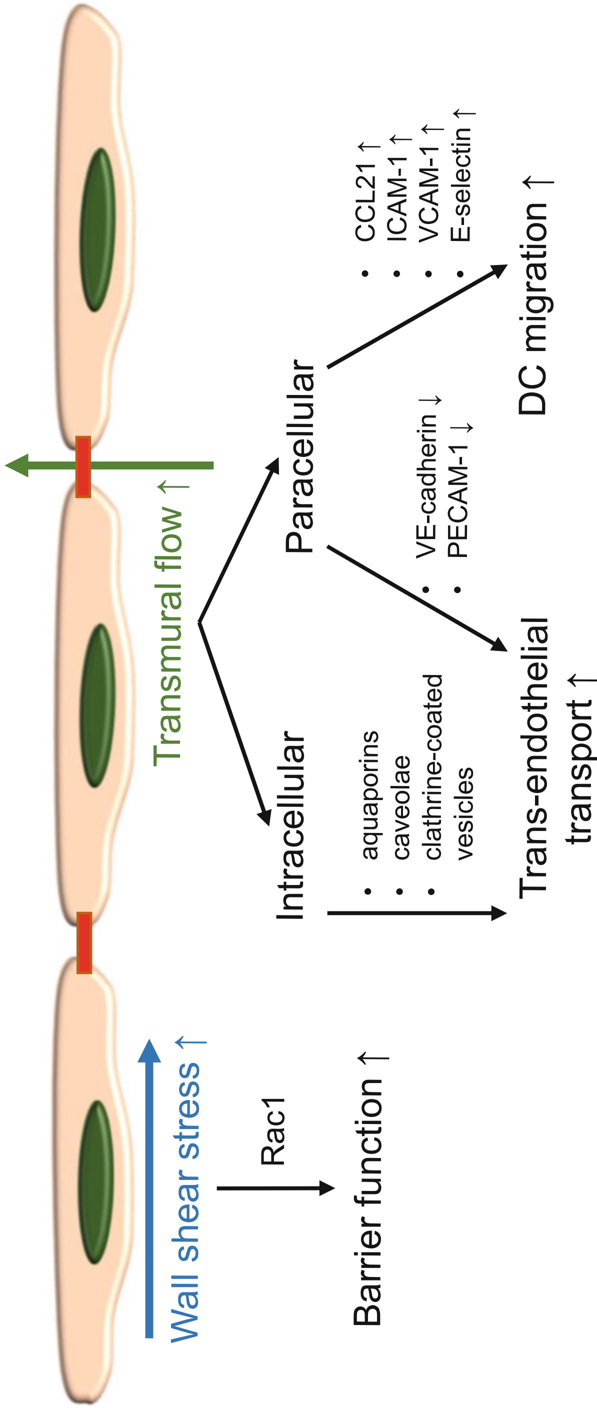

Fig. 8.5 The effect of wall shear stress and transmural flow on the lymphatic barrier function. Wall shear stress has the effect of increasing the barrier function. Transmural flow can enhance trans-endothelial transport and DC migration through both intracellular and paracellular pathways

proteins most abundantly expressed at the regions of the primary lymphatic valves) decreased in response to an elevated transmural flow, which signifies an increase in the endothelial permeability.

Besides trans-endothelial transport between the endothelial junctions, LECs have also been shown to uptake solutes through intracellular pathways (e.g., LECs scavenge albumin from the interstitium), thus implying that intracellular solute transport is a viable method of movement of solutes across the endothelial layer. This uptake can take place through caveolae and/or clathrin-coated vesicles. Transmural flow, which was found to increase the effective permeability of lymphatic vessels, was found to preferentially stimulate the transcellular pathway over the paracellular pathways of solute transport [95].

Another important effect of transmural flow is to increase the migration of dendritic cells (DCs) across the LEC layer [94]. This is at least partly caused by an increase in the secretion of CCL21 by LECs in response to the transmural flow. CCL21 is a chemokine that attracts DCs toward lymphatic vessels and facilitates trans-endothelial migration of DCs. LEC adhesion molecules that facilitate trans-endothelial migration of DCs, such as ICAM-1, E-selectin, and VCAM-1, were also found to be upregulated in response to transmural flow. Of these, ICAM-1 and E-selectin were found to be necessary for the flow-induced transmigration of DCs. Additionally, the lymphatic vessel permeability can also be controlled by CCR7 expressed by mature DCs, with CCR7-deficient mice showing an increased collecting lymphatic permeability. Thus, CCR7-expressing DCs also control lymphatic permeability and regulate the transmural flow across the collecting lymphatics [96].

Summary The lymphatic mechanical microenvironment can modulate the barrier function and, consequently, the trans-endothelial transport function of LECs. Shear stress increases the barrier function of the LEC layer through a tightening of the endothelial cell–cell junctions. The actin cytoskeleton is important in this shear-induced increase in barrier integrity. Transmural flow can increase endothelial permeability and has been implicated in affecting the integrity of the primary lymphatic valves formed by the LECs in the initial lymphatics. Transmural flow was found to preferentially stimulate the transcellular pathways over the paracellular pathways of trans-endothelial solute transfer. The transmural flow was also found to increase DC transmigration across the LEC layer by increasing the secretion of CCL21 by LECs. LEC adhesion molecules were also found to be upregulated by transmural flow, aiding DC transmigration across the lymphatic endothelium.

8.5 Lymphatic Contractility

A hallmark of collecting lymphatic vessels is their ability to intrinsically contract and push lymph through the lymphatic vasculature. In contrast to blood vessels that exhibit only slow, tonic contractions, lymphatic vessels can exhibit fast, phasic contractions as well. The lymphangions, the functional units of the collecting

vessels, are exposed to a highly oscillatory pressure and flow environment during a contraction cycle [23]. This oscillatory microenvironment of the lymphangion regulates the contractility of the lymphangion to a large extent, affecting the pump function of the lymphangion, lymph transport, and even the synchronized contractions between the lymphangions [97–99]. The contractile activity has also been seen to be dependent on the region of the body from where the lymphatics have been isolated [100, 101]. The mechanical microenvironment of the lymphangion can be transduced through the lymphatic endothelium, or directly stimulate the LMCs to change their contractile force, thus changing the pump function of the lymphatics.

The two primary mechanical forces that affect the contractility of lymphangions are the luminal wall shear stress (WSS) and the transmural pressure, although there is also evidence that axial force may also play a role in lymphatic contractions [102, 103]. The WSS has the effect of applying a shearing force to the LECs lining the lumen of the lymphangion, which acts in the direction of the flow and parallel to the endothelial cell layer. The WSS is dependent on the rate of flow of the lymph (higher flow produces higher WSS) and the geometry of the lymphangion (a smaller cross-sectional area corresponds to a higher WSS for the same flow rate). Transmural pressure is the difference between the intralymphatic pressure and the interstitial pressure. Transmural pressure exerts a radial force on the walls of the lymphangion, effectively stretching the lymphangion. It is important to note that the transmural pressure is affected by both the intrinsic contractility of the lymphatics as well as extrinsic forces resulting from muscle movements, stimulation by the autonomous nervous system, and atrial natriuretic peptides. A higher basal transmural pressure corresponds to an elevated stretch on the lymphatic endothelial cells and muscle cells. Collecting lymphatics are also exposed to a basal axial stretch, with additional stretch possibly affecting lymphatic function. These forces have a significant impact on both the normal functioning of the lymphatic vasculature, as well as pathological conditions that may arise out of chronically elevated mechanical forces and/or reduced sensitivity to these mechanical forces [17, 104]. While mechanical forces are important, the autonomous nervous system is also crucial in regulating lymphatic contractility and will be discussed in detail, drawing connections to its interaction with mechanical forces wherever relevant.

This section will go in-depth into the mechanoregulation of lymphatic contractility, detailing the functional changes in lymphatic vessels in response to mechanical stimuli and the molecular mechanisms that mediate these changes. The metrics for quantifying the function of lymphangions are usually adapted from the cardiovascular literature since the lymphangions exhibit intrinsic contractility much like the heart [105, 106]. Hence, the following functional metrics are usually investigated in the context of lymphatic function:

- Contraction frequency (Hz or contractions/min).
- Contraction amplitude (μm).
- Diastolic diameter (μm)—the maximum diameter of the vessel over a contraction cycle.

- Systolic diameter (μm)—the minimum diameter of the vessel over a contraction cycle.
- Vessel tone (%)—the diastolic diameter of the vessel compared to the diameter of the vessel when it is maximally dilated (under calcium-free condition).
- Stroke volume (μm^3)—the volume of lymph pumped by a unit length of lymphangion per contraction.
- Ejection fraction (%)—the percentage of lymph pumped by a unit length of lymphangion per contraction.
- Lymphatic pump flow ($\mu m^3/s$)—the volume of lymph pumped by a unit length of lymphangion per second. Mathematically, this is equivalent to

$$\text{stroke volume} \times \text{contraction frequency}.$$

- Fractional pump flow (%/s)—the percentage of lymph pumped by a unit length of lymphangion per second. Mathematically, this is equivalent to

$$\text{ejection fraction} \times \text{contraction frequency}.$$

8.5.1 Luminal Shear Stress

The luminal WSS is one of the most important regulators of lymphatic pump function, acting through the lymphatic endothelium to control the contraction of the LMCs. The molecule that has been most extensively studied in this context is NO. Early studies performed with cultured LECs showed that endothelial cells can produce NO and upregulate iNOS expression in response to agonist stimulation [107]. The increased upregulation was proposed to cause lymphatic relaxation and hence control lymphatic tone, similar to the role played by NO in blood vessels. Stimulation of a lymphangion with acetylcholine (Ach) reduced or inhibited contractions completely [108], which was proposed to be due to the Ach-dependent relaxation of smooth muscles by the endothelium that has been investigated previously [109]. Exogenous blockage of NO production was found to have a reverse effect, increasing the contractile frequency. Stimulation of NO production also reduced spontaneous transient depolarizations (referred to as pacemaker potentials in the context of lymphatic vessels) in LMCs, and this effect was reversed by blocking NO.

Early studies on the effect of flow rate on the contraction of lymphangions suggested that the contractile frequency increased and the contraction amplitude decreased in rat iliac lymphatic vessels in response to a pressure gradient [110]. The direction of the flow was also implicated as a deciding factor in whether the pump function is decreased or increased, according to whether the pressure gradient is negative or positive [111]. Gashev et al. [112] quantified the effect of an imposed pressure gradient on the lymphatic pump function of rat mesenteric lymphatic vessels and thoracic ducts. An increase in the pressure gradient caused the frequency, ejection fraction, and fractional pump flow to decrease for both thoracic ducts and mesenteric vessels, with the thoracic ducts showing a more marked

decrease in comparison to the mesenteric lymphatics. The magnitude of the changes in contraction amplitude was also affected partially by the direction of the flow, while the rate of change of the pressure gradient while reversing the flow affected the contraction frequency. Interestingly, blocking NO with an exogenous NOS inhibitor, L-NMMA, could not completely reverse the flow-dependent contraction inhibition, thus implying that other molecules are also involved in this response. The flow-dependent relaxation of lymphangions was also reiterated in the canine thoracic duct, where NO was found to play an important role.

In vivo experiments have shown that the inhibition of NOS using an exogenous blocker (L-NMMA) reduces the lymph flow in initial lymphatics without affecting their structure [113]. The flow reduction by blockage of NO was reduced when the collecting lymphatic vessels were ligated. The findings suggest that NO is required for normal collecting vessel function, which affects the fluid transport across the lymphatic network. This role of NO in the regulation of lymphatic function was further investigated by Gasheva et al. [114], where the authors used phasically contracting vessels to generate flow (instead of an imposed flow) and compared them against phasically non-contractile vessel segments. The phasically contractile vessels showed a lower tone than their non-phasically contracting counterparts, which was consistent across transmural pressures ranging from 1 to 5 cm H_2O. NO was also found to be the most important regulator of this flow-dependent vasodilation. Thus, one can see a picture of lymphatic self-regulation forming from all these studies, where the lymphatics can regulate their own function in a shear-dependent manner, with NO acting as a major player in this self-regulation. This idea was formalized as the pump–conduit duality of the lymphatic vessel using computational modeling and in vitro studies [115, 116]. Under this framework, the lymphangions act as pumps when exposed to physiologically relevant pressure gradients, which are typically characterized by an elevated afterload. However, during pathological conditions such as edema, the direction of the pressure gradient can reverse, resulting in the lymphatic vessels acting more like conduits, characterized by a reduction in lymphatic pumping metrics. The transition to a conduit configuration reduces the resistance of the lymphatic vessels to flow, thus resulting in more efficient conduction of lymph through the lymphatic network.

Follow-up studies have confirmed the inhibitory role played by NO in lymphatic contractility by genetically blocking basal NO production, which increased lymphatic contractility [117]. Two important studies have attempted to isolate the regional variation of NO production in the lymphatic vessels [87, 118]. The studies have looked at the difference in eNOS expression and NO production in the tubular region of the collecting lymphatics and the valve region, comprising of the secondary lymphatic valves and the bulb region surrounding the valves. It was found that while eNOS expression and NO production in response to lymphatic contractions were increased throughout the lymphatic vessel segment, the valve regions showed the most increase. Specifically, the valve leaflets showed the most production of NO, followed by the bulb region and the tube region.

While the pathways leading to the release of NO by shear stress in lymphatics are not completely understood, a few mechanisms have been suggested. Fluid-sensitive cilia on endothelial cells have been shown to transduce shear stress signals into the LEC cytoplasm [119]. Cytoplasmic levels of NO and calcium (Ca^{2+}) were increased

in response to shear stress in wild-type LECs, while this increase was abolished in LECs that are deficient in the proteins polaris and polycystin-1. Shear stress has been found to increase the release of ATP from LECs [120]. A possible mechanism for shear stress transduction that has been suggested involves the binding of ATP to the purinergic P2X/2Y receptor, which leads to an increase in cytosolic Ca^{2+} due to their release from calcium stores by stimulation of the IP3 receptor. This is concurrent with an increase in endothelial constitutive NOS (ecNOS) expression. The stimulation of K^+ channels by the increase in cytosolic Ca^{2+} may also increase ecNOS expression.

Other molecules identified to play a role in the shear-induced functional changes in lymphatic vessels are endothelial prostanoids [110], histamine [121], and reactive oxygen species (ROS) [122, 123]. Histamine was shown to be an important endothelium-derived relaxation factor (EDRF) that acts along with NO to inhibit lymphatic pumping [121]. While previous studies have shown that inhibition of NOS, and consequently NO production, was not able to completely abrogate the flow-mediated relaxation in lymphatic vessels, blocking histamine and NO together completely eliminated the flow-dependent lymphatic vessel relaxation. Exogenously applied ROS have been shown to inhibit the contractile function of mesenteric lymphatics significantly [123]. Chronically increased flow in vivo has been shown to increase the ROS concentration in LECs [122]. This is affected through a decrease in PPAR-γ signaling due to an increase in KLF-2 activity. Since PPAR-γ regulates the production of ROS by NADPH oxidase, inhibition of its activity can lead to overproduction of ROS which can have long-term pathological effects.

Ca^{2+} is an important small molecule that is upregulated in the LEC cytosol in response to mechanical forces. In the cardiovascular literature, an increase in cytosolic Ca^{2+} is coincident with the phosphorylation and activation of eNOS, leading to NO production. Ca^{2+}/calmodulin complexes also modulate the cytoskeletal remodeling induced by mechanical forces, and hence control the permeability of the endothelial layer. A similar role of Ca^{2+} has also been found in LECs where an increase in cytosolic Ca^{2+} is concurrent with an increase in eNOS activity. This is possibly mediated by a shear-dependent release of ATP from LECs, as mentioned before. The increase in cytosolic Ca^{2+} with shear stress was found to depend on the magnitude of the applied shear stress and depended on both the extracellular calcium source and intracellular ER stores [124].

More recently, the lymphangions have been found to entrain to externally applied oscillatory flow waveforms at constant transmural pressures, i.e., their contraction frequency matches the frequency of the externally applied oscillatory flow [125, 126]. The entrainment was found to occur only for large amplitude oscillatory flow waveforms, and the blocking of NO or histamine did not block the entrainment. The oscillatory flow when applied on denuded vessels did not cause the vessel to entrain, thus showing that the endothelium is necessary for entrainment. A recent study has found that the extent of the entrainment is dependent on the amplitude and frequency of the applied flow waveform, as well as the intrinsic contraction frequency of the vessel and the critical shear threshold for flow-mediated relaxation of the vessel [127]. The vessels entrain maximally to the external oscillatory shear

stress when the intrinsic contraction frequency is similar to the applied frequency. The entrainment was also found to be correlated to the amplitude of the applied shear stress when it is below the critical shear stress for the lymphangion. Applied frequencies close to the intrinsic frequency were also found to improve the ejection fraction. The findings suggest a mechanism by which lymphangions can respond to their oscillatory microenvironment and modulate their contractility to function at an optimal capacity. Further studies are required to delineate the molecular mechanisms behind the entrainment phenomenon.

8.5.2 Transmural Pressure

Increasing transmural pressure has the effect of increasing the pumping frequency and pump function of the lymphatic vessels until an optimum pressure is reached, and then subsequently decreasing the pumping metrics above the optimum pressure. This behavior is expressed as a bell-curve of lymphatic pumping metric versus transmural pressure which has been recapitulated in a number of different animals including bovine, rat, and mouse lymphatics [105, 106, 128], and is thought to be driven by an optimal length–tension relationship that has been documented in most muscle cells [101, 129, 130]. The effect of preload and afterload on lymphatic contractility has also been delineated. Increasing preload has the effect of increasing the end-diastolic diameter and stroke volume when increased slowly, but a sharp increase in preload is associated with a reduced stroke volume that slowly recovers [131]. Complementary to the ex vivo perfused isolated vessel experiments, the length–tension relationship of lymphatic vessels has been studied using wire myography and have revealed an increase in the active wall tension generated by isolated lymphatics in response to an increase in stretch [101, 129, 130, 132]. An acute increase in downstream pressure was found to increase both tonic and myogenic constrictions in the upstream section of the isolated lymphatic vessels [133, 134]. While the endothelium is essential for flow-mediated changes in lymphatic contractility, it was shown that the endothelium is not required for the transmural pressure-dependent modulation of lymphatic pumping [135]. Thus, the focus of transmural pressure, and hence stretch, on collecting lymphatic function has mostly been focused on the lymphatic muscle cells, and the presence of stretch enabled ion channels that may lead to a transmural pressure-dependent control of contractility [136].

The spontaneous transient depolarizations that are characteristic of lymphatic vessels have the ability to generate action potentials (AP), concurrent with the release of Ca^{2+} from intracellular stores [137]. The Ca^{2+} release can cause membrane depolarization by opening Cl^{-} channels [138], which can lead to the opening of voltage-dependent calcium channels, causing vessel contraction [139]. The frequency of the generated APs was found to increase in response to the increase in the circumferential stretch, with a simultaneous increase in the contraction strength and intracellular calcium [140, 141]. Thus, it is reasonable to hypothesize that the stretch experienced by lymphatic vessels can modulate their contractility by activating voltage-gated Ca^{2+} channels in a stretch-dependent manner. The

involvement of extracellular and intracellular calcium was established in the modulation of lymph flow by transmural pressure, specifically implicating the L-type calcium channels [142, 143]. Lee et al. [144] delineated the roles of the L-type and T-type Ca^{2+} channels in the stretch-sensitive response. L-type channels controlled the amplitude and strength of the contractions, while T-type channels controlled the resting membrane potential and frequency of the contractions. The amount of stretch was found to influence the level of activation of these channels. Both the L-type and T-type channels were also found to be localized on the smooth muscle cells, thus reiterating the endothelium-independent response of lymphatic vessels to circumferential stretch. The interplay between intracellular and extracellular Ca^{2+} release (calcium induced calcium release, CICR) has been proposed as a coupled oscillator that controls the hyperpolarization of the LMCs, followed by an AP and subsequent contraction [139].

The isolated lymphatic vessels were also found to exhibit myogenic constriction and dilation, similar to that seen in blood vessels, where the diastolic diameter of the vessel slowly decreases after a sharp increase following an increase in transmural pressure [145]. The amount of constriction was found to be higher when the pressure increase was effected from a lower baseline pressure, and enhancing lymphatic contractility increased the magnitude of the constriction. A ramped increase in transmural pressure elicited a rate-dependent response, where the contraction frequency (but not the amplitude) was found to be enhanced in response to a faster ramp. The mechanism behind these responses is not well established and needs further investigation.

While the response of lymphatic vessels to an acute increase in transmural pressure is well investigated, their response to an elevated level of transmural pressure for an extended duration has only been sparsely studied [146]. In response to partial occlusion of a mesenteric vessel in vivo in a bovine model for 3 days, the upstream segment of the vessel that has been exposed to an elevated pressure was found to have a higher systolic and diastolic diameter, as well as higher pump function than downstream vessels. However, there were no detectable changes in the thickness or microstructure of the lymphatics, and the long-term remodeling of lymphatic vessels to increased transmural pressures need to be delineated.

The pressure gradient across a lymphatic valve is important in determining its open or closed state [18, 147]. The funnel-like structure of the valves, formed by the leaflets, has been visualized and it is widely believed that lymphatic valves are opened or closed passively depending on the transmural pressure gradient existing across it [148–150]. The pressure gradients governing the passive gating have been studied and are found to increase substantially with an increase in transmural pressure and hence vessel distension [151].

Compared to shear stress and transmural pressure, axial stretch is relatively less studied. It is known that axial stretch can affect lymphatic muscle contraction, and that transmural pressure can also control the amount of axial stretch in isolated collecting vessels and vice versa [102, 152]. Lymphatic vessels are always under a basal level of axial stretch under in vivo conditions [103]. Increasing the axial stretch in isolated lymphatic vessels can result in a reduction in the amplitude, frequency,

and pump function of the vessel. Multiphoton microscopy has revealed that collagen fibers are aligned axially to the lymphatic vessels and the LMCs are orientated both circumferentially and axially. These findings suggest that the lymphatic vessels have developed to withstand axial loads, and the active contraction of lymphatic muscles can increase both the circumferential and axial stretch. Further studies are needed to understand how axial stretch can affect lymphatic function in vivo and whether a degradation of axial load-bearing capabilities of collecting lymphatics may be a hallmark of lymphatic dysfunction.

Several mathematical models have attempted to simulate the dynamics between the smooth muscle cells and endothelial cells that lead to spontaneous contractions of lymphatic vessels. It has been shown through mathematical modeling that stretch-induced Ca^{2+} and shear-induced NO release can act as a biological oscillator, sustaining lymphatic contractility. These models have been successful at simulating the behavior of lymphatic vessels in response to a large number of pressure and shear conditions [153, 154]. Although all possible mechanisms behind the control of lymphatic contractility have not been incorporated into these models, they are an important first step in understanding how simple mechanical cues can lead to the coordinated contraction of large networks of lymphatic vessels. Mathematical models have also attempted to capture the calcium dynamics and electrophysiology of LECs [155]. Lumped parameter models have attempted to capture the synchronized contraction of multiple lymphangions in series and their valve dynamics by incorporating the interdependence between lymphatic pumping and the transmural pressures that the lymphangions are exposed to [156–161]. The pumping by collecting lymphatics have been shown to generate a suction pressure, by experimental and computational models, which enables the uptake of lymph by the initial lymphatics and hence lymph propagation [162].

8.5.3 Nervous Stimulation

It is worth noting that mechanical forces are not the only factors governing the contractile activity of the collecting lymphatics. In fact, nervous stimulation plays a very important role in modulating lymphatic contractility. It has been known for a long time that collecting lymphatics are innervated [163], and the presence of both adrenergic and cholinergic nerve fibers have been confirmed in LMCs [164]. α adrenergic stimulation has the effect of increasing the amplitude, frequency, and tone of lymphatic contractions [163, 165]. The distribution of the adrenoreceptors is varied depending on the species and the location of the lymphatic vessels, with α_1 and α_2 adrenoreceptors having been shown to be involved in modulating contractility in dog thoracic ducts [166], and α_2 adrenoreceptors being shown to be involved in noradrenaline mediated increase in lymphatic contractile frequency in bovine collecting lymphatics [167].On the other hand, β adrenoreceptor stimulation was shown to have an inhibitory effect on contractility [168, 169]. β adrenoreceptor stimulation can stimulate the relaxation of LMCs through the phosphorylation of eNOS in LECs and the consequent production of NO [170]. A later study showed

that β_2 adrenoreceptors were directly expressed in LECs and stimulation of the same could induce lymphatic relaxation through nitric oxide [170].

On the parasympathetic side, stimulation of collecting lymphatics with acetylcholine caused a relaxation of the LMCs, suggesting the role of muscarinic receptors in the control of lymphatic vessel relaxation and dilation, with a possible involvement of endothelium-derived relaxation factors (EDRF) that act through GCaMP [171]. This EDRF was identified as NO, a well-known and potent vasodilator, that was previously shown to also be activated in response to shear stress [172]. Thus, both nervous and mechanical cues may modulate lymphatic contractility through the same pathway. However, the relative importance of shear stress or other means of mechanical loading over cholinergic stimulation in controlling lymphatic contractility is yet to be parsed out. Bachmann et al. showed in a follow-up study that muscarinic stimulation may also increase lymphatic contractility, and this increase may be dependent on the relative distribution of muscarinic receptors between the LMCs (increases contractility) and LECs (decreases contractility) [170].

Summary Lymphatic vessels exhibit spontaneous contractility, which can be modulated by the luminal shear stress and transmural pressure exerted on the vessel. Luminal shear stress has the effect of reducing the contraction frequency and lymphatic pumping metrics. The magnitude of the reduction is dependent on the region from where the lymphatic vessel has been isolated (e.g., thoracic duct vs mesenteric lymphatics). The major molecules that are involved in the shear-induced relaxation of the lymphatic vessels are NO produced by NOS (eNOS and iNOS), endothelial prostanoids, histamine, and ROS. The major sources of NO production in lymphatic vessels are in the valves and the surrounding bulb region, and Ca^{2+} is involved in the regulation of NO production. Lymphatic vessels can also entrain their contraction to an externally applied oscillatory shear stress, and the entrainment is dependent on the dynamics of the applied shear stress and the intrinsic contractility and mechanosensitivity of the vessel. NO and histamine have not been found to mediate the entrainment, and further studies are required to delineate the molecular mechanisms of this response of lymphatic vessels to an oscillatory flow.

Lymphatic vessels also respond to the magnitude of the transmural pressure on their wall in an endothelium-independent way. The response to transmural pressure is mediated by depolarizations induced by the stretch, which are closely related to the intracellular and extracellular Ca^{2+}. CICR from the ER, as well as T-type and L-type calcium channels, have been implicated in controlling the contraction frequency and strength of the lymphatic contractions in response to stretch. Lymphatic vessels also exhibit myogenic constriction in response to stretch and modulate their contractility depending on the rate of an imposed ramped transmural pressure. Extended exposure of lymphatic vessels to elevated transmural pressures can cause an increase in their diastolic and systolic diameter, as well as their pump function. The broader effects of transmural pressure and wall shear stress on lymphatic contractility have been summarized in Fig. 8.6. Axial stretch is intrinsically tied to the transmural pressure and can modulate lymphatic contractility in ex vivo conditions. Mathematical models have attempted to capture the stretch and shear-

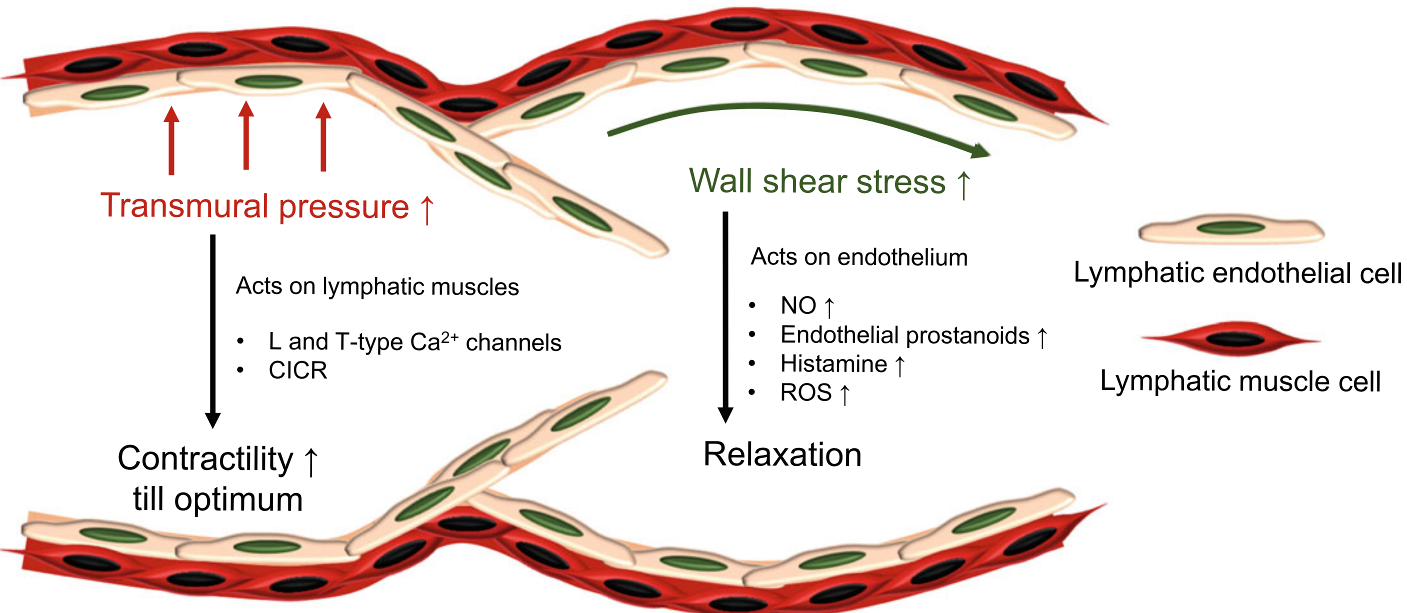

Fig. 8.6 Collecting lymphatic vessels respond to the wall shear stress and transmural pressure acting on the LECs and LMCs, respectively. The wall shear stress exerts a force parallel to the LEC layer and causes relaxation and reduction in contractility. The transmural pressure acts perpendicular to the wall of the lymphatic vessel and controls the contractility by affecting the lymphatic muscle cell layer

induced modulation of lymphatic contractility by modeling the Ca^{2+} and NO dynamics in LMCs and LECs, respectively.

The autonomous nervous system controls lymphatic contractility through both the sympathetic and parasympathetic systems. α adrenergic stimulation increases lymphatic contractility while β adrenergic stimulation has the effect of lymphatic relaxation. Muscarinic receptors in lymphatic muscle cells and endothelial cells interact with cholinergic agents to either increase or decrease lymphatic contractility, respectively. The parasympathetic relaxation of lymphatics can act through the LECs, phosphorylizing eNOS and producing NO, similar to the effect of shear stress on lymphatics. Hence, mechanical forces and nervous stimulation may act on lymphatics through similar pathways and interact with each other in a complex manner.

8.6 Lymphatic Vasculature in Disease

As has been discussed until now, lymphatic vessels are highly sensitive to their mechanical microenvironment. The mechanical forces acting on the lymphatic vessels can modulate their pump function and ensure that the lymphatic vasculature is performing at an optimum condition. Dysfunction of lymphatic vessels is frequently accompanied by pathological shear stresses and transmural pressures. The elevated mechanical forces are usually the result of some form of injury to the lymphatic system, or a pathological condition in the body. The effect of the pathological forces is seen in a maladaptive remodeling of the lymphatic vessels, leading to a loss of function or a loss in sensitivity to the mechanical forces, abnormal

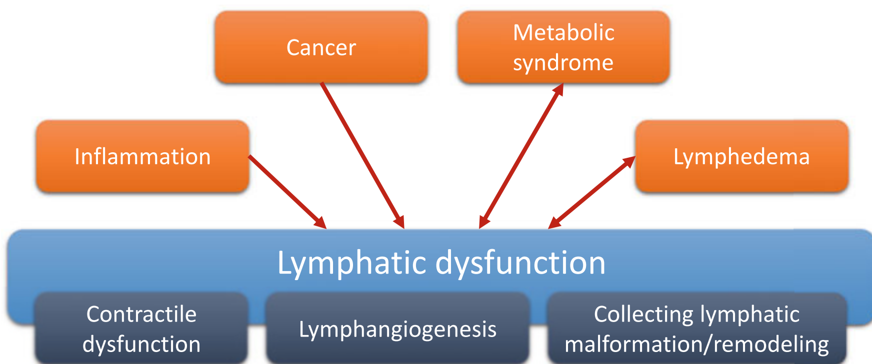

Fig. 8.7 The various pathologies (orange boxes) that can lead to lymphatic dysfunction. For some pathologies such as inflammation and cancer, lymphatic dysfunction occurs as a result of the pathology. For metabolic syndrome and lymphedema, the pathology can drive lymphatic dysfunction and also vice versa. Lymphatic dysfunction can manifest as contractile dysfunction of the collecting vessels, abnormal lymphangiogenesis, and/or the malformation and remodeling of collecting lymphatic vessels

lymphangiogenesis, and/or malformations and remodeling (Fig. 8.7). Lymphatic system function that is disrupted in this way can lead to various pathologies in the human body including lymphedema, one of the most prominent lymphatic-related diseases. This section will look at the effect of aberrant mechanical forces on the lymphatic vasculature, and discuss how it affects the three major functions of the lymphatic system; maintaining the interstitial fluid balance, immune cell transport, and lipid transport.

8.6.1 Contractile Dysfunction

The intrinsic contractile activity of lymphatics is crucial for the maintenance of interstitial fluid volume, and proper functioning of the immune system and lipid transport. Accumulation of interstitial fluid due to insufficient lymphatic drainage is called lymphedema. Secondary lymphedema is the most common form of lymphedema [173, 174], with breast cancer-related surgery being the leading cause in most developed countries and lymphatic filariasis being a major cause in most developing countries. Secondary lymphedema resulting from a surgical procedure is usually caused by the removal of lymphatic tissue (e.g., lymph nodes) and a secondary insult (e.g., radiation therapy) that can damage the lymphatic vasculature and impair lymphatic pump function [174, 175].

The damage caused to the lymphatic vasculature in lymphedema can lead to contractile dysfunction of the surviving lymphatics. In a murine model of tail lymphedema, it was found that chronic edema induced by a lymphatic injury had the effect of reducing fluid uptake by lymphatic capillaries, as well as a reduction in contraction amplitude of the collecting lymphatics [176]. A reduction in contractile function has also been observed in humans with secondary lymphedema [177, 178]. Studies on lymphatic contractile function at various stages of lymphedema have revealed a decrease in lymphatic pumping pressure and contraction frequency [177, 179]. A study involving lymphatic injury in the hind leg of sheep showed that lymphatic injury can cause functional remodeling of collecting lymphatics, increasing the frequency and force generation, while also decreasing the flow-mediated contraction inhibition [180]. Secondary lymphedema caused by lymphatic filariasis is characterized by a significant impact on lymphatic contractile function, resulting in dysfunctional lymphatic valves, vessel dilation, and reduction in muscle contractility [181, 182]. All these effects lead to an overall reduction in lymphatic pump function. What is less clear is whether this loss of contractile function is driven by a biological inflammatory response, abnormal mechanical loading, or some combination of the two. Recent work investigating the maladaptive remodeling of the aorta in hypertension has provided a computational framework and paradigm to integrate both mechanics and inflammation, but such an approach has not been implemented in lymphatics. However, a study proposing a mechanically mediated growth and remodeling framework within lymphatics has suggested that the growth rate in the remodeling response drastically influences the overall effect of the remodeling response on lymphatic function [183]. These simulations provide an important illustrative case that remodeling of lymphatic structure—and

the corresponding consequence to function—are intimately related. This is probably even more so than in blood vessels, as the lymphatics serve as their own source of flow generation.

Increased lymph flow during pathological conditions can impair lymphatic contractility. Contractile activity of lymphatic vessels has been seen to be impaired during mesenteric venous hypertension, which is characterized by intestinal edema formation and a substantial increase in lymph flow. Lymphatic vessels exposed to a chronic mesenteric venous hypertension, and hence a higher lymph flow, were found to remodel to become weaker pumps, characterized by a decrease in pump function and contraction frequency, and lower active tension [184, 185]. The opposite effect was seen when the mesenteric vessels were exposed to an elevated transmural pressure for a prolonged duration (3 days) whereby their pump function, and diastolic and systolic diameters, were both found to increase, resulting in a stronger pump [186]. Somewhat in disagreement to the aforementioned findings, edemagenic stress created by artificially increasing the total fluid volume by infusion of saline into the venous circulation resulted in an acute increase in lymphatic contraction frequency and flow in a rat model [187]. Exposure of lymphatic vessels to chronically increased pulmonary flow has been shown to decrease the availability of NO and reduced NO-mediated relaxation of the thoracic duct [188]. This is probably mediated through a KLF-2 dependent increase in ROS, which competes with NO for bioavailability [122]. ROS has been seen to inhibit contractions in isolated lymphatic vessels [189]. Prolonged exposure of lymphatic vessels to ROS can also lead to endothelial dysfunction similar to that seen in blood vessels [190].

In addition to these experimental conditions of pathologically elevated lymph flow, there is compelling evidence to suggest that certain patients who have higher baseline rates of lymph formation, and thus lymph flow, may be predisposed to a greater lymphedema risk [191–194]. While several studies have reported that later stage lymphedema is associated with a reduction in pumping pressure in the lymphatics [178, 195], it is interesting to note that patients with elevated lymphatic pumping pressure prior to cancer treatment had a higher risk of developing lymphedema after the treatment [191]. This work lends credence to the hypothesis that in many cases of lymphedema the disease is driven by overworking, via abnormal mechanical loading, of a lymphatic system that is already close to operating at maximum capacity. Demonstrating this further, and elucidating the molecular mechanisms involved, is a crucial area of future study that could provide new molecular targets in lymphedema therapy.

Obesity has been implicated in the reduction of lymphatic pump function [196]. High-fat diet has been shown to impact lymphatic pump function acutely, reducing contraction amplitude and frequency, but increasing the lymph flow and viscosity [197]. A chronic high-fat diet and predisposition to obesity in rodent models have also been shown to reduce lymph flow, impair DC migration, and drive a slight remodeling of lymph nodes [198–200]. Metabolic impairments resulting from obesity, such as in metabolic syndrome, impaired the flow-mediated reduction in lymphatic contractility [201]. This resulted in lymphatic vessels that were less sensitive to their mechanical microenvironment. The pathology was concurrent with a decrease in eNOS expression. A decrease in lymphatic function

was also seen in a mouse model of lymphedema, where the obese animals showed a lower baseline lymphatic contractile function, which was further exacerbated by lymphatic injury [202].

Inflammation has the effect of reducing lymphatic contractile function [203, 204], as has been seen in a model of ileitis and acute inflammation induced by the application of oxazolone. The molecular markers of inflammation such as prostanoids, histamine, and NO have all been implicated in reducing the contractile function of lymphatic vessels [110, 112, 114, 121, 205]. While the reduction in contraction amplitude and frequency may signal a decrease in lymphatic function, the overall transport of fluid may be improved, since a less contractile vessel offers lower resistance to the lymph flow. However, the extent that this inflammatory milieu integrates with mechanically mediated mechanisms driving lymphatic physiology and remodeling remains ill-defined.

Lymphatic contractility also adjusts to accommodate for changes in lymph volume in response to pathologies such as hypervolemia. Hypervolemia, an increase in the fluid retention of the body, is associated with the release of atrial natriuretic peptides (ANP) in response to atrial distension. ANPs have the effect of vasorelaxation and an increase in vascular permeability of blood vessels. When it comes to the collecting lymphatics, ANPs exert a similar effect on their contractility and permeability. In isolated lymphatic vessel setups, the addition of ANP caused an inhibition of contraction, reducing both the frequency and the force generated by the LMCs [206] and causing a consequent decrease in lymphatic transport [207]. The inhibition happens independent of the endothelium, directly relaxing the LMCs possibly through a GCaMP pathway [208]. The other effect of ANPs on lymphatic function is their increased permeability which enables the lymphatics to extravasate proteins and fluid into the interstitium [209, 210]. The interplay between the decreased contractility and increased permeability of the collecting lymphatics possibly mitigates the volume overload that is effected by an increase in fluid uptake by the lymphatic capillaries.

Recent studies have also implicated tumor-induced altered microenvironments in affecting lymphatic contractility. A tumor microenvironment can remodel the surrounding lymphatic vasculature and increase its propensity for tumor metastasis. Chronic stress can induce an increase in the diameter of collecting lymphatics near the tumor site and increase tumor metastasis [211, 212]. This stress-induced increase in metastasis may possibly be effected by the sympathetic nervous system since the blocking of β adrenoreceptors reversed the adverse effects of stress. A recent study has shown that collecting lymphatic vessels near tumor sites exhibit increased contractility, concurrent with an increase in the innervation density [170]. Blocking of α adrenergic receptors and stimulation of β adrenoreceptor inhibited this increase in contractility, suggesting a role of the sympathetic nervous system in the tumor-induced increase in lymphatic function and indicating potential therapeutic targets. Most importantly, the studies show the complicated ways in which sympathetic nervous stimulation can affect collecting lymphatic function and tumor metastasis, exemplified by the fact that both blocking and stimulating β adrenoreceptors can cause a decrease or increase in lymphatic metastasis by affecting different aspects of

lymphatic function. A further delineation of the role of the different components of the sympathetic nervous system in modulating lymphatic function is needed before they can be useful as therapeutic targets for tumor metastasis.

8.6.2 Lymphangiogenesis

The previous sections have discussed how lymphangiogenesis can be induced by mechanical forces, primarily by the interstitial flow and shear stress, in the lymphatic microenvironment. However, lymphangiogenesis is also the hallmark of three frequently encountered pathologies: inflammation, cancer, and lymphedema. In all these cases, the mechanical microenvironment of the lymphatic vasculature is altered to induce lymphangiogenesis and can lead to the amelioration or progression of the disease.

Inflammation is always associated with edema, whereby there is an accumulation of interstitial fluid in the inflamed region because of excess extravasation of fluid from capillaries compared to lymphatic drainage [44]. Lymphatic vessels can undergo lymphangiogenesis in response to inflammation [213, 214], which motivates the hypothesis that an increased density of lymphatic vessels can result in increased clearance of the interstitial fluid, including activated dendritic cells (DCs) and antigens from the site of the inflammation to the lymph node, thus aiding in the immune response as well. VEGF-C is an important molecule involved in the proliferation of LECs and is upregulated during inflammation [215]. Chronic inflammation is characterized by the stasis of interstitial fluid and cells due to inadequate drainage and can lead to the production of VEGF-A which stimulates lymphangiogenesis at the lymph nodes [216]. TGF-β has also been found to be an important factor in inflammation-induced lymphangiogenesis, as observed in rodent models of unilateral ureteral obstruction [217]. Arthritis is frequently characterized by acute or chronic edema of the joints and is associated with increased lymphangiogenesis [218]. In fact, treatment with VEGF-C can be a possible therapeutic option for chronic inflammatory disorders, as they have been shown to increase lymphangiogenesis and lymphatic drainage in rodent models of arthritis [219, 220], and are potential targets for inflammatory bowel diseases such as ulcerative colitis and Crohn's disease [205, 221–223].

Somewhat related to the inflammation-induced lymphangiogenesis is the remodeling of the lymphatic vasculature surrounding a tumor site. The tumor microenvironment is characterized by elevated interstitial pressure (IP), which is correlated with an increase in capillary permeability surrounding the tumor site [224]. The high interstitial pressure supposedly also results in poorly functional lymphatic vessels within the tumor site [225, 226], which in turn might lead to poor fluid clearance and hence a persistence of the high IP. The tumor microenvironment is also characterized by increased lymphangiogenesis in the peritumoral lymphatics, which are functional and might be involved in metastasis by enabling the tumor cells to infiltrate the lymphatics through the endothelium [227]. The relationship between the high IP and increased peritumoral lymphangiogenesis is not clear and needs to be

delineated. VEGF-C and VEGF-D are also involved in tumor-induced lymphangiogenesis and tumor metastasis [228–231], which makes them potential therapeutic targets for inhibiting tumor metastasis. Sequestering VEGF-C and VEGF-D, or blocking VEGFR-3 has been found to reduce tumor lymphangiogenesis and metastasis [232–234].

The role of VEGF-C and lymphangiogenesis in resolving secondary lymphedema has not yet reached a consensus. Early studies found that VEGF-C gene transfer in murine models of ear and tail lymphedema resulted in a reduction in swelling, which was concurrent with increased lymphangiogenesis [75, 235]. A later study found that blocking VEGFR-3, and consequently lymphangiogenesis, did not have any effect on the reduction in swelling in a mouse model of lymphatic injury [236]. More recent studies have concluded that chronic edema can lead to upregulated VEGF-C and lymphatic vessel hyperplasia [237], and blocking of VEGF-C can actually help in the resolution of edema that has been induced due to lymphatic injury [238]. Increasing the interstitial flow and not VEGF-C has been seen to cause an ameliorative effect on tail swelling due to lymphatic injury [236]. It should be noted that most of these studies have ignored the mechanical microenvironment in which these VEGF-C dependent mechanisms are taking place. The extent that mechanotransduction pathways also influence the functional responsiveness of VEGF-C therapy warrants future investigation, and could shed insight into the contradictory results regarding VEGF-C therapy.

8.6.3 Collecting Lymphatic Malformation and Remodeling

Lymphatic malformations, resulting from genetic defects, can impact lymphatic function [239]. Primary lymphedema can be caused by a malformed lymphatic vasculature including absent or nonfunctional lymphatics, obstruction of lymph node, or incompetent valve formation [51, 240, 241]. Abnormalities in VEGFR-3, FOXC2, and GATA-2 genes, among others, have been suggested in the pathogenesis of primary lymphedema [55, 242, 243]. Malformation of the lymphovenous valve, which is located at the intersection of the lymphatics and blood circulation, have been found in four different models of primary lymphedema in mice [54]. RASA1 mutation has been found to lead to malformation of the lymphatic vessels (lymphatic vessel hyperplasia and vessel dilation) and lymphatic valves, which results in inefficient lymphatic pumping due to increased backflow, leading to chylothorax [244, 245]. This is possibly due to RASA1 acting as a negative regulator of VEGFR-3, promoting lymphatic quiescence and thus preventing lymphatic hyperplasia [246]. Other lymphatic abnormalities have been associated with somatic mutations in specific populations of LECs at some point in development, and the severity and presentation of the disease in these cases often depend on the anatomical site of the mutation [247]. While the underlying cause of disease in these cases is genetic, these mutations are occurring in cells that exist in various different mechanical environments and, presumably, cell behavior will be influenced by the mechanics as the disease progresses.

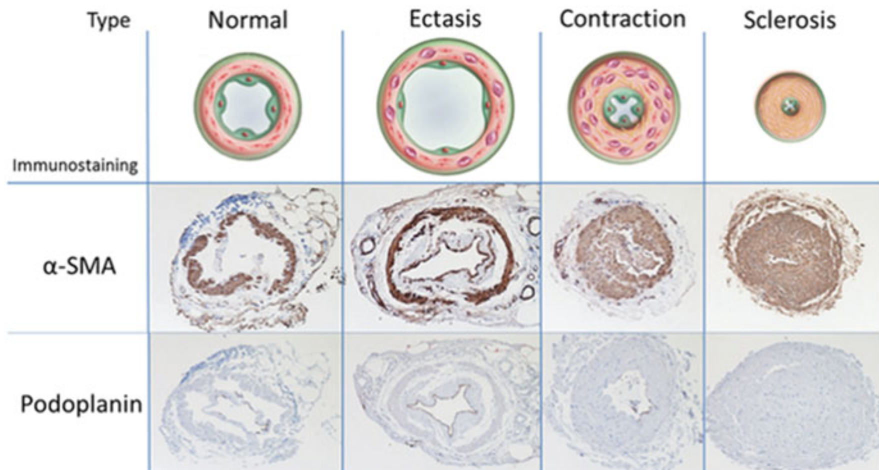

Fig. 8.8 The various stages through which the collecting vessels undergo maladaptive remodeling in response to a pathological microenvironment are shown. The α-SMA staining, specific for LMCs, shows an initial thinning of the muscle layer followed by a gradual thickening till the lumen gets almost completely occluded. The podoplanin staining, specific to LECs, is initially positive till the ectasis stage but is absent at later stages [250]

Lymphatic vessels also show a remodeling response to elevated mechanical forces during pathological conditions such as secondary lymphedema, which is frequently caused by lymph node removal following breast cancer surgery. The intact collecting lymphatic vessels experience elevated transmural pressures in case of lymphatic injury [248, 249]. The elevated pressure in the lymphatics can lead to their remodeling, which progresses through ectasis (enlargement of the lumen and thinning of muscle layer), contraction (narrowing of lumen and thickening of muscle layer), and eventually sclerosis (absence of lumen, thickening of muscle layer and fibrosis), depending on the severity and stage of lymphedema [250] (Fig. 8.8). The phenotype of the LMCs changes depending on the stage of lymphedema, progressing from a contractile phenotype to a more proliferative phenotype that also has an increased rate of ECM production. The phenotypic switching from a contractile to a proliferative phenotype has also been found in cultured LMCs in response to cyclic mechanical stretching [251]. An interesting and recent study showed that inducing an elevated pressure by ligating lymphatics produced collateralization that was not due to lymphangiogenesis but due to the remodeling of preexisting pre-collecting lymphatics into collecting lymphatics [252].

Summary Dysfunctions in lymphatic vessels are related to many major pathological conditions such as chronic inflammation, cancer, metabolic syndrome, and lymphedema. The primary diseases that may impact the lymphatic system are inflammation and pathologies bearing the hallmarks of inflammation such as venous hypertension, edemagenic stress, obesity and metabolic syndrome, and cancer. The

impairment of lymphatic vasculature can lead to primary or secondary lymphedema. Lymphatic contractility and lymphangiogenesis are the two primary functions of the lymphatic vasculature that may be affected by pathological conditions. Aberrant mechanical forces can also lead to the malformation and maladaptive remodeling of lymphatic vessels.

Secondary lymphedema is associated with reduced contractility of lymphatic vessels. Increased lymph flow by mesenteric venous hypertension impairs lymphatic contractility, while edemagenic stress can acutely enhance lymphatic contractility. Long-term exposure to elevated pulmonary flow increases ROS and decreases NO availability, which can lead to endothelial dysfunction. High-fat diet and obesity, and associated diseases like metabolic syndrome, impair lymphatic contractility. Inflammation can impair lymphatic contractility and increase the production of molecules that are involved in the regulation of lymphatic contractility. Hypervolemia can reduce lymphatic contractility and permeability through the action of atrial natriuretic peptides. Tumor microenvironment can increase lymphatic diameter and contractility, possibly through adrenergic receptors on the lymphatics. Chronic stress has also been shown to increase tumor metastasis through the lymphatics by possibly stimulating β adrenoreceptors in the LECs. Thus, adrenoreceptors can be possible therapeutic targets in treating tumor metastasis.

Lymphangiogenesis can be enhanced by inflammatory conditions, and VEGF-C induced lymphangiogenesis can be a therapeutic target for the treatment of chronic inflammatory conditions such as arthritis and inflammatory bowel diseases. The tumor microenvironment can also cause lymphangiogenesis in the periphery of the tumor, which is associated with increased tumor metastasis. VEGF-C and VEGF-D are thus also therapeutic targets for inhibiting tumor metastasis. The role of VEGF-C and lymphangiogenesis in secondary lymphedema is not yet clear, although recent studies suggest that inhibiting VEGF-C can ameliorate the swelling induced by lymphatic injury. Lymphatic malformations resulting from genetic defects, such as malformed lymphatic valves, can lead to impaired lymphatic function and, consequently, primary lymphedema.

8.7 Chapter Summary

The lymphatic system is a system of vessels and nodes that act as a parallel circulatory system to the cardiovascular system. They are primarily involved in the clearance of interstitial fluid, immune response, and lipid transport. This is achieved by a network of initial lymphatics that take up the interstitial fluid and a downstream network of collecting lymphatic vessels that pump the fluid back into the blood circulation. In the absence of a centralized pump, like the heart, the lymphatic circulation is dependent on the contraction of spontaneously contractile units called lymphangions that push the lymph through the lymphatic network, while secondary lymphatic valves ensure unidirectional flow. The lymphatic vasculature is sensitive to a range of mechanical forces that act upon them and regulate their anatomy and

physiology. These forces are the interstitial flow, interstitial pressure, transmural pressure, axial stretch, and shear stress.

Interstitial pressure and flow regulate the embryonic development of initial lymphatic vasculature by regulating lymphatic proliferation. Shear stress regulates lymphatic valve development through transcription factors PROX1, FOXC2, and GATA-2, and the cation channel PIEZO1. Matrix stiffness also plays a role in LEC proliferation. Postnatal lymphangiogenesis can be caused by interstitial and luminal flow. VEGF-C and MMPs are involved in the directional migration of LECs leading to the formation of luminal structures. NO is an important molecule involved in the proliferation of lymphatics. The luminal shear stress can reduce lymphatic barrier permeability while the transmural flow can increase the permeability. Transmural flow also has the effect of increasing DC migration across the lymphatic barrier.

The contractility of lymphatic vessels is modulated by the transmural pressure and shear stress. Shear stress reduces lymphatic contractility by causing relaxation. NO, endothelial prostanoids, histamine, and ROS are some of the molecules that have been identified in this shear-dependent relaxation. Lymphangions also entrain their contractions to external oscillatory flows, depending on their intrinsic contractility and mechanosensitivity. The transmural pressure acts in an endothelium-independent manner to modulate lymphatic contractility by directly affecting the LMCs. CICR plays an important role in the contractility of lymphangions, with L-type and T-type calcium channels being involved. Various computational models have successfully captured many aspects of the mechanosensitivity of lymphatic vessels. Nervous stimulation also controls lymphatic contractility through sympathetic and parasympathetic agents and frequently acts through similar pathways as mechanical forces such as shear stress.

Various pathological conditions such as inflammation, cancer, metabolic diseases, and lymphedema are associated with dysfunctional lymphatics. Dysfunction of the lymphatic vasculature can occur as contractile dysfunction, abnormal lymphangiogenesis, and/or malformations and maladaptive remodeling. An understanding of lymphatic dysfunction has also allowed for the identification of therapeutic targets for treating lymphatic diseases such as lymphedema, with VEGFR-3 being one of the foremost molecules of interest. Attempts at therapeutic lymphangiogenesis have yielded mixed results, but better treatments may be possible with the advancement of our understanding of the lymphatic system.

Conflict of Interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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Mechanical Regulation of Epigenetic Modifications in Vascular Biology and Pathobiology

9

Shu-Yi Wei and Jeng-Jiann Chiu

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Abstract

Shear stress and cyclic stretch are mechanical forces on the vessel wall exerted by blood flow and luminal pressure. These forces regulate gene expression and function in vascular cells, including endothelial cells (ECs) and smooth muscle cells (SMCs), thus affecting vascular biology in health and pathobiology in disease. Epigenetics refers to the study of sequence-independent heritable DNA alterations that modulate gene expression, including DNA methylation, histone modification/chromatin remodeling, and RNA-based mechanisms. Recently, the roles of mechanical force-induced epigenetic modifications in vascular gene expression and function have been intensively investigated. This chapter presents a critical concept: vascular gene expression can be mechanically modulated without DNA sequence change. By elucidating the relationship between mechanical forces and epigenetic modifications in gene expression, cell proliferation, angiogenesis, migration, and pathological status, this review provides a conceptual framework for understanding how mechanical force-induced epigenetic modifications modulate gene expression and cellular function in vascular biology in health and pathobiology in disease. This review contributes to our knowledge of how the mechanical microenvironment affects epigenetic changes in vascular cells and modulates their functions and behaviors, with the consequent modulation in vascular diseases.

Abbreviations

3′-UTR	3′-Untranslated region
5mC	5-Methylcytosine
ABs	Apoptotic bodies
Ago	Argonaute
AMPK	AMP-activated protein kinase
ANRIL	Antisense noncoding RNA at the Ink4 locus
$ApoE^{-/-}$	Apolipoprotein E-deficient genotype
BMP3	Bone morphogenetic protein 3
CTGF	Connective tissue growth factor
CVD	Cardiovascular disease
DNMT	DNA methyltransferase
EC	Endothelial cell
ECM	Extracellular matrix
EV	Extracellular vesicle
eNOS	Endothelial nitric oxide synthase
FAK	Focal adhesion kinase
GAX	Growth arrest-specific homeobox
GSK-3β	Glycogen synthase kinase-3β
H	Histone
HAT	Histone acetyltransferase
HDAC	Histone deacetylase

HUVEC	Human umbilical vein endothelial cell
ICAM-1	Intercellular adhesion molecule-1
IL	Interleukin
IRAK	IL-1 receptor-associated kinase
KLF	Krüppel-like factor
LISPR1	Long intergenic noncoding RNA antisense to S1PR1
LncRNA	Long noncoding RNA
MALAT1	Metastasis-associated lung adenocarcinoma transcript 1
MBD	Methyl CpG-binding domain protein
MCP-1	Monocyte chemotactic protein-1
MEF2	Myocyte enhancer factor 2
MiRNA	MicroRNA
MiR-21	MicroRNA-21
MMP	Matrix metalloproteinase
mRNA	Messenger RNA
mTOR	Mammalian target of rapamycin
NAD	Nicotinamide adenine dinucleotide
NcRNA	Noncoding RNA
NF-κβ	Nuclear factor-κβ
NO	Nitric oxide
NQO1	NADPH quinine oxidoreductase 1
Nrf2	NF-E2-related factor 2
OSS	Oscillatory shear stress
Ox-LDL	Oxidized low-density lipoprotein
PPARα	Peroxisome proliferator-activated receptor α
PRC	Polycomb repressive complex
PRKD2	Protein kinase D2
PSS	Pulsatile shear stress
PTM	Posttranslational modification
Rb	Retinoblastoma protein
RNAi	RNA interference
ROS	Reactive oxygen species
S1P	Sphingosine-1-phosphate
S1PR	Sphingosine-1-phosphate receptor
SAM	S-adenyl methionine
SHR	Spontaneously hypertensive rat
SIRT	Sirtuin
SMC	Smooth muscle cell
STEEL	Spliced-transcript endothelial-enriched lncRNA
TET	Ten-eleven translocation methylcytosine dioxygenase
TNF-α	Tumor necrosis factor
USS	Unidirectional shear stress
VCAM-1	Vascular cell adhesion molecule-1
VE-Cad	VE-cadherin
VEGF	Vascular endothelial growth factor

VEGFR2	Vascular endothelial growth factor receptor 2
WKY	Wistar Kyoto rat

9.1 Introduction

The development of the normal vessel wall involves a highly regulated process of cell proliferation, migration, differentiation, and relaxation in vascular cells, which comprises endothelial cells (ECs) and smooth muscle cells (SMCs), that are constantly exposed to various types of hemodynamic forces [1]. Hemodynamic forces are generated by blood flow and luminal pressure, which can be characterized as shear stress, cyclic stretch, and hydrostatic pressure [2]. ECs are mainly exposed to shear stress resulting from blood flow parallel to the vessel wall, whereas SMCs and ECs are subjected to cyclic stretch caused by pulsatile blood flow and pressure [2]. Another mechanical force, i.e., hydrostatic pressure, exerted by a fluid at rest, usually affects the capillaries of the circulatory system and is less extensively studied. Mechanical force-induced signals are received by mechanoreceptors in the cell membrane, such as ion channels, integrins, receptors of tyrosine kinases, G protein-coupled receptors, junction proteins, membrane lipids, and primary cilia, and these are in turn transmitted to the interior of the cell. The mechanoreceptors act on adaptor molecules (e.g., Src homology 2 domain-containing protein and growth factor receptor-bound protein 2) and trigger a series of intracellular signaling cascades, which consequently modulate gene expression; cell proliferation, differentiation, and migration; and angiogenesis [3]. This process, known as mechanotransduction, eventually leads to functional and morphological changes that contribute to physiological homeostasis [4]. Unbalanced regulation of these cellular functions causes vascular cell dysfunction and leads to a pathological cell state, which consequently contributes to the development of cardiovascular disease (CVD) [5].

In recent decades, epigenetics, which is the study of sequence-independent heritable DNA changes, has made the connection between gene expression and environmental stimuli and linked their relationship to disease susceptibility [6, 7]. It provides a perspective new to the public by showing that gene function can be altered in ways other than by changes to the DNA sequence. The different epigenetic processes, including DNA methylation, histone-mediated chromatin remodeling, and RNA-based mechanisms, modulate gene expression to cause changes in cellular function [8]. These changes subsequently lead to the adaptability of the organism or disease. DNA methylation, the best-known epigenetic process, is the addition of a methyl group (CH3) from S-adenyl methionine (SAM) to the fifth carbon of a cytosine residue to form 5-methylcytosine (5mC) in the CpG pair [9]. The hypermethylation of CpG islands results in the recruitment of protein complexes that remove acetyl groups and repress gene expression [9]. DNA demethylation is a mechanism that reverses gene silencing and is involved in embryo development, germ cell differentiation, and neuronal functions [10]. Another important epigenetic process is histone-mediated chromatin remodeling. Chromatin, which is composed

of DNA and histones, can be modified by histone acetyltransferase (HAT)-mediated acetylation and histone deacetylase (HDAC)-mediated deacetylation. Histone acetylation/deacetylation causes the conformation change of chromatin, thereby influencing gene transcription. Noncoding RNAs (ncRNAs), including microRNAs (miRNAs) and long noncoding RNAs (lncRNAs), have recently emerged as epigenetic regulators. MiRNAs, which consist of 18–22 nucleotide single-stranded RNAs, cause the degradation of messenger RNA (mRNA) by binding to the 3′-untranslated regions (3′-UTRs) of target genes or induce translational repression by binding to imperfectly complementary sequences [11]. LncRNAs, which are single-stranded RNAs containing more than 200 nucleotides, modulate gene expression by diverse mechanisms [12, 13]. Many cellular behaviors, such as cell proliferation and morphological changes, and the development of diseases, including cancer, CVD, and autoimmune disease, have been shown to be associated with epigenetic modulation [13–18]. Recently, the role of epigenetics in CVD has recently been intensively studied and has provided important insights into the diagnosis and therapeutic intervention of CVD [17]. Epigenetic factors, including HDACs [19], miRNAs [20, 21], and DNA methyltransferases (DNMTs) [22], have been shown to play vital roles in modulating vascular function and dysfunction and hence the development of atherosclerosis. In general, epigenetics offers a new perspective on gene regulation, which is not exerted by cis/trans-acting transcription but through multiple diverse processes that do not involve alterations to the DNA sequence.

In this chapter, the role and mechanism of epigenetics in regulating vascular biology and pathobiology in response to mechanical stimuli are discussed. This chapter shows the importance of mechanical forces (i.e., shear stress and cyclic stretch) and the corresponding signaling pathways in modulating vascular function and disease. Moreover, different epigenetic processes and their modulation of cellular responses, particularly those relating to vascular biology and pathobiology, are also described. This chapter summarizes the evidence that mechanical force-induced epigenetic modification influences homeostasis and pathology of the vessel wall. Such information provides new insights into the mechanisms by which epigenetic modification modulates gene expression, cellular function, and disease development in response to mechanical forces. Thus, epigenetic regulators have great potential as molecular targets or biomarkers that can be developed for the diagnosis and therapeutic intervention of vascular disorders associated with perturbed mechanical forces, such as atherosclerosis.

9.2 Vascular Mechanobiology

Blood vessels are subjected to sustained hemodynamic forces derived from blood flow and luminal pressure, which generate shear stress and cyclic circumferential stretch [2]. Another internal stress caused by both blood flow and pressure is known as hydrostatic pressure (i.e., normal stress), which arises from stationary fluid flow. Shear stress applied to vascular ECs is a frictional force parallel to the vessel wall, whereas normal stress is the force perpendicular to the vessel wall on which it acts.

Blood flow-induced shear stress has been reported to be involved in atherosclerosis, as revealed by the tendency of atherosclerotic lesions to be localized in the curves and branches of arterial trees, where the local flow is usually disturbed due to low and oscillatory shear stress (OSS). Conversely, blood flow in the straight segments of the arteries is unidirectional and pulsatile, generating pulsatile shear stress (PSS), which has been shown to play an atheroprotective role in the vessel wall [23, 24]. Tensile force generated by pulsatile blood flow and luminal pressure leads to circumferential stretching of the vessel wall and affects gene expression in, and the function of, vascular cells. Stretch force acts on both vascular ECs and SMCs and play an important role in maintaining the contractile phenotype of SMCs and regulating vascular tone when the stretching is at the physiological level [25]. Abnormal stretching caused by hypertension or flow overload can disturb biochemical homeostasis, leading to vascular remodeling and altered vasorelaxation [26]. In contrast to its role in the SMCs, the role of cyclic stretch in vascular ECs has not been fully investigated. Hydrostatic pressure is determined by the density of liquid, the acceleration of gravity, and the height of fluid, which may play an important role in cellular physiology. However, it has received relatively less attention than shear stress and cyclic stretch in the field of vascular biology. In this section, the cellular responses of vascular ECs and SMCs to shear stress and cyclic stretch are discussed.

9.2.1 Shear Stress

9.2.1.1 Shear Stress Modulates Vascular Morphogenesis

Shear stress has been shown to modulate vascular morphogenesis and heart development during embryogenesis [27, 28]. The early formation of shear stress is produced by the heartbeat, which causes the reorganization and migration of ECs to form an efficient vascular network [28]. By using in vivo imaging and quantitative analyses of intracardiac flow forces in zebrafish embryos, a study showed that high-velocity vertical flow exists at two key stages in the developing heart [27]. Flow that is blocked at either the cardiac inflow or outflow tracts results in abnormal development of the third chamber and impaired valve formation in the heart [27]. The zinc finger-containing transcription factor Krüppel-like factor 2a (KLF2a), which is activated by the reverse flow in the atrioventricular canal, is highly associated with valve development. It has been reported that protein kinase D2 (PRKD2) causes the derepression of KLF2a through HDAC5 phosphorylation [28, 29]. Thus, KLF2a expression is greatly reduced in PRKD2 mutants, which do not form valves. Blood flow also activates the expression of miRNAs, such as miRNA-21 (miR-21) and miR-143, which leads to the cell proliferation that induces valve formation in the zebrafish heart [30]. In addition, there is evidence that fluid shear stress modulates vessel remodeling via endothelial nitric oxide synthase (eNOS) [31, 32]. Fernández-Varo et al. analyzed the vascular properties of cirrhotic rats with ascites and determined that they cause vascular remodeling [31]. Specifically, they found that the vessels in cirrhotic rats have higher levels of eNOS and a dramatic reduction in wall thickness and area compared to the vessel thickness and area in control rats,

indicating that eNOS is required for the regulation of vessel compliance and vascular remodeling [31]. Another study also showed the critical role of eNOS in modulating ischemia-induced arteriogenesis, angiogenesis, and blood flow recovery in mice [32]. Taken together, these studies demonstrate the importance of mechanical forces in the modulation of epigenetic factor expression in embryonic cardiogenesis and vasculature development.

9.2.1.2 Shear Stress Regulates Physiological Functions

In addition to its functions in embryonic vascular morphogenesis and cardiogenesis, shear stress plays an important role in endothelial morphological changes and cellular functions, including cell proliferation, differentiation, and migration, through biochemical and biological events [26, 33]. It has been shown that the physiological level of PSS exerts protective functions by inducing nitric oxide (NO) production. Conversely, OSS-disturbed flow impairs biochemical homeostasis and leads to vascular remodeling and dysfunction (e.g., altered vasorelaxation, vascular tone, and stiffness). Shear stress-induced mechanotransduction in ECs has been investigated by using both in vitro and in vivo approaches. For in vitro studies, researchers have developed several devices to generate different types of shear stresses that stimulate blood flow in the human body. A parallel-plate flow channel is created by using a gasket with a thin silicon membrane that produces steady, unidirectional shear stress (USS, 12 dynes/cm^2), PSS (12 $\pm$ 4 dynes/cm^2), and reciprocating shear stress (i.e., OSS, 0.5 $\pm$ 4 dynes/cm^2) on cultured ECs [33]. In vivo studies have been performed to evaluate the applicability and relevance of the in vitro findings to physiological and pathophysiological conditions. In nature, blood flow in the curves and branches of the arterial trees is disturbed, whereas flow in the straight segments of the arteries is pulsatile and unidirectional [34]. Transcription factor KLF2, which is an abundant molecule in ECs, promotes endothelial survival in response to oxidized low-density lipoprotein (ox-LDL) stimuli [35]. The continued application of PSS on ECs for 24 h led to a sustained expression of KLF2, which protected the ECs against oxidative stress stimuli [35]. In addition to KLF2, a number of genes have been shown to be induced by USS in ECs and are involved in EC survival, angiogenesis (e.g., Tie2 and fetal fiver kinase 1), and vascular remodeling (e.g., matrix metalloproteinase-1, MMP-1) [36]. Physiological levels of flow also maintain the gene expression profile in ECs in a nonproliferative state by increasing the expression of the growth arrest proteins GADD45, p21cip1, and p53 and inhibiting retinoblastoma protein (Rb) phosphorylation [3]. Application of USS to ECs induces a transient expression of monocyte chemotactic protein-1 (MCP-1) through the modulation of the Ras-mitogen activated protein kinases pathway [37]. Continuous application of USS causes downregulation of MCP-1 and that of various pro-inflammatory molecules such that their expression levels fall below the basal levels and the ECs remain in a noninflammatory state [37]. Shear stress can modulate intercellular junction proteins, such as VE-cadherin (VE-Cad), connexin19, and platelet endothelial cell adhesion molecule-1 [38], all of which play important roles in modulating the integrity and permeability of ECs. The continuous distribution of VE-Cad staining has been shown at the cell borders in the region

affected by PSS [39, 40]. The cytoskeleton is reorganized via the Rac1, cdc42, and Rho signaling pathways, leading to cell morphological changes in response to shear stress stimuli [41–43]. EC exposure to USS or PSS induces the alignment of cytoskeletal fibers in the flow direction [44]. Taken together, the redistribution of cellular junctions and cytoskeletal proteins in regions subjected to PSS contributes to the maintenance of the cellular integrity and the physiological functions of ECs.

9.2.1.3 Shear Stress Is Involved in the Development of Vascular Pathologies

ECs are generally in a quiescent state unless stimulated by some pathophysiological conditions. Disturbed flow is involved in endothelial dysfunction and leads to a pathophysiological state, thereby contributing to the development of vascular disorders, including atherosclerosis and thrombosis and the complications related to them [2, 5]. In native circulation, disturbed flow generally occurs at arterial branches and curves, such as carotid bifurcations, branch points of the coronary, and the infrarenal and femoral arteries, as well as the inner curvature of the aortic arch, where atherosclerotic lesions preferentially develop [2]. ECs with a pathological status are changed structurally and functionally. Their morphology can be changed, such that they are enlarged and/or acquire an irregular shape. ECs can also lose the regulatory roles that are characterized by the endothelium layer becoming more permeable, by endothelial inflammation, and within the ECs, by oxidative stress. Disturbed flow can cause EC dysfunction through the expression of adhesion molecules, such as intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and E-selectin; proinflammatory cytokines, such as interleukin (IL)-1, IL-6 and tumor necrosis factor-alpha (TNF-α); and proinflammatory chemokines, such as IL-8 and MCP-1 [2, 5]. These molecules attract leukocytes and monocytes to the surface of the activated ECs, thereby enabling lipoprotein penetration and inflammatory cell infiltration, which initiate a pro-inflammatory process within the vessel wall. This process is the first step of atherogenesis. KLF2, a key PSS-induced transcription factor, has been shown to have anti-inflammatory and anticoagulant roles in ECs [45]. Disturbed flow suppresses the expression of KLF2 and causes the dysfunction of ECs [35]. Disturbed flow also induces the sustained activation of transcription factor nuclear factor-$\kappa\beta$ (NF-$\kappa\beta$), which induces the expression of proinflammatory genes in ECs [46]. Increased intracellular oxidative stress is another indicator of EC dysfunction. It has been shown that USS plays a protective role in vessels by inducing antioxidant enzymes, such as superoxide dismutase, heme oxygenase-1, and NADPH quinine oxidoreductase 1 (NQO1), which provide a homeostatic oxidative balance [47]. Homeostasis can be disrupted by disturbed flow, which causes the enhanced expression of reactive oxygen species (ROS), which damage the vessel wall [47]. Taken together, these studies show that disturbed shear stress induces differential cellular responses compared with that induced by PSS, leading to endothelial dysfunction and atherosclerotic lesion formation at preferential sites.

9.2.2 Stretch Force

9.2.2.1 Cyclic Stretch Regulates Physiological Functions in Vascular SMCs and ECs

Cyclic stretch is the circumferential stretching of the vessel wall, which plays an important role in regulating vascular tone and maintaining the contractile phenotype of SMCs under physiological conditions [48, 49]. Unlike shear stress, which is mainly sensed by ECs, both ECs and SMCs are subject to cyclic stretch [49, 50]. However, the role of cyclic stretch in endothelial function has not been fully investigated. Cellular responses may vary depending on whether the cell is subjected to physiological or pathological stretch. Normal stretching can establish homeostatic oxidative balance and maintain vascular integrity to support vessels in the physiological state. When cyclic stretching is perturbed, such as when stretching is excessive due to high blood pressure, homeostasis is disrupted, which leads to vascular remodeling, arterial stiffness, and calcification [51]. Although vascular cell roles in and responses to stretching are less clear than their roles in and responses to shear stress, these two forms of mechanical forces induce processes that share many similar features. Mechanoreceptors can sense stretch force and transmit mechanical stimuli to intracellular signaling pathways to regulate cellular behaviors [52]. Several studies have described the important role of stretching on SMC gene expression and cellular functions, such as proliferation/apoptosis, migration/alignment, and the phenotypic switching of SMCs [53, 54]. MMPs are calcium-dependent zinc-containing endopeptidases that can degrade extracellular matrix proteins and thus mediate the extracellular matrix (ECM) [55]. Applying cells with a physiological level of cyclic (1 Hz) uniaxial stretch was shown to repress the expression of MMP-2 and MMP-9 in human cultured SMCs [56]. This report demonstrated that SMCs responded to physiological stretching by altering MMPs, which led to the subsequent remodeling of the ECM surrounding the vasculature. Physiologic stretching also inhibits apoptosis and mitosis in vascular ECs and SMCs, respectively [57, 58]. The magnitude of the cyclic stretch can cause cytoskeletal rearrangement, which increases EC permeability, indicating an important role for cyclic stretch in the regulation of mass transport through the vessel wall [59].

9.2.2.2 Cyclic Stretch Regulates Pathophysiological Changes in Vascular SMCs and ECs

SMC hypertrophy, hyperplasia, and ECM remodeling are considered to play roles in the development of hypertension. Pathological stretching caused by hypertension disrupts vessel homeostasis and leads to vascular remodeling, phenotypic switching of SMCs (from the contractile type to the synthetic type), arterial stiffness, and calcification, which are involved in the pathogenesis of CVDs [51]. It has been suggested that hypertension reduces myocardin activity in SMCs, resulting in the initial switch from the contractile phenotype to the synthetic SMC phenotype [60]. Another study showed that cyclic stretch (30 cycles/min; 15% elongation) induces SMC proliferation [54]. These studies indicate a role for cyclic stretch in the modulation of SMC gene expression and phenotypic changes. Moreover, uniaxial

cyclic stretch causes SMCs to align perpendicular to the direction of the stretching, which affects cytoskeleton rearrangement. However, the mechanism by which stretching induces cytoskeletal rearrangement remains unclear [53]. In addition to morphological changes, cyclic stretch also plays a role in SMC migration by promoting the translocation of a key intracellular signal transducer, protein kinase C-δ, to the cytoskeleton [61]. Chronic high-magnitude cyclic stretch also plays a role in the modulation of the inflammatory response in vascular ECs via vascular endothelial growth factor receptor 2 (VEGFR2) signaling and matrix remodeling [50, 62]. Gawlak et al. reported that human pulmonary ECs subjected to chronic cyclic stretch (18% cyclic stretch) had induced VEGFR2 expression and tyrosine phosphorylation, which led to an increase in the expression of ICAM-1 and VCAM-1. Collectively, these studies show that cyclic stretch plays an important role in SMC phenotypic switching, vessel homeostasis, and vascular functions.

9.3 Epigenetics

Epigenetics gradually emerged in the 1980s in the course of researchers studying many phenomena that were incompatible with classical Mendelian genetics. It has been defined as the study of heritable changes in gene expression and cellular phenotype and has provided a relatively new explanation for gene function that is altered in ways other than solely by changes in DNA sequence [8]. Epigenetics has been linked to many cellular behaviors, such as cell proliferation and morphological changes, and diseases, including cancers, CVDs, and reproductive, autoimmune, and neurobehavioral conditions [13–18]. Epigenetics includes two types of modifications: selective transcriptional regulation, such as DNA methylation and histone covalent modification/chromatin remodeling, and posttranscriptional regulation, including RNA-based mechanisms. The ncRNA-based mechanisms, which involve miRNAs, lncRNAs, and antisense RNAs, are the most recently recognized epigenetic modifications by which gene expression is regulated.

9.3.1 Methylation

DNA methylation, the most well-known epigenetic process, is the addition of a methyl group to the fifth carbon of a cytosine residue to form 5mC in the CpG pair [9]. CpGs tend to aggregate to form CpG islands and are quite rare in mammalian genomes (~1%), which are generally unmethylated. The hypermethylation of CpG islands results in the recruitment of protein complexes that remove acetyl groups and repress gene expression [9, 63]. DNA methylation is catalyzed by a family of DNMTs. DNMT1, DNMT3a, and DNMT3b have methyltransferase activity [64]. DNMT-3L is able to interact with DNMT3a and DNMT3b to methylate retrotransposons [65]. DNA methylation-associated proteins, including methyl CpG-binding domain proteins (MBDs) and ubiquitin-like PHD, and RING finger domain-containing proteins, are bound to DNA-containing methylated CpG

dinucleotides and recruit repressor complexes to methylated promoter regions to inhibit transcription. DNA methylation plays roles in many illnesses and health conditions [66, 67]. During early embryonic development, CpG islands undergo differential methylation, which confers their totipotency or pluripotency [68]. In addition to embryonic development, CpG island methylation plays a crucial role in genomic imprinting [9]. In vascular ECs, the CpG islands are methylated at the promoters of eNOS and VEGFR2, from where they recruit MBD2 to suppress gene expression at these methylated CpG islands [69]. DNA demethylation catalyzed by Ten-eleven translocation (TET) methylcytosine dioxygenases is a mechanism for reversing DNMT-mediated gene repression. TETs, including TET1, TET2, and TET3, which convert 5mC into 5-hydroxymethylcytosine [10], has been reported to inhibit DNMT1 expression and global DNA methylation in atherosclerotic plaques [70, 71]. Moreover, TET2 has also been shown to regulate the phenotype changes of SMCs, contributing to endothelial dysfunction and macrophage-induced inflammation [72]. These studies demonstrate the importance of DNA methylation/ demethylation in the modulation of vascular function.

9.3.2 Histone Modification and Chromatin Remodeling

Chromatin is composed of DNA and proteins, including four core histone proteins (H2A, H2B, H3, and H4) that are wrapped around 147 base pairs of DNA [73]. Histones are subjected to covalent posttranslational modifications (PTMs), including lysine acetylation, lysine and arginine methylation, serine and threonine phosphorylation, lysine ubiquitylation, and lysine sumoylation on their tails [74]. Histone PTMs affect gene expression by recruiting histone modifiers and inducing chromatin remodeling. In general, tightly folded chromatin tends to inhibit gene expression, while more open chromatin leads to gene expression. Although gene transcription that is regulated by histone PTMs can be turned on and off, histone acetylation directly contributes to gene expression. Histone acetylation has been the most intensively studied modification, and the results have shown that this modification is caused by HATs transferring an acetyl group from acetyl-CoA to form ε-N-acetyllysine [74, 75]. HATs are divided into three families, namely, Gcn5-related N-acetyltransferases, MYSTs, and CREB-binding proteins (CBP/p300) [74, 75]. HDACs have an opposite action to that of HATs in that they remove acetyl groups from histones. There are three distinct families of HDACs: class I (HDAC1/ 2/3 and HDAC8), class II (HDAC4/5/6/7 and HDAC9/10), and class III [sirtuin (SIRT) family (SIRT1/2/3/4/5/6/7)] [76]. Class I HDACs are mostly expressed in the nucleus and display high enzymatic activity, whereas HDAC3, which is similar to the class IIA HDACs (HDAC4/5, HDAC7, and HDAC9), contributes to nuclear-cytoplasmic shuttling, providing a mechanism for linking extracellular signals with gene expression [77, 78]. Class IIB HDAC6 is the primary cytoplasmic deacetylase found in mammalian cells, whereas the functions of HDAC10 are poorly understood. Class III HDACs/SIRTs are the highly conserved protein family of nicotinamide adenine dinucleotide (NAD)-dependent histone deacetylases located in

different subcellular compartments, such as the nucleus (SIRT1/3/6/7), cytoplasm (SIRT1/2), and the mitochondria (SIRT3/4/5) [79, 80]. SIRTs exert their functions by transferring an acetyl group to the cofactor NAD to generate O-acetyl ADP-ribose and nicotinamide, which serve as feedback inhibitors of enzymatic reactions [80]. Histone acetylation is a reversible process that is controlled by the antagonistic actions of HATs and HDACs. In general, hyperacetylation is involved in the upregulation of transcription, whereas hypoacetylation contributes to the downregulation of gene expression [81]. The balance between acetylation and deacetylation represents a critical regulatory mechanism for gene expression, developmental processes, and disease progression, such as those of CVDs [82]. It has been shown that HATs and inhibitors of HDAC attenuate CVDs by mediating certain cellular processes, including myocyte hypertrophy, apoptosis, oxidative stress, and inflammation [79, 82–84]. These results implicate HATs and other inhibitors of HDACs as novel agents useful for treating CVD patients [82, 85].

9.3.3 RNA-Based Mechanisms

Epigenetic modulation of gene expression through ncRNAs is a newly discovered regulatory mechanism [86]. ncRNAs, including miRNAs, small interfering RNAs, piwi-interacting RNAs, small nucleolar RNAs, and lncRNAs, are functional RNAs that are not translated into proteins [87, 88]. RNA-based epigenetic regulation of gene expression is divided into RNA interference (RNAi)-mediated transcriptional gene silencing and RNA-dependent histone methylation without RNAi. MiRNAs regulate chromatin structure and inhibit transcription via Argonaute (Ago) complexes that bind to the 3′-UTRs of complementary nascent RNAs or via the translational repression induced by the pairing of imperfect complementary sequences, leading to the degradation of the mRNA and the recruitment of histones and DNMTs [11, 89]. LncRNAs, distinct from other small ncRNAs, modulate gene expression by diverse mechanisms, which have been categorized into signaling, acting as molecular decoys, guiding ribonucleoprotein complexes to specific chromatin sites, and serving as scaffolds in the formation of transcriptional complexes. In general, many lncRNAs are able to bind to particular genomic sites, implying their ability to modulate chromatin activities. In addition, lncRNAs can also act posttranscriptionally by regulating translation and splicing and affecting mRNA stability. Xist, a 17 kb nuclear lncRNA, is expressed exclusively on the inactive X chromosome and mediates global inactivation of a randomly chosen X chromosome in an early developmental process in females [90]. This process is known as X chromosome inactivation, which transcriptionally silences the X chromosome coated by Xist, thereby providing equivalent gene expression between males and females. Xist may exert its function by directing the polycomb repressive complex (PRC) 2 to chromatin and catalyzing histone methylation to repress gene transcription [86]. Several studies have revealed that ncRNAs are involved in the regulation of various processes, such as metabolism; development, particularly vascular development; cell proliferation; and various diseases, including vascular diseases [88, 91,

92]. Chen et al. have shown that miR-146a plays a critical role in the inhibition of vascular inflammation and neointimal lesion formation in rat or mouse carotid artery [93] (Fig. 9.1). Shear- and synthetic SMC-induction of miR-146a in ECs via integrins/Nrf-2 targets IL-1 receptor-associated kinase (IRAK) to inhibit NF-κB signaling, which exerts negative feedback control in the biogenesis of itself. Moreover, EC miR-146a expression has been shown to modulate synthetic SMC phenotype toward a contractile state [93]. Taken together, atheroprotective shear stress-induced miR-146a expression inhibits EC inflammation and neointima formation in injured arteries. This regulation is highly correlated with clinical symptoms such as vascular remodeling after injury. MiR-126 has been reported to play roles in angiogenesis and anti-inflammation in vascular ECs. The angiogenic signaling and integrity of the vessel wall are regulated by miR-126 during embryogenesis [94]. MiR-126 has also been reported to attenuate TNF-α-induced VCAM-1 expression to affect leukocyte adhesion [95]. MiR-143/145 is involved in the modulation of SMC phenotypic switching in vessels [96]. The expression of the antisense noncoding RNA at the Ink4 locus (ANRIL) is regulated by DNA polymorphisms in this region, which is highly associated with the incidence of CADs [97]. The NF-κB-induced expression of ANRIL modulates the expression of IL-6 and IL-8 by recruiting the transcription factor Yin Yang 1, which also leads to endothelial inflammation [98]. In addition, ANRIL promotes the proliferation of SMCs by recruiting PRC complexes to cyclin-dependent kinase inhibitor 2A/B, which downregulates ANRIL [99]. In contrast to ANRIL, the expression of another lncRNA, lincRNA-p21, is decreased in the atherosclerotic plaques in $ApoE^{-/-}$ mice and humans, indicating its protective roles in vessels [100]. LincRNA-p21 inhibits SMC proliferation, neointima formation, and atherosclerosis by enhancing p53-mediated apoptosis in SMCs [100]. LncRNA metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) is considered to be associated with vascular disease because its expression is low in atherosclerotic plaques of the coronary artery [101, 102]. Mice with Malat1 deficiency ($Malat^{-/-}$) in an $ApoE^{-/-}$ background ($ApoE^{-/-}$ $Malat^{-/-}$) possess an increased number of inflammatory cells and atherosclerotic lesions compared with the number in the $ApoE^{-/-}$ $Malat1^{+/+}$ control mice [102]. Taken together, these studies demonstrate that ncRNAs are not genetic waste but play critical roles in the pathophysiology of vascular diseases.

9.4 Mechanical Force-Induced Epigenetic Modifications in Vascular Health and Disease

The roles of mechanical force-induced epigenetic modifications in vascular gene expression and various functional regulations have been intensively studied. In this section, the roles and related mechanisms of mechanical force-induced DNA methylation, histone acetylation/deacetylation, and miRNA/lncRNA expression in modulating vascular cell function are discussed (Tables 9.1, 9.2, and 9.3).

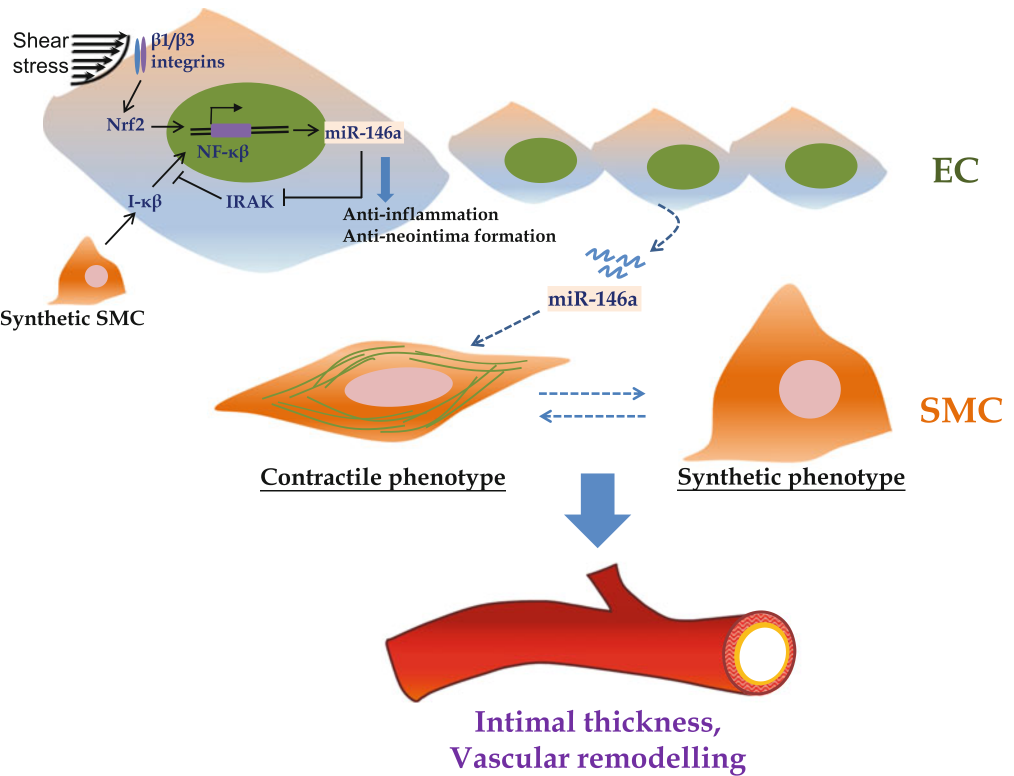

Fig. 9.1 Schematic diagram showing the mechanisms underlying the shear- and synthetic SMC-mediated miR-146a expressions and their inhibition in EC inflammation and neointimal lesion formation in rat or mouse carotid artery. Application of unidirectional shear stress (12 dynes/cm^2) to ECs co-cultured with synthetic SMCs for 24 h induces EC miR-146a expression through integrins/Nrf-2 signaling cascade. miR-146a targets IRAK to inhibit NF-κB signaling, which

Fig. 9.1 (continued) exerts negative feedback control on the biogenesis of itself. In vivo, silencing either Nrf-2 or miR-146a leads to increased neointima formation of injured rat carotid artery under physiological flow. Overexpressing miR-146a inhibits neointima formation of rat or mouse carotid artery. Unidirectional shear stress applied to ECs can modulate synthetic SMC phenotype toward a contractile state through EC miR-146a induction. Taken together, atheroprotective shear stress-induced miR-146a expression inhibits EC inflammation and neointima formation of injured arteries, leading to vascular remodeling

Table 9.1 DNMTs and TETs are involved in hemodynamic force-modulated vascular cell function and dysfunction

DNMT & TET	Target gene	Type of mechanical force	Cell type	Function	Reference
DNMT1 ↑	HoxA5, KLF3	Disturbed flow (±5 dyne/cm^2, at 1 Hz)	Arterial endothelium	Inflammation ↑	[103]
DNMT1 ↑	Cyclin A, CTGF	Oscillatory shear stress (0.5 ± 4 dyne/cm^2)	Vascular endothelium	Proliferation ↑	[104]
DNMT3a ↑	KLF4	Disturbed flow (0.4 ± 1.6 dyne/cm^2)	Human aortic EC	Inflammation ↑/ vascular tone	[105]
TET2 ↓	?	Low shear stress (5 dyne/cm^2)	EC	Autophagy dysfunction/ vascular tone	[106]

9.4.1 Methylation

Dysregulation of DNA methylation, including both hypermethylation and hypomethylation, has been reported to occur in various diseases, including CVDs [133, 134]. Genetically atherosclerosis-prone apolipoprotein E-deficient (ApoE$^{-/-}$) mice show alterations in DNA methylation profiles in their aortas and monocytes before histologically detectable vascular lesions appear [133]. The hypermethylation of the miR-145 promoter and its decreased expression in SMCs are caused by DNMT1 upregulation or TET2 downregulation, leading to the activation of the nucleotide-binding oligomerization domain-like receptor protein 3, which facilitates the inflammatory response and induces plaque formation [135]. This study indicated that the DNA methylation dynamically regulated by DNMTs and TETs plays a crucial role in atherosclerosis. Furthermore, several studies have demonstrated that DNMTs and TETs are modulated by mechanical forces that regulate vascular cell function (Table 9.1). DNMT1 has been shown to play a role in the regulation of endothelial functions in response to blood flow and may contribute to atherogenesis [103, 104]. Disturbed flow induces DNMT1 expression via the integrin/focal adhesion kinase (FAK)/mammalian target of rapamycin (mTOR)/p70S6 kinase signaling pathways to induce global DNA methylation, including on HoxA5, KLF3, Cyclin A, and connective tissue growth factor (CTGF), both in vivo and in vitro [103, 104]. The downregulation of DNMT1 by using 5-aza-2′-deoxycytidine or by inhibiting mTOR reduced the disturbed flow-induced endothelial inflammation and proliferation, thereby attenuating plaque formation in the ApoE$^{-/-}$ mice [103, 104]. The expression of eNOS regulated by the basal transcription machinery in the core promoter plays an important role in modulating endothelial functions such as vascular tone via NO production. However, eNOS mRNA is destabilized when ECs are under pathological conditions such as inflammation, proliferation, ox-LDL treatment, or hypoxia [136]. DNMT3a stimulated by OSS binds to the promoter of the transcription factor KLF4 and causes DNA methylation of the KLF4

Table 9.2 HDACs and HATs are involved in hemodynamic force-modulated vascular cell function and dysfunction

HDAC & HAT	Co-factor	Type of mechanical force	Cell type	Function	Reference
HDAC1	p53	Unidirectional shear stress (12 dyne/cm^2)	ECs	Proliferation ↓	[107]
HDAC1 ↑	Nrf2	Oscillatory shear stress (0.5 ± 4 dyne/cm^2)	ECs	Proliferation ↑/ inflammation ↑/ oxidation ↑	[108]
HDAC2 ↑	Nrf2	Oscillatory shear stress (0.5 ± 4 dyne/cm^2)	ECs	Proliferation ↑/ inflammation ↑/ oxidation ↑	[108]
HDAC3 ↑	Nrf2/ MEF2	Oscillatory shear stress (0.5 ± 4 dyne/cm^2)	ECs	Proliferation ↑/ inflammation ↑/ oxidation ↑	[108]
HDAC3	Akt	Oscillatory shear stress (4.5 dyne/cm^2 at 0.5 Hz)	ECs	Survival ↑	[109]
HDAC3 ↓		Cyclic stretch (1 Hz at 10% elongation)	VSMCs	Migration ↓	[110]
HDAC4 ↓		Cyclic stretch (1 Hz at 10% elongation)	VSMCs	Migration ↓	[110]
HDAC4 ↓		SHRs	Arteries	Hypertension	[111]
HDAC5	MEF2	Unidirectional shear stress (24 dyne/cm^2)	ECs	Vascular tone	[112]
HDAC5 ↓		SHRs	Arteries	Hypertension	[111]
HDAC5 (Cyto) ↑		Pulsatile shear stress (12 ± 4 dyne/cm^2)	ECs	Inflammation ↓/ oxidation ↓	[108]
HDAC5 (Nucle) ↑	MEF2	Oscillatory shear stress (0.5 ± 4 dyne/cm^2)	ECs	Inflammation ↑/ oxidation ↑	[108]
HDAC6 ↑	Tubulin	Unidirectional shear stress (15 dyne/cm^2)	ECs	Cytoskeletal remodeling/ migration ↑	[113]
HDAC7 (Cyto) ↑		Pulsatile shear stress (12 ± 4 dyne/cm^2)	ECs	Inflammation ↓/ oxidation ↓	[108]
HDAC7 (Nucle) ↑	MEF2	Oscillatory shear stress (0.5 ± 4 dyne/cm^2)	ECs	Inflammation ↑/ oxidation ↑	[108]
HDAC7 ↑		Cyclic stretch (1 Hz at 10% elongation)	VSMCs	Migration ↓	[110]
SIRT1 ↑	eNOS	Unidirectional shear stress (12 dyne/cm^2)	ECs	Vascular tone	[114]
p300	NF-κβ	Unidirectional shear stress (15 dyne/cm^2)	ECs	Vascular tone	[115]

Table 9.3 NcRNAs are involved in hemodynamic force-modulated vascular cell function and dysfunction

NcRNA	Target gene	Type of mechanical force	Cell type	Function	Reference
miR-126 ↑	KLF2	Blood flow	ECs	Angiogenesis	[116]
miR-663 ↑		Oscillatory shear stress (0.5 ± 4 dyne/cm^2)	ECs	Inflammation ↑	[117]
miR-21 ↑	PPARα	Oscillatory shear stress (0.5 ± 4 dyne/cm^2)	ECs	Inflammation ↑	[118]
miR-10a ↓		Athero-susceptible regions	ECs	Inflammation ↑	[119]
miR-10a ↓	GATA6/VCAM-1	Oscillatory shear stress (0.5 ± 4 dyne/cm^2)	ECs	Inflammation ↑	[120, 121]
miR-10a ↑	GATA6/VCAM-1	Pulsatile shear stress (12 ± 4 dyne/cm^2)	ECs	Inflammation ↓	[120, 121]
miR-92a ↓	KLF2	Unidirectional shear stress (12 dyne/cm^2)	ECs	Vascular tone	[122]
miR-19a ↑	Cyclin D	Unidirectional shear stress (12 dyne/cm^2)	ECs	Cell cycle arrest at G1/S transition	[123]
miR-23b ↑	p-Rb/E2F	Pulsatile shear stress (12 ± 4 dyne/cm^2 at 1 Hz)	ECs	Proliferation ↓	[124]
miR-101 ↑	mTOR	Unidirectional shear stress (12 dyne/cm^2)	ECs	Proliferation ↓	[125]
miR-26a ↑	GSK-3β	Cyclic stretch (1 Hz)	Human airway SMCs	Airway SMC hypertrophy	[126]
miR-130a ↑	GAX	SHRs	VSMCs	Proliferation ↑	[127]
Let-7d ↓	K-RAS	SHRs	VSMCs	Proliferation ↑	[128]
miR-33 ↓	BMP3	Arterial stretch (1.25 Hz at 10% elongation)	Venous SMCs	Proliferation ↑	[129]
miR-146a	IRAK	Unidirectional shear stress (12 dyne/cm^2)	ECs-VSMCs	Inflammation ↓/neointima formation ↓/VSMC contractile phenotype	[93]

(continued)

Table 9.3 (continued)

NcRNA	Target gene	Type of mechanical force	Cell type	Function	Reference
Extracellular miR-143/145 ↑		Unidirectional shear stress (20 dyne/cm^2)	ECs-VSMCs	VSMC contractile phenotype	[130]
Extracellular miR-126-3p ↑		Disturbed flow (0.5 ± 4 dyne/cm^2)	ECs-VSMCs	VSMC synthetic phenotype	[131]
MANTIS ↑	ICAM-1	Unidirectional shear stress	ECs	Inflammation	[132]

promoter. This process results in the inhibition of KLF4 transcription and eNOS expression, leading to inflammation in ECs [105]. The hypermethylation of the KLF4 promoter as well as the downregulation of KLF4 and eNOS are also observed in the endothelium of OSS-affected regions in the porcine aorta [105]. In addition to DNMTs, TETs have also been shown to participate in the initiation and progression of atherosclerosis [106]. TET2 levels and TET2-mediated endothelial autophagy are decreased in response to low shear stress (5 dyne/cm^2), which may contribute to atherogenesis. Moreover, overexpressed TET2 enhances eNOS expression and reduces endothelin-1 levels. This result implies that TET2 may play a role in vessel constriction [106]. Taken together, these studies provide new insight into the mechanism by which shear stress-mediated DNA methylation in ECs influences vascular cell function and, hence, atherosclerosis.

9.4.2 Histone Modification

9.4.2.1 Class I HDACs

Mechanical force-induced histone modifications cause chromatin remodeling that regulates gene transcription, which is responsible for the modulation of endothelial function (Table 9.2, Fig. 9.2). An in vitro study showed that USS facilitated the association of p53 with HDAC1 to cause the deacetylation of p53 at Lys-320 and Lys-373 in ECs [107]. The USS-mediated deacetylation of p53 induced p21 expression, leading to cell cycle arrest. In our previous study, class I HDACs were found to play a role in modulating OSS-induced cell proliferation and oxidation in ECs [108]. OSS stimulus caused an increased nuclear accumulation of HDAC1/2/3 and thus induced cyclin A expression but inhibited p21 expression, leading to the upregulation of EC proliferation [108]. OSS stimulation also induced the association of HDAC1/2/3 with NF-E2-related factor 2 (Nrf2) and HDAC3 with myocyte enhancer factor 2 (MEF2), which resulted in the deacetylation of Nrf2 and MEF2 and the inhibition of NQO1 and KLF2 expression [108]. By using the in vivo rat stenosis model, in which a U-clip was applied to the abdominal aorta to produce a 65% constricted diameter [35], we found increased expression of HDAC2/3/5 and

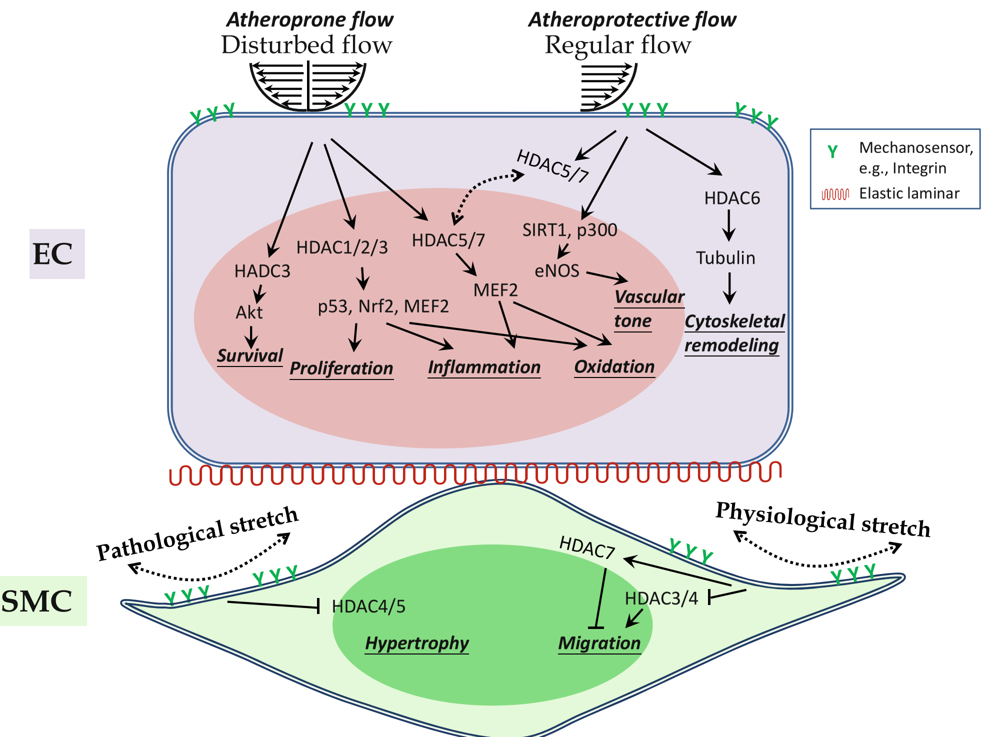

Fig. 9.2 Schematic diagram of HDACs, HATs, and related molecules involved in hemodynamic force-induced vascular cell function and dysfunction. Atheroprone flow, i.e., disturbed and oscillatory flow, induces the expression and nuclear accumulation of class I HDAC1/2/3 and class II HDAC5/7 in ECs. Subsequently, this accumulation causes HDAC1/2/3-mediated deacetylation of Nrf-2 to inhibit NQO1 expression, which contributes to antioxidation in ECs. Furthermore, HDAC1/2/3 are involved in oscillatory flow-induced cell cycle progression, endothelial proliferation, and cell survival. Disturbed flow also causes

Fig. 9.2 (continued) HDAC3/5/7-mediated deacetylation of MEF2 to inhibit KLF2, thereby promoting endothelial anti-inflammatory responses and antioxidation. In contrast, atheroprotective flow, i.e., regular and pulsatile flow, induces the phosphorylation-dependent nuclear export of HDAC5/7 in ECs, thereby increasing the expression of NQO-1 and KLF2. Regular flow also enhances the expression of HDAC6, SIRT1, and P300, which play roles in the modulation of vascular tone and EC cytoskeletal remodeling. Another mechanical force, cyclic stretch, mainly mediates the regulation of SMC hypertrophy and migration through HDAC4/5 and HDAC3/4/7 signaling

incorporation of BrdU in ECs located in downstream of stenosis where OSS occurs. This OSS-dependent BrdU incorporation was attenuated in the ECs by the class I-specific HDAC inhibitor valproic acid (VPA) [108]. In addition, HDAC3 expression and the OSS-stimulated phosphorylation of its serine/threonine residues were shown to play an essential role in the survival and integrity of cultured ECs [109]. Inhibition of HDAC3 expression via specific short hairpin RNA reduced EC survival, by reducing Akt activity, which led to vessel rupture and atherosclerosis in ApoE$^{-/-}$ mice.

9.4.2.2 Class II HDACs

HAT p300 has been reported to cooperate with p65 to bind to the shear stress response B element of the eNOS promoter, leading to eNOS expression under laminar flow [115]. In addition to p300, other HDACs have been shown to play roles in mechanical force-induced eNOS expression [112]. USS stimuli induce the phosphorylation of class II HDAC5 and its nuclear export through a calcium/calmodulin-dependent pathway [112]. This export subsequently caused the dissociation of HDAC5 from MEF2 and promoted MEF2 transcription, resulting in KLF2 and eNOS expression. Moreover, class II HDAC6 has been reported to modulate USS-induced cytoskeletal remodeling in ECs co-cultured with SMCs [113]. Tubulin is an important cytoskeletal protein, and its acetylation stabilizes microtubules and retards cell migration. USS stimulus activates HDAC6 to inhibit tubulin acetylation, leading to cytoskeletal remodeling and cell migration in ECs co-cultured with SMCs [113]. Furthermore, we have identified the roles of class II HDACs in modulating endothelial oxidation and inflammation in response to OSS and PSS stimuli [108]. OSS stimuli induce the expression and nuclear accumulation of class II HDACs in ECs, which causes HDAC3/5/7 to interact and form a complex with MEF2 to suppress KLF2 expression contribute to anti-inflammatory responses [108]. Another mechanical force, cyclic stretch (1 Hz at 10% elongation), has been shown to inhibit SMC migration through the hyperacetylation of histone H3, increased expression of HDAC7, and downregulation of HDAC3/4 [110]. The role of cyclic stretch-mediated histone modifications in CVDs have been studied in vivo. Western blotting analysis of the proteins from the aortas and mesenteric arteries of spontaneously hypertensive rats (SHRs) and Wistar Kyoto rats (WKYs) showed that the expression of HDAC4 and HDAC5 were decreased in the SHRs compared to their expression in the WKYs [111]. The downregulation of HDACs caused by VPA greatly reduced the blood pressure, cytokines, ROS, and angiotensin II in the SHR mouse model [137].

9.4.2.3 Class III HDACs

The class III HDAC SIRT1, interacting with AMP-activated protein kinase (AMPK), has been shown to influence eNOS expression in vitro and in vivo [114]. The USS-induced mechanical stimulation enhances the association of SIRT1 and eNOS, resulting in eNOS deacetylation and expression through AMPK signaling [114]. In addition to eNOS, SIRT1 also deacetylates p65 at lysine 310 in macrophages and suppresses macrophage binding to aortic ECs, thereby inhibiting

NF-κβ signaling and reducing the expression of the adhesion molecules ICAM-1 and VCAM-1 [138]. An in vivo study showed that overexpressed endothelial SIRT1 in ApoE$^{-/-}$ mice maintained vascular cells in a physiological state and hence attenuated the formation of atherosclerotic plaques [138]. Furthermore, SIRT1 also plays roles in the proliferation and inflammation of SMCs. SIRT1 promotes the mitosis of senescence-resistant cells by suppressing p21. Moreover, the inhibitor of MMP-3 in tissues is enhanced by SIRT1 overexpression, which causes the downregulation of MMPs and induces anti-inflammatory responses in SMCs. Taken together, these studies indicate that mechanical forces mediate the activation and expression of HDACs and hence contribute to vascular cell function as well as the development of atherosclerosis.

9.4.3 MicroRNA

9.4.3.1 miRNAs Are Regulated by Shear Stress

The functions of mechanical force-induced miRNAs in modulating cellular angiogenesis, inflammation, proliferation, and migration in vascular biology and disease have been extensively studied (Table 9.3, Fig. 9.3). It has been shown that KLF2 plays a critical role in flow-induced angiogenesis in zebrafish embryos through the miR-126/VEGF signaling pathway [116]. To elucidate the mechanisms by which miRNAs regulate cellular functions in response to mechanical stimulation, the miRNA expression profiles of cultured ECs subjected to differential flow were analyzed [117]. miR-663 was found to play a role in endothelial inflammation but not in apoptosis upon OSS stimulation. miR-21 activated by OSS in cultured ECs also triggered an inflammatory response by downregulating peroxisome proliferator-activated receptor α (PPARα) [118]. The role of miRNAs in the regulation of endothelial inflammation has been studied in vivo. The decreased expression of miR-10a in atherosclerosis-susceptible regions (i.e., the inner curvature of the aortic arch and aorta-renal branches) in swine vessels indicates that miR-10a may play a protective role during atherosclerosis [119]. miR-10a confers protection to the vascular system through its anti-inflammatory effect in ECs [119]. Moreover, miR-10a can be induced by PPS and OSS stimuli via HDAC signaling to modulate anti-inflammatory and inflammatory responses, respectively, through GATA-binding factor 6 and VCAM-1 expression [120, 121]. In addition to endothelial inflammation, miRNAs have been shown to participate in KLF2-mediated eNOS expression in response to shear stress [122, 139]. USS (12 dyne/cm^2) but not OSS (0.5 $\pm$ 4 dyne/cm^2) stimuli can downregulate miR-92a expression to increase KLF2 expression and thus facilitate eNOS induction and NO production, which contributes to the modulation of vascular tone [122]. Moreover, it has been shown that USS can induce miR-19a, miR-23b, and miR-101 expressions to inhibit the expressions of cyclin D, E2F, and mTOR, as well as the phosphorylation of Rb, which leads to cell cycle arrest and the inhibition of EC mitosis [123–125].

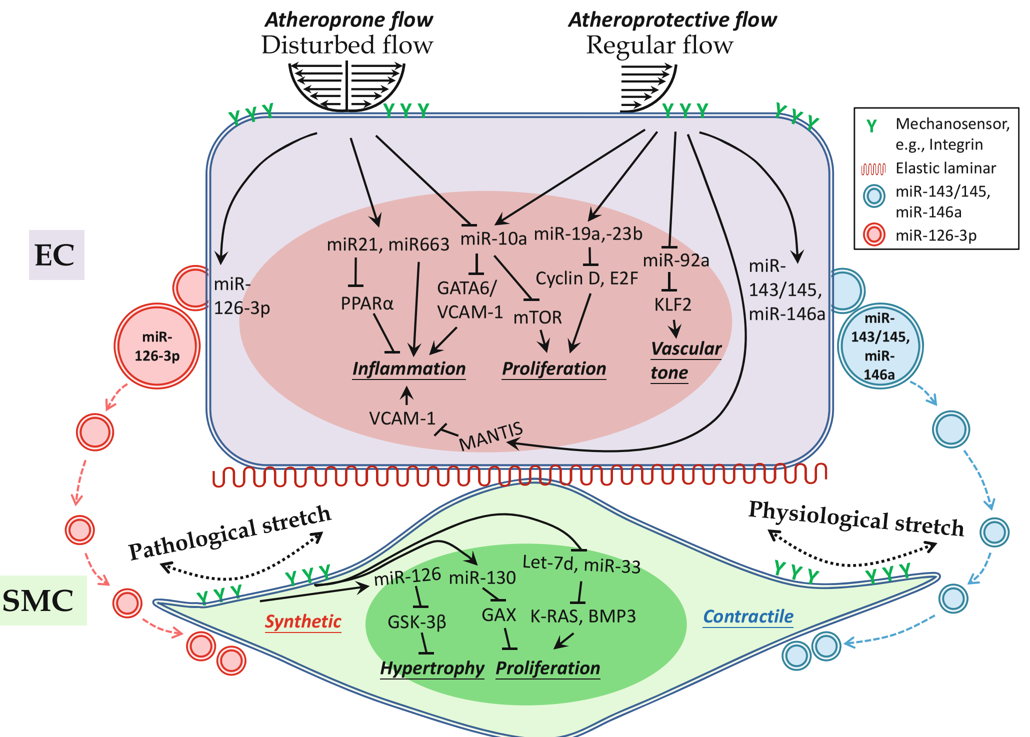

Fig. 9.3 Schematic diagram of ncRNAs and related molecules involved in hemodynamic force-modulated vascular cell function and dysfunction. Disturbed flow induces the expression of miR-21 and miR-663 to downregulate PPAR-α expression, leading to an inflammatory response in ECs. Moreover, disturbed flow inhibits miR-10 expression to upregulate the expression of GATA6 and VCAM-1, leading to an inflammatory response in ECs. Conversely, regular flow induces the expression of miR-10a, miR-19a, and miR-23b, causing the downregulation of mTOR, cyclin D, and E2F and contributing to endothelial

Fig. 9.3 (continued) proliferation. Disturbed flow and regular flow also play roles in SMC phenotype switching via the secretion of miR-126-3p and miR-143/145 from ECs. In addition to miRNAs, regular flow also induces the expression of lncRNA MANTIS, which inhibits VCAM-1 levels and inflammation in ECs. Another mechanical force, cyclic stretch, mainly regulates SMC hypertrophy and proliferation. Pathological stretching causes the upregulation of miR-126 and miR-130 but downregulation of let-7d and miR-33, resulting in the inhibition of GSK-3β and GAX but increased expression of K-RAS and BMP3. These processes promote hypertrophy and proliferation in SMCs

9.4.3.2 miRNAs Are Regulated by Cyclic Stretch

Stretch force has also been shown to cause miR-26a expression, which serves as a hypertrophic gene in SMCs [126]. miR-26a subsequently downregulates glycogen synthase kinase-3β (GSK-3β), an anti-hypertrophic protein, to enhance SMC hypertrophy. The roles of miRNAs in modulating cellular functions during cyclic stretching have been studied in vivo. miR-130a and let-7d have been shown to be correlated with vascular remodeling in SHRs [127, 128]. The expression of miR-130a is upregulated, which suppresses the expression of growth arrest-specific homeobox (GAX) to promote SMC proliferation in these hypertensive rats. Conversely, let-7d is significantly downregulated in the SMCs of the SHRs. Let-7d can bind oncogene K-RAS to downregulate it, leading to the suppression of SMC mitosis. Recently, Huang et al. proposed that miRNA-33 protects against venous SMC proliferation in response to arterial mechanical stretch in a grafted vein [129]. The expression of miR-33 in venous SMCs subjected to a 10% 1.25 Hz arterial stretch in vitro is decreased, which upregulates bone morphogenetic protein 3 (BMP3), and increases phosphorylation of smad2 and smad5. These modulations and modification result in venous SMC proliferation and neointimal hyperplasia.

9.4.3.3 Extracellular miRNAs

Although active in cells, few miRNAs are found in extracellular biofluids, such as serum and saliva, or in cultured media [140–144]. In contrast to RNA, these released miRNAs are relatively stable in the extracellular environment, where they are in the continual presence of RNases, suggesting that they may have a protective mechanism that enables them to bypass areas with high RNase activity [145, 146]. Extracellular miRNAs might be conjugated with proteins, included in the lipid complexes, or wrapped with membrane vesicles to avoid degradation [147, 148]. The roles and mechanisms of miRNA exported to the extracellular environment have been studied. Biofluid miRNAs can be detected in several vesicles and complexes, indicating that the release of miRNAs to the extracellular region is mediated by extracellular vesicles (EVs), including exosomes, microvesicles, and apoptotic bodies (ABs), and by high-density lipoproteins and the Ago2 protein complex [140, 144, 147, 148]. These released miRNAs are transported to the recipient cells via specific pathways through which they modulate the expression of target genes/molecules by serving as signal transducers in cell–cell communications. The role and function of extracellular miRNAs in vascular biology have been investigated [131, 149, 150]. Application of unidirectional shear stress (12 dynes/cm^2) to ECs co-cultured with synthetic SMCs for 24 h induces EC miR-146a expression to inhibit neointima formation and modulate synthetic SMC phenotype toward a contractile state [93]. This study provides evidence that miR-146a is secreted from ECs to act on the adjacent SMCs. ABs containing miR-126 have been reported to deliver cargo to recipient vascular cells and reduce atherogenesis in mice [149]. Human umbilical vein endothelial cells (HUVECs) were transfected with the KLF2 plasmid or subjected to PSS to generate miR-143/145-abundant EVs that contributed to SMC phenotypes. Coculturing HUVECs with SMCs led to the reduction of target gene expression in recipient SMCs, which attenuated the development of

atherosclerosis [130]. Conversely, disturbed flow-induced expression and secretion of endothelial miR-126-3p promoted the phenotypic switch of SMCs, causing SMC hyperplasia and, hence, atherogenesis [131]. Taken together, these studies demonstrate that shear stress-mediated extracellular miRNAs play critical roles in EC–SMC interactions and vascular diseases. Thus, extracellular miRNAs may have the potential to be developed as noninvasive clinical biomarkers for atherosclerosis.

9.4.4 Long Noncoding RNA

LncRNAs have been shown to modulate vascular cell function and CVDs [151, 152]. Studies regarding mechanical force-mediated lncRNA function in vascular biology have recently emerged [132, 153]. The role of spliced-transcript endothelial-enriched lncRNA (STEEL) in angiogenesis has been identified [153]. STEEL transcriptionally upregulates eNOS and KLF2 expression via STEEL-mediated recruitment of the poly-ADP-ribosylase to the KLF2 promoter. Moreover, STEEL receives inhibitory feedback from both eNOS and KLF2 in response to USS stimulus [153]. Leisegang et al. demonstrated that the beneficial effects of HMG-CoA-reductase inhibitors (statins) and laminar flow on ECs are conferred by lncRNA MANTIS (also known as lncRNA n342419) [132] (Table 9.3, Fig. 9.3). Laminar flow and statins have been shown to activate MANTIS expression via the transcription factors KLF2 and KLF4, which causes a reduction in the association of the SWI/SNF chromatin remodeling factor BRG1 and the ICAM-1 promoter, thereby preventing the development of atherosclerosis [132]. Sphingosine-1-phosphate (S1P) is a potent signaling lipid activated by the S1P receptor (S1PR). Recently, long intergenic noncoding RNA antisense to S1PR1 (LISPR1), which is highly expressed in ECs and lung tissue but expressed at low levels in human lung diseases, plays an essential role in S1P signaling by regulating S1PR1 expression, thereby regulating endothelial migration and sprouting [154]. Downregulated LISPR1 inhibits S1P-induced migration and sprouting in ECs. Moreover, LISPR1 and S1PR1 expression are upregulated by the increased association of KLF2 with the S1PR1/LISPR1 shared promoter in response to USS and statins. Taken together, these studies suggest the possibility that lncRNAs can be developed as clinical biomarkers of vascular diseases and may be potential therapeutic drugs for CVDs.

9.5 Conclusions and Future Perspectives

Mechanical forces, including shear stress and cyclic stretch, can modulate gene expression, cellular functions, and morphological changes in vascular health and disease. Normal shear stress and cyclic stretch maintain vascular homeostasis, whereas disturbed flow and pathological cyclic stretch cause vascular cell dysfunction and thus promote the occurrence of vascular diseases. Epigenetic factors,

including DNMTs, HDACs, miRNAs, and lncRNAs can modulate gene expression without altering the DNA sequence. Mechanisms by which mechanical forces act on vascular gene expression and cellular functions via differential intracellular signaling pathways are widely explored. However, studies investigating the role of mechanical force-induced epigenetic modifications in vascular biology have emerged only in recent decades. In this chapter, in vitro and in vivo studies on mechanical force-induced DNA methylation, histone modification/chromatin remodeling, and ncRNA-dependent modification in the regulation of gene expression, cellular function, and pathology are summarized. Studies regarding the mechanical regulation of vascular gene expression, proliferation/migration, angiogenesis, antioxidation, inflammation, and vascular disorders are discussed. Furthermore, the roles and regulations of critical vascular molecules such as eNOS, KLF2, ICAM-1, VCAM-1, NF-κβ, p21, and p53 are also described.

Functional roles of shear-induced DNMTs, TETs, HDACs, HAT, miRNAs, and lncRNAs in the regulation of gene expression and vascular cell function and dysfunction are well studied. However, the mechanisms by which shear stress and stretch force induce DNMTs, TETs, HDACs, and HAT expressions remain unclear and warrant further investigation. Mechanoreceptor integrin and its downstream FAK/mTOR/p70S6 signaling pathway have been shown to be involved in the disturbed flow-induced DNMT1 expression [103, 104]. In comparison to shear stress, the role of another mechanical force, cyclic stretch, in the epigenetic regulation of vascular physiology and pathology remains unclear. Recently, the effect of cyclic stretch on vascular ECs has been investigated [50]. There is increasing evidence that cyclic stretch serves as a potential trigger for the induction of the inflammatory response of ECs and inflammatory cells, leading to ECM remodeling [50]. Mechanical force-mediated endothelial function and its interplay with ECM are highly associated with the programming of abdominal aortic aneurysm [50]. Unlike shear stress caused by blood flow, the generation of mechanical stretch is more complicated. Matrix remodeling, which is involved in the interaction of the ECM with MMPs, alters the mechanical properties of vessels and therefore causes altered stretching [155]. The stretch force caused by ECM remodeling may play roles in epigenetic-mediated vascular health and disease, but these roles need to be elucidated. Extracellular miRNAs and lncRNAs are emerging epigenetic regulators that modulate vascular function in response to mechanical forces. Although extracellular miRNAs constitute a small portion of all miRNAs, their characteristics, such as circulating in biofluids and mounting resistance to RNAase, make them potential targets to develop as noninvasive clinical biomarkers of atherosclerosis and other CVDs. LncRNAs, distinct from small ncRNAs, modulate gene expression by diverse mechanisms. In addition to modulating gene transcription, lncRNAs can also regulate translation and RNA splicing and affect mRNA stability. However, studies on mechanical force-induced lncRNAs suggest that they rarely regulate vascular biology. Therefore, the role of these ncRNA-mediated epigenetic modifications in modulating vascular gene expression and the corresponding cellular functions need to be further investigated.

In conclusion, epigenetic studies increase our knowledge of mechanical forces that transcriptionally and posttranscriptionally regulate gene expression in vascular

physiology and pathology without altering DNA sequences. These studies provide new insights into the dynamic regulation of vascular functions and the ways they alter the vascular biology or pathobiology, findings that are expected to lead to the development of diagnostic and therapeutic approaches for treating vascular diseases.

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Abstract

Enhanced mechanical forces are imposed on small and large vessels in hypertension. The enhanced transmural pressure increases predominantly circumferential wall stress that is returned toward control by adaptive mechanisms such as active constriction and eutrophic remodeling with concomitant increases of wall thickness. However, other hemodynamic, mechanical stresses are enhanced by such adaptive responses. Specifically, wall shear stress rises by pressure-induced constriction in smaller vessels provoking an endothelium-dependent dilation. A fine balance between these two homeostatic mechanisms that control wall stress and wall shear stress determines vascular tone in small resistance vessels which is

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shifted in hypertension toward higher vascular tone with enhanced peripheral resistance. Wall shear stress equals the frictional pressure loss during blood flow and must be larger to keep downstream capillary pressure stable, in the face of an increased pressure head. In this light, adaptive responses that decrease luminal diameter to control wall stress appear as maladaptive and energy-consuming. In large arteries, wall thickening is also observed in hypertension. However, the main impact of hypertension in large arteries, specifically elastic proximal vessels, is the profound consequence on pulse wave transmission. Pressure distends elastic arteries and consequently changes their capacity to store further volume during cardiac ejection in systole. This capacity depends on distensibility or compliance (the inverse of stiffness) which is decreased solely due to higher pressure. Changes in stiffness attributable to structural changes in the vessel wall are only found in young hypertensive individuals. Nevertheless, pulse wave velocity is largely increased due to the less compliant arteries at the prevailing pressure. This impacts hemodynamics in the pulsatile compartment of the vascular system that is governed by the Moens–Korteweg equation and wave reflections with dramatic consequences on other organs in the long run.

10.1 Introduction

Hypertension is a very frequent pathology with a prevalence amounting to 30–40% in adults that is of high interest because it considerably amplifies the risk of cerebrovascular and coronary heart disease [1, 2]. In addition, other diseases that are even more linked to the vascular system are regarded to be consequences of long-standing hypertension such as chronic renal disease or aortic aneurysms [3, 4]. Sub-optimal arterial pressure is considered to be the number one attributable risk for death throughout the world [1]. These consequences are very likely predominantly related to the enhanced mechanical stresses that are imposed onto the vascular tree during hypertension. In this brief chapter, I will emphasize the mechanical impacts and consequences as well as resulting biological adaptations without detailing invoked signaling pathways.

10.2 Mechanical Force Exerts Stress That Creates Tension in the Tissue

In terms of mechanics, forces acting on materials in a continuum exert stresses, which is a measure of force intensity and therefore given as force per area (N/m^2 or Pa). Stress can be represented by stress tensors which can be oriented in the three dimensions of space. Notwithstanding the complexities of in vivo mechanics, an artery may be compared to a perfused cylinder that is subjected to three primary mechanical stresses: (1) wall shear stress (τ) induced by the flow of blood,

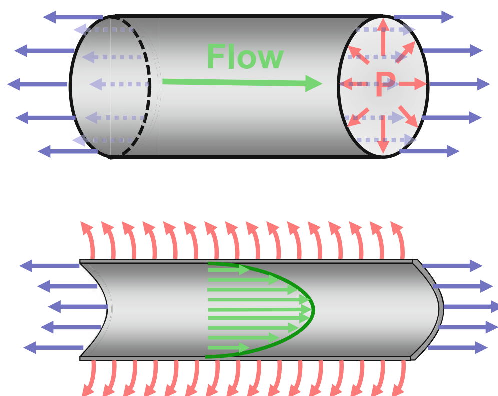

Fig. 10.1 Forces acting in a perfused vessel. Forces acting in a perfused vessel exert stresses which are calculated as force per area (N/m^2 or Pa). The flow of blood creates a force parallel to the cross-section of the material that is imparted on the boundary of the wall, in case of the vessel onto the endothelial cells which have to withstand this force. The stress is exerted in the direction of flow and is termed wall shear stress (depicted in green). Its magnitude (τ) depends on flow (Q), luminal radius (a), and viscosity of the fluid (μ) (formula 1). The transmural pressure (P) is a force that expands the vessel in all perpendicular directions of its circumference (upper panel). It exerts so-called circumferential wall stress in the perpendicular direction of the longitudinal axis of the vessel (depicted in red) that creates circumferential tension in the vessel wall. The amplitude (σ_θ) depends on the pressure difference across the wall (P), luminal radius of the vessel (a), and wall thickness (h) (formula 2). Furthermore, forces act in the longitudinal direction of the axis of the vessel (depicted in blue) that tend to lengthen the vessel and exert a stress named axial wall stress. The amplitude of axial wall stress (σ_ζ) depends on wall thickness (h), luminal vessel radius (a), and the force acting in this direction (f) that is more difficult to infer from hemodynamic forces directly (formula 3)

(2) circumferential wall stress (σ_θ, also called hoop stress) induced by the pressure difference across the vessel wall (transmural pressure), and (3) axial wall stress (σ_ζ) which tends to lengthen the vessel and is more difficult to infer directly from hemodynamic forces (Fig. 10.1). At stationary and simplified conditions, i.e., excluding such factors as pulsatility or the non-Newtonian behavior of blood, these stresses can be estimated as:

(1) Wall shear stress: $\tau = (4\mu Q)/(\pi a^3)$
(2) Circumferential wall stress: $\sigma_\theta = Pa/h$ (Laplace)
(3) Axial wall stress: $\sigma_\zeta = f/[\pi h(2a + h)]$

where μ is the viscosity, Q the volumetric flow, a the luminal radius, h the wall thickness, P the transmural pressure, and f the force directed in the direction of the

length axis of the vessel [5, 6]. The thickness to lumen ratio (*h/a*) is of specific interest since this ratio defines whether the more simple formula (2) can indeed be applied in which the vessel wall is regarded as a surface. This is true for thin-walled vessels with a thickness to lumen ratio in the range of 0.1 which is the case for larger arteries (and veins). However, in small arteries, arterioles, and capillaries this ratio increases up to values of 0.5 or even higher and the vessel wall cannot be regarded anymore as a surface. At these conditions, wall stress varies considerably between the outside and the inside surfaces of the wall. However, for practical purposes formula (2) may also be applied for such smaller vessels bearing in mind that wall stress is not equally distributed across the wall. If the vessel lining is considered as a wall, the stress is higher at its inner side.

From these basic physical considerations, it becomes obvious that hypertension with high transmural pressure increases circumferential wall stress (other variables being constant). This can be returned to the initial value by either increase of the wall thickness (*h*) or a reduction of the vascular diameter (*a*). The latter, short-term adaptation of the vessel decreases circumferential wall stress again [7], but also increases wall shear stress (as well as axial wall stress). These simple considerations exemplify the close interaction of the different stresses that are imposed onto the vessel wall by mechanical forces. Nevertheless, returning to its initial stress values is the well-described response of vessels examined in multitudes of experimental settings in vivo or in vitro. These responses are diameter reduction and/or enlarging wall thickness upon pressure increases [7] or vessel dilatation upon enhanced levels of wall shear stress [8]. The adaptation responses act on different time scales ranging from minutes to weeks and involve the interaction of actin and myosin, changes in the cytoskeleton and integrins, extracellular matrix changes as well as growth highlighting the manifold cellular processes being invoked in these responses that even include transcriptional processes [9–14].

The stress exerted onto the vessel wall creates tension in the tissue that resists the exerted stress. In fact, the tension within the tissue counterbalances exactly the stress and is generated by the physical interaction of (protein) molecules inside the wall to withstand the stress. The tension in the tissue can be transferred across anchoring points that attach the cell structure to its underlying foundation, the extracellular matrix, comparable to architectural systems [15]. By this means, tension can also be transferred from cell to cell (through externally located proteins) to enable a concerted action of a multitude of cells in response to stress. Sensors are required to detect the tension within these structural pathways in order to react to mechanical stimuli and possibly offset any imposed alteration. In order to function, these sensors do not necessarily need to quantify the tension in absolute terms. It seems reasonable to assume that rough information ("too low" or "too large") would suffice to enable a functional feedback system that keeps itself in homeostasis and to always offset any deviation from the equilibrium. Another interesting aspect in this model is the capability of cells to initiate a "prestress" or "pretension" imposed through active mechanisms onto those structures that bear tension, for example through the interaction of actin and myosin [15]. This may be compared to tuning a piano or a violin string which generates the right tone if the tension is set correctly or "in

homeostasis." This concept was initially developed from cell models and is termed "tensegrity" or "tensional homeostasis" [5, 16].

10.3 Vascular Adaptations in Small Arteries to Enhanced Wall Tension During Hypertension

10.3.1 Acute Response: Myogenic Constriction

The increased transmural pressure that occurs during hypertension enhances circumferential wall stress in those vessels that are exposed to it. The wall tension can be reduced to the initial level by either a decrease in diameter or an increase in wall thickness (formula 2, see above). An active constriction is a well-known physiologic response upon pressure increase in arterial vessels that was initially described more than a century ago by Bayliss [17] and is accordingly named Bayliss effect or myogenic response due to its myogenic origin. It emanates from a mechanical stimulus arising from the enhanced wall tension which, in turn, generates intracellular signals that enhance the contractile state of the vascular smooth muscle cells and decrease diameter [18]. Modeling suggests that indeed wall tension (rather than radius or distension) is the parameter that drives the myogenic response and the sensor should be arranged in series (rather than in parallel) with the contractile elements. Thereby, the vessel response (constriction) abrogates the initiating stimulus (wall tension) in a negative feedback loop mechanism providing a limit for the response [7]. Such negative feedback is in line with the idea of a homeostatic system. Interestingly, this setup provides "a relatively close regulation of blood flow even though flow is not the regulated variable" [7, 19]. Moreover, the myogenic response limits pressure increases in the capillaries preserving their important function during fluid filtration into the extravascular space. This highlights the important physiologic function of acute myogenic responses which are reviewed in detail in numerous publications [20–28].

A reduced diameter in resistance vessels is often found in hypertension (in midlife) as indicated by enhanced systemic vascular resistance [29]. Assuming that organ blood flow remains constant (serving tissue needs) it follows that wall shear stress is enhanced at conditions with reduced diameters in arterioles (formula 1, see above). This assumption is in line with energy dissipation since pressure decreases because of the frictional forces that oppose blood flow, i.e., wall shear stress. Only if wall shear stress is increased, the pressure drop attains larger values along a certain length of the vascular tree resulting in unchanged pressure at the capillary level despite a larger pressure head acting on resistance vessels as is the case in hypertension. However, enhanced wall shear stress triggers in an endothelium-dependent manner vasodilation, the so-called flow-induced dilation [30–32]. In case wall shear stress is a regulated homeostatic parameter (similar to circumferential wall stress), it follows that opposing regulatory effects within these two control loops are exerted, vasoconstriction to keep circumferential wall stress constant and vasodilation to maintain wall shear stress. In fact, experiments on

isolated vessels as well as in vivo have suggested that myogenic responses in arterioles enhance wall shear stress and are opposed by vasodilations mediated by enhanced endothelial nitric oxide release [33–37]. Consequently, an intermediate vascular diameter may prevail that results in small changes of both regulated parameters [38–40], and vascular tone is further modulated by metabolic signals from tissues substantially contributing to vascular tone as is suggested by modeling [41].

The obvious deviation from the above mentioned homeostatic feedback system may be due to several reasons. Firstly, these two control loops may act at distinct sites along the vascular tree with differential efficacy as was proposed in several reviews according to experimental evidence [38, 42, 43]. The feedback regulation of wall shear stress is more efficient at upstream sites thereby providing the important physiologic function to keep pressure drop stable at these upstream sites despite large increases of flow that occur during enhanced oxygen demand of the tissues (e.g., during exercise). In contrast, circumferential wall stress is more effectively controlled along the arteriolar tree at downstream sites just upstream of the capillaries which must be protected from pressure increases by enhancing upstream pressure drop. In fact, in vivo experiments demonstrated that myogenic responses are opposed by nitric oxide-mediated vasodilation (likely being elicited by enhanced wall shear stress) preferentially in larger arterioles [36]. Secondly, the efficacy of the control loops may be altered during hypertension, i.e., wall shear stress elicited vasodilations are reduced or myogenic responses are enhanced or a combination of both.

In fact, experimental evidence supports both ideas. It was well demonstrated in a multitude of investigations that flow-induced vasodilations are reduced in hypertension, in humans as well as in experimental settings [44–48]. A main contributor is a reduced biological efficacy of nitric oxide due to enhanced oxidative stress, commonly known as endothelial dysfunction [49–51]. An initial compensatory mechanism may be a shift in the mediator of the endothelial dilator mechanism as reported in isolated human vessels at high intraluminal pressure [52, 53]. In any case, hypertension may render the opposing wall shear stress-induced counterregulatory mechanism inoperative and result in enhanced vasoconstriction. Very recently, exaggerated vasoconstrictions upon phenylephrine in small arteries isolated from hypertensive animals were demonstrated to be due to impaired endothelial Ca^{2+} signaling [54]. Phenylephrine elevates not only smooth muscle cell Ca^{2+} levels (leading to constriction) but also elicits endothelial Ca^{2+} signaling, thereby activating a concomitant dilator response invoking NO release that limits the vasoconstriction [55]. The endothelial activation is proposedly elicited by a transfer of inositol 1,4,5-trisphosphate (IP_3) through myoendothelial gap junctions [56, 57]. Such local endothelial IP_3-mediated Ca^{2+} signals were smaller in amplitude, shorter in duration, and occurred less frequently in vessels from hypertensive animals. The authors concluded from these observations that the organization of local Ca^{2+} signaling circuits residing at myoendothelial projections is disrupted in hypertension which results in enhanced vasoconstriction [54].

As a matter of fact, it is difficult to clearly distinguish and separate alterations in one from the other mechanically triggered feedback loop in vivo. In more general terms, any defect in these feedback loops alters the overall systemic resistance under the assumption of constant volumetric flow: An impaired wall shear stress response results in enhanced levels of frictional resistance (as wall shear stress is not kept constant) and an exaggerated myogenic response may decrease circumferential wall stress below the "control point" but likewise increases the levels of frictional resistance. The immediate consequence of such exaggerated frictional resistance is an enhanced pressure drop along the vessels resulting in decreased downstream pressure. This can be prevented by an enhanced upstream pressure head which is invariably established if cardiac output at rest (reflecting tissue needs) remains unchanged.

10.3.2 Chronic Adaptations: Vascular Remodeling

A characteristic feature in humans with essential hypertension and in animal models of hypertension is small artery inward remodeling [58–63]. During this process, the vessel lumen decreases (inward remodeling), and inward remodeling is usually associated with a likewise decreased external diameter [64]. The most common form is eutrophic inward remodeling which is characterized by an unchanged media surface area (total wall tissue) and thus reflects a rearrangement of the cells in the vascular wall. Since the lumen decreases in size and media tissue assembles in a smaller total area (reduced external diameter), the media-to-lumen ratio increases which is the hallmark of inward remodeling. It is also found in spontaneously hypertensive rats and may represent the earliest form of damage in hypertension [65, 66]. Arteries or arterioles that have undergone eutrophic inward remodeling may appear as vessels exhibiting a high level of constriction. However, the structural changes are uncovered by demonstrating the inability of the vessel to dilate if the vessel is pressurized and subjected to full relaxation, for example, by exposing it to calcium-free conditions [67]. With respect to circumferential wall stress, this chronic structural adaptation represents an effective means to reduce circumferential wall stress in the face of high transmural pressure. Both adaptive changes, diameter reduction as well as wall thickness increase, contribute to reduce circumferential wall stress. In this regard, they may be classified as protective adaptations. However, the decrease in luminal diameter enhances wall shear stress at constant volumetric blood flow as outlined above, highlighting once more the inability of the endothelium to oppose these (possibly maladaptive) responses.

In case remodeling is associated with an increased total wall mass area (hypertrophic inward remodeling) the cells in the vascular wall have undergone a growth process either by enlarged volume of the smooth muscle cells without cell number increase (hypertrophy) or by an increase in cell number (hyperplasia). This is often observed in secondary hypertension such as renovascular hypertension [68]. It is interesting to speculate that, in addition to enhanced vascular tone resulting from enhanced pressure, growth stimuli (such as angiotensin II) exert an additional effect.

Upon activation of the angiotensin type 1 receptor, angiotensin II activates receptor and non-receptor tyrosine kinases as well as the NAD(P)H oxidase and increases vascular smooth muscle growth, contractility, and collagen deposition [64, 69, 70]. Specifically, hypertrophic remodeling is associated with enhanced deposition of extracellular matrix proteins (mainly collagen) which is related to a synthetic phenotype of smooth muscle cells. The volume density of collagen is increased in hypertensive rats as well as in subcutaneous small arteries obtained from patients with essential hypertension [71, 72]. Herein, stimulating factors such as angiotensin II may be involved [73]. Adhesion molecules, specifically integrins, build the junctions between the extracellular matrix proteins and the cytoskeletal components. During these processes, integrins that act as cellular anchorage sites are also rearranged and may in fact trigger the restructuring of smooth muscle cells [73].

Remodeling was elicited in vitro by subjecting isolated small arteries for a prolonged period (days) to vasoconstrictory protocols using different vasoconstrictors such as endothelin-1 [67, 74]. These observations argue against a major role for increased circumferential wall stress as the initiating stimulus. Rather a prolonged vasoconstriction or enhanced vascular tone itself seems to induce eutrophic inward remodeling (see also Chap. 6) [75, 76]. In line with this hypothesis are studies in hypertensive patients that compare the effect of different therapeutic regimes on lumen-to-media ratio [61, 77]. It seems that drugs targeting vascular tone directly, such as angiotensin-converting enzyme (ACE) inhibitors, angiotensin receptor blockers, and calcium-channel blockers are more effective to reduce this ratio than diuretics. Likewise, calcium-channel blockers and angiotensin receptor blockade were effective in preventing inward remodeling in an ex vivo culture system or in vivo [71, 78, 79]. At this point, it needs to be stressed once more that at these conditions (more dilated vessels) circumferential wall stress increases which strongly argues against the fact that this stress is the crucial stimulus inducing remodeling. Further details on vascular remodeling are available in Chap. 6 and is reviewed also elsewhere [67, 80–83].

10.4 Structural and Mechanical Alterations in Large Arteries in Hypertension

Proximal large elastic arteries such as the aortic root, the common carotid artery, or the femoral artery are enlarged in hypertensive patients at advanced age [84–88]. This is commonly attributed to the mechanical destruction of elastin fibers that are bearing a large part of the additional mechanical load due to the enhanced transmural pressure. Physiologically, elastin fibers allow vascular elasticity and recoil [6, 89]. It is of specific interest that the local pulse pressure (estimated with applanation tonometry) was a strong independent determinant of the enlargement of the lumen of the common carotid artery (an elastic artery) whereas mean blood pressure was not [90]. The same held true for the enlargement of the aortic root (commonly known as aortic root dilatation) as the augmentation index was the only predictor of aortic root dilatation [88]. In contrast, the internal diameter of the radial

artery was only correlated with age but not with radial pulse pressure which suggests that the cyclic stretching due to local pulsatile pressure increases contribute to the structural changes in elastic arteries, but not in muscular arteries.

Structural damage of the elastic laminae is well-known for their thinning, splitting, and fraying and occurs with advanced age suggesting that the exposure to cyclic stress over decades induces their damage in line with a process called "stress fatigue" [91]. It is interesting to note that elastin exhibits a very slow turnover. The expected longevity is in rodents even many times longer than the lifespan of the host organism and also in humans its longevity is considered to be as long as lifespan [92]. This longevity may prevent the replacement of elastin molecules that are mechanically damaged by the fatiguing effect of enhanced pulsatile stress load or by degradation through proteases [89] similar to lung tissue in which the synthesis of elastin was shown to be essentially non-existent during adulthood [93].

In both vessel types, intima-media thickness was also increased indicating remodeling. This increase correlated in elastic arteries with local pulse pressure whereas it correlated in muscular arteries with mean blood pressure suggesting that also these changes are elicited in elastic arteries by the rhythmic loading of the vessels but in muscular arteries by the steady elevation of circumferential wall stress [90].

Interestingly, rhythmic stretching exhibits a stronger effect than the static load on the phenotype of smooth muscle cells, their growth including DNA synthesis and the production of matrix components [94, 95]. The focal adhesion protein zyxin is a key regulator of stretch-induced gene expression in smooth muscle cells [96] and its loss promotes a synthetic phenotype of smooth muscle cells [97]. Additionally, cyclic stretch was demonstrated to negate the activity of myocardin which is a critical transcriptional determinant of the contractile phenotype of smooth muscle cells as its knockdown is sufficient to induce smooth muscle cell proliferation. Elimination of the regulatory effects of myocardin by exaggerated cyclic stretch as observed in vitro may thus also promote the aforementioned shift in smooth muscle function toward a more synthetic phenotype in hypertension [98].

With respect to circumferential wall stress the observed changes elicit opposite effects: luminal diameter widening increases wall stress whereas enhanced wall thickness decreases it (formula 2, above). In distal muscular arteries, such as the radial artery, the diameter was found unchanged in hypertension [86] and thus the increase in wall thickness compensates for the increased transmural pressure. In contrast, in large elastic arteries, the increase in wall thickness was insufficient to compensate for the pressure enhancement, and the concomitant lumen widening [99]. This indicates that in elastic vessels the circumferential wall stress is not a (strictly) regulated parameter. It also seems to be at odds with the function of such vessels which is to store part of the stroke volume momentarily during systole and provide this volume during diastole thereby dampening pulsatility of flow and pressure. During systole part of the energy is stored in the wall of elastic arteries and this energy recoils the vessel releasing the stored volume ensuring more continuous flow. The term elastic describes a mechanical behavior that does not

dissipate energy. The material (vessel) rather returns to its original geometry upon unloading thereby releasing the stored energy. This feature is called windkessel function and reduces total cardiac energy expenditure. A requirement for this function is a compliant vessel (in conjunction with recoil) that allows to store a certain amount of volume (ΔV) at a given pressure change (ΔP), i.e., compliance ($\Delta V / \Delta P$). Assuming a reduced volume distensibility [$\Delta V / (\Delta P \times V)$] of vessels in hypertensive individuals, an enlargement of the vessel with increased volume may compensate for any reduction in volume distensibility and maintain compliance (which is the product of volume and volume distensibility). However, a mechanical sensor and feedback system to control this parameter (compliance) and adjust vessel volume accordingly is hard to imagine.

10.5 Large Artery Stiffness and Pulse Wave Velocity

Stiffness is a measure of the resistance offered by an elastic body to deformation induced by stress. The deformation of the body is measured as a strain that is normalized to an initial condition and is therefore dimensionless or given as a percent change. Stress-strain relationships characterize the stiffness of a material. In a physiologic sense, stiffness is the inverse of compliance and increased vascular stiffness therefore relates to changes in vessel wall properties characterized by reduced compliance. The measurement of the pulse wave velocity is generally accepted as the most simple, noninvasive, robust, and reproducible method to determine arterial stiffness in different regions (i.e., between different points of measurement). In contrast, ultrasound devices allow the determination of arterial stiffness in a locally confined area by echo tracking, for example in the carotid artery or the radial artery [100].

Specifically, carotid-femoral pulse wave velocity has emerged as the gold standard for assessing aortic stiffness [101]. Age and blood pressure are the main determinants for pulse wave velocity which increases to the second power with age and linearly with arterial pressure (in the relevant pressure range). The increase with age was more pronounced in individuals of the higher pressure category demonstrating that higher pulse wave velocities at advanced age are not simply attributable to higher arterial pressure [84, 102]. Although changes in pulse wave velocity are generally directly attributed to arterial stiffness, pulse wave velocity is in fact determined by the elastic modulus and fluid density: $c_o = \sqrt{(\kappa / \rho)}$, where c_o is the pulse wave velocity, ρ the density of the fluid, and κ the elastic modulus. The latter is given by [$V \times \Delta P / \Delta V$] which is the inverse of volume distensibility (see above). Therefore, pulse wave velocity increases if vessel compliance [$\Delta V / \Delta P$] decreases but also if the vessel volume [V] increases. As outlined above, large elastic arteries are characteristically enlarged during hypertension, which consequently contributes in part to the higher pulse wave velocity found in hypertension.

Another feature that has to be considered in this context is the shape of the compliance curve of large elastic arteries. This curve was assessed in human thoracic aortas ex vivo in which diameter changes were measured in response to stepwise

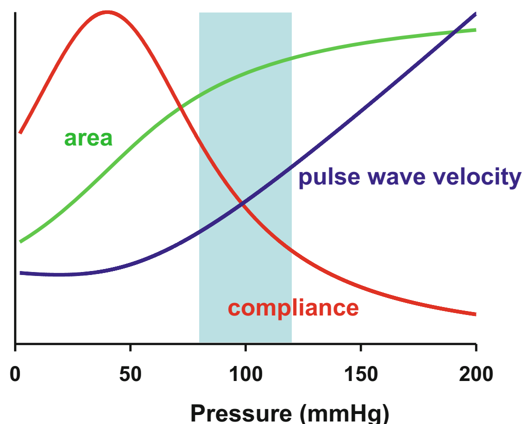

Fig. 10.2 Aortic area, compliance, and pulse wave velocity are changing with pressure. Aortic area (depicted in green) is calculated using the formula published by Langewouters, Wesseling, and Goedhard [103] that was derived from experimental data obtained in human aorta studied ex vivo. Assuming a certain length aortic volume can be derived from cross-sectional area. Accordingly, compliance (depicted in red) was calculated as the first derivative of the pressure–area function because compliance is the slope of the pressure–volume curve (ΔV / ΔP). The pressure–area function was also used to extrapolate the distensibility coefficient ((ΔA / A) / ΔP) in order to allow calculation of the pulse wave velocity (depicted in blue) by means of the Bramwell–Hill formula [104]. Physiologic pressure is highlighted in bright blue. Pressure increases expand the aorta in a characteristic S-shaped function and, therefore, decreases compliance and increases pulse wave velocity dramatically

pressure increases [103]. The cross-sectional area was obtained from diameter changes and compliance computed. Assuming a certain length, cross-sectional area relates to volume, and pressure–volume curves can be derived. This function (volume/area over pressure) is smooth, monotonic, and exhibits a typical curvilinear, sigmoidal shape for large elastic arteries. During pressure rise, the volume increase is initially at very low pressures (below the physiologic range) small. Thereafter, volume increase reaches a maximal value (infliction point) that already occurs below the physiologic pressure range. At higher pressures including the physiologic pressure range, the volume increase declines again reaching very low values at the end (Fig. 10.2). The slope of this curve represents the vessel's compliance (ΔV / ΔP). Consequently, vessel compliance is initially low, reaches a maximum at pressure levels below the physiologic range, and decreases more and more with rising pressure before finally reaching zero at very high (supraphysiologic) pressures [91, 103, 105, 106].

This behavior is due to the progressive loading and uncurling of elastic fibers. Eventually (with increasing volume), stiff collagen fibers are recruited that minimize or prevent further volume increases. Hence, two structural phases determine the nonlinear behavior of arterial wall stiffness [91]. Taking this behavior into consideration it follows that pulse wave velocity increases during hypertension solely due to the fact that vessel compliance is decreased at higher pressures even though the

compliance (or stiffness) function of the vessel in itself has not changed at all. These considerations make it very clear that despite an elevation of the pulse wave velocity observed during hypertension the compliance or stiffness of the vasculature may be, in fact, unaltered.

This raises the question if large arteries indeed exhibit an altered compliance or stiffness for example due to structural changes in hypertension as is often assumed by the observation of enhanced pulse wave velocity. Local echo tracking of arteries during the cardiac cycle provides insight into the distensibility of the vessel by measuring internal diameter, its change during the cardiac cycle as well as the intima-media thickness while additionally monitoring local pulse pressure. From such measurements, local compliance or distensibility can be determined for different pressure values prevailing during the cardiac cycle. As outlined above compliance changes with pressure and accordingly the distensibility–pressure relationship is likewise a curvilinear function but in this case with a negative slope, i.e., arteries exhibit a high distensibility at low pressures that declines progressively (not linearly) with increasing pressure. Such distensibility–pressure curves measured in the common carotid artery are not altered in hypertensive as compared to normotensive subjects [64, 87]: The curve obtained in hypertensives was shifted to a higher pressure range (because higher pressure ranges are found in hypertensives) but still overlapped with the curve obtained in normotensives for a wide range. In fact, both curves lay on top of each other for this overlapping part with equal pressures indicating a similar distensibility of this artery in these two cohorts (Fig. 10.3). Similarly, distensibility was not reduced in spontaneously hypertensive rats compared to normotensive controls, in fact, it was slighter larger in hypertensive rats [106, 107].

This same procedure can be applied in a more distal artery (radial artery) which in itself is less distensible than the proximal elastic arteries. Again the curve obtained in hypertensives was found to be shifted rightwards (to higher pressures, as expected). Surprisingly, the arterial distensibility curve obtained in patients with essential hypertension was shifted upwards (toward larger values, Fig. 10.3) in the overlapping pressure range [86] or was mostly unaltered [106]. This demonstrates a higher distensibility of this distal artery in hypertensives, which is in contrast to the common assumption that vessels are stiffer in hypertensives. Thus, local echo tracking in elastic and muscular arteries did not demonstrate an impaired distensibility (or enhanced stiffness) of vessels in hypertensive subjects. Taken together, this suggests that the commonly known property of vessels (compliance decrease with higher pressures as outlined in the previous paragraph, Fig. 10.2) is the largest contributor for the observed "enhanced stiffness" of vessels in hypertension (as measured by enhanced pulse wave velocity) rather than a structural alteration of the vessel itself (due to hypertension). In line with this conclusion is the demonstration that aortic impedance changes seen in hypertensive subjects revert with a reduction in arterial pressure and can, conversely, be induced in normotensive subjects by pressure increase [108].

Another measure reflecting the mechanical properties of a material is the Young's elastic modulus. In contrast to the aforementioned distensibility (or compliance)

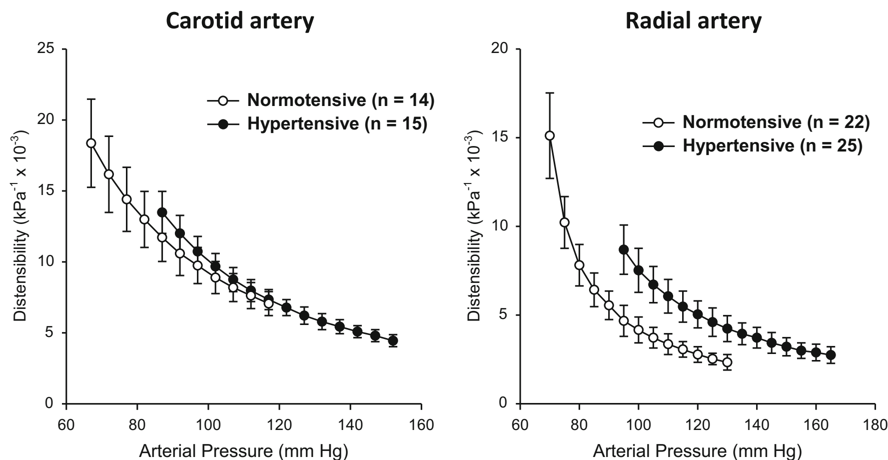

Fig. 10.3 Distensibility of arteries in normo- and hypertensive subjects. Distensibility was assessed in normo- and hypertensive individuals (age- and sex-matched) using echo tracking and simultaneous pressure recordings during the cardiac cycle. An elastic artery (carotid) is shown on the left (adapted with permission from Laurent et al. [87]) and a muscular artery (radial) on the right (adapted with permission from Laurent et al. [86]). The curves were not different between the two groups in the carotid artery. The curve obtained in the radial artery in hypertensive individuals was shifted upwards ($p < 0.05$) as compared to normotensive subjects toward higher distensibility. Values are mean ± SEM

which takes also into consideration the hollow structure of the vessel wall (structural stiffness), Young's elastic modulus only reflects the properties of the material of the vessel wall itself (material stiffness). It provides information about the properties of the material independent of the way in which the material is arranged (i.e., its geometry) and, thus, specifies the stiffness of the material itself, i.e., its deformation (strain) upon pressure (stress). This parameter was analyzed in follow-up studies in large elastic arteries [64, 99]. In normotensives, the elastic modulus increased with circumferential wall stress (depending on pressure and diameter) in a curvilinear fashion. However, the function is nearly linear, i.e., its slope rises only moderately with increasing wall stress (Fig. 10.4). With advanced age, the elastic modulus increased and also the rise in the elastic modulus with wall stress was steeper indicating stiffer material in the vessel wall at an advanced age. In a group of age-matched hypertensives that were never treated for their hypertension, these curves were shifted rightward to higher wall stress values (due to higher pressure values), but they were not steeper and also not shifted upwards toward higher elastic modulus values in old and middle-aged hypertensives (Fig. 10.4). Only in young hypertensive, an upward shift and a steeper rise of the elastic modulus with increasing wall stress was observed (Fig. 10.4). The curve obtained in these individuals lay on top of that obtained in middle-aged subjects [99]. This demonstrates that in hypertension the wall material is only stiffer in young subjects (<45 years) but not in middle-aged or old individuals indicating that the disadvantageous effects of

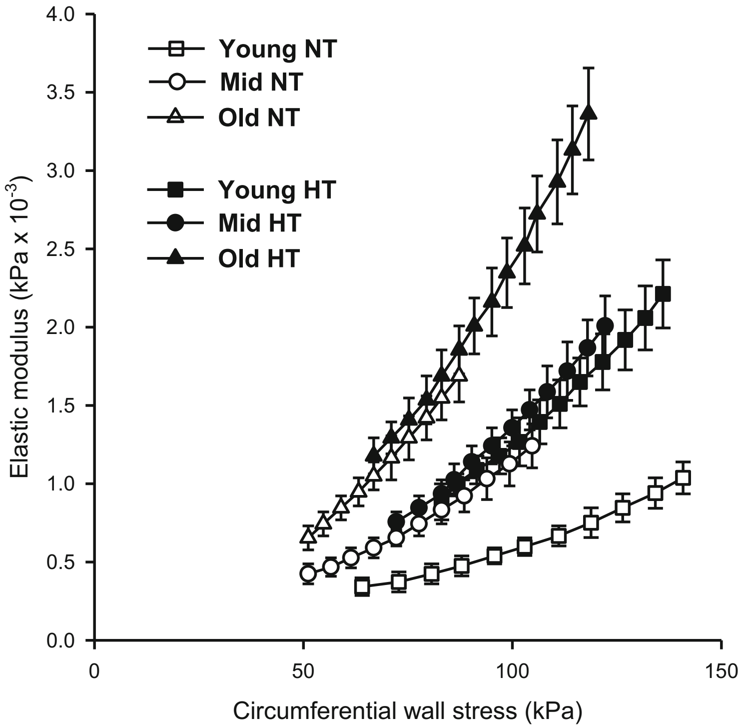

Fig. 10.4 Elastic modulus of the carotid artery in normo- and hypertensive subjects at different ages. Young's incremental elastic modulus was assessed in the carotid artery in normotensive (NT) and hypertensive (HT) subjects and is depicted as a function of circumferential wall stress (adapted with permission from Bussy et al. [99]). Individuals were grouped according to age tertiles into young (36 ± 7 years), mid-age (age 46 ± 7 years), and old subjects (age 62 ± 7 years). Values are mean ± SEM. The modulus–stress curves are shifted upwards and became steeper with aging with overlaps of normo- and hypertension in the mid- and old-age groups. However, the curve in the young age group was shifted upwards in hypertensive individuals compared to normotension ($p < 0.01$) indicating increased stiffness of the wall material in hypertension only in the young age group

aging are not further upscaled by hypertension [64, 99]. The enhanced stiffness observed in younger individuals may relate to spatial reorganization and distribution of extracellular matrix and vascular smooth muscle cells [15].

In summary, the data do not indicate that hypertension exerts a major effect on the mechanical properties of the vascular wall in humans. Therefore, the well-known higher pulse wave velocity is largely due to the prevailing higher pressures and the related shift along the volume–pressure curve that results in an actual alteration in effective compliance and effective distensibility. High pressure subjects elastic large vessels to larger stress which enhances the volume load and recruits stiffer wall material (e.g., collagen) to bear this stress. There is hardly any evidence that the vessels themselves are stiffer due to structural changes in the vessel wall that

modulate the mechanical behavior of the vessel. Only recently, evidence indicates that smooth muscle cells from hypertensive rats are stiffer than those from normotensive controls which may also enhance aortic stiffness in artificially reconstituted aortas using such cells [109]. Thus, the role of vascular smooth muscle cells in arterial stiffness is being uncovered and emerging [15].

10.6 Functional Consequences of the Change in Effective Distensibility

The pressure at which the large arteries operate is higher in hypertension and at such higher levels vessel distensibility is decreased which is specifically the case for elastic arteries. Consequently, the distensibility decrease that usually prevails along the vascular tree (from elastic toward muscular arteries) is reduced or lacking in hypertension. At locations with changes in distensibility, the pulse wave is partially reflected because of according changes in impedance [110]. It follows that pulse wave reflections along the large arteries are reduced in hypertension and consequently pulsatile energy is transmitted more efficiently (without reflections) into downstream arteries and possibly even down to resistance vessels. At this site, the higher energy input may induce damage in the microcirculation. The enhanced energy transmission into small arteries due to the mechanisms discussed above is suggested to be a major factor in the organ target damage that is observed as a consequence of hypertension [64, 111]. Therefore, it is not surprising that prevailing, effective arterial stiffness and central pressures (systolic pressure and pulse pressure) have a predictive value for cardiovascular events and renal complications in hypertensive individuals [112–114].

A further, well-known consequence of the effective change in distensibility and the resulting rise in pulse wave velocity is that pressure wave augmentation due to wave reflection from the periphery is not only seen in more peripheral (e.g., brachial artery) but also in central arteries. Consequently, the amplification of the pulse wave and the increase in systolic pressure from central to peripheral arteries is reduced [91]. More importantly, the reflected waves return to the aortic root during late systole when the heart is still ejecting blood and not in early diastole as observed at lower pulse wave velocities. The premature arrival of reflected waves occurs specifically at low heart rates with a prolonged systole and results in the aforementioned change in the contour of the pressure wave in the aorta. The supplementary increase in systolic pressure is called augmentation and the augmentation index that is calculated for its quantification is an independent predictor of cardiovascular risk [114–116]. The term amplification is actually misleading as energy is not generated in the aorta as implied by the term “amplification.” Boosting aortic pressure in systole enhances myocardial workload as well as oxygen demand and hinders ventricular ejection. Moreover, the detrimental effects of cyclic stress on the wall structure of elastic arteries are inextricably upscaled accelerating fatigue as outlined above [91]. At the same time pressure during diastole is reduced which compromises coronary perfusion. Hence, arterial hypertension is not merely a burden to the heart

due to enhanced systemic vascular resistance but it dramatically changes hemodynamics in large arteries which further impacts cardiac function. So, it comes with only little surprise that arterial hypertension is a major factor in progressive myocardial hypertrophy and cardiac failure.

In conclusion, arterial hypertension inevitably changes “effective arterial stiffness” (or distensibility) in elastic arteries in a passive manner through a shift along the pressure–volume curve and, hence, enhanced stiffness is a direct consequence of hypertension. The increase in stiffness in itself further boosts central systolic pressure and, thus, stiffness enhancement is also a cause for (central) hypertension [114]. This occurs in two ways. Firstly, the amplitude of the initial pressure wave generated by ventricular ejection is enhanced (due to the reduced distensibility). Secondly, the concomitant increase in pulse wave velocity amplifies systolic pressure further because the reflected wave arrives centrally at an earlier time point. On the other hand, wave reflections during the travel of the pressure wave from elastic into muscular arteries are reduced because the usual characteristic impedance (distensibility) changes are offset. This increases energy transmission into the microcirculation which may impose functional damage at this site.

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Mechanosensing and Mechanotransduction in Pulmonary Hypertension

11

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Abstract

Pulmonary hypertension (PH) is a devastating disease with a poor outcome. Progressive remodeling of the pulmonary microvasculature leads to an increase in pulmonary vascular stiffness as well as to an increase in pulmonary vascular resistance. These alterations in mechanical characteristics of the pulmonary vasculature further contribute to the progression of pulmonary vascular disease. This chapter will focus on how changes in vascular stiffness affect mechanical forces acting on the endothelium and smooth muscle cells and how these changes in mechanical forces in turn contribute to the development and progression of pulmonary vascular disease with a focus on the role of mechanosensing and mechanotransduction in these processes.

11.1 Introduction

Pulmonary hypertension (PH), defined as a pulmonary artery pressure above 20 mmHg, is a devastating disease, which—when left untreated—leads to right heart failure and death. PH is divided into five categories by the World Health Organization, based on the etiology of the disease [1]. The most severe form of PH is pulmonary arterial hypertension (PAH), which originates in the distal pulmonary arterial microvasculature. In other forms of PH, pulmonary arterial pressure is increased secondary to other diseases, such as left heart disease or congenital heart disease, chronic hypoxia, thrombi in the pulmonary vasculature, or inflammatory (lung) diseases. In all forms of PH, the progression of pulmonary vascular disease (PVD) involves endothelial dysfunction, vasoconstriction, and progressive remodeling of the pulmonary vasculature, which is characterized by increased muscularization of the distal pulmonary arterioles and perivascular fibrosis. Altogether, PVD results in an increased pulmonary vascular resistance (PVR) and increased pulmonary vascular stiffness (decreased pulmonary vascular compliance (PVC)). For all precapillary forms of PH (i.e., PH that is not solely due to changes in pulmonary venous pressure), PVR $\geq$ 3 Woods Units has recently been added to the definition of PH [1].

Contrary to the systemic vasculature, in which vascular resistance is the main determinant of the afterload of the left ventricle, both the mean pulmonary artery pressure and its pulsatility, and hence both pulmonary vascular resistance and stiffness, contribute to workload of the right ventricle (RV) [2]. In the initial phase of the disease, the RV is capable of coping with the increased afterload, initially by increasing its contractility, and in the long term by RV hypertrophy. However, with the progression of PVD, the RV can no longer cope with the increased afterload and starts to fail [3].

Although it is well-known that mechanical forces such as shear stress and wall stress have a direct impact on the endothelium and vascular smooth muscle cells,

respectively, and could thereby affect vascular remodeling, these mechanical forces acting on the distal pulmonary vasculature of healthy subjects and patients with pulmonary hypertension have only recently been actually measured and modeled [4]. Furthermore. it has only recently been recognized that small changes in vascular stiffness impact the pressure and flow characteristics throughout the entire pulmonary vasculature and thereby alter the mechanical forces acting on both the proximal and distal pulmonary vasculature [5, 6]. It has now become evident that increased pulmonary stiffness not only presents an early disease marker in PH but also contributes to the progression of PVD [5, 6].

This chapter will describe (*i*) the alterations in mechanical characteristics of the pulmonary vasculature during the development and progression of PVD, (*ii*) how these alterations affect mechanical forces acting on the endothelium and smooth muscle cells, and (*iii*) how these changes in mechanical forces in turn contribute to the development and progression of PVD, with a focus on the role of mechanosensing and mechanotransduction in these processes.

11.2 Changes in Mechanical Characteristics of the Pulmonary Vasculature in PH

The healthy pulmonary vasculature comprises a system with a low resistance and a high compliance to allow the cardiac output to be pumped through the pulmonary vasculature at low pressure. PVR and PVC are inversely related and are evenly distributed across the vasculature [7]. PVR is calculated as (pulmonary artery pressure − pulmonary capillary wedge pressure)/cardiac output. PVC can be estimated as stroke volume/(systolic pulmonary artery pressure − diastolic pulmonary artery pressure). This results in a slight overestimation of PVC, as it does not take flow into the distal pulmonary vasculature into account [8]. Contrary to the systemic vasculature, in which the aorta determines the largest part of the total compliance, in the healthy pulmonary vasculature, only 15–20% of PVC resides in the proximal pulmonary vasculature, due to the large number of branching vessels [8, 9].

Pulmonary vascular compliance is important because a considerable part of the energy delivered by the RV is turned into pulsatile power. Thus, the energy required to be delivered by the RV to pump the cardiac output through the pulmonary vasculature, i.e., the hydraulic power, consists of the sum of the mean hydraulic power and the pulsatile (or oscillatory) hydraulic power. Mean hydraulic power is used to propel blood through the pulmonary vasculature and equals the product of mean pulmonary artery pressure and cardiac output. Conversely, pulsatile power is related to pulsatile pressure and is not used for forward movement of flow. In the pulmonary vasculature, pulsatile power is estimated to be 23–33% of total power generated by the RV [5, 7], and hence contributes significantly to the workload of the RV.

PVD results in narrowing and stiffening of the pulmonary vasculature, thereby increasing PVR and reducing PVC, through increased muscularization and perivascular fibrosis. In early PVD, vascular remodeling mostly impacts PVC,

with relatively small changes in PVR. Changes in PVC are even already observed prior to the development of overt PH [10]. Furthermore, stiffening has been shown to start in the distal pulmonary vasculature, whereas stiffening of more proximal vessels evolves later in disease development [11]. With more severe PH, PVC is low and PVR is high and disease progression only results in minor additional decreases in PVC, which are accompanied by large changes in PVR. Interestingly, it has recently been suggested that not only vascular remodeling, but also rarefaction contributes significantly to the increase in PVR [12] and, given the contribution of the distal pulmonary vasculature, likely also to the decrease in PVC. It has been estimated that in patients with advanced PH, PVR increases 18-fold, and PVC decreases 20-fold [7].

Because PVR and PVC are inversely related in health and disease, the RC-time of the pulmonary vasculature is constant and the pulmonary pulse pressure is linearly related to mean pulmonary artery pressure. This means that, with increasing disease severity, mean and pulsatile power increase to the same extent, both contributing equally to the increased afterload of the RV [9]. The importance of changes in PVC for the progression of PVD is further underscored by several studies showing that PVC is a better predictor of mortality than PVR, not only in PAH, but also in PH associated with congenital heart disease and heart failure [8]. The importance of the pulsatile component of RV afterload is further underscored by the observation that pulmonary arterial impedance, a measure of opposition to pulsatile flow, correlated better with prognosis than PVR in patients with PH [13, 14].

11.3 Changes in Mechanical Forces Acting on the Pulmonary Vasculature in PH

Decreased PVC results in increased pulse wave velocity, and hence pressure wave reflections from the distal pulmonary vasculature return faster to the heart. Thus, in the healthy pulmonary vasculature, reflected pressure waves reach the proximal pulmonary vasculature in diastole, whereas in the diseased pulmonary vasculature, pressure waves appear during mid- or late systole, thereby further augmenting the pulse pressure in the proximal pulmonary artery and contributing to the increased RV afterload [15] (Fig. 11.1). Furthermore, these returning pressure waves also impact the flow-profile resulting in a shortened time to peak and an enhanced mid and late systolic deceleration [10]. However, as the large PVC also acts to dampen the pressure and flow pulsations that enter the pulmonary microvasculature (i.e., vessels smaller than 100 μm in diameter), the decreased PVC not only impacts the RV but also results in increased pressure and flow pulsations into and throughout the distal pulmonary vasculature [5, 6, 10]. Computational modeling reveals that time-averaged wall shear stress is lower in the large pulmonary arteries of PAH patients [16–18]. Similarly, time-averaged wall shear stress in the proximal pulmonary vasculature (diameter above 500 μm) decreased with increasing disease severity in pediatric PH patients (age 4–17 years) (from 20 to 6 dynes/cm^2), whereas shear stress increases in the distal small arteries (with a diameter between 100 and 500 μm)

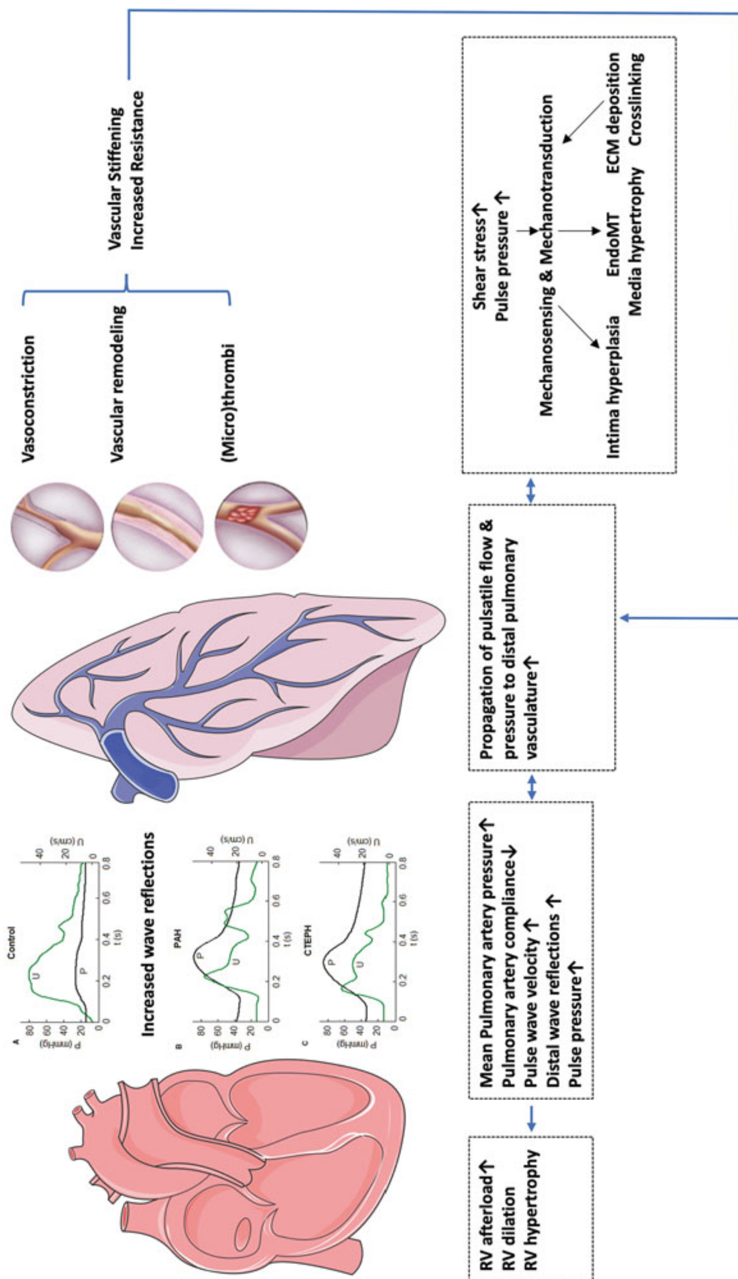

Fig. 11.1 Changes in vascular stiffness initiate alterations in pressure (P) and flow velocity (U) profiles within the pulmonary vasculature that promote pulmonary vascular remodeling, thereby increasing pulmonary vascular resistance and further augmenting right ventricular afterload. Waveforms are from [15]

(from 20 to 116 dyn/cm^2) and the microvasculature (from ~50 to ~300 dyn/cm^2). Furthermore, the oscillatory shear index in the main pulmonary artery increased from 0.13 to 0.20 [4]. Conversely, despite the increase in pulmonary artery pressure, wall strain of the main pulmonary artery tended to decrease (from 0.16 to 0.11) but was not different in the left and right pulmonary artery and their branches. Vessel stiffness, as indicated by Young's modulus increased (from 1.26×10^6 to 3.0×10^6 dyn/cm^2).

11.4 Impact of Altered Mechanical Forces on Pulmonary Vasculature

Altered hemodynamics were already proposed to play an important role in the development of pulmonary microvascular lesions in patients with congenital heart disease in 1957 [19]. The response of cells within the pulmonary microvasculature to these altered hemodynamics plays a key role in the (mal)adaptive response of vascular remodeling and lesion formation. Both changes in mechanical forces and the capability to respond to these mechanical forces through mechanosensing and mechanotransduction are altered in PH [6, 10, 20, 21].

Pulmonary microvascular remodeling in PH encompasses all layers of the vasculature. Thus, endothelial plexiform lesions, (in situ) thrombotic lesions, medial hypertrophy/hyperplasia, muscularization of the distal pulmonary arterioles as well as intimal and adventitial fibrosis contribute to PVD [22].

11.4.1 Altered Mechanotransduction in Pulmonary Endothelial Cells

Particularly PAH is characterized by intimal hyperplasia in the so-called plexiform lesions. Within these lesions, endothelial cells lining the vasculature appear quiescent, whereas the network of channels in the center of the lesions consists of hyperproliferating endothelial cells [23, 24]. These hallmark lesions of PAH are found at the branching point of the so-called supernumerary arteries [23, 24], and have recently been found to be associated with bronchopulmonary anastomoses [25]. The location of these lesions suggests that the altered, hyperproliferative, endothelial phenotype is, at least in part, influenced by mechanical stimuli.

The main mechanical stimulus for endothelial cells, that are directly in contact with the blood flowing through the vasculature is shear stress. Endothelial cells respond to changes in shear stress with changes in secretion of the vasoactive mediators nitric oxide (NO), prostacyclin (PGI2), thromboxane, and endothelin (ET-1) [26]. Furthermore, endothelial cells exposed to unidirectional shear stress possess the capability of aligning with the flow direction. Kruppel-like factor 2 and 4 (KLF2 and 4) are considered the master regulators of the anti-inflammatory, anti-proliferatory, and antithrombotic signaling pathways characteristic of healthy endothelial cells under unidirectional shear stress [27] (Fig. 11.2, see also below).

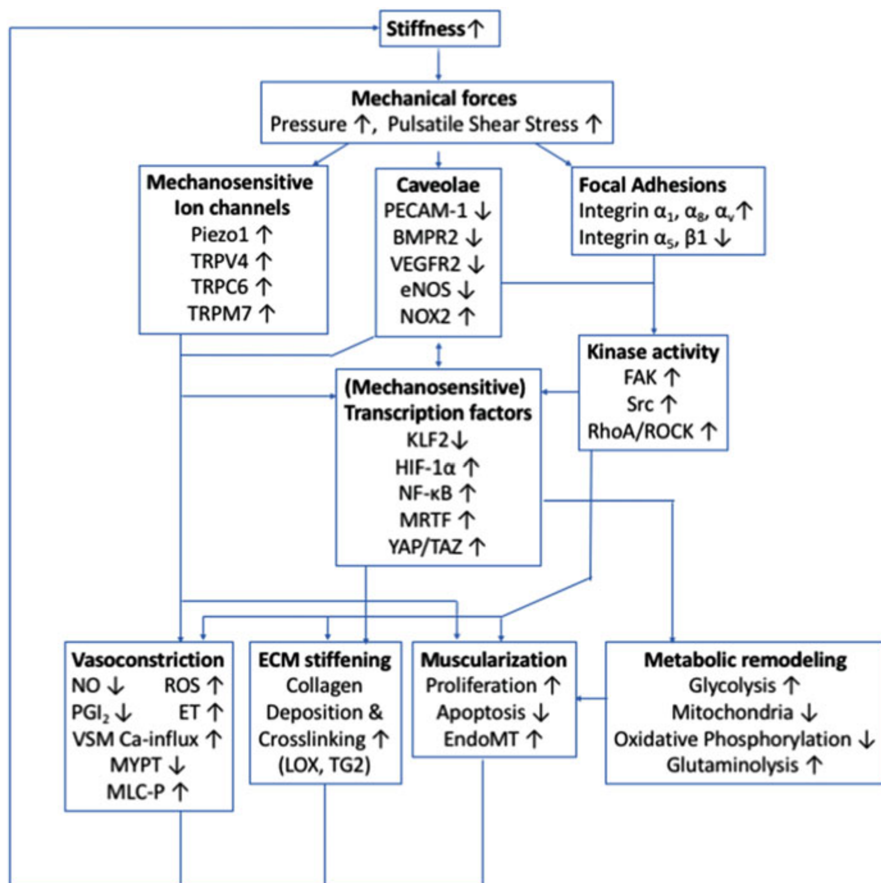

Fig. 11.2 Mechanosensing, involving mechanosensitive ion channels, caveolae and focal adhesions, and mechanotransduction, involving both changes in kinase activity as well as transcriptional regulation are altered in pulmonary hypertension, leading to metabolic remodeling vasoconstriction, increased muscularization, and ECM stiffening. These processes constitute a positive feedback loop, culminating in worsening of vascular remodeling and aggravation of pulmonary hypertension

As outlined above, both average shear stress and the oscillatory shear index are increased in the pulmonary microvasculature in PH [4], due to changes in stiffness of both the proximal and distal pulmonary vasculature. These chronic changes in shear stress are accompanied by endothelial dysfunction in PAH patients [22]. Studies in isolated pulmonary microvascular endothelial cells, exposed to different flow profiles show that pathological high and pathological low flow induce changes in vasoactive pathways (impaired NO-production, enhanced ET-1 production) favoring vasoconstriction, with increased production of reactive oxygen species (ROS) [26], whereas increased pulsatility induces increased mRNA expression of adhesion molecules [E-selectin, MCP-1, intercellular adhesion molecule-1 (ICAM1), vascular

cell adhesion molecule 1 (VCAM1)] and release of pro-inflammatory cytokines and chemokines [28]. The resulting vasoconstriction, impaired angiogenesis, and defective repair mechanisms contribute to the development and progression of PH. To investigate the role of defective endothelial mechanotransduction in response to shear stress in the development of PH, pulmonary arterial endothelial cells (pAECs), and pulmonary microvascular endothelial cells (pMVECs) from healthy control subjects and patients with PAH were exposed to shear stress. Both pAECs and pMVECs align to shear stress. However, when comparing pAECs and pMVECs obtained from healthy lungs with those from patients with PAH, the response of pAECs was similar but the alignment of pMVECs obtained from patients with PAH was delayed from 72 to 120 h after shear onset, and some cells detached and were washed away, suggesting that the strength of adherence to the fibronectin-coated surface was reduced [29].

11.4.2 Altered Mechanotransduction in Pulmonary Vascular Smooth Muscle Cells

In the normal pulmonary circulation, the phenotype of pVSMCs varies throughout the vasculature, being different in the proximal and distal vasculature [30]. Furthermore, the population of pVSMCs is more homogeneous in the distal as compared to the proximal pulmonary arteries [30]. Within the proximal pulmonary arteries, both fully differentiated pVSMCs as well as less differentiated smooth muscle-like cells have been identified, characterized by α-smooth muscle actin expression. In the distal pulmonary vasculature, pVSMCs exhibit a more uniform, differentiated, phenotype, characterized by expression of smooth muscle myosin heavy chain, h-caldesmon, and metavinculin [30]. In the proximal pulmonary vasculature, the main function of the pVSMCs is to provide strength to the vessels to withstand pressure, while maintaining distensibility to accommodate stroke volume with minimal increases in pulse pressure. In the distal pulmonary vasculature, the main function of pVSMCs is to regulate pulmonary vascular tone and resistance [30]. The main mechanical stimulus for pVSMC is stretch, to which they respond with contraction (short-term) as well as proliferation (long-term). Different subtypes of pVSMCs exhibit site-specific and unique responses to pathologic hypertensive stimuli.

In PH, wall thickness of the proximal pulmonary vasculature increases commensurate with the increase in pressure, whereas the increase in media-thickness of the distal pulmonary vasculature precedes (intra-acinar vessels) or follows (hilar arteries) the increase in pressure. Furthermore, muscularization of previously non- or partially muscularized distal pulmonary arterioles occurs in PH [30]. The increased muscularization is only partially derived from the proliferation of preexisting pVSMC. In addition, at the border of muscularized and non-muscularized arterioles, a population of progenitor cells arises which is derived from resident cells, recruited from circulating progenitor cells, or derived from perivascular inflammatory cells, macrophages, and/or endothelial cells. These cells

migrate distally and are clonally expanded and differentiated to contribute to muscularization. These processes are regulated by HIF-1α, KLF-4 and PDGF-B [31, 32].

In addition to medial thickening, α-SMA positive mesenchymal-like cells are increased in obstructive pulmonary intimal lesions. These VSM-like cells are thought to be derived from endothelial cells, in a process called endothelial to mesenchymal transition (EndoMT) [33, 34]. This process is orchestrated by the transcription factors, Snail, Twist1 and Slug, which are activated in response to low shear stress [30, 35, 36].

The cross-talk between the different cell types in the vascular wall influences the pVSMC phenotype. In a co-culture with pulmonary endothelial cells and smooth muscle cells, high shear stress on the endothelium increases smooth muscle actin as well as smooth muscle myosin heavy chain expression in the vascular smooth muscle cells. In the absence of endothelial cells, however, high shear stress on the pVSMCs induced a decrease in smooth muscle actin as well as smooth muscle myosin heavy chain [37].

11.4.3 Mechano-metabolic Coupling

Recently, mechanical sensing has been coupled to endothelial phenotypic changes through metabolic signaling [27]. In the lung, very little research in this area has been performed. However, it is known that shear stress can alter substrate utilization and mitochondrial biogenesis and that disturbances in these processes are implicated in PAH [27] (Fig. 11.2). Endothelial cell quiescence is associated with a phenotype of mitochondrial respiration, whereas proliferation is associated with a glycolytic phenotype. Unidirectional flow activates KLF2, which increases mitochondria biosynthesis, reduces glycolysis, and increases oxidative phosphorylation [27]. Conversely, a reduced mitochondrial mass and upregulation of glycolysis were shown in endothelial cells under disturbed flow. These changes were mediated by an increase in hypoxia-inducible factor (HIF)-1α, an increase in ROS production and nitric oxide deficiency. Upregulation of NOX4 and the resultant increase in ROS prevent HIF-1α degradation, which in turn activates glycolysis while impairing the mitochondrial electron transport chain at complex 1 and initiating inflammatory gene expression [38].

Glycolysis is further promoted by activation of the hippo pathway, with Yes-associated protein (YAP) and transcriptional coactivator with PDZ-binding motif (TAZ) as downstream transcription factors. Activation of this pathway occurs in disturbed flow (see below), further linking mechanotransduction to metabolism [27]. Furthermore, glutaminolysis, the conversion of glutamine to glutamate which feeds into the tricarboxylic acid (TCA) cycle and contributes to the synthesis of amino acids, fatty acids, and purines and pyrimidines, is stimulated by YAP/TAZ signaling by promoting the expression of glutaminase-1 [27]. Glutaminolysis is associated with a hyperproliferative phenotype and glutaminolytic reprogramming was observed in vascular lesions of patients and animal models with PAH [27, 38].

11.4.4 Mechanosensors

Several structures in the pulmonary vasculature contribute to sensing of the mechanical forces acting on the endothelial and smooth muscle cells.

11.4.4.1 Caveolae

Also in the pulmonary vasculature, caveolae are thought to be the main platform for sensing and transducing changes in shear stress (Fig. 11.2). Indeed, mutations in the CAV-1 gene have been associated with the development of PAH [39] and caveolin-1 is reduced in endothelial cells of patients and animals with PH [40]. It is important to note that bone morphogenetic protein receptor 2 (BMPR2), which is well-known for its role in the development of PAH, colocalizes with caveolae [41]. Although endothelial cells from BMPR2$^{+/-}$ mice have higher numbers of caveolae, increased Sarcoma (Src) activity prevents the trafficking of the caveolae to the cell membrane [41].

At the cell membrane, the caveolin-1-rich invaginations form a scaffold that brings together several proteins essential in shear sensing and mechanotransduction, including platelet endothelial cell adhesion molecule 1 (PECAM-1), ATP-dependent potassium channels (K_{ATP}), endothelial nitric oxide synthase (eNOS), and NADPH-oxidase 2 (NOX2) (Fig. 11.2) [21, 42, 43]. Furthermore, the mechanosensitive cation-channel, transient receptor potential vanilloid 4 (TRPV4), colocalizes with caveoli [44].

A direct molecular interaction between caveolin-1 and PECAM-1 is required for phosphorylation and activation of PECAM-1 [43]. Downstream signaling involves the closing of K_{ATP} channels, membrane depolarization, activation of NOX2 and superoxide production [21, 43] as well as phosphorylation of Src, thereby decreasing its activity, resulting in inhibition of ERK1/2-phosphorylation [29].

Culture of pulmonary endothelial cells on a fibronectin coating under unidirectional shear stress up to 21 dynes/cm^2 increases expression of VE-cadherin, PECAM-1, and vascular endothelial growth factor receptor 2 (VEGFR2) as compared to static conditions [29]. However, in pMVECs from patients with PAH with delayed alignment to shear stress, both caveolin-1 and PECAM-1 were reduced as compared to healthy controls and cells were more prone to detachment from the culture slides. Downstream effectors of PECAM-1 signaling were also altered in that phosphorylation of Src was decreased, thereby increasing its activity, resulting in increased phosphorylation of ERK1/2 in these cells. Conversely, shear-dependent pathways not activated via PECAM-1 (i.e., adenosine monophosphate activated kinase (AMPKα) and protein kinase B (AKT)) were not altered [29].

An acute change in shear stress occurs in vivo with pulmonary embolism, i.e., when a blood clot gets stuck in the pulmonary vasculature. In a series of experiments, Chatterjee and co-workers investigated mechanosensing and mechanotransduction in the lung associated with such embolism and stop of flow. Their experiments involved a combination of experiments in isolated pulmonary endothelial cells, isolated perfused lung preparation subjected to cessation of flow, and in vivo micro-embolization [21, 42]. The cessation of flow was sensed by a

complex involving caveolae, PECAM-1 and NOX-2, and resulted in ROS production. Furthermore, it was shown that this stop of flow resulted in an increased neutrophil influx as well as a pro-angiogenic phenotype shift of the endothelial cells that was consistent with increased VEGF-expression. Both neutrophil influx and VEGF-expression were dependent on PECAM-1 and NOX-2 activity [43].

Taken together, caveolae serve as scaffolds for several proteins involved in mechanosensing and mechanotransduction in pulmonary endothelial cells. Aberrant signaling involving caveolae has been shown to be present in PH and likely contributes to endothelial dysfunction in PH.

11.4.4.2 Mechanosensitive Ion Channels

Mechanical changes can be converted into chemical signals through mechanosensitive channels in the cell membrane. In pulmonary endothelial cells, TRPV4, PIEZO1, and inward rectifying potassium channels (Kir, particularly Kir6.x, comprising the pore-forming unit of K_{ATP} channels) induce activation of eNOS in response to shear stress [6]. Both TRPV4 and PIEZO1 are permeable to Ca^{2+}. TRPV4 is activated by ROS from dysfunctional mitochondria, resulting in Ca^{2+} entry and migration and proliferation of pMVECs from rats with PAH. TRPV4 blockade normalized these responses, suggesting a role for TRPV4 in the hyperproliferative lesions in the pulmonary vasculature [45].

PIEZO1 activation results in an increase in intracellular Ca^{2+}, and activates eNOS as well as Gq/G11, which results in the release of ATP from endothelial cells and activation of P2Y2-receptors [46, 47]. PIEZO1 activation causes vasodilation [47] and is involved in the regulation of vascular barrier function [48]. However, the development of PH in response to chronic hypoxia is not altered by endothelial deletion of PIEZO1 [47], and PIEZO1 expression is unaltered in endothelial cells from patients with PAH [49]. Hence, a definitive role for these endothelial mechanosensitive channels in the development and progression of PH remains to be established.

In contrast, the role of mechanosensitive channels in the pVSMCs in PH is more clear. pVSMCs from mice with chronic hypoxia-induced PH showed enhanced Ca^{2+} influx in response to osmotic swelling, which could be blocked by Gd^{3+} as well as GsMTx-4, both blockers of mechanosensitive channels [50]. Similarly, TRPV4, TRPM7, and TRCP6 are upregulated in VSMCs from patients with PAH and contribute to an enhanced Ca^{2+}-influx in response to shear stress [51]. Furthermore, knockdown of TRPV4 attenuated the development of PH in response to chronic hypoxia [52]. Since Ca^{2+} is an important factor in both VSMC contraction and proliferation, upregulation of these mechanosensitive channels in pVSMCs likely contributes to the development and/or progression of PH (Fig. 11.2).

Given the dual role for upregulation of TRPV4 in the development of PH, having detrimental effects in both endothelial and smooth muscle cells, therapeutic targeting of this channel may delay or even prevent the progression of PH.

11.4.4.3 Integrins and Cytoskeleton

Focal adhesions are multi-protein structures that connect the cell's cytoskeleton to the ECM. Within those focal adhesions, clusters of integrins, transmembrane proteins consisting of heterodimers of various α and β subunits, form the interface between the ECM and the cytoskeleton. Currently, 18 α- and 8 β-integrin subunits have been identified, that can form various combinations. In the pulmonary vasculature, α_1, α_5, α_7, α_8, and α_v as well as β_1, β_3, and β_4 were shown to be expressed. Integrin expression is altered in PH, in that both chronic hypoxia and monocrotaline-induced PH are accompanied by increased expression of α_1, α_8, and α_v, whereas α_5 and β_1 expression is decreased in the pulmonary vasculature (Fig. 11.2) [53].

Intracellularly, integrins connect via adaptor proteins such as talin and vinculin to the actin structure of the cytoskeleton. Both Src and focal adhesion kinase (FAK) co-localize with focal adhesions and modulate integrin-cytoskeletal links, thereby altering mechanical force transmission [6]. Activation of FAK is Src-dependent and FAK activation is required for mechanosensing in pVSMCs. FAK activation is increased in pVSMCs of PAH patients, where it decreases apoptosis, promotes proliferation and migration [54]. Similarly, FAK activation is increased in pVSMCs of rats with hypoxia-induced PH, but only when these cells were cultured on collagen, and not on fibronectin. These findings are consistent with the elevation of α_1-integrin, which is required for contact with collagen, and the downregulation of α_5, which is required for binding fibronectin in the ECM [53]. Importantly, FAK inhibition can attenuate PH development and pulmonary vascular remodeling in the monocrotaline rat model. Altogether, these data indicate a causal role of FAK activation in the development of PAH [54].

Small Rho-GTPases, including RhoA, are key players in mechanotransduction [55], activated downstream of integrin signaling (Fig. 11.2). These Rho-GTPases can subsequently alter the dynamics of the actin cytoskeleton and influence cell migration and proliferation. RhoA and its downstream effector RhoA-associated protein kinase (ROCK) have been shown to be altered in animal models of PAH as well as in ECs and VSMCs of patients with PAH. In VSMCs, ROCK enhances phosphorylation (and thereby activation) of the myosin light chain (MLC) as well as of myosin phosphatase (MYPT), thereby inactivating it and reducing MLC dephosphorylation. The resultant phosphorylation of MLC increases the contractile force exerted by myosin II on actin [56]. In ECs, ROCK activation can downregulate eNOS, increase inflammatory markers, and is responsible for the cytoskeletal rearrangement in response to shear stress [6]. ROCK inhibition with fasudil attenuates both monocrotaline [57] and hypoxia-induced [56] PH in rats and decreased PVR in patients with PH [58].

The activity of Rho-GTPases is regulated by guanine nucleotide exchange factors (GEFs), GTPase activating proteins (GAPs), and Guanine nucleotide dissociation inhibitors (GDIs). GEFs catalyze the exchange of GDP (inactive form) to GTP (active form), GAPs promote intrinsic GTPase activity, leading to inactivation, and GDIs extract membrane-bound GTPases into the cytosol where they are kept inactivated [55]. Both Src and FAK are implicated in activation of GEFs, and hence in activation of RhoA [55]. Intriguingly, PDE5-inhibition increased RhoA

phosphorylation and association to its cytosolic inhibitory protein, GDI, in pulmonary arteries, MYPT activation, and loss of stress fibers in pVSMCs of rats with PH due to chronic hypoxia [56].

Hence, RhoA/ROCK activation is required for focal adhesion assembly and regulates stress fiber formation, while ROCK inhibition and vascular smooth muscle-specific knockout of ROCK2 protected against the development and/or progression of PH [6]. Interestingly, we recently found that the vasodilation in response to ROCK inhibition was blunted in pulmonary small arteries from swine with chronic thromboembolic PH [59], suggesting that ROCK activation in the pulmonary vasculature may depend on the type of PH.

11.4.5 Mechanotransduction: Transcriptional Regulation

Different transcription factors play a role in translating mechanical forces to changes in gene expression. Yet, different pathways of mechanotransduction converge on a few transcription factors that were shown to be altered in PH, including KLF2, β-catenin, nuclear factor kappa B (NFκB), myocardin related transcription factor (MRTF), YAP, and TAZ (Fig. 11.2) [6].

KLF2 is considered the master transcriptional regulator of the response to unidirectional shear stress that mediates the vasodilatory, anti-inflammatory, and antithrombotic properties of endothelium [27]. KLF2 is involved in the regulation of eNOS expression and reduced expression of KLF2 is associated with inflammation. Furthermore, KLF2 modulates substrate utilization in endothelial cells and increases mitochondrial biosynthesis [27]. Interestingly, a mutation in KLF2 has recently been implicated in the development of PAH [60]. KLF2 is also regulated by the apelin-APJ receptor axis [61], which may allow pharmacological modulation of this mechanosensitive transcription factor.

Activation of β-catenin occurs downstream of RhoA and ROCK activation and subsequently alters Wnt signaling. Although β-catenin activation in response to mechanical stimuli has not been described in the pulmonary vasculature, both endothelial upregulation of β-catenin in pulmonary small arteries of PAH patients and β-catenin downregulation in proliferating smooth muscle cells of animals with hypoxia-induced PH have been shown [6].

NFκB is transiently activated in endothelial cells in response to physiological levels of unidirectional shear stress but is highly activated in response to pulsatile flow. Its activation is dependent on the integrity of the cytoskeleton, involved activation of Toll-like receptor 2 (TLR2), and resulted in an inflammatory endothelial phenotype associated with EndoMT [10]. Both NFκB and TLR2 have been shown to be activated in PAH [6, 10].

Evidence is accumulating that mechanobiological signaling in the pulmonary vasculature involves activation of the transcription factors YAP and TAZ, which are part of the Hippo pathway. YAP and TAZ translocate from the cytoplasm to the nucleus depending on matrix stiffness [20, 62]. Thus, the culture of pVSMCs on a stiff matrix enhances nuclear translocation of YAP and TAZ [63]. YAP and TAZ are

increased in the lungs of patients and animal models of PAH. In addition to altering metabolism, activation of YAP and TAZ induces activation of microRNA (miR) 130/310, leading to alterations in ECM secretion by pVSMCs. Furthermore, inhibition of YAP and TAZ signaling reduces the expression of lysyl oxidase (LOX) as well as transglutaminase 2 (TG2), enzymes involved in the cross-linking of ECM proteins. In addition, inhibition of YAP and TAZ signaling attenuates pVSMC migration and contraction and the proliferative response of pVSMCs cultured on a stiff matrix [63]. Hence, activation of YAP/TAZ signaling in pVSMCs promotes a shift toward a pro-remodeling phenotype.

11.5 Role of Extracellular Matrix

As outlined above, stiffening of the distal pulmonary vasculature has been proposed as the initiating event of pulmonary vascular remodeling. Vascular stiffness is determined by the composition and cross-linking of the extracellular matrix (ECM) in combination with vascular tone and media-thickness. Vascular stiffening in PH was shown to start with disruption of the internal elastic lamina. Loss of elastic fibers and increased deposition of collagen in PH are evidence of ECM remodeling, resulting in a stiffer ECM [11, 63]. The ECM of a normal human pulmonary artery consists of collagens, elastins, laminins, fibronectin, tenascin C, and proteoglycans. In PAH, there is increased collagen deposition and cross-linking in all layers of the vessels (Fig. 11.2) [64].

Collagen deposition is highest in the intima of PAH patients, which is associated with increased expression of different collagen subtypes (Col14A1, Col4A5, and Col18A1) in endothelial cells [64]. pVSMCs in the media and fibroblasts in the vessel adventitia are generally identified as the cellular source for this regional collagen accumulation, although endothelial cells that undergo endoMT likely also contribute [5]. Stiffness is further increased by increased fibronectin and osteopontin, as well as the breakdown of the internal elastic lamina and a reduced elastin content in PAH [64].

The alterations in ECM structure are not only caused by changes in expression of its components, but the balance between proteolytic enzymes, such as metalloproteases [a disintegrin and metalloproteinases (ADAMs)], serine elastases, matrix metalloproteinases (MMPs), and their endogenous inhibitors, tissue inhibitors of metalloproteinase (TIMPs), also influences ECM composition. In addition, changes in the activity of cross-linking enzymes such as LOX and TG2 also modulate ECM stiffness. The pulmonary arteries from animals as well as from patients with PAH show increased expression of MMPs, ADAMs, serine elastases, LOXs, TG2, and TIMPs [64]. Endothelial cells undergoing EndoMT have been proposed to be the main cells contributing to these changes in ECM composition and cross-linking. Inflammation, disturbed flow, hypoxia, and alterations in the TGF-β-BMP balance promote this aberrant signaling [64]. Importantly, prevention of ECM remodeling by inhibition of serine elastase or LOX, as well as through administration of proline analog cis-4-hydroxy-L-proline, an inhibitor of collagen

synthesis reduces the progression of PAH in animal models, suggesting that indeed vascular stiffness is a prime determinant of disease progression [64]. Altogether, changes in the balance between collagen deposition and breakdown increased cross-linking of collagens and enhanced elastin breakdown in the intravascular and perivascular compartments of the pulmonary arteries all contribute to an increased stiffness of the pulmonary vasculature.

To study the effect of alterations in vascular stiffness on the different cell types within the pulmonary vasculature, cells have been cultured on matrices with varying stiffness. Culturing pAECs and pVSMCs on a stiffer ECM resulted in enhanced proliferation and decreased apoptosis of both cell types [11, 63]. Furthermore, culture of pAECs and pVSMCs as well as pulmonary arterial adventitial fibroblasts (pAAF) on a stiffer matrix altered expression of genes implicated in hereditary PAH; i.e., matrix stiffening significantly increased Cerebellin2 precursor (CBLN2) and decreased activin receptor-like kinase 1 (ACVRL1), BMPR2, growth differentiation factor 2 (GDF2), and potassium channel subfamily K member (KCNK3) in all three cell types. Also, the expression of other genes known to be involved in hereditary PAH was altered by ECM stiffening in either one of these cell types. Thus, CAV1 was upregulated in pAECs and pVSMCs, but downregulated in pAAFs [65], cation-transporting ATPase (ATP13A3) was upregulated in pAECs and pAAFs, and Mothers against decapentaplegic homolog 9 (SMAD9) was upregulated in pAECs. Many of these factors modulate expression of miR 130/301; reducing CAV1, BMPR2, GDF2, and ATP13A3 with siRNA resulted in upregulation of miR 130/301, while reducing CBLN2 downregulated expression of miR 130/301 in at least two cell types. Conversely, a miR 130/301 mimetic upregulated CBLN2 and downregulated BMPR2, GDF2, endoglin (ENG), and ATP13A3, while inhibition of miR 130/301 had the opposite effect in all three cell types. Furthermore, these genes were shown to be involved in the regulation of genes such as Col1, Col3, and LOX, thereby affecting ECM stiffening. These data suggest an enforcing loop in which miR 130/301 plays a key role in linking genes associated with hereditary PAH with genes linked to changes in vascular stiffness [65].

In accordance with the existence of such an enforcing loop, the effect of stiffening of the matrix on the proliferative response of pVSMCs was further enhanced in pVSMCs with a BMPR2 mutation, that predisposes to PH development, as well as in pVSMC obtained from rodents with hypoxia-induced PH [11]. Furthermore, smooth muscle cells cultured on a stiffer matrix showed increased production of collagen and fibronectin [11], suggesting a positive feedback loop between ECM stiffening and pVSMC proliferation, leading to enhanced muscularization of the distal pulmonary vasculature.

Consistent with the reduced circulating levels of prostacyclin in patients with PH [66], pVSMCs cultured on a stiff ECM had reduced cyclo-oxygenase 2 (COX2) expression and reduced production of prostacyclin [11]. This effect occurred secondary to upregulation of YAP/TAZ signaling as enhancing YAP/TAZ activity resulted in downregulation of COX-2 [11, 63] while interfering with YAP/TAZ signaling prevented downregulation of COX-2 induced by culture of pVSMCs on a stiff matrix [63]. Importantly, administration of the prostacyclin analog treprostinil

in cell culture attenuated pVSMC proliferation and reduced secretion of the ECM proteins. In addition, treprostinil lowered mRNA expression of collagen 1 and 3 as well as the ECM cross-linking enzyme LOX and slowed progression of PH development in response to monocrotaline in vivo [11]. Altogether, these findings suggest that prostanoid therapy, which is now applied only late in PVD, could be more beneficial early in the disease, by interfering with mechanosensitive processes in a critical phase of disease progression.

11.6 Conclusions and Perspectives

Changes in pulmonary vascular stiffness are now recognized as early drivers of pulmonary vascular disease. Vascular stiffening occurs both in the proximal and distal pulmonary vasculature. Subsequent alterations in pressure and flow patterns activate mechanosensitive pathways, leading to inflammation, endothelial dysfunction, EndoMT, clonal expansion of progenitor cells into smooth muscle cells, muscularization, metabolic changes predisposing to proliferation and further ECM remodeling, thereby initiating a positive feedback loop leading to progression of pulmonary hypertension. Whether novel therapeutic strategies, intervening within this loop, can ameliorate pulmonary hypertension remains to be established.

Compliance with Ethical Standards

Conflict of Interest Authors declare that they have no conflict of interest.

Ethical Approval This article does not contain any studies with human participants or animals performed by any of the authors.

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Mechanobiology of Atherosclerosis

12

Andreas H. Wagner

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Abstract

Vascular mechanobiology deals with the question of how different physical forces and changes in the mechanical properties of single cells and entire tissue structures contribute to cell differentiation, physiology, initiation, and progression of disease development. This review surveys new findings and progress in the research field of atherosclerosis in recent years. Moreover, it aims to integrate different aspects to demonstrate the interlacing and integration of certain mechanisms in the pathogenesis of atherosclerosis.

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Abbreviations

ApoE	Apolipoprotein E
ICAM-1	Intercellular adhesion molecule-1
LIM	Named after their initial discovery in the proteins **L**in11, **I**sl-1, **M**ec-3
NADPH	Nicotinamide adenine dinucleotide phosphate hydrate
NOS3	Endothelial nitric oxide synthase (eNOS)
NOX	NADPH oxidase
ROS	Reactive oxygen species
Smad	Acronym refers to the Caenorhabditis elegans *Sma* (**sma**ll worm phenotype) and the Drosophila *Mad* (**m**others **a**gainst **d**ecapentaplegic) gene family
SUMO	**S**mall **U**biquitin-related **Mo**difier
TAZ	Transcriptional co-activator with PDZ-binding motif
TNF-α	Tumor necrosis factor-α
VCAM-1	Vascular cell adhesion protein-1
VSMCs	Vascular smooth muscle cells
YAP	Yes-associated protein

12.1 Introduction

Blood vessels and their constituent endothelial cells are constantly exposed to mechanical stimuli. A striking feature of atherosclerosis is its highly heterogeneous distribution within the arterial tree [1]. Atherosclerotic lesions predominantly develop at branches and bifurcations. These sites are exposed to low or disturbed blood flow causing low or oscillatory shear stress on the vessel wall, respectively [2, 3]. In addition to this flow evoked wall shear stress, circumferential stress driven by the pulsatile blood pressure plays an important role in mechanical processes including angiogenesis, vascular remodeling, and atherogenesis [4]. Potential mechanosensors of endothelial cells, e.g., surface glycocalyx [5], integrins, mechanosensitive ion channels, or stretch sensitive focal adhesion proteins [6] sense different physiological or pathological intensities of mechanical stimuli on the cell surface. These different intensities are converted inside the cell into the activation of various pathways determining the fate of cells. Besides, changes in extracellular matrix proteins and mechanical properties of the vessel wall are related to arterial stiffening which may activate several mechanisms also involved in the process of atherosclerosis [7, 8]. This in turn enables the recruitment and diapedesis of circulating immune cells, predominantly monocytes, and T lymphocytes, starting the inflammatory process of atherosclerosis [9].

12.2 The Endothelial Glycocalyx is an Amplifying Mechanosensor

The glycocalyx not only represents a physical barrier to leukocyte–endothelium adhesion but rather plays an important role in sensing and transducing the magnitude and direction of mechanical forces into biochemical signals [5], thus governing the phenotype of endothelial cells at atherosclerosis-prone branch regions [10]. Glycocalyx thickness, which ranges from 200 to 2000 nm in thickness [11], and cell surface coverage were significantly decreased in the atheroprone region of the brachiocephalic artery in a high fat-fed ApoE$^{-/-}$ mouse model [12]. In ApoE/ low-density lipoprotein receptor (LDLR) deficient mice glycocalyx degradation and multiple manifestations of endothelial dysfunction coincide in the early phase of endothelial dysfunction before atherosclerotic plaque development was detectable [13]. The observed glycocalyx shedding increased lipid permeability and facilitated macrophage infiltration promoting lipid retention and the development of atherosclerotic plaques [12, 14, 15]. Present results indicate that biglycan as a small leucine-rich repeat proteoglycan plays a protective role during the progression of atherosclerosis by inhibiting thrombin activity, platelet activation, and finally macrophage-mediated plaque inflammation [16].

Histomorphometric analysis of vascular walls suggest a remodeling induced by low flow and high-pressure loading [17]. These findings are consistent with the classic hypertensive aortic phenotype characterized by a thicker and more rigid vascular wall as well as an increased aortic diameter [17]. It has been suggested that glycocalyx degradation occurs in atheroprone regions by TNF-α-dependent mechanisms involving activation of endothelial heparanase [18]. As a consequence, the normal nitric oxide/NF-κB negative feedback loop, i.e., nitric oxide production and NF-κB inhibition [19], is disrupted. Thus, decreased nitric oxide availability results in sustained activation of NF-κB in response to shear and increased intercellular adhesion molecule-1 (ICAM-1) expression. ICAM-1 interaction with the integrin LFA-1 (leukocyte function-associated antigen-1) found on leukocytes is crucial in mediating its transmigration [10].

The glycocalyx is a prerequisite for unidirectional shear stress-induced nitric oxide generation [20], thus modulating redox signaling in endothelial cells. Locally produced reactive oxygen species (ROS) generated by xanthine oxidoreductase has been shown to induce glycocalyx reduction [21]. Xanthine oxidase has been shown to bind to the glycocalyx through its heparin-binding domain [21]. Prolonged inhibition of NADPH oxidase with apocynin decreased xanthine oxidase protein levels and prevented endothelial superoxide generation in response to oscillatory shear stress [22]. These data suggest firstly that NADPH oxidases maintain endothelial cell xanthine oxidase levels and secondly that xanthine oxidase is responsible for increased ROS production in response to oscillatory shear stress. Interestingly, the NOX family of ROS-generating NADPH oxidases has been found to modulate the endothelial redox state in response to different forms of wall shear stress [23]. Unidirectional shear stress activates the NOX2–p47phox complex to activate endothelial nitric oxide synthase (NOS3) phosphorylation and nitric oxide formation

[23]. In contrast, oscillatory shear stress activates the NOX1–NOXO1 (NADPH oxidase organizer 1) complex to uncouple NOS3 increasing the NOS3-dependent generation of ROS [23].

One has to keep in mind that in isolated endothelial cells cultured for several passages, the glycocalyx appearance is altered or collapsed due to the treatment with enzymatic solutions containing collagenase or trypsin to separate cells from the vessel wall or tissue-culture plates. Interestingly, glycocalyx appears predominantly on the edge of endothelial cells in the early days in culture after seeding and within 1 week in the apical area of the cell membrane [24]. Most of the published research using isolated endothelial cells as a model is presumably performed with cells grown to confluence for 3–7 days. Thus, estimating the status and importance of the glycocalyx in cultured endothelial cells under these conditions seems to be meaningful only to a very limited extent. This limitation needs to be kept in mind extrapolating such in vitro experimental results to pathophysiological or clinical settings.

12.3 Mechano-redox Regulation of Antioxidant Enzymes

It is widely assumed that low and oscillatory wall shear stress plays a key role in the initiation and development of atherosclerosis but evidence for this is less robust than commonly assumed [2]. In addition to common atherosclerosis risk factors increasing ROS formation by endothelial and vascular smooth muscle cells, other physical forces seem to regulate vascular production of ROS [25]. Cyclic stretch modulates redox enzyme expression in endothelial cells through an increased ROS formation, mainly superoxide anions and hydrogen peroxide [26]. Superoxide anions and nitric oxide rapidly react to peroxynitrite, hence reducing the level of biologically active nitric oxide even further [27]. This reaction not only weakens the nitric oxide inhibition of pro-inflammatory gene expression but may exert additionally a plethora of potentially deleterious effects [28]. On exposure to cyclic stretch, there was a transient increase in intracellular ROS in cultured HUVECs returning to control levels after 24 h [29]. The observed concomitant rise in glutathione peroxidase-1 (GPx-1) and heme oxygenase-1 (HO-1) expression may comprise an adaptive mechanism through which the cells maintain their anti-atherosclerotic properties despite a decreased bioavailability of nitric oxide [30]. GPx-1 is a selenium-dependent enzyme that inactivates hydrogen peroxide as well as various lipid hydroperoxides [26]. The expression of the inducible stress protein HO-1 can be markedly augmented by a wide range of substances causing transient changes in the cellular redox state [31]. The sialic acid component of the glycocalyx and the HO-1 expression were differentially regulated by unidirectional and oscillatory shear stress compared to static cultures [32]. Removal of sialic acid with neuraminidase before unidirectional shear stress exposure abrogated HO-1 induction mediated by the transcription factor nuclear factor E2-related factor 2 (Nrf2). Subsequently, mitochondrial superoxide response to fluid shear stress was enhanced [32]. These findings demonstrate that fluid shear stress-sensitive Nrf2 signaling is dependent on mechanotransduction through the glycocalyx and restoration may normalize

redox homeostasis in atheroprone vascular regions [32]. Moreover, in response to unidirectional shear stress, an adaptive reorganization of the endothelial glycocalyx associated with changes in membrane rafts and the actin cytoskeleton has been reported [27].

Although the glycocalyx is essential for maintaining vascular homeostasis, none of the common appropriate treatments for atherosclerosis including lipid-lowering and anti-platelet therapies target the endothelial glycocalyx [33]. Monitoring and protecting the endothelial glycocalyx in patients may lower the risk of cardiovascular comorbidities [34]. Current pharmacologic approaches aim at inhibiting multiple adverse factors and enzymatic attacks or reassembling glycocalyx components and have only been tested in animals (reviewed in [34]). The effectiveness of such strategies remain to be determined in clinical experiments while accounting for unpredictable compensatory responses [34].

12.4 Mechanical Force-Induced CD40 Signaling Results in Endothelial Dysfunction

CD40 receptor expression by endothelial cells and the pro-inflammatory response of these cells after receptor stimulation by its ligand CD154, suggest an important role for CD40-CD154 co-stimulation in atherosclerosis and other vasculopathies [35]. Using an appropriate ROS scavenger and a protein kinase inhibitor revealed that stretch-induced CD40 expression is ROS-dependent and is mediated via the JNK/AP-1 signaling pathway [28]. In areas of disturbed blood flow with oscillatory shear stress and increased circumferential wall stress, endothelial cell CD40 expression, which is normally suppressed by unidirectional shear stress, is disinhibited [28]. Peroxynitrite is a potent oxidant with deleterious tissue-oxidant effects, formed from the reaction between superoxide radicals and nitric oxide. Interestingly, in this case, this oxidative protein modification seems to be protective. CD40-tyrosine nitration by peroxynitrite functionally inactivates and disconnects the receptor from the intracellular signal transduction cascade to the nucleus, followed by internalization of the nitrated protein and subsequent accelerated degradation [28]. Internalized CD40 exhibits different patterns of tumor necrosis factor receptor-associated factors TRAF2/3/6 recruitment and Akt (also known as protein kinase B) phosphorylation from the membrane-anchored CD40 complex [36]. Through this posttranslational oxidative modification, endothelial cells may adapt to unfavorable hemodynamic conditions and maintain their anti-inflammatory phenotype.

Otherwise, ligation of endothelial CD40 by platelet-bound CD154 or soluble CD154 triggers the release of ultra-large von Willebrand factor multimers stored in Weibel–Palade bodies of endothelial cells [30]. Platelets rapidly adhere to these multimers even under flow conditions and become activated. Presumably, through P-selectin–PSGL-1 interaction, they can trap circulating monocytes facilitating their diapedesis and differentiation into macrophages [30]. Interestingly, platelet hyaluronidase-2, stored in α-granules translocates to the surface upon activation [37] possibly to function in glycocalyx degradation facilitating monocyte transmigration, as discussed above.

12.5 Mechanosensitive Transcription Factors

Disturbed blood flow and unidirectional laminar flow have been shown to differently modulate a variety of mechanosensitive transcription factors on various levels, as reviewed recently [38]. In particular, the occurrence of spatial shear stress gradients may represent important local modulators of endothelial transcription factor expression and consecutively activation at sites predisposed to atherosclerotic development [39, 40]. Thus, it is hardly surprising that emerging studies show that transcription factors, e.g., KLF2 or NFR2, represent promising therapeutic targets for the prevention and treatment of atherosclerosis [38, 41].

Some transcription factors have not yet been a focus within the biomechanics research interest so far. Biomechanical stretch activates NFAT5 (nuclear factor of activated T cells-5), also known as TonEBP (tonicity-responsive enhancer-binding protein), a transcription factor regulating the expression of genes involved in osmotic stress [42]. In high extracellular tonicity environments, the COOH-terminal transactivation domain of NFAT5 becomes phosphorylated, resulting in nuclear translocation of the activated transcription factor [43]. NFAT5 has been shown to regulate the expression of tenascin-C [44] and ACTBL2 (beta-actin-like protein 2) in native and cultured VSMCs [45] contributing to an enhanced migration of these cells promoting maladaptive vascular remodeling processes. Interestingly, NFAT5 seems to be continuously expressed and degraded in resting VSMCs while expression and accumulation of the NFAT5 isoform C in the nucleus is facilitated during biomechanical stress [46]. Nuclear translocation required palmitoylation and specific phosphorylation at Y143 but was inhibited by phosphorylation at S1197. Finally, VSMC-specific knockout of NFAT5 in mice inhibited the proliferation of VSMCs and the thickening of the arterial wall during both flow-induced collateral remodeling and hypertension-mediated arterial hypertrophy [47].

Besides the activation of transcription factors by phosphorylation and translocation from the cytosol to the nucleus, mechanical signals can propagate through mechanically stiff structures like focal adhesions. The LIM domain protein Zyxin is localized primarily at focal adhesion plaques [48]. Zyxin binds Ena/VASP proteins (enabled/vasodilator-stimulated phosphoprotein) that, in turn, promote actin polymerization [49]. Growing evidence suggests that zyxin is a vital mechanotransductor and key regulator of stretch-induced gene expression as zyxin translocated into the nucleus of cultured cyclic stretched VSMCs [50]. Loss of zyxin drives VSMCs toward a synthetic phenotype switch, a process further consolidated by exaggerated stretch. VSMCs phenotypic modulation plays a key role in atherosclerosis and is classically defined as a switch from a contractile phenotype to a synthetic phenotype [51].

In cultured human endothelial cells and perfused femoral arteries isolated from wild-type and several knockout mouse strains, a multistep signaling pathway leading to zyxin activation has been characterized [52]. Cyclic stretch led to a transient receptor potential channel 3-mediated release of the endothelial vasoconstrictor peptide endothelin-1 (ET-1). Through autocrine activation of its B-type receptor, ET-1 elicited release of pro–atrial natriuretic peptide (ANP) causing autocrine

activation of the ANP receptor guanylyl cyclase A (GC-A). GC-A activation provoked protein kinase G-mediated phosphorylation of zyxin at serine 142, thereby leading to translocation of zyxin into the nucleus and inducing stretch-dependent changes in gene expression. Almost all zyxin-dependent genes can be attributed to a set of well-defined pathways that, on the one hand, inhibit apoptosis and proliferation and strengthen cell–matrix interactions but, on the other hand, contribute to a broad range of pro-inflammatory responses, e.g., Interleukin-8, ICAM-1 and VCAM-1 [53].

Focal adhesion plaques are integrin-containing, multi-protein structures, and sites of transmembrane interaction between the extracellular matrix and the actin cytoskeleton. The discovery of a connection between endothelial cell integrins, extracellular matrix, and signaling events opened a new perspective in the understanding of the molecular mechanisms regulating vascular responses to the changes in blood flow [54]. Excessive accumulation of the endothelial glycocalyx component hyaluronan around the vascular smooth muscle cells results in increased aortic stiffness and strength and accelerated atherosclerosis in ApoE-knockout mice [55]. Changes in extracellular matrix proteins and the mechanical properties of the vessel wall related to arterial stiffening may activate several mechanisms involved also in the initiation of atherosclerotic lesions [7, 56]. Extracellular matrix proteins like fibronectin and laminin differentially regulate the Smad2 activation in vascular endothelial cells in response to disturbed flow [57]. Oscillatory shear stress-induced a sustained activation of Smad2 in endothelial cells cultured on fibronectin, but only a transient activation of Smad2 in endothelial cells on laminin resulting in a transient induction of NF-κB and pro-inflammatory gene expression [57].

Recent genome-wide association studies identified that the JCAD (Junctional cadherin 5 associated, also known as *KIAA1462*, encoding a junctional protein associated with CAD) locus is associated with the risk of coronary artery disease and myocardial infarction [58]. JCAD deficiency attenuated high-fat diet-induced atherosclerosis in ApoE-deficient mice. Mechanistically, JCAD regulated the YAP/TAZ pathway, thus promoting endothelial dysfunction and the expression of downstream proatherogenic genes in human coronary artery endothelial cells [58]. YAP and TAZ are both effectors of the Hippo pathway. Dysregulation of the Hippo signaling pathway leads to different kinds of cardiovascular diseases, such as myocardial infarction, cardiac hypertrophy, neointima formation, and atherosclerosis [59]. Emerging evidence indicates that YAP and TAZ sense different blood flow patterns and regulate atherosclerotic lesions [60]. Disturbed flow leads to a significant decrease in YAP phosphorylation and marked increased adhesion molecule and TAZ expression [60]. Moreover, disturbed flow resulted in YAP/TAZ nuclear localization, whereas increased YAP/TAZ cytoplasmic retention was observed in HUVECs subjected to unidirectional shear stress [60]. In the latter study, methotrexate at therapeutically relevant concentrations inhibited disturbed flow-induced endothelial YAP/TAZ activation. Thus, it has been proposed that methotrexate can be regarded as an important therapeutic agent not only to treat rheumatic diseases but also to reduce cardiovascular risk and mortality [61].

12.6 Flow-Induced Epigenetic Mechanisms of Endothelial Gene Expression

Epigenetic mechanisms involve the interplay between signal-transduction pathways, transcription factors, and the genome chromatin packaging, determining the gene expression pattern of a cell. Recent research demonstrates that blood flow and pressure are hemodynamic cell environments that have been demonstrated to influence transcription via epigenetic mechanisms (c.f. recent review [62] and Chap. 9). Principal mechanisms are chemical modifications, e.g., methylation of cytosine DNA residues and amino acids of histone proteins associated with DNA in nucleosomes [62]. The promoter of several mechanosensitive genes, such as HoxA5, Klf3, and Klf4, were hypermethylated by disturbed blood flow but rescued by DNA methyltransferase inhibitors [63].

In addition, posttranslational modifications, including phosphorylation and SUMOylation, provide new perspectives on the disturbed flow-induced pathogenesis of atherosclerosis [64]. It has been shown that SUMOylation of DNA methyltransferase is induced by disturbed flow but not by steady laminar flow signaling [64]. Disturbed flow induces peroxynitrite production, which in turn activates protein kinase C ζ and its binding to the E3 SUMO (small ubiquitin-like modifier) ligase PIASy (protein inhibitor of activated STATy) [64, 65]. Thus, determining the interplay of each PTM and epigenetic event will provide a new paradigm to elucidate the difference between disturbed flow and steady laminar flow, which may lead to novel therapeutic intervention strategies to inhibit plaque formation [64].

In recent years, noncoding RNAs as a mode of epigenetic-related regulation at the transcriptional and post-transcriptional level have become an area of intensive investigation. Targeting mRNA by short microRNAs (miRNAs, 20–26 nucleotides) and long noncoding RNAs (lncRNAs, >200 nucleotides) facilitate transcript degradation. Since noncoding RNAs make up >97% of the transcriptome [66], there is huge potential for dynamic epigenomic regulation of gene expression including biomechanical stimulation of the epigenomic regulation. So far, several miRNAs such as miR-10a, miR-19a, miR-23b, miR-101, and miR-143/145 have been identified to be induced by high shear stress mediating an atheroprotective role [67]. Interestingly, changes in the expression profile of miR-21 and miR-92a by high shear stress are associated with an atheroprotective function, while low shear stress-induced expression of miR-21, miR-92a, and miR-663 results in a pathological endothelial cell phenotype [67]. This contradiction could be explained by the fact that these microRNAs are differentially regulated by diverging shear stress modes or additional factors beyond shear stress [67]. For example, overexpressing miR-21 in endothelial cells on one hand decreases apoptosis and increases NOS3 phosphorylation and subsequently nitric oxide formation. On the other hand, oscillatory shear stress and overexpression of miR-21 enhances VCAM-1, and monocyte chemotactic protein-1 (MCP-1) expression and thereby monocyte adhesion to the endothelial cells [68]. MiR-155 fulfills a wide range of functions in different regions of the vasculature. When exposed to different modes of shear stress it is elevated in

pro-inflammatory macrophages and atherosclerotic lesions in vivo [69]. Flow disturbance induced by partial carotid ligation led to a lower expression of miR-23b and a higher endothelial cell proliferation in comparison with the pulsatile flow regions of unligated vessels [70].

In the cardiovascular system, lncRNA expression has been detected and characterized under normal physiological conditions and in disease states (c.f. recent review [71, 72]). The pro-angiogenic lncRNA MANTIS was tightly regulated by the mechanosensitive transcription factors KLF2 and KLF4 and limits the ICAM-1 mediated monocyte adhesion to endothelial cells and thus potentially atherosclerosis development in humans [73]. In contrast, the role of flow-dependent lncRNAs regulation in vascular dysfunction and atherosclerosis is widely unknown and an emerging research field [74]. First results about the flow-sensitive lncRNA STEEL along with other lncRNAs studied in the context of vascular pathophysiology and atherosclerosis have been reviewed very recently [74]. The role of lncRNAs as potential biomarkers in cardiovascular disease especially in atherosclerosis is still at its beginning [74].

12.7 Conclusions and Remaining Questions

So obviously, on the cellular level, there appear to exist several mechanical force-dependent pro-atherosclerotic but also in parallel protective, closely integrated mechanisms to locally protect endothelial cells under unfavorable conditions present at atherosclerotic predilection sites (Fig. 12.1). So why do some people develop atherosclerotic plaques and others do not? At first, the individual lifestyle associated with a balanced diet may be important. Several epidemiological studies report promising protective effects of antioxidant foods. However, many unclear points are remaining regarding the contribution of the nutritional elements found in antioxidant foods to the prevention of atherosclerotic disease [31]. Thus, among other risk factors, genetic polymorphisms should be more prominently included in the individual risk profiling to develop atherosclerosis or coronary heart disease. For example, the C allele of a functional polymorphism in the Kozak consensus sequence of the CD40 gene has been associated with enhanced translational efficiency correlating to increased CD40 protein expression and an overall increased atherosclerosis risk [75]. The polymorphism genotype frequency of the oxidative stress detoxifying glutathione S-transferase mu 1 (gene name GSTM1) was significantly higher in individuals diagnosed with atherosclerosis and reported to smoke or being former smokers [76]. Single polymorphisms in other protective genes like superoxide dismutase (SOD2) [77] or NOS3 [78], or an association between NOS3 and GPx-1 gene polymorphisms [79] are independent risk factors for susceptibility to develop atherosclerosis or coronary artery disease in men. Interestingly, a fluid shear stress-induced rise in SOD2 expression [80] and enhanced release of the anti-inflammatory metabolite of the arachidonic acid pathway 15-deoxy-Δ12,14-prostaglandin J2 (15d-PGJ2) [81] in NOS3 dysfunctional genotype endothelial cells effectively stabilizes their anti-atherosclerotic phenotype [80]. Thus, it must be

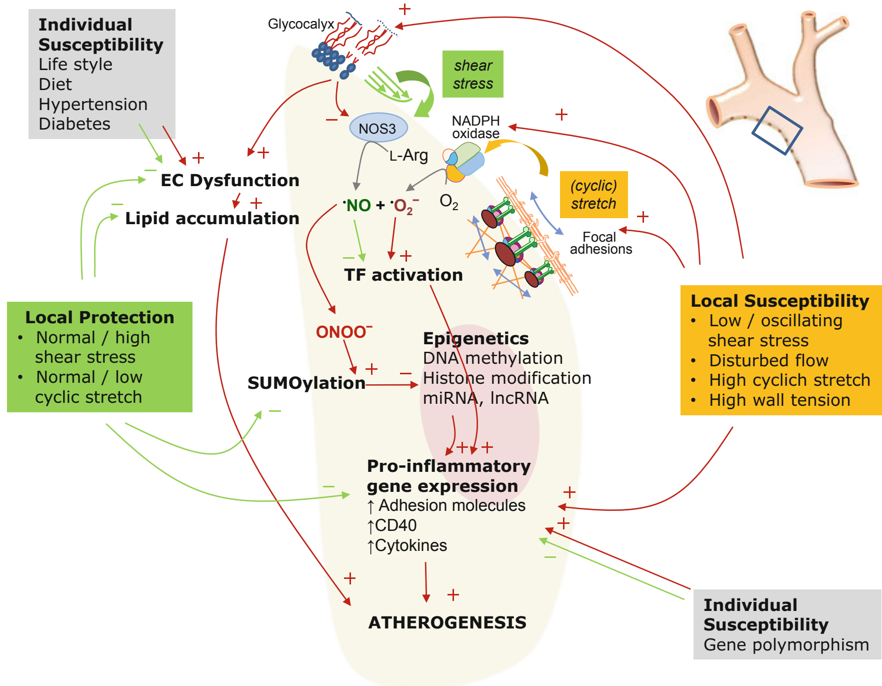

Fig. 12.1 Schematic summary demonstrating how individual risk factors along with local biomechanical forces provide a local predilection for the initiation of atherosclerosis at arterial bifurcations by stimulating (red) or inhibiting (green) atherogenic processes in endothelial cells (*L-Arg*, Arginine, *CD40* cell surface receptor, *EC* endothelial cells, *lncRNA* long noncoding RNA, *miRNA* microRNA, *NO* nitric oxide, *NOS3* nitric oxide synthase 3, $(O_2^{\bullet -})$ superoxide, *ONOO*$^-$ peroxynitrite, *TF* transcription factor)

concluded that there are individual and summed effects of high-risk genetic polymorphisms on the development of atherosclerosis.

Compliance with Ethical Standards

Conflict of Interest The author declares that he has no conflict of interest.

Ethical Approval This chapter does not contain any studies with human participants or animals.

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Exploitation of Vascular Mechanobiology for Therapy Innovations

13

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Abstract

This chapter provides an overview of the recent endeavors taken by bioengineers to exploit mechanobiology for the improvement of existing cardiovascular treatments and the development of novel strategies in cardiovascular therapy. It starts with a summary of mechanobiological mechanisms that can be utilized as the foundations for the development of future cardiovascular therapies. This is followed by discussing how mechanobiology has been or will be exploited to

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change various cardiovascular therapeutic strategies, which include vascular devices such as stent and graft, medication, regenerative medicine, disease management, and preventions for cardiovascular diseases. For each therapeutic strategy, mechanobiological applications in its existing treatment options, emerging approaches, explanation of treatment outcomes, and/or addressing prominent problems or needs are examined.

13.1 Introduction

Cardiovascular diseases are the most common cause of death in the USA, accounting for nearly 29% of all deaths each year [1], and rise above 25% worldwide [2]. Traditionally, biomedical research studies on cardiovascular diseases and their treatment solutions have largely focused on biochemical signaling cascades, with the goal of improving disease understandings and developing innovations in interventions or treatments targeting critical signaling molecules. In the last two decades, much of the attention has shifted to vascular mechanobiology. Novel mechanotransduction mechanisms, together with new mechanobiological approaches, emerge to advance our understandings of cardiovascular physiology and pathology. These new approaches include engineered biomaterials with biomimetic stiffness for the culture of vascular cells [3–8], bioreactors with vascular-like mechanical loading [9–15], computational modeling of vascular flow dynamics [16–21], as well as mechanobiological modeling of vascular diseases and regeneration [22–27]. Despite significant advances in mechanistic understanding, researchers have just started the journey of exploiting mechanobiological principles for novel therapies for cardiovascular diseases. This chapter reviews the established foundations and recent trends in this specialized area, with our suggestions about existing challenges and opportunities.

Our main focus here is to review recent endeavors of bioengineers to exploit mechanobiology for the development of novel strategies in cardiovascular therapy, which may significantly improve clinical outcomes in the near future. Specifically, our review centers around the following questions: (1) Which mechanobiological mechanisms may be utilized as the foundations for developing next-generation cardiovascular therapies? (2) Has mechanobiology been exploited to advance any existing or emerging cardiovascular therapies such as medications, implants, and disease management? (3) What are the prominent problems or specific needs for each mainstream therapy? Will it be possible for us to leverage the mechanisms in vascular mechanobiology to address these needs, so as to improve therapeutic outcomes, expedite the discovery of medications, or innovate disease management? Nevertheless, before mechanobiological concepts may ultimately advance or alter existing cardiovascular therapy, it is expected that significant efforts must be made to translate recent research into clinical practices.

13.2 Mechanobiological Foundations for Therapeutic Innovations

The cardiovascular system is a dynamic, pressure-driven flow system, involving ongoing mechanical feedback loops directing cell response and extracellular matrix (ECM) remodeling. These mechano-responsive events sustain homeostasis of the system, guide adaptive remodeling, or perpetuate the disease progression. Mechanotransduction thus represents the most critical mechanism in determining cardiovascular health, disease, and treatment response. For example, physiologically relevant flow stresses (e.g., shear stress) within the vasculature are beneficial (e.g., atheroprotective) to healthy blood vessels, while pathologically relevant stresses initiate or exacerbate vascular lesions in diseased vessels [28–33]. Another example is that highly elastic, compliant arteries are the necessary condition for reducing cardiac afterload and flow pattern disturbance.

Biomolecules mediating healthy and/or unhealthy flow stresses have become key targets for vascular mechanobiology investigations and relevant pharmacological studies. The identified mechanical sensors in vascular cells, which sense and respond to mechanical stimuli, are mainly either structural sensors and transducers, such as primary cilia, glycocalyx, caveolae, nucleus, focal adhesions, and ion channels [34–38], or molecular sensors and transducers, such as PECAM-1, VE-cadherin, and VEGFR2 [39–43]. Through these sensors, cyclic loading of hemodynamic forces (i.e., vascular flow-induced shear stress, wall tension, and compression) has marked effects on vascular cell morphology and migration patterns [44, 45]. Blocking of these sensor functions may reverse the influences of adverse mechanical conditions. Vascular mechanobiology studies are continuously seeking to elucidate the mechanisms, in which coupling of mechanical stimuli and sensors regulate cellular functions within the vasculature. Figure 13.1 summarizes the current understanding of vascular mechanobiology.

In recent years, attention has gradually shifted to the central roles of the ECM in coordinating vascular responses to mechanical loads. Mechanical loads acting on the vessel, along with vessel stiffness changes (i.e., cellular tractions through cell–cell and cell–ECM adhesions), are perceived by vascular cells as stimuli that are transmitted through constituents of the ECM, ECM receptors, interfibrillar and intracellular structures. Intriguingly, mechanosensing, and mechanoregulation of the ECM are integrated across different length and time scales to achieve mechanical homeostasis of the ECM, as reviewed by Humphrey et al. [46]. One central idea proposed around the concept of mechanical homeostasis requires that cells sense the initial ECM mechanics and then regulate to maintain it. The mechanoregulation process of cells includes the deposition, rearrangement, or removal of the ECM to maintain overall form and function [46–49]. However, as cells undergo dynamic remodeling by continually reading and responding to environmental cues for homeostasis, complementary homeostatic processes often lead to fibrosis, mechanical failure, or other pathologies. Computational models suggest that the form and function of tissues can be maintained only if the structural constituents of the degraded ECM

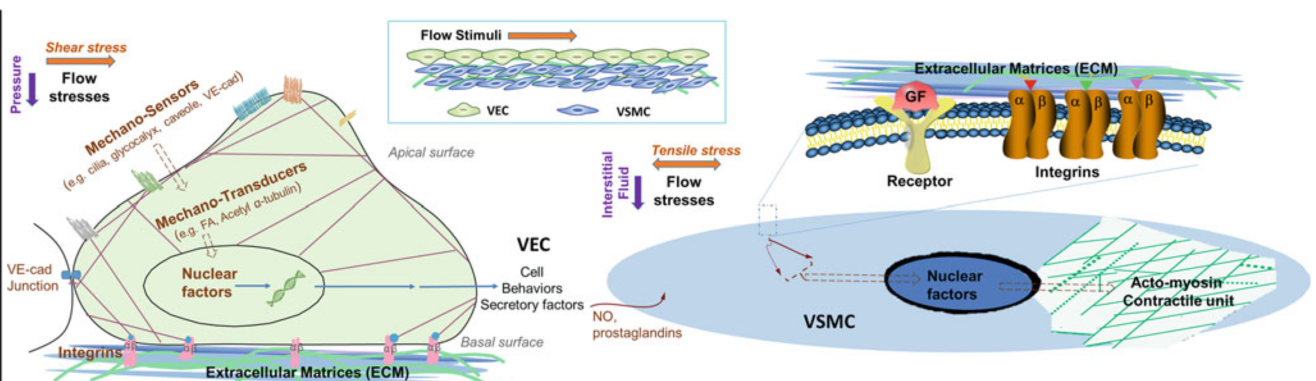

Fig. 13.1 An overview of the current understanding of vascular mechanobiology. Vascular cells, VEC and VSMC, are capable of effectively sensing and transducing mechanical stresses from blood flow and/or mechanical signals from the vessel wall ECM. The flow-initiated mechanical forces include fluid shear stress, tensile stress, pressure-induced compressive stress, and interstitial fluid stress. *VEC* vascular endothelial cell, *VSMC* vascular smooth muscle cell, *NO* nitric oxide, *FA* focal adhesion, *Acetyl a-tubulin* acetyl alpha tubulin. Herein, mechanosensors refer to the molecules or structures on or around the cell membrane, which sense mechanical stimuli, while mechano-transducers refer to intracellular structure or molecules that generate a measurable response to mechanical stimulation

are replaced with the same properties including the same level of prestress-induced stiffness [50].

An up-to-date knowledge and understanding of vascular mechanobiology can always be extended to improve existing treatment options. For example, arterial grafts and stents, in many cases, result in stenosis and/or thrombosis. To enhance their performances, the device designs were optimized by considering the impacts of device placement and mechanics, on the mechanical loads on neighboring vessel cells [51–53]. With increasing interest in applying regenerative approaches to graft and stent innovation, the investigations into the roles of the ECM constituents in coordinating vascular responses will be continuously primed [54].

13.3 Mechanobiology-Driven Innovations in Cardiovascular Therapy

Cardiovascular diseases encompass a number of severe and even fatal health problems. Because the cardiovascular system is so complex and far-reaching, diseases can appear anywhere in the body in many different forms. Vascular diseases often lead to or occur along with heart disease. The focus in this book chapter is mainly vascular diseases, i.e., disorders of the vast blood vessel network. The most common vascular diseases are coronary artery disease (often called atherosclerosis), peripheral artery disease, carotid artery disease, aortic aneurysm, critical limb ischemia, blood clots, and venous diseases. Failure to properly treat vascular diseases leads to myocardial infarction, stroke, heart failure, and death. Mechanobiological principles are increasingly applied to innovate current therapeutic devices, medications, materials design, or disease management. This section thus elaborates such mechanobiological applications on the following treatments: (1) vascular stent; (2) vascular graft; (3) vascular regeneration; (4) discovery of new medications; and (5) disease management and prevention. Figure 13.2 provides an overview of the current status of applying mechanobiological principles to these applications.

13.3.1 Vascular Stent

Vascular stenting is routinely used to treat patients with a number of vascular diseases such as atherosclerosis. It helps to restore the blood flow through narrowed or blocked vessels. Atherosclerosis, narrowing of vascular tissues through plaque formation and successive hardening, alone is the leading cause of death in America, killing 365,914 people in 2017 [55]. The most common treatment for this condition has been the implantation of vascular stents, with over 1 million procedures performed in the USA annually [56].

Stenting, however, is often associated with two major complications, namely in-stent thrombosis and in-stent restenosis, both of which eventually lead to the re-occlusion of arterial lumen. The former is caused by platelet accumulation, while

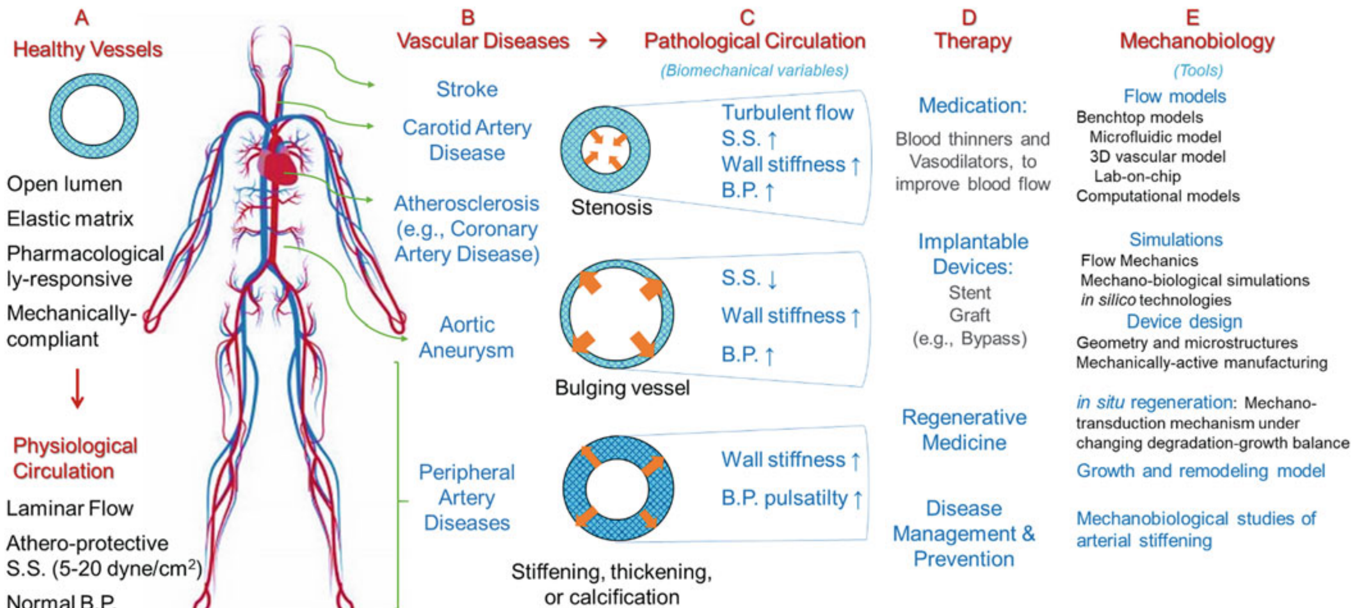

Fig. 13.2 An overview of the current status of applying mechanobiological principles to the applications of vascular devices, vascular regeneration, discovery of new medications, and disease management and prevention. Vascular mechanobiology and its derived mechanomedicine covers the understanding of biomechanical variables in normal, diseased, and treated circulation as well as the computational and benchtop models using molecular-, cellular-, and tissue-level mechanobiological approaches to improve existing therapy. Several most common vascular diseases are listed as examples in panel (**b**). *SS* shear stress, *BP* blood pressure

the latter is caused by the inflammation-induced overgrowth of vascular smooth muscle cells (VSMCs) [57]. The three major types of stents, which have been on the market for clinical uses, are metal stent, drug-eluting stent, and bioresorbable stent [58]. Bioresorbable stents manufactured by Abbott Laboratories, once thought to revolutionize the stent field by providing temporary support to open the vessel lumen, were found to unexpectedly increase stent thrombosis [59].

Despite the bad news, there has been no shortage of interest in bioresorbable stent technologies [60]. A number of companies continue their journey to develop bioresorbable stents and to unravel the mechanisms potentially associated with their risk of thrombosis. Two mechanically relevant mechanisms, the device structure and "scaffold dismantling," were proposed as major contributors to the failures of bioresorbable stents. Experimental models and computer analysis demonstrated that the thickness of stent struts influences turbulence of blood flow after stent implant [61]. "Scaffold dismantling" implies the collapse of the bioresorbable struts inside the vessel lumen during the reabsorption process of struts [62]. Dynamic changes of such stents, in particular during the first 3 months, should be comparable to tissue repair and remodeling around stents.

Mechanobiology concepts explored in the stent design involve mostly computational approaches. Chen and colleagues have investigated the stent mechanobiology using fluid and solid mechanics, linking computational methods with animal outcomes. They showed that inflammatory responses were triggered by either decreased shear stress due to increased residence time for low-density lipids and inflammatory cells or increased compressive stress due to stent struts compressing the tissue [63]. Boyle et al. used computational modeling to study inflammatory responses to various stenting designs [64]. Their findings suggest that the patient sensitivity to the stent design can arise from the inflammatory phenotype; such a model may serve as a prediction tool for long-term efficacy of stents in the preclinical setting. Hence, the use of computational simulation technique suggests a platform to forecast mechanobiological responses of arteries to stenting [51–54]. As a future therapeutic option in the clinical setting, computational outcomes could suggest alternative treatment requirements in replace of stenting for patients with high inflammation.

Regarding the mechanobiological effect of stent designs, the stent strut, length, and diameter are the main design parameters examined, in terms of their influences on wall shear stresses and vascular damage [65–68]. In general, longer implanted stents increase the rates of in-stent restenosis and thrombosis. Choi et al. observed patients treated with a stent length of $\geq$32 mm had a greater risk of restenosis than those treated with a stent of $<$32 mm [69]. This effect is speculated to be due to the increase in mechanical damage to the vessel wall during both the implantation and regular use of the longer stent segments in the body, causing inflammatory factors to the site of injury as well as the increased proliferation, migration, and aggregation of VSMCs. As a result of this trend, stenting of only the most severely damaged portions of a target blood vessel has been proposed in order to circumvent the shortcomings of longer bare-metal stents [70]. The diameter of the target blood

vessel has also been linked to increased restenosis rates, in particular when the vascular diameter was less than 3 mm, likely due to flow disturbance.

Overall speaking, stent mechanobiology studies, which have involved computational approaches and in vitro models, with inputs from animal models and clinical data, provide insights into the influences of fluid–solid mechanics on stent outcomes. The goals of such studies are to understand mechanisms, predict clinical outcomes, and improve geometrical designs of stents. So, what is lacking now and should come next? First, though underlying cellular and molecular mechanisms are always suggested in these studies to justify the results, limited experimental studies have been done to unravel such mechanobiological mechanisms. Therefore, despite incremental improvements in stent designs, the fundamental causes of restenosis underlying the success or failure of vascular stenting are still poorly understood and should be the focus of future stent mechanobiology studies. More cellular and molecular investigations might in turn promote and verify mechanobiological models as a predictive tool for the long-term efficacy of stents. Second, the matrix materials of stents may play yet-to-be-determined roles in stent mechanobiology. For example, will the stiffness of the stent coating materials alter the rate of in-stent restenosis and/or thrombosis? [71–73]

13.3.2 Vascular Graft

Vascular grafting is yet another major option for long-term therapy of occluded vessels, besides stent. Grafts such as coronary artery bypass, peripheral artery bypass, and vascular access, are vascular prostheses employed through surgery in the replacement of blood vessels. Large-diameter grafts made of Dacron and Teflon have been successfully deployed by surgeons, but grafts for medium and small diameter vessels often failed in clinical trials [74]. The current gold standard for vascular grafts with a diameter less than 6 mm are autologous vessels such as saphenous veins and internal thoracic arteries [75]. However, these grafts have limited availability. To overcome this, tissue-engineered vascular grafts (TEVG) are vastly investigated. The past and current states as well as future directions of TEVG and various approaches to generate TEVGs with their results in in vivo studies and clinical trials have been extensively reviewed [74, 76].

Important to the TEVG development are the biomechanical, biophysical, and biochemical cues of the materials used for TEVG. Scaffold materials range from decellularized tissues to natural and synthetic polymers [77, 78]. These polymers are versatile in chemical and mechanical properties. The main goals of the TEVG scaffold development have been the resistance to thrombosis and stenosis by forming a confluent luminal endothelium layer and being mechanically compliant like native vessels [79]. The properties of scaffold materials must be tailored to meet the technological needs. To that end, developed TEVG technologies include: (1) pre-seeding a scaffold material with vascular endothelial cells (VECs), VSMCs or stem cells, which is followed by cell culture in vitro under mechanically active environments for weeks to create vessel-equivalent grafts, (2) culturing TEVG

in vivo, often in the subcutaneous location or peritoneal cavity using the body as a bioreactor, and (3) self-assembly approaches, which do not employ supporting scaffold in TEVG creation but rather rely on cell production of tissue materials [80–83]. In particular, different forms of self-assembly approaches that have been developed include sheet-based tissue engineering, which involves layering a 2D cell sheet, rolling it around a mandrel and then forming into a tubular shape, as well as microtissue aggregation and cell printing, both of which assemble microtissues or cells layer by layer to build up a tubular structure encouraging ECM production and tissue maturation.

As the development and/or maturation of TEVG are often performed under mechanically active culture environments such as pressurized fluid perfusion, mechanobiological mechanisms have been studied extensively [84]. One example is the mechanical homeostasis, which examines the crucial roles of mechanics along with macro- and microstructures of scaffolds and tissues. Such mechanisms are essential to the rational design of not only micromechanical properties toward scaffold microstructure (e.g., fiber diameter, elasticity, alignment, pore size, and topography), and macromechanical properties (e.g., graft diameter, geometry, stiffness, material, strength, and fatigue), but also material degradation and biochemical properties (e.g., surface chemistry and bioactivity) [85, 86].

To study TEVG mechanobiological mechanisms or to optimize TEVG processes, the cumbersome, manual laboratory procedures for TEVG manufacturing, with difficulty to be scaled out, have been recognized as a major bottleneck and a cost driver of the graft device development. Thus, digital tissue engineering, or so-called in silico (modeling) technology, has emerged [87, 88]. In silico technology ranges from data-driven technologies to mechanistic modeling technologies. The former uses efficient data generation methods such as high-throughput modality, where only an experimentally generated model is used; the latter is developed based on concepts and hypotheses informed by current biological knowledge and insights. Though mechanistic models may provide insight into the system under consideration, the models are difficult to parameterize. Furthermore, the complexity of the biological processes is often too high, or an understanding of the process is too limited in order to describe them fully by mechanistic models [89, 90]. Because of their black box nature, data-driven models, on the other hand, provide less insight into the system but are straightforward to develop. An example is to use high-throughput material devices with the appropriate statistical and digital tools to quantify and integrate the results [91]. Ultimately, hybrid strategies, resulting in the formation of a cross talk and integration between data-based and mechanistic models for TEVG may provide an efficient framework to gain the best of both worlds for models usable in clinical settings [92].

13.3.3 Regenerative Medicine

In recent years, arterial regeneration (often in the setting of TEVG) and microvascular regeneration have become the main streams in the studies of cardiovascular

regeneration. The primary goal is to regenerate functional vascular tissue or network in situ, with minimal processing in vitro. This thus requires one to recapitulate the complex structure and function of native vessels, in a state of quiescent homeostasis [93]. Such a state may be achieved by presenting optimal inflammatory, physical, and biomechanical microenvironments for cells. These local microenvironments are constituted by the initial scaffold parameters but will change over time due to scaffold degradation and new tissue formation under hemodynamic conditions. Temporal understanding and control of these processes is one of the main current challenges for in situ regeneration. Consequently, the development of appropriate models is required in order to predict and tune the regenerative process, and to assess the robustness of the technique depending on patient demographics. To this end, mechanistic modeling of mechanobiology for in situ regeneration has been developed [16, 92, 94]. One example is growth and remodeling models, which were used for designing combinations of scaffold parameters in order to achieve evolving luminal radius and graft compliance matched to that of the native vein [16].

To accelerate the regenerative process and ensure success, the biological and mechanical cues in regenerative scaffolds and the cell types present are key determinants. The biomaterial-driven regenerative process starts upon implantation, by interplay between host immune cell, stem/progenitor cells, tissue cells, and scaffold microenvironment in the hemodynamic environment [95–97]. Regenerative scaffolds not only should have adequate mechanical properties to resist against hemodynamic loadings but also have the potential to remodel or grow into a native-like vascular structure. Thus, selecting an appropriate scaffold material receives the most attention in in situ vascular regeneration. Natural polymers do not generally meet the requirements for bearing mechanical load and controlled biodegradation, but they do not activate chronic inflammation or toxicity. In contrast, the properties of synthetic polymers may be manipulated for potential needs, but they can have unexpected inflammatory responses. In terms of cells possibly involved in in situ regeneration, although the underlying mechanism is yet unclear, the cell type can be highly diverse, ranging from immune cells such as granulocytes, monocytes, macrophages, lymphocytes, and mast cells, to stem cells such as mesenchymal stem cells and vascular progenitor cells, and to vascular cells such as VECs and VSMCs. Therefore, the precise control over the in situ regeneration requires the considerations of various cell–scaffold interactions.

Maintaining a delicate balance among scaffold degradation, tissue repair, and vascular regeneration, via appropriate mechanotransduction is key to the success of in situ regeneration. Rapid resorption should be balanced by timely mechanotransduction for tissue remodeling into physiological-like vessels with biomimetic ECM production. In particular, timely resolution of inflammation in vivo is critical. Naito et al. characterized the time course of ECM development during in situ graft remodeling, showing an initial surge in the production of fibrillary collagen, possibly isolating the polymer [98]. Such collagen I-rich scar tissue formation was reduced upon degradation of the scaffold, after which vascular ECM proteins (e.g., GAGs, elastin, and collagen type IV) increased proportionally, and the mechanical properties of the graft converged to regenerating those of the

native vessels [99, 100]. On the other hand, insufficient regeneration, with merely reparative mechanism or even adverse remodeling (e.g., calcification), can be related to delayed degradation and/or lack of regenerative signals [101]. A paradoxal challenge during tissue repair around scaffolds is the roles of M2 macrophages and fibrocytes in both tissue regeneration and excessive fibrosis, which is dependent on a delicate balance of resorption, biochemical and mechanical factors [102]. In the future, applying developmental principles may guide the materials' designs and improve parametric studies for in situ regeneration [103, 104]. An additional regenerative challenge for the future is a valvular structure such as an aortic valve.

On the molecular level, vascular regeneration research has shifted the focus from the quantity of regenerated ECM constituents (e.g., collagen) for sufficient strength, to the ECM quality (particularly the elastin content and structure) for arterial-like elasticity. Indeed, a major difference between fibrous graft and regenerated graft such as vein graft is the elastin network [105]. However, the extremely challenging task is the restoration of a highly organized native elastic network. The hemodynamic loading, inflammatory milieu, and signaling molecules present during vascular remodeling can all significantly hamper the elastin expression or network formation.

In summary, the formation of regenerated vascular tissues and the degraded biomaterials at the onset of implantation should be well-balanced to avoid chronic inflammation due to the prolonged presence of foreign materials and avoid loss of structural integrity due to fast degradation, respectively. Therefore, in order to optimize the vascular regeneration process under hemodynamic forces, mechanobiological mechanisms and modeling tools are important for establishing scaffold design principles over parameters, such as material degradation, surface chemistry and topography, fiber diameter and alignment, and mechanical properties.

13.3.4 Medications

Vascular diseases, if not properly treated, may progress quickly, continuously increasing the workload of the heart and eventually leading to heart failure. In the clinic, prescribed medications are often used to treat vascular diseases and heart failure by lowering the blood pressure, lessening the heart workload, and improving the flow circulation. To that end, often used are blood thinners, anticoagulants or vasodilators, including beta-blockers (e.g., carvedilol, metoprolol, bisoprolol), digoxin (e.g., lanoxin), hydralazine and nitrates (e.g., Apresoline, Nitrobid, Imdur, Isordil), ACE inhibitors (e.g., lisinopril, captopril), and angiotensin receptor blockers (e.g., losartan). A combination of medicines is often used to manage the patient's condition. But there are still tremendous needs for new or improved cardiovascular medications or combinations.

In the development of new cardiovascular drugs, staggeringly high drug attrition rates in clinical trials for cardiovascular diseases have resulted in enormous time, cost, and risk. It is now recognized that many failures toward new treatment development and mechanistic understanding can be attributed to the disease

heterogeneity and lack of predictive disease models. Cell and rodent models represent traditional research methods to discover a new biological signaling mechanism or to test a new medication. However, both models fail to faithfully recapitulate certain disease characteristics, particularly mechanobiological characteristics, of clinical diseases. Using the inputs from precise disease measures and mechanistic modeling, the 3D biomanufacturing process can generate novel bioengineering models, which may ultimately recapitulate various pathologic abnormalities of clinical vascular diseases, including hemodynamic forces, vessel microstructures, and reconstruction of ECM properties and other vessel features [106]. These novel disease-specific vascular models, considering biomechanical stimulation of cells, can provide great insights into drug-based therapy.

These bioengineering models, in particular disease-specific ones such as the aneurysm model, atherosclerosis model, or pulmonary hypertension model, can be instrumental to the mechanobiological investigation of drug effects on the cardiovascular system and the discovery of novel therapeutic targets. To discover new therapeutic targets or medications via mechanobiological mechanisms, two benchtop model approaches exist: one introduces 3D biomimetic cell cultures and the other uses flow devices [107]. The latter further evolve into a lab-on-chip platform of drug screening for comprehensive therapeutic efficacy study of microenvironmental impact [108].

These two types of benchtop models can be combined into a fluid 3D vascular model for better prediction of the effects of molecular targets or drugs [109, 110]. For example, 3D in vitro microfluidic devices, in which one can seed VECs to form perfusable vascular networks, have been developed [111]. The inherent similarity (i.e., vessel geometry, ECM composition and dimensionality, cellular architectures, flow profiles) between in vivo microvascular networks and these 3D in vitro vascular models provides an attractive approach to begin to investigate in-depth questions related to drug–tissue interactions. Measurements may include the drug effects on the multifaceted roles of VECs, including their roles as a selective barrier for macromolecules, regulatory function during vascular remodeling via the secretion of growth-promoting and inhibiting molecules, modulating blood coagulation through the production of both pro- and anti-coagulating factors, mediating inflammatory responses via the regulation of cytokine releasing surface proteins, and regulate VSMCs contraction through the release of vasodilators and constrictors.

Further integration with computational models for disease-specific or even personalized models Integration of fluid models further expands our ability to model sophisticated biological processes (e.g., thrombosis and stenosis) for detailed molecular mechanistic characterization of the mechanotransduction processes. For example, on these devices, irregularities in flow, shear stress, and cyclic loading can compound with other risk factors such as genetics, biochemical factors, and lifestyle to induce endothelial dysfunction and pathophysiological states such as thrombosis, atherosclerosis, and other complications [112, 113]. The future may see a more sophisticated integration of bioengineering mechanobiological models, computational and benchtop models, being customized to each cardiovascular disease profile

for the drug discovery or for maximizing drug regimens for a certain group or an individual.

13.3.5 Disease Management

Aside from the traditional cardiovascular disease management using lifestyle changes, medications, and devices, the revolution that vascular mechanobiology can bring to the patients suffering from cardiovascular diseases will include disease management and prevention, a currently underdeveloped field.

As Benjamin Franklin advised, "an ounce of prevention is worth a pound of cure." The incidence of cardiovascular diseases increases markedly with advancing age. In fact, age is the strongest predictor of cardiovascular risk in most risk estimates [114, 115]. It is largely due to arterial stiffening. Arterial stiffness is primarily determined by the intrinsic elastic properties of the arterial wall. Structural changes in the arterial wall, particularly decreased density of elastin with corresponding increases in collagen content, are thought to play a major role in age-associated increases in central arterial stiffness. The ability to modify arterial stiffness over a period of days or weeks is thought to be because of, at least in part, modulation of the contractile states and intrinsic stiffening of the vascular smooth muscle cells in the arterial wall [116]. It has been well established that regular aerobic exercise is associated with reduced cardiovascular events and mortality, because it modifies arterial stiffness [117, 118]. Aerobic exercise has been shown to improve arterial compliance/stiffness in healthy young and middle/older persons and is an effective treatment for cardiometabolic diseases and vascular dysfunctions associated with advancing age.

Another example of applying mechanobiological principles to cardiovascular disease management is external counter pulsation therapy (EECP) of refractory angina [119]. It uses three sets of pneumatic cuffs that sequentially contract during diastole, increasing aortic diastolic pressure, augmenting coronary blood flow, and central venous return. EECP improves anginal symptoms and exercise tolerance in coronary artery disease patients. It is also safe and beneficial in patients with symptomatic stable congestive heart failure.

Despite clinical uses, the precise mechanisms underlying the beneficial effects of both physical activity and EECP are unclear. It has been postulated that the benefits of physical activity are through both vasoactive substances and modulations of vessel elastin/collagen content [115], while the benefits of EECP are mediated through nitric oxide- and vascular endothelial growth factor-mediated vasodilatation and angiogenesis, respectively [120, 121]. In order to make these mechanotherapies more clinically accessible on a larger scale, future investigations can apply mechanotransduction mechanism to study how the type, magnitude, frequency, and timeline of mechanical forces influence vessels of varied stiffness on the molecular, cell and tissue levels, and correlate these findings to clinical outcomes in disease prevention and management.

13.4 Vascular Mechanomedicine: Perspectives and Challenges

"Mechanotherapy" and "mechanomedicine" are two relatively new terms, referring to the use of mechanobiology principles by integrating biochemistry, mechanics, and clinical aspects. Mechanotherapy involves physical therapy applying engineering interventions [122], while mechanomedicine broadly focuses on the field encompassing studies of the pathology and treatment of various diseases based on the knowledge obtained from mechanobiological studies [123, 124]. Mechanomedicine seeks to understand molecular, cellular, tissue, organ, and individual responses to mechanical stimuli, and then to apply the gained knowledge to improve health. The fundamental understandings of the collaborative roles between biochemistry, physiology, and mechanics offer innovative translational perspectives for improving the effectiveness and efficiency of modern medicine. The use of mechanobiology as a discovery tool for novel treatments has already motivated some collaborative efforts between the clinical and engineering fields [125]. Those synergies are necessary to complement the basic mechanobiology science and to elevate it to effective clinical solutions, opening a door to a new era of pharmacological and engineering interventions. Overall, mechanomedicine holds the great potential to innovate clinical treatment, cardiovascular devices, vascular regeneration and drug discovery, disease prevention, and management.

While mechanobiological studies have already contributed immensely to the understanding of cardiovascular pathologies, only a few have been translated into clinical solutions. Major challenges include the complexity of interpretation of biological data, the assessment of the isolated and synergistic roles of mechanosensitive molecules in the disease process, the identification of effective, but safe, molecular inhibitors aimed at blocking the mechanobiological cascade, and the design of effective procedures and devices to normalize blood flow.

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