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The role of extracellular vesicles in neointima formation post vascular injury

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Abbreviations: adventitial fibroblasts, (AFs); coronary artery bypass grafting, (CABG); drug eluting stents, (DES); endothelial cell, (EC); endothelial progenitor cell, (EPC); early-sorting endosome, (ESE); endosomal sorting complexes required for transport, (ESCRT); extracellular vesicle, (EV); extracellular matrix, (ECM); intraluminal vesicles, (ILVs); late-sorting endosome, (LSE); matrix metalloproteinase, (MMP); mesenchymal stem cell, (MSC); multivesicular bodies or endosomes, (MVBs or MVEs); percutaneous coronary intervention, (PCI); vascular smooth muscle cells (VSMC); vein graft disease, (VGD); vein graft failure, (VGF)

Abstract

Pathological neointimal growth can develop in patients as a result of vascular injury following percutaneous coronary intervention and coronary artery bypass grafting using autologous saphenous vein, leading to arterial or vein graft re-occlusion. Neointimal formation driven by intimal hyperplasia occurs as a result of a complex interplay between molecular and cellular processes involving different cell types including endothelial cells, vascular smooth muscle cells and various inflammatory cells. Therefore, understanding the intercellular communication mechanisms underlying this process remains of fundamental importance in order to develop therapeutic strategies to preserve endothelial integrity and vascular health post coronary interventions. Extracellular vesicles (EVs), including microvesicles and exosomes, are membrane-bound particles secreted by cells which mediate intercellular signalling in physiological and pathophysiological states, however their role in neointimal formation is not fully understood. The purification and characterisation techniques currently used in the field are associated with many limitations which significantly hinder the ability to comprehensively study the role of specific EV types and make direct functional comparisons between EV subpopulations. In this review, the current knowledge focusing on EV signalling in the neointimal formation post vascular injury is discussed.

Key words: intimal hyperplasia, neointimal formation, extracellular vesicles, coronary artery bypass grafting, percutaneous coronary intervention, vein graft failure, arterial restenosis.

1. Vascular injury and intimal hyperplasia/neointimal formation

Intimal hyperplasia is defined as a reparative process of cell population expansion within the innermost layer of an artery or vein, where different coagulation and inflammatory factors and cells stimulate proliferation and migration of vascular smooth muscle cells (VSMCs) from the tunica media [1]. Neointimal formation or thickening driven by intimal hyperplasia is a common pathological feature observed in different vascular pathologies including atherosclerosis, vein graft failure (VGF) following coronary artery bypass grafting (CABG) surgery (Figure 1) and arterial restenosis following percutaneous coronary intervention (PCI) (Figure 2) [1–3].

CABG and PCI are both invasive revascularisation procedures used to treat severe cases of advanced atherosclerotic disease [4,5]. However, despite advancements in coronary artery interventions, both PCI and CABG procedures are associated with considerably high rates of clinical stenosis arising from intimal hyperplasia [6–9]. In the stent era, the incidence of arterial restenosis following the advent of drug eluting stents (DES) has been reduced to less than 10% with both the frequency and extent of intimal hyperplasia being reduced [10,11]. In comparison, between 10% and 25% of vein grafts have been reported to fail within 1 year of surgery primarily due to the development of intimal hyperplasia with the overall vein graft patency rate decreasing to approximately 50% at 10 years due to a combination of factors including enhanced graft intimal hyperplasia and the development of atherosclerosis-like lesions within the graft [12].

1.1. Mechanical injury-associated vascular changes and thrombosis

The pathophysiological mechanisms underlying the development of VGF and arterial restenosis post-stent implantation include similar molecular and cellular processes involved in vessel remodelling and neointimal formation that occur largely due to induced-vascular injury [1,6–8]. In the case of CABG procedures, early graft failure occurs predominantly due to technical mishandling leading to acute thrombosis [13,14], although the “no touch” technique for harvesting the venous conduit has reduced this incidence [15]. Immediately after harvesting, the venous tissue undergoes a period of ischaemia and reperfusion following engraftment which causes significant endothelial cell (EC) damage and subsequently EC activation resulting in a prothrombotic phenotype in the first month. As a result of endothelial denudation, extracellular matrix (ECM) components underlying the endothelial layer become exposed to the circulation, tissue factor is released and prostacyclin and nitric oxide (NO) bioavailability is reduced, all of which contribute to enhanced platelet activation, fibrin deposition and ultimately thrombus formation [6,16]. Engraftment in the arterial circulation exposes the venous graft to augmented pressure, flow and longitudinal and circumferential shear stress which may additionally cause damage to ECs and VSMCs consequently triggering an inflammatory response (Figure 1) [17–19]. Similarly, the mechanical damage induced by stent implantation and/or balloon angioplasty also leads to EC injury, platelet activation and thrombus formation along with an inflammatory reaction

