Chapter

The Role of Fibroblasts in Atherosclerosis Progression

Tadeja Kuret and Snežna Sodin-Šemrl

Abstract

The following chapter addresses vascular fibroblasts in a healthy, quiescent state, as well during vascular inflammation, focusing on atherosclerosis. The development of atherosclerosis, an inflammatory disease of medium- and large-sized arteries, has traditionally been viewed as an "inside-out" mechanism, with prominent roles of the innermost layer of the artery, consisting of endothelial cells. However, emerging evidence suggests a new paradigm of "outside-in" mechanism, including an earlier role for fibroblasts, constituents of the outermost adventitial layer of the artery. Phenotypic and functional changes of fibroblasts in adventitia may even occur prior to, or alongside endothelial activation. Activated adventitial fibroblasts, implicated in atherosclerosis progression, begin to transform into myofibroblasts, upregulate production of different proinflammatory cytokines, chemokines, growth factors, proteolytic enzymes, extracellular matrix proteins and reactive oxygen species, leading to extensive matrix remodeling, chemotaxis and recruitment of immune cells. Due to their suitable location for drug delivery systems, preventing fibroblast activation, modulating their activity or inducing myofibroblast dedifferentiation could represent a promising therapeutic approach for atherosclerosis regression.

Keywords: atherosclerosis, fibroblasts, inflammation, disease progression

1. Introduction

Fibroblasts are mesenchymal cells that are morphologically characterized as adherent, flat, elongated (spindle-shaped) cells with leveled, oval nuclei. One of their major roles is to produce and integrate structural proteins, such as collagen, elastin, and proteoglycans into the extracellular matrix (ECM) of most mesenchymal tissues and thus maintain their structural integrity [1].

In healthy arteries, fibroblasts can be found in the adventitia, the outermost layer of the vessel wall. Adventitial fibroblasts display numerous subtypes, even in a quiescent state, however, very little is known about their exact involvement in atherosclerosis development and progression [2]. Most of the attributed functions of adventitial fibroblasts have been largely extrapolated from findings describing fibroblasts in different tissues and organs, such as the skin. However, fibroblasts from different anatomic sites and tissues are functionally and phenotypically distinct. For example, cultured fetal and adult human skin fibroblasts derived from different anatomical sites expressed distinct transcriptional patterns of genes involved in extracellular matrix synthesis, lipid metabolism, and cell signaling pathways regulating proliferation, cell migration and fate determination [3].

The discovered topographic differences of fibroblasts might be connected to the positional memory since adult fibroblast maintain key features of the HOX gene pattern expression, established during embryogenesis. Indeed, many HOX genes that encode a family of evolutionarily conserved transcription factors, are differentially expressed in fibroblasts derived from different anatomical sites, indicating that fibroblasts from each topographic site express a unique HOX gene expression pattern [3, 4].

Fibroblasts are metabolically active cells that play a central role in, not only matrix maintenance and remodeling and regulating ECM, but also in managing interstitial fluid volume and pressure, new tissue formation and wound healing. They have been found to be associated with many connective tissue pathologies, either due to their direct implication in the disease mechanism or due to the resulting fibrosis associated with damage in other cell types [5]. Recently, novel mechanisms proposed a prominent role of fibroblasts also in the development and progression of atherosclerosis. Atherosclerosis is a chronic, fibro-proliferative disease of the arterial vessel walls that underlies the development of many cardiovascular diseases (CVDs) and affects the structure and function of the involved arteries [6].

Vascular inflammation, leading to atherosclerosis, has been traditionally viewed as an "inside-out" response, beginning with the activation of endothelium and an inflammatory response that spreads outwards, from the intima towards media and adventitia, ultimately forming fibrous plaque and damaging all three vessel wall layers [7, 8]. The classical mechanism of atherogenesis has been challenged recently with emerging evidence supporting a new hypothesis of an "outside-in" mechanism, in which vascular inflammation actually begins in the adventitia and progresses inward towards the media and intima [8, 9], suggesting a more prominent role of fibroblasts than previously thought.

So, in order to pinpoint, the potential role of fibroblasts during atherosclerosis progression, we need to first look at the arterial wall composition and function.

2. Vessel wall structure and fibroblasts

2.1 Arterial vessel wall structure

Characterization of the resident, stromal cell populations and subpopulations in a blood vessel is an important step in understanding cellular contribution to vascular development and disease. Since atherosclerosis is prevalently a disease of large- and medium-sized arteries [10], we will focus here on the description of the structure of these vessels and corresponding vascular stromal cell populations.

The walls of large- and medium-sized arteries are a heterogeneous three-layered structure consisting of the tunica intima, media and adventitia. Each layer is unique in its histologic, biochemical and functional properties and is differentially involved in maintaining vascular homeostasis and regulating the vascular response to stress or injury [8]. The tunica intima or innermost layer represents a monolayer of endothelial cells, which are in direct contact with the blood flow. The intima is separated from the tunica media by a basement membrane and an internal elastic lamina. The tunica media consists of multiple layers of vascular smooth muscle cells (VSMC). The tunica adventitia or the outermost layer is separated from the media by an external elastic lamina and represents the most complex layer of the blood vessel [11]. The adventitia is composed primarily of fibroblasts in a loose connective tissue matrix, and it also contains resident immune cells (e.g. dendritic cells, macrophages, mast cells), pericytes, small blood vessels with endothelial cells (*vasa vasorum*), several progenitor cells and adrenergic nerve cells (**Figure 1**) [12].

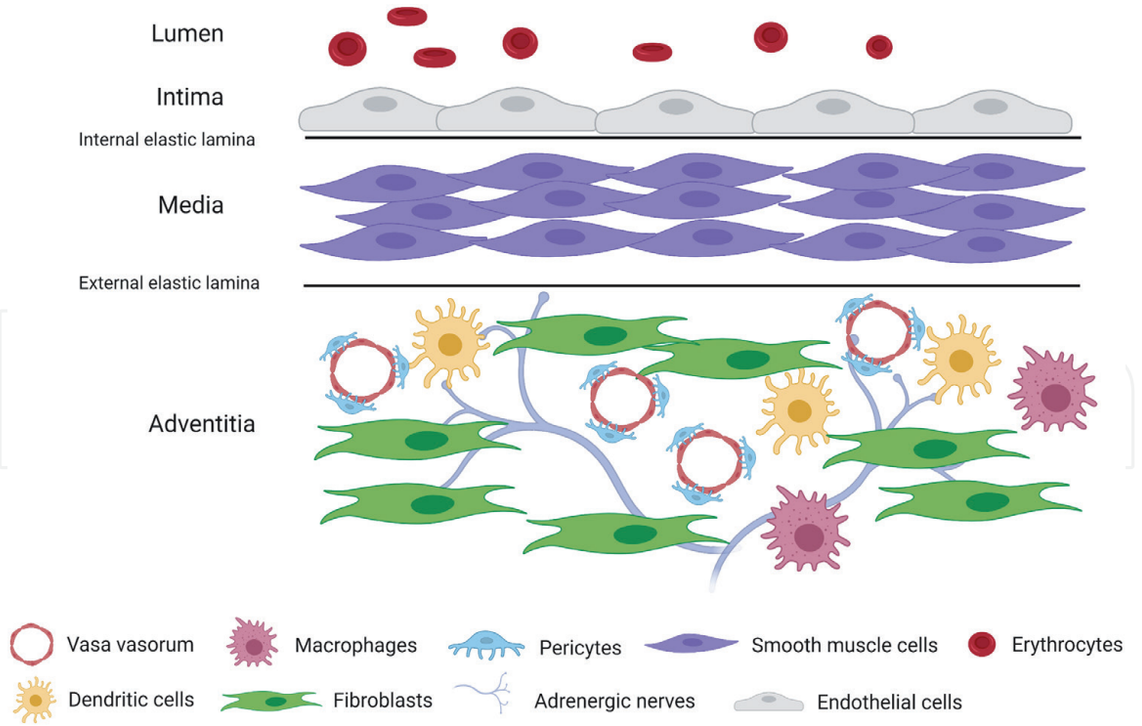

Figure 1.
Heterogeneous cellular composition of the vascular adventitia. Compared to arterial intima and media, which are composed of endothelial and smooth muscle cells, respectively, the arterial adventitia is composed of a variety of heterogeneous cell populations, including fibroblasts, immunomodulatory cells (e.g. dendritic cells, macrophages), vasa vasorum*, pericytes and adrenergic nerves. The figure was adapted from [13] and created using Biorender.*

For many years, adventitial cells were thought to have a limited physiological "static" function serving as structural support to the blood vessel, supplying oxygen and nutrients to the media of large vessels, and sustaining sympathetic innervation of the vessel wall [14]. However, latest *in vivo* and *in vitro* studies have shown that the adventitia represents a dynamic microenvironment that regulates both structural and functional properties of all three arterial layers [15]. The adventitia is an important source of cells that migrate towards the intima and media. For example, stem and progenitor cells that function and reside in the adventitia can transform into medial and intimal cells, such as VSMCs, and endothelial cells [16–18]. The adventitia of coronary arteries also contains cholinergic nerve terminals that release acetylcholine, diffusing to the intima layer of endothelium, where it induces the release of nitric oxide, causing VSMC relaxation and vasodilatation [19]. After stimulation with angiotensin II, adventitial fibroblasts can synthesize and release endothelin-1 (ET-1) that is important in mediating VSMC contraction [20]. The important role of adventitia is further supported by the role of *vasa vasorum* network, serving as a pipeline for inflammatory cell infiltration during vascular inflammation. In atherosclerosis, inflammation contributes to increased neovascularization and enhanced permeability of the adventitial *vasa vasorum*, allowing more inflammatory cells to enter the atherosclerotic plaque. Indeed, by suppressing the neovascularization of *vasa vasorum*, Moulton et al. [21] observed reduction in numbers of macrophages in atherosclerotic plaques and inhibition of atherosclerosis progression in experimental mice models [21]. Additionally, adventitial resident cells participate in initiation and regulation of vascular development, response to injury and tissue repair and thus, importantly contribute to disease development, especially intimal hyperplasia. This is mediated by their ability of responding to external physiological stress with intensive tissue repair or arterial remodeling [9, 16, 22]. Importantly, resident adventitial cells (e.g. fibroblasts) are

often the first cells in the vascular wall to become activated in response to hormonal and inflammatory stimuli, as well as environmental stress, such as hypoxia/ischemia and hypertension [8, 15].

2.2 Fibroblasts in healthy arterial vessel walls

Understanding the role of fibroblasts in normal and pathologic conditions is often obstructed by the lack of reliable and specific markers. The fibroblast is therefore still one of the most difficult cell types to define *in vivo*, likely due to their heterogeneity (multiple subtypes) and plasticity [23].

In a healthy artery, adventitial fibroblasts are found in a non-active, quiescent state, and are usually defined by their location in the vessel wall since they can be separated from the more generally recognized smooth muscle cell layer by an external elastic lamina [24, 25]. All currently used markers to identify fibroblasts, including vimentin, platelet derived growth factor receptor α (PDGFRα), fibroblast specific protein 1 (FSP1), discoidin-domain receptor, and prolyl-4-hydroxylase, are potentially problematic, as they are also expressed in other cell types and are not present in all fibroblasts [3, 26, 27]. Therefore, to identify fibroblasts, investigators have to rely on the lack of markers for other cell lineages (e.g. non-lymphoid, non-endothelium, and non-epithelium), along with morphologic, functional, and biochemical characteristics [3].

Adventitial fibroblasts show differences in morphology, size, function and activity in the healthy, as well as stressed conditions or disease states [15]. For example, An et al. [28] found two major fibroblast subpopulations in the adventitia of rat thoracic aorta. The two populations were described as epithelioid-like cells and spindle-like cells, however only epithelioid-like fibroblasts were sensitive to stimulation with angiotensin II, a hormone involved in the development of hypertension and atherosclerosis [28]. Studying fibroblasts from the adventitia of bovine pulmonary artery, Das et al. [29] concluded that numerous phenotypically and biochemically distinct fibroblast subpopulations can be found and only a selective increase in the number of resident fibroblast subpopulations with enhanced growth capability was observed under hypoxic conditions [29].

With the development and increasing popularity of single cell RNA sequencing technology, it might now be possible to find markers, specific to adventitial fibroblasts, as well as to characterize in depth, their subpopulations in normal and diseased states [30]. Kalluri et al. [31] explored the cellular atlas of healthy mice aortas using single cell RNA sequencing. They showed that fibroblasts represent approximately 33% of all aortic cells and were defined by higher expression of PDGFRα and collagens/collagen-binding proteins (e.g. Col1a1, Col1a2, Dcn, Lum) whereas the expression of VSMC-associated contractile proteins (e.g. Myh11, Cnn1) was reduced. These fibroblasts clustered into two subpopulations and are probably derived from the adventitia, however their exact location needs to be confirmed by immunohistochemistry or in-situ hybridization [31]. Another study using the high resolution single cell analysis approach, was performed by Gu et al. [32] in aortic adventitial cells from wild type and apolipoprotein E-deficient (*ApoE−/−)* mice. They determined four heterogeneous mesenchymal populations with differential gene expression suggesting potential functions in ECM organization, immune regulation and bone formation. Furthermore, interaction of resident, mesenchymal cells with immune cells was enhanced in the adventitia of *ApoE−/−* mice. These data revealed a heterogeneous cellular landscape of the adventitia and confirmed fibroblast variability present already in the healthy, quiescent state [32].

3. Adventitial fibroblasts in vascular pathology

3.1 Contribution of adventitial fibroblasts to vascular pathology

In response to injury or environmental stress, adventitial fibroblasts can become activated, displaying altered phenotypic and functional properties. Activated fibroblasts intensely proliferate and increase production and deposition of ECM proteins, as well as proinflammatory cytokines, chemokines, adhesion molecules, matrix metalloproteinases (MMPs) and tissue inhibitors of MMPs (TIMPs), and growth factors, such as vascular endothelial growth factor (VEGF) [13, 33]. These molecules directly affect the phenotype of other resident cells in the vessel wall, such as VSMC and endothelial cells, promote neointima formation, regulate *vasa vasorum* expansion and affect the recruitment of infiltrating immune cells [8, 24]. Despite an excessive proliferation of activated fibroblasts, no hyperplasia of adventitia is usually observed in vascular pathologies. It is likely that many different mediators can also activate replication repressor signals in fibroblasts to limit or control replication. For instance, protein tyrosine phosphatases that regulate growth factor signaling in vascular remodeling, are upregulated in adventitial fibroblasts in response to vascular injury presumably to mitigate proliferative responses [34]. It was also demonstrated that adventitial fibroblasts under hypoxic conditions activate protein kinase C-zeta and MAPK phosphatase-1 that repress proliferative signals and limit the proliferation of fibroblasts [35, 36].

Vascular fibroblasts may also produce large amounts of reactive oxygen species (ROS) that seem to regulate their proliferation and ECM deposition [37, 38]. Activated fibroblasts increasingly interact with other cell types in the arterial wall, such as endothelial cells and VSMC and regulate their functions, as well as recruit immune cells into the vessel wall [39]. After their activation, some fibroblasts differentiate into myofibroblasts that contain bundles of stress fibers and can be identified by expression of contractile proteins, such as α-smooth muscle actin (αSMA). Multiple stimuli in the microenvironment, such as mechanical stress, growth factors (e.g. TGF-β), proinflammatory cytokines (e,g, IL-1β, IL-6, TNF-α), adhesion molecules, and ECM molecules can cause differentiation of a fibroblast towards the myofibroblast phenotype. Myofibroblasts contribute to extensive remodeling, intimal hyperplasia, and luminal stenosis due to their invasion and migration into the intima and increased production and secretion of ECM proteins [40–43].

The recognition that fibroblasts are not only able to generate, but also to sustain inflammatory responses, provides insight into why vascular inflammatory responses, in certain situations, fail to resolve. It is suggested that chronic inflammation occurs due to dysregulated fibroblast activity in which they fail to switch off their inflammatory programme, leading to the inappropriate survival and retention of leukocytes within inflamed tissue [44]. It is also clear that the activated adventitial fibroblasts play an important role in regulating *vasa vasorum* growth, which can contribute to ongoing vascular remodeling by acting as a conduit for delivery of inflammatory and progenitor cells [45].

Recent studies shed light on the implication of adventitial fibroblasts in different vascular pathologies, characterized by arterial remodeling and neointimal formation [43]. One of the most persistent findings in experimental *in vitro* and *in vivo* models is intensive adventitial remodeling, found very early in response to vascular injury or stress [46–48]. Adventitial remodeling in the vasculature has been characterized by increased proliferation of fibroblasts, which appear to be the first cells in the vessel wall that respond to different stimuli by their activation [49].

Direct evidence of fibroblast migration into the intima was provided in a study, performed by Li et al. [50], in which primary syngeneic adventitial fibroblasts were transduced with β-galactosidase (*LacZ*) and introduced into the adventitia of rat carotid arteries immediately after baloon injury. MRNA expression of *LacZ* and in situ enzymatic activity of β-galactosidase were detected in the media and the neointima, 7 days after injury. On the contrary, in the arteries that were not injured, the expression of *LacZ* and enzymatic activity of β-galactosidase were restricted to the adventitia [50]. Similar findings were later reported by Han et al. [51] showing that adventitial fibroblasts migrated to the media and intima on seventh day after balloon injury in the rat carotid artery. The results were obtained by direct labeling of adventitial fibroblasts using *in vivo* gene transfer technique, as well as transmission electron microscopy [51]. Furthermore, Dutzmann et al. [52] discovered that early activation of adventitial fibroblasts after wire-induced injury in C57BL/6 mice stimulated their proliferation and release of proinflammatory cytokines and growth factors, and the subsequent proliferation of VSMC, resulting in neointima formation [52].

Pulmonary artery hypertension (PAH) is one of the vascular pathologies, characterized by extensive arterial remodeling and neointima formation, in which fibroblasts were shown to play an important role [53]. For example, in the neonatal bovine hypoxic PAH model, adventitial fibroblasts were found to undergo the earliest and most significant increases in proliferation, among all the vascular wall cell types [54]. Fibroblasts derived from experimental hypoxia-induced PAH and patients with PAH, display a hyperproliferative, apoptosis-resistant, and proinflammatory phenotype, defined by increased production of IL-6, IL-1β, CCL2/MCP1, CCL12/SDF1, VCAM1 and osteopontin [13, 55]. Moreover, when naïve bone marrow derived macrophages were exposed *in vitro* to conditioned medium generated by adventitial fibroblasts from human PAH patients and hypoxia induced PAH animals, they increased the transcription of several markers of activation (e.g. Cd163, Cd206, Il4ra and Socs3) [56]. These findings suggest that activated adventitial fibroblasts in PAH secrete various soluble factors required for macrophage activation and polarization leading to the propagation of inflammation from adventitia towards media and intima, supporting the "outside-in" hypothesis [53].

3.2 The role of fibroblasts in atherosclerosis and potential fibroblast-targeted therapy

Several findings suggest a role of fibroblasts in all stages of atherosclerosis, from initial phase to fibrous cap and plaque formation. It is becoming evident that adventitial cells, including adventitial fibroblasts are one of the first cells to respond to injury and become activated in the initial stage of atherosclerosis, even before the formation of atherosclerotic lesions, supporting the new "outside-in" hypothesis [46, 48].

Studies have shown that in various presentations of CVDs, the adventitia becomes heavily populated with multiple immune cell types, including monocytes, macrophages and T-cells, while adventitial fibroblasts proliferate increasingly and differentiate into myofibroblasts [33, 57–59]. Furthermore, several studies reported on increased *vasa vasorum* neovascularization in early atherosclerosis prior to the development of endothelial dysfunction [60, 61]. Neovascularization may act as a pipeline, allowing the entry of immune cells into the site of injury, as the density of *vasa vasorum* is highly correlated with the extent of inflammatory infiltrates in *ApoE−/−* mice [21]. These studies indicate that increased neovascularization of *vasa vasorum* in adventitia, that promotes inflammatory response and plaque angiogenesis, can occur before the endothelial activation and dysfunction in the intima [8]. Adventitial

fibroblasts can regulate the growth and neovascularization of *vasa vasorum* through the release of soluble angiogenic growth factors, such as VEGF, TGF-β and platelet-derived growth factor (PDGF). Furthermore, with the release of chemokines, such as monocyte chemoattractant protein (MCP1), fibroblasts facilitate infiltration of circulating leukocytes, further increasing the growth of the *vasa vasorum* and perpetuating the inflammatory response [37].

Xu et al. [62] investigated the role of adventitial fibroblasts in atherosclerotic lesion formation by comparing the characteristics of adventitial fibroblasts from *ApoE−/−* and wild type mice. They found αSMA expressing adventitial fibroblast in *ApoE−/−* mice, but not in wild type mice. The gene expression of collagen I and collagen III was upregulated in adventitial fibroblasts from *ApoE−/−* mice, compared to the wild type mice. Furthermore, adventitial fibroblasts from *ApoE−/−* mice synthesized more TGF-β, MCP1, and PDGFβ and exhibited proliferatory and migratory properties [62]. MCP1 is important in regulation of migration and infiltration of monocytes into the vessel wall, which differentiate into macrophages, form foam cells and importantly contribute to fatty streak formation [63]. The effects of adventitial fibroblasts are also ascribed to ROS produced by adventitial fibroblast NADPH oxidases that play important roles in neointimal formation and growth in vascular pathologies, including atherosclerosis [37]. Xu et al. [64] studied ROS production and expression of NADPH oxidase subunit p47phox in the hyperlipid diet-induced atherosclerosis in the *ApoE−/−* mouse model. The activated fibroblasts from aortas of *ApoE−/−* mice displayed upregulated NADPH oxidase activity, augmented ROS production, and increased p47phox levels, compared with wild-type mice. ROS production was also associated with the increased proliferation and migration of adventitial fibroblasts. In addition, silencing of p47phox decreased the proliferation and migration of fibroblasts from *ApoE−/−* mice [64]. Fibroblasts, in response to ROS proliferate and release a number of growth factors and other mediators that influence vascular function, including ET1, PDGF, endothelial growth factor (EGF), fibroblast growth factor2 (FGF2), prostaglandin H2 (PGH2), and cyclophilins [65, 66]. In addition, ROS can also stimulate phenotypic switch of VSMC from the contractile to the proliferative and migratory one, suggesting that fibroblasts can indirectly influence other cell types inside the vessel wall [38].

In the initial phase of fibrosis, injured arteries start with tissue remodeling, and the formation of initial fibrous plaque actually represents a protective process; however, as in all chronic inflammatory conditions, fibrotic components in the plaque produce surplus levels of cytokines and proteolytic enzymes, causing excessive remodeling and tissue damage [59]. In advanced stages of atherosclerosis, fibrosis plays a central role and fibroblasts are the major cell population involved in remodeling of ECM in the fibrous plaque [59]. The most important functions of fibroblasts in progressed atherosclerosis include regulation of the inflammatory response, ECM protein production, and maintenance of the structural integrity of the plaque as well as regulated balance of MMP production, to enable beneficial tissue remodeling, alongside preventing plaque rupture, for instance [59, 67]. The potential role of fibroblasts in the development of atherosclerosis is shown in **Figure 2**.

Regulation of fibroblasts activities might be beneficial in controlling or reversing the progression of atherosclerosis, hence, the adventitial fibroblast may be an attractive target for therapeutic intervention. Furthermore, the location of the adventitia as the outermost arterial layer makes it suitable for drug delivery and gene therapy [24]. It has already been shown that local adventitial drug delivery into coronary arteries results in better efficiency compared to luminal or intimal delivery methods [71]. Low efficiency of gene transfer to cells in adventitia by intraluminal administration has been reported and efficient transfection of these

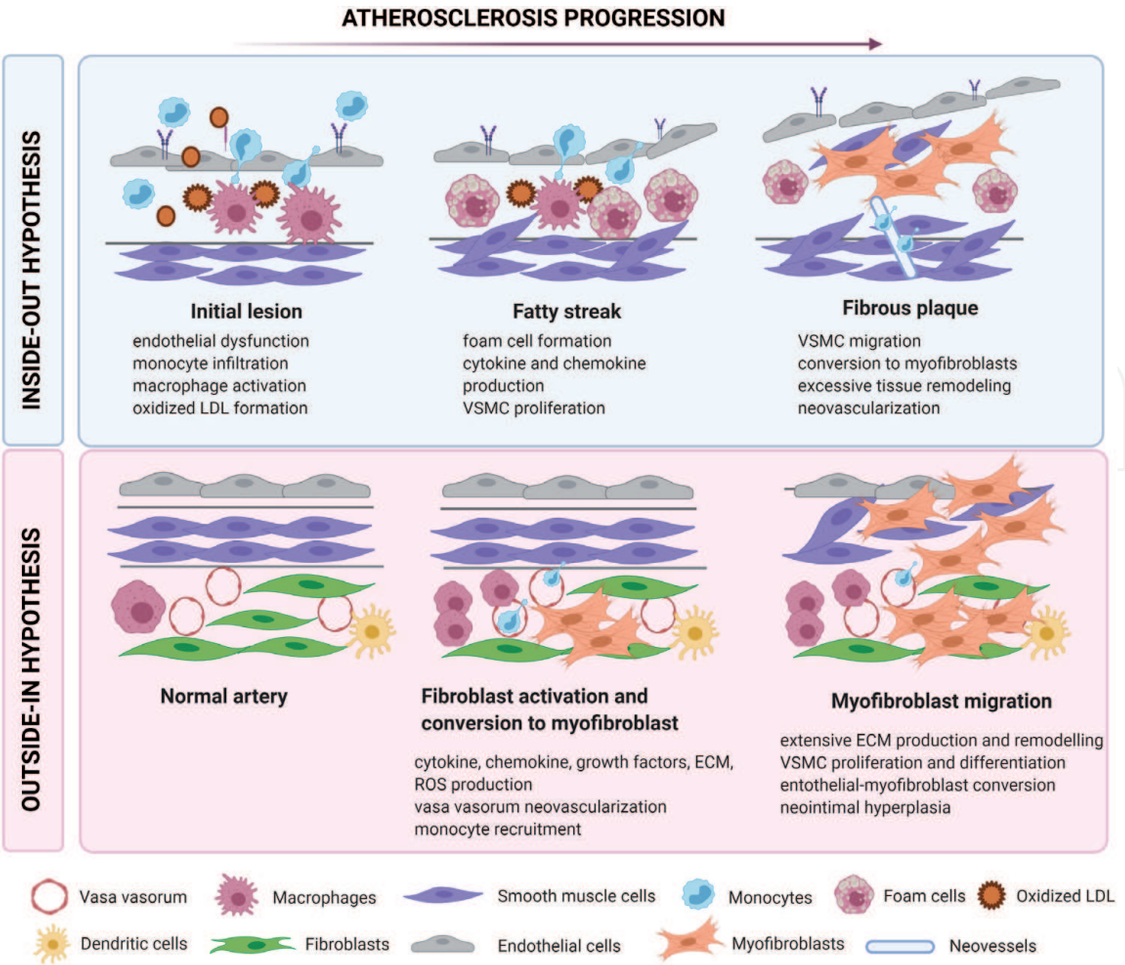

Figure 2.
The potential role of fibroblasts during atherosclerosis progression. Emerging evidence suggests that adventitial fibroblasts are activated in the initial stage of atherosclerosis, supporting the new "outside-in" hypothesis, which proposes that vascular inflammation begins in the adventitia and progresses inward towards the media and intima. In contrast, the original "inside-out" hypothesis of atherosclerosis proposes that the inflammatory response spreads from the intima outward towards adventitia with a more prominent role of endothelial and smooth muscle cells. The figure was adapted from [68–70] and created using Biorender. Legend: ECM, extracellular matrix; LDL, low density lipoprotein; ROS, reactive oxygen species; VSMC, vascular smooth muscle cells.

cells is achieved only when endothelium is denuded or damaged. In atherosclerotic arteries, intimal hyperplasia might present an additional barrier for intraluminal delivery. To overcome these problems, delivery from the adventitial side might be considered. On the other hand, numerous attempts to transfect or deliver the therapeutic agents to the media from the adventitial side of large blood vessels have failed because of the impenetrable nature of the external elastic lamina, separating adventitia from the media layer. This barrier hence allows for selective adventitial delivery and specific targeting of cells residing in adventitia [72].

Perivascular delivery of an adenoviral vector expressing a NADPH oxidase inhibitor in the rat carotid artery adventitia significantly reduced neointimal formation after balloon angioplasty. This specific vector targeted adventitial fibroblasts, and it did not affect VSMC in the media [73]. Targeting proteins, expressed by activated fibroblasts could attenuate vascular inflammatory responses and ameliorate vascular disease, including atherosclerosis. Recently, it was discovered that inhibition of expression or activity of fibroblast activation protein (FAP), that is expressed in activated but not in quiescent fibroblasts and was found to be associated with atherosclerotic plaques, can attenuate progression of atherosclerosis by increasing plaque stability in experimental mice models of atherosclerosis [74].

Reports on animal models of cardiomyopathy have indicated that reversibility of fibrosis was possible, with losartan (a selective angiotensin II type 1 receptor antagonist), which suppressed TGF-β expression [75, 76]. However, angiotensin II type 1 receptor is expressed also on VSMC and TGF-β can be produced by multiple cell types, in addition to fibroblasts.

A substantial number of adventitial fibroblasts can differentiate into myofibroblasts during initial stages of atherosclerosis, upon the influence of proinflammatory cytokines, chemokines adhesion molecules, growth factors and ECM proteins [33, 77]. For example, TGF-β induces the transition of a fibroblast into the myofibroblast by stimulating αSMA expression and collagen production [41, 42]. These highly proliferative αSMA-positive cells were found to be widely distributed in atherosclerotic plaques [24]. However, myofibroblasts in the atherosclerotic plaques can derive from multiple other sources, including VSMC [39], the endothelial to mesenchymal transition [78], as well as resident macrophages [79] (**Figure 3**). Myofibroblasts can contribute to changes in the function and structure of the vessel wall that occur during atherosclerosis (i.e arterial remodeling) due to their contractile properties and enhanced ECM protein production [80]. Myofibroblasts migrate from the adventitia to the media and intima and contribute to intimal hyperplasia [81, 82].

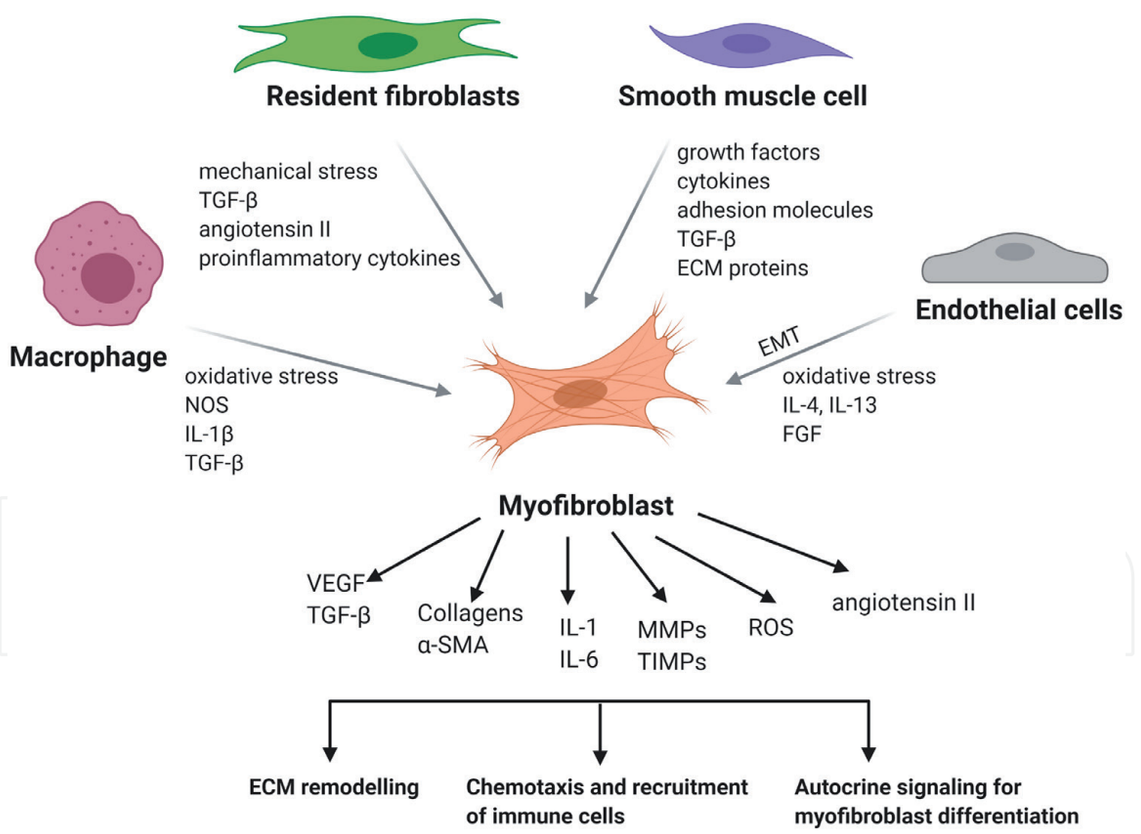

Figure 3.
Various cells from the arterial wall can differentiate into myofibroblasts in atherosclerosis. Macrophages, resident fibroblasts, smooth muscle cells and endothelial cells can differentiate into myofibroblasts, depending on tissue microenvironment and inflammatory mediators. Myofibroblasts are characterized by increased expression of α-smooth muscle actin and synthesize and release large amounts of ECM proteins, growth factors, proinflammatory cytokines, proteolytic enzymes and their inhibitors, as well as reactive oxygen species. They are responsible for extensive ECM remodeling, increased chemotaxis and recruitment of immune cells and provide signals for further myofibroblast differentiation. The figure was adapted from [41] and created using Biorender. Legend: αSMA: α-smooth muscle actin; ECM: extracellular matrix; EMT, endothelial mesenchymal transition FGF: fibroblast growth factor; IL: interleukin; NOS: nitric oxide synthase; MMPs: matrix metalloproteases; ROS: reactive oxygen species; TGF-β: transforming growth factor-β; TIMPs: tissue inhibitors of MMPs; VEGF: vascular endothelial growth factor.

Although myofibroblasts were previously considered to be terminally differentiated cells, their capacity for dedifferentiation, defined as the loss of αSMA, is now well recognized and necessary to resolve idiopathic pulmonary fibrosis [83]. Several factors, such as prostaglandin E2 (PGE2), nuclear factor erythroid 2-related factor2 (Nrf2) and FGFs have shown the ability to dedifferentiate established lung and corneal myofibroblasts and might be promising therapeutic targets also for adventitial myofibroblasts in atherosclerosis [84]. For example, treatment with PGE2 was shown to inhibit proliferation and collagen I expression in fibroblasts extracted from histologically normal lung tissue [85]. In TGF-β or ET-1-activated lung myofibroblasts, treatment with PGE2 induced a dose-dependent decrease in αSMA and collagen I expression that was associated with inhibition of focal adhesion kinase signaling [86]. Sulforaphane, a Nrf2 activator, induced myofibroblast dedifferentiation in cultured lung fibroblasts from patients with idiopathic pulmonary fibrosis, as well as inhibited TGF-β-mediated profibrotic effects [87]. Corneal myofibroblasts that were grown in the presence of FGF1 or FGF2 and heparin reduced expression of αSMA, TGF-β receptors, and cadherins, thus promoting the quiescent fibroblast phenotype [88]. FGF21 has been shown to induce angiotensin-converting enzyme 2 (ACE2), and thus inhibit vascular remodeling, hypertension and fibrosis, and when stimulating with adiponectin, FGF21 may inhibit aortic inflammation in atherosclerosis, as well as decrease cardiac dysfunction in myocardial infarction, attenuate smooth muscle cell proliferation and migration and lower macrophage oxidizes low-density lipoprotein uptake [89]. In addition, Fgf21 knock-out mice have been reported to show impaired lipid metabolism [90]. So, FGF21 has shown promise, as a potential therapeutic for atherosclerosis, but would need further investigation in regard to its effects on leukocytes and activities of its receptors.

3.3 The origin of fibroblasts in atherosclerosis

Fibroblast heterogeneity in quiescent and diseased state might be a result of their various origins, as well as plasticity, since they can transform into different cell types, subsequently to their adaptation to stress or injury. Evidence suggests that fibroblasts involved in atherosclerosis may originate from different adventitial mesenchymal stem/progenitor cells, however, recent studies revealed they can also originate from VSMC or endothelial cells [30].

Several distinct progenitor/stem cell populations with the capacity to differentiate into endothelial cells, VSMC, fibroblasts, and macrophages reside in a specialized niche in the adventitia at the media-adventitia border [91]. A population of vascular progenitor cells in the aortic adventitia of *ApoE−/−* mice expressing the stem cell markers Sca1 and CD34 was described that might differentiate to vascular fibroblasts [92]. However, the exact identity of adventitial progenitor/stem cells is still controversial, since fibroblast also have the ability to acquire stem cell properties by upregulating Sca1 [93–95]. Moreover, mesenchymal stem cells and fibroblasts are similar in terms of morphology and share the expression of a number of surface markers, such as CD90, CD73, CD105, vimentin and FSP1. Some researchers therefore suggest that these adventitial stem cells positive for Sca1 are actually fibroblasts [30, 93]. For example, Tang et al. [96] showed that 40% of Sca1-positive adventitial stem cells also express PDGFRα, found to be expressed on the surface of fibroblasts [96]. Using single cell RNA sequencing, Gu et al. [32] identified four mesenchymal clusters in the aortas of *ApoE−/−*, as well as wild type mice, but did not annotate them as stem or progenitor cells. However, one of the clusters displayed high expression of Sca1 indicating stem cell properties [32]. The separation between adventitial fibroblasts and progenitor/stem cells seem to be much smaller than previously thought and cell transition of stem cells into fibroblasts or vice versa appears to be

common in atherosclerosis [30]. It would be interesting to further investigate how this transdifferentiation would affect the pathological process of atherosclerosis and whether it could be targeted to reverse atherosclerosis progression.

The evidence that fibroblasts might originate from VSMC came from Wirka et al. in 2019 [97]. They reported that VSMC can transform into fibroblast-like cells (termed "fibromyocytes"), found in the arteries of *ApoE−/−* mice, as well as atherosclerotic human coronary arteries. Fibromyocytes expressed lower levels of VSMC differentiation markers and increased expression of genes, associated with fibroblast cluster, such as lumican, decorin and biglycan. However, fibromyocytes were transcriptionally different from the fibroblasts indicating that either they will further dedifferentiate into fibroblasts or they might represent another distinct population of fibroblasts [97]. Furthermore, it is currently still unclear, whether fibroblasts might also transform into VSMC.

Endothelial cells can serve as a possible source of atherosclerotic fibroblasts since they can undergo endothelial-mesenchymal transition, promoting atherosclerosis progression [98]. This was elegantly shown in 2016 by Evrard et al. [78] using a tamoxifen-inducible endothelial lineage tracking system in *ApoE−/−* mice. After 8 weeks of high fat diet, the mouse atherosclerotic plaques consisted of one third endothelial-derived cells positive for FAP and a range of other fibroblast markers. These cells further expanded in number in advanced atherosclerotic plaques. *In vitro* modeling confirmed that endothelial-mesenchymal transition is driven by TGF-β signaling, oxidative stress and hypoxia that are all characteristic for atherosclerosis. Furthermore, the extent of this transition correlated with an unstable plaque phenotype in humans, driven by altered collagen and MMP production that might be associated with clinical events [78].

Whether functional differences between fibroblasts, originating from different sources exist and what is their exact contribution to development and/or progression of atherosclerosis, still remain two important and open questions.

4. Conclusions

Recently, the well-established "inside-out" hypothesis of atherosclerotic development and progression has been revitalized to involve an "outside-in" component, including an earlier role for fibroblasts in the tunica adventitia layer. It is now thought that early "outside-in" events, with more prominent roles of fibroblasts in adventitia may even occur prior to, or alongside endothelial activation. Adventitial fibroblasts involved in atherosclerosis comprise a very heterogeneous population, due to their differential origins (mesenchymal stem/progenitor cells, smooth muscle cells, endothelial cells, macrophages) and an extensive repertoire of possible cell transitions into αSMA^+ myofibroblasts, or even back to stem cells. It is thought that when resident fibroblasts begin to transform into αSMA^+ myofibroblasts, this allows for intense release of VEGF, TGF-β, collagens, IL-1, IL-6, MMPs, ROS and angiotensin II, among other factors, that could lead to perpetual autocrine differentiation and inflammatory, proliferative states, responsible for ECM remodeling, chemotaxis and recruitment of immune cells. Their heterogeneity and consequently, a general lack of specific markers, both contribute to difficulties in studying their exact phenotypes and functions in atherosclerosis. The recent development and accessibility of single cell RNA sequencing technology provides new opportunities to find answers to the remaining questions in an unbiased manner. Furthermore, modulating fibroblast activity, preventing their activation or inducing myofibroblast dedifferentiation in atherosclerotic arteries could represent a promising therapeutic approach for atherosclerosis regression. The plasticity of

the atherosclerotic plaque may reform and dynamically remodel many times, before either rupturing or, on the other hand, stabilizing with a plaque cap or even regressing, depending on, among many factors, also molecules in the microenvironment (micro-exoproteome) and presence of certain cellular profiles that may help lean the process either way.

Conflict of interest

The authors declare no conflict of interest.

Author details

Tadeja Kuret[1,2] and Snežna Sodin-Šemrl[2,3]*

1 Institute of Cell Biology, Faculty of Medicine, University of Ljubljana, Slovenia

2 FAMNIT, University of Primorska, Koper, Slovenia

3 Department of Rheumatology, University Medical Centre Ljubljana, Slovenia

*Address all correspondence to: ssodin1@yahoo.com

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