(Figure 2) [8,20]. Indeed, delayed endothelial recovery after the deployment of DES has been recognised as a major contributing factor to late stent thrombosis [21–23] with strut coverage being an important determinant of the risk of thrombosis [22]. However, it is important to note that the implementation of dual anti-platelet therapy has significantly reduced the risk of thrombosis after both CABG and stent implantation [14,24]. Re-endothelialization following venous or arterial injury begins quickly after the initial damage with proliferating ECs observed during the first 14 days after vein graft implantation or an arterial balloon injury in experimental animal models [25,26]. Repairing the damaged endothelial layer is thought to play an important part in limiting vascular remodelling where enhanced re-endothelialisation has been associated with decreased neointimal formation in experimental rat models [27,28]; thus, highlighting the process of endothelial cell monolayer restoration as potential therapeutic target in vein graft disease (VGD) and arterial restenosis.

Figure 1. Schematic representation of neointimal formation and accelerated atherosclerosis leading to vein graft failure. Within hours of vein graft implantation, the endothelial cell layer becomes damaged leading to fibrin deposition at the site of injury. Circulating inflammatory cells including neutrophils, monocytes, and lymphocytes start to accumulate and infiltrate the intima. Similarly, VSMCs in the media become activated and start migrating towards the intima where they are further stimulated to proliferate by different growth factors and cytokines released by cells in the vessel wall. This process of cell population expansion and extracellular matrix deposition results in neointimal formation. Under atherogenic conditions, years after graft implantation, accelerated atherosclerosis can develop due to a continuous lipid uptake by macrophages turning into foam cells. As a result, due the presence of dying cells and cholesterol depositions, a necrotic core is formed. The formation of neovessels is also observed in the late stage of VGD.

1.2. Intimal hyperplasia/neointimal formation

Weeks after implantation, the vein graft undergoes structural vessel wall remodelling and intimal thickening in order to adapt to its new arterial environment, where moderate intimal hyperplasia is physiological and is required for appropriate arterialisation and long-term graft patency [29–31]. ECM turnover, VSMC proliferation, migration and apoptosis within the vessel wall post coronary interventions are all important aspects of the development of intimal hyperplasia and

1 consequently narrowing and stiffening of the vessel (Figure 1) [1,6,8]. One of the
2 theories explaining the origin of the observed large number of highly proliferative
3 VSMCs within the intima suggests that under certain conditions fully matured
4 quiescent 'contractile' VSMCs migrate from the media to the intima where they
5 change their phenotype to a dedifferentiated, proliferating 'synthetic' phenotype
6 (Figure 1) [19,32,33]. It is believed that, at the site of injury, activated ECs, platelets,
7 monocytes and leukocytes express and/or secrete pro-inflammatory molecules such
8 as E- and P-selectin, IL-6, IL-8, platelet-derived growth factor (PDGF), transforming
9 growth factor beta 1 (TGF- β 1) and basic fibroblast growth factor (bFGF) thereby
10 stimulating local inflammation and VSMC dedifferentiation, proliferation and
11 migration - cellular processes that are all crucial for the development of intimal
12 hyperplasia (Figure 1) [26,34–44]. It has previously been shown that targeting those
13 growth factors and/or their receptors *in vivo* affects the development of both venous
14 and arterial neointimal hyperplasia [45–48]. Early inward remodelling appears to be
15 predictive of subsequent VGF [49] and limiting intimal hyperplasia in both *ex vivo*
16 and *in vivo* studies has been demonstrated as a potentially successful approach to
17 improving graft patency [50,51]. In addition to cell proliferation and migration, ECM
18 production and deposition are important in neointimal formation. Matrix
19 metalloproteinases (MMPs) have the ability to substantially modify the ECM
20 surrounding vascular cells [52]. During the structural remodelling of the vessel wall,
21 vein grafts produce various MMPs including MMP2 and MMP9 capable of degrading
22 collagen and other ECM components [53,54]. MMPs have also been implicated in
23 the pathogenesis of in-stent restenosis after coronary stent implantation [55,56].

24
25 In clinical settings, a therapeutic strategy targeting local VSMC proliferation and
26 migration with DES coated with specific inhibitors of cell proliferation such as
27 plumericin, xanthohumol, paclitaxel and sirolimus, has been shown to successfully
28 limit intimal hyperplasia after PCI in both experimental animal models and also in
29 clinical studies [57–59]. Although, the introduction of DES greatly improved PCI as a
30 treatment option for advanced atherosclerotic disease, it has been previously shown
31 that drugs used in this technology could indiscriminately target both ECs and VSMCs
32 leading to severe side effects because of impaired re-endothelialisation, increasing
33 the risk of late thrombosis [22].

1 Another important aspect of the development of intimal hyperplasia is the immune
2 system which is involved in all phases of VGD and arterial restenosis [6,60,61].
3 Early post-graft implantation damage of the vessel wall results in increased selectin-
4 mediated leukocyte adhesion on vascular endothelium and subsequent endothelial
5 dysfunction and vessel wall infiltration [26,62]. Similarly, following PCI, neutrophils
6 start to infiltrate the vessel wall through the damaged endothelial layer [63]. With
7 their ability to secrete a wide range of pro-inflammatory molecules such as cytokines,
8 growth factors and proteases, including MMP2 and MMP9, activated neutrophils
9 have the capacity to function as major determinants of diverse pathologies including
10 neointimal formation [64–67]. Additionally, shortly after graft or stent implantation,
11 monocytes start to accumulate in the vessel wall where they become macrophages
12 actively contributing to the inflammatory process by secreting various cytokines and
13 growth factors mainly targeting VSMCs [68–70]. Macrophages become the most
14 common type of inflammatory cells present in the vein graft which may be able to
15 serve as an early marker of graft occlusion [69]. The late stages of vein graft failure
16 and arterial restenosis are characterised by the development of accelerated
17 atherosclerosis and accelerated neoatherosclerosis respectively [71–73].

Figure 2. Schematic representation of neointimal formation and accelerated neoatherosclerosis leading to arterial restenosis. Stent implantation and/or balloon angioplasty cause mechanical damage to the vessel wall leading to EC injury, platelet activation and potentially thrombus formation. Additionally, an inflammatory reaction is also triggered leading to immune cell infiltration of the vessel wall. Activated VSMCs in the media start migrating towards the intima. Growth factors and cytokines released by cells within the vessel wall further promote VSMCs phenotype switching resulting in the accumulation of highly proliferative VSMCs. Accelerated neoatherosclerosis can also develop due to a continuous lipid uptake by macrophages turning into foam cells, eventually forming a necrotic core.

2. Biogenesis and heterogeneity of extracellular vesicles (EVs)

Extracellular vesicles (EVs) are membrane cargo carriers secreted by cells which have the capacity to exchange various components between cells, including nucleic acids, lipids and proteins, thus acting as signalling vehicles in both physiological and pathophysiological conditions. It is now recognised that cells secrete a highly heterogeneous population of EVs varying in size, composition and subcellular origin (Figure 3), which has introduced many challenges in the process of their characterisation [74–76]. Initially, EVs were thought to have a simple protein-

recycling function, as originally reported for the transferrin receptor in reticulocytes maturation [77,78]; however, it is now clear that EVs have multiple functions all depending on not only their cellular origin but also metabolic status and environment of the cells [79]. Based on current knowledge of the biogenesis of EVs, they can be generally categorised into two distinct classes: ectosomes, including microvesicles, microparticles and other larger vesicles, and exosomes. Ectosomes are generally larger in size (~50-1000 nm in diameter) compared to exosomes (~40-160 nm in diameter) with a significant overlap in size ranges between the two classes (Figure 3) [79]. The terms ectosomes, microvesicles and microparticles are commonly used interchangeably across studies. There is now evidence that individual cell types modify the biogenesis of EVs depending on their physiological state, resulting in the release of EVs with specific lipid, protein and nucleic acid compositions [75].

The biogenesis of exosomes involves the formation of multiple endosomal structures including early-sorting endosome (ESE) followed by late-sorting endosome (LSE) and finally intracellular multivesicular bodies or endosomes (MVBs or MVEs) that contain several intraluminal vesicles (ILVs) which are ultimately secreted to the extracellular space as exosomes [75,79]. Several distinct mechanisms appear to be involved in the biogenesis of exosomes which inevitably contributes to the heterogeneity of secreted exosomes [74]. The components of the endosomal sorting complexes required for transport (ESCRT) system have been shown to be involved in the biogenesis of ILVs, by not only affecting exosome secretion but also the selection of specific exosomal cargo [80]. Exosomes could also be formed in an ESCRT-independent manner through the generation of ceramide by neutral type II sphingomyelinase [81,82] and/or the engagement of the tetraspanin family of proteins, more specifically CD63 [83,84]. Additionally, other tetraspanins such as CD81, CD82 and CD9 appear to also be directly involved in the organisation of specific cargoes into exosomes [85,86].

The biogenesis of microvesicles, on the other hand, involves a direct blebbing and pinching of the plasma membrane resulting in the release of vesicles into the extracellular space [74,75]. Similarly to exosomes, different cargo molecules, including protein and nucleic acids, are also selectively packaged into microvesicles [87]. Although microvesicles are not formed in endosomal compartments, the

1 endosomal machinery is still utilised in the formation of microvesicles with both the
2 ESCRT system and the Ras-related GTPase ADP-ribosylation factor 6 (ARF6) being
3 involved in the process [88,89]. Nabhan *et al.* described a mechanism of
4 microvesicle budding in an epithelial cell model resulting from the interaction of
5 arrestin domain-containing protein-1 (ARRDC1) with the late endosomal protein -
6 tumour susceptibility gene 101 (TSG101) [88]. It was suggested that due to this
7 interaction and the re-localisation of TSG101 from endosomal membranes to the
8 plasma membrane, localized changes in membrane curvature are induced resulting
9 in the release of microvesicles that contain TSG101, ARRDC1, and other cellular
10 proteins but no markers of late endosomal compartments [88]. Another possible
11 protein-driven mechanism of vesicle budding is crowding of proteins unrelated to
12 membrane curvature at the cell periphery and lateral pressure generation through
13 protein-protein interactions which may also contribute to membrane shape changes
14 resulting in microvesicle formation [90]. Additionally, plasma membrane lipid
15 components have been also found to play a fundamental role in vesicle budding
16 processes [74], with phosphatidic acid (PA) specifically demonstrated to be directly
17 involved in generating membrane curvature in cooperation with brefeldin-A ADP-
18 Ribosylated Substrate (BARS) [91]. Interestingly, microvesicle fission and release
19 from the cell surface has been shown to occur following activation of the ARF6-
20 mediated signalling cascade [89] where ARF6 has also been specifically implicated
21 in ILV formation and exosome biogenesis and possibly LSE trafficking [92].

22
23 Exosomes and microvesicles are both commonly found in various biological samples
24 including conditioned culture medium (CCM), serum, plasma, broncho-alveolar
25 lavage fluid, saliva and urine [93–98] as they may be produced by the same cell type
26 [99]. Although microvesicles and exosomes are generated through distinct pathways
27 within the cell, common intracellular mechanisms and components are involved in
28 the biogenesis of both EV types which further complicates the possibility of
29 distinguishing between the different vesicle subpopulations [75]. Tetraspanins, for
30 example, have been proposed as potential exosome-specific markers as members
31 of the tetraspanin family including CD53, CD63, CD37, CD81 and CD82 have been
32 found to be enriched in the membrane of exosomes [100,101]. For many years,
33 CD63 has been considered a classical exosome marker due to its typical association
34 with intracellular compartments of endosomal/lysosomal origin [102]. However, as

1 many tetraspanins are widely distributed in the plasma membrane, their presence in
2 other subpopulations of EVs is highly possible. It has been suggested that CD63 and
3 CD81, the classical exosome markers, are also highly enriched in exosome-like
4 vesicles which originate through plasma membrane budding and could not be
5 distinguished from exosomes [103] with one study demonstrating their presence in
6 both microvesicles and exosomes secreted by three different cell lines [104]. CD9
7 has also been detected in larger EVs suggesting that it cannot be used as an
8 exosome specific marker [105]. Additionally, a recent proteomic study has identified
9 four subcategories of small EVs (sEVs) pelleting at high ultracentrifugation speed
10 where some sEVs can be characterised by the co-enrichment of CD63, CD81 and
11 CD9 whereas others entirely lack those protein markers further suggesting that
12 tetraspanins alone could not be used to distinguish between different types of EVs
13 [106]. Nevertheless, tetraspanins appear to play an important role in target cell
14 binding for exosomal message transfer with exosomes exposing the tetraspanin-
15 integrin (Trans8-alpha4) complex shown to be selectively taken up by ECs and
16 pancreatic cells [107]. Such findings suggest the existence of subpopulation of
17 exosomes expressing specific tetraspanins that may be particularly important in the
18 context of vascular remodelling processes in different cell types, including in the re-
19 endothelization stage post vascular injury.

20
21 Furthermore, the existence of exosomal subpopulations defined on the basis of
22 distinct size ranges has also been demonstrated using refined fractionation methods
23 [108]. Analysis of the microRNA (miRNA) content of exosomes revealed that not all
24 exosomes contain the same amount of certain cargo and miRNA-rich subpopulations
25 of exosomes may exist within a population [109]. EV populations secreted by cells
26 could be highly heterogeneous owing to not only their size and content but also
27 subcellular origin and biological effect on recipient cells [79,110,111]. The current
28 limitations of purification and characterisation techniques hinders the ability to
29 comprehensively study the role of specific EV types and make direct functional
30 comparisons between EV subpopulations [110].

31
32 Throughout this review, primarily the term EVs will be used referring to both
33 ectosomes/microvesicles and exosomes since different EV isolation methods and
34 analytical procedures have been used in the various studies discussed which may

have potentially resulted in the isolation of heterogeneous population of EVs that include exosomes. Therefore, it is important to consider that some of the findings discussed in this review may reflect those of exosomes mixed with other EVs.

Figure 3. Biogenesis of EVs. There are two main categories of EVs – ectosomes and exosomes. Ectosomes are also commonly called microvesicles or microparticles and these are formed through plasma membrane budding with a size range of ~ 50-1000 nm in diameter. Exosomes are formed through the endosomal pathway within a series of endosomes including ESE, LSE and finally MVB/MVE which contain multiple vesicles released in the extracellular space as exosomes (size range ~40-160 nm in diameter). Abbreviations: ESE, early sorting endosome; LSE, late sorting endosome; MVB, multivesicular body; MVE, multivesicular endosome;

3. The role of EVs in intimal hyperplasia/neointimal formation

Since pathological neointimal growth post invasive coronary intervention develops as a result of a complex interplay between molecular and cellular processes involving different cell types including ECs, VSMCs and various inflammatory cells, understanding the intercellular communication mechanisms remains of fundamental importance in order to develop therapeutic strategies to preserve endothelial integrity and vascular health. The role of EVs in intercellular communication as well as their therapeutic potential in neointimal formation after vascular injury has been investigated to some extent, however, the specific involvement of distinct EV types

remains to be fully elucidated. The most commonly used *in vivo* models for studying neointimal hyperplasia following vascular injury caused by PCI or vein grafting include carotid artery ligation mouse model and wire injury mouse model [112–114].

3.1. Platelet-derived EVs

In human blood, EVs derived from platelets account for 70-90% of all circulating EVs whereas only 5% originate from ECs, red blood cells and monocytes [115]. Platelet-derived EVs, for example, have been found to influence the biology of both vascular ECs and VSMCs. Interestingly, although platelets lack a nucleus, many of the proposed contrasting effects of platelet-derived EVs can be attributed to transfer of miRNAs to recipient cells [116–118]. *In vitro* studies showed that platelets secrete inflammatory signals in the form of EVs which can directly interact with resting VSMCs causing them to switch to a proinflammatory synthetic phenotype resulting in increased VSMC proliferation, migration and also monocytic cell recruitment [119]. Additionally, platelet-derived EVs have also been found to influence the function of vascular ECs by increasing EC surface activation [120,121], an effect that could at least partially be explained by miR-320b transfer via EVs [121].

Further highlighting their enhanced state of activation in response to platelet-derived EVs, ECs start to secrete various pro-inflammatory cytokines including IL-8, IL1 and IL-6 [120]. The proapoptotic effects of platelet-derived EVs was demonstrated *in vitro* where platelet-released miR-223 was shown to promote EC apoptosis induced by advanced glycation end products via targeting insulin-like growth factor 1 receptor [122]. Similarly, miR-142-3p transfer to ECs via platelet-derived EVs caused an increase in EC apoptosis possibly by suppressing *BCL2L1* (BCL2 Like 1) gene expression [123]. However, when mir-142-3p mimic and inhibitor were both assessed *in vitro*, the authors reported that EC apoptosis appeared to be suppressed. A further study also investigating the effects of miR-142-3p transfer via platelet-derived EVs demonstrated an increase in EC proliferation *in vitro* through targeting *BCLAF1* (BCL2-Associated Transcription Factor 1) gene [124]. Such inconsistent findings may be due to the fact that a single miRNA usually regulates the expression of multiple genes and miR-142-3p has been found to have many apoptosis-related targets including *BCL2L1*, *BCLAF1* and *PRKCA* (Protein Kinase C Alpha) [123,124]. Furthermore, in these studies, platelet-derived EVs were isolated via centrifugation

at a relatively low speed of 20,500g [123,124] which may have resulted in the isolation of highly heterogeneous population of EVs [106] potentially containing a wide-range of bioactive molecules with various functions. Additionally, the isolated EV populations were also not comprehensively characterised to allow more confident interpretation of the results regarding the type of EV responsible for the observed effects. Nevertheless, these findings suggest that platelet-derived EVs are important message carriers that can deliver different signals to vascular cells. As such they have the potential to interfere with EC homeostasis and VSMC behaviour [119–121]. Platelets play an important role in the early stages of VGF due to acute thrombosis as well as the development of neointimal formation after vascular injury [7,125]. Additionally, platelet-derived EVs have been associated with 50-100 times higher coagulation activity in comparison to activated platelets [126]. Taken together these findings suggest that improving our understanding of how platelet-derived EVs may be involved in the development of vascular pathologies/remodelling processes such as intimal hyperplasia/neointimal formation, remains of significant importance.

3.2. Endothelial cell-derived EVs

As ECs are important mediators of vessel remodelling processes, EC homeostasis requires to be tightly regulated in order to avoid inappropriate activation and a shift towards a pro-inflammatory state which could have deleterious effects [127]. EC monolayer restoration post mechanical injury of the vessel wall represents a critical step in limiting the neointimal growth [27] and endothelial-derived EVs have been shown to be involved in the regulation of vascular remodelling processes [128,129]. Over the years, the exact mechanisms of EC repair after injury have been the focus of many studies with different research approaches being explored including strategies focusing on the investigation of the regenerative capacity of endothelial progenitor cells (EPCs) [130–134] as well as strategies investigating the complex interaction of mature vessel-wall ECs with other surrounding cells [6,26,67,68]. More recently, understanding the role of both EC- and EPC-derived EVs in vascular remodelling processes has been of central importance for many studies.

The well-known therapeutic benefit of EPCs is related to their ability to differentiate into ECs, however, recently EPCs have also been shown to exert other therapeutic effects via additional indirect mechanisms involving the production of EVs [135,136].

1 The first study comparing the EV populations secreted by mature ECs and EPCs,
2 including their potential therapeutic effects in the context of vascular injury, was
3 published in 2019 [137]. The authors reported that, although, EPCs released
4 significantly more EVs within the exosome range compared to mature ECs, no
5 significant difference was detected between the two EV populations in terms of
6 morphology, size distribution, and presence of protein markers as well as in their
7 ability to promote EC proliferation and migration *in vitro* and early stage re-
8 endothelialization after endothelial injury *in vivo* [137]. Additionally, another group
9 reported that a similar population of EPC-secreted EVs promote vascular repair post-
10 mechanical injury *in vivo* by inducing changes in the expression levels of various
11 angiogenesis-related molecules (endothelial nitric oxide synthase, eNOS; vascular
12 endothelial growth factor A, VEGFA and vascular endothelial growth factor receptor
13 2, VEGFR-2) in ECs, thus, enhancing their function [135]. Consistent with these
14 findings, Kong *et al.* also provided evidence from *in vitro* and *in vivo* studies
15 supporting the idea that EPC-derived EVs (within the exosome range) inhibit
16 neointimal hyperplasia by promoting EC proliferation and migration; however, when
17 the effect of the same EVs on VSMCs were studied *in vitro*, it was found that the EVs
18 had the opposite effect and stimulated VSMCs proliferation and migration [138].
19 These inconsistent findings regarding the protective effects of EPC-derived EVs in
20 the context of vascular injury and neointimal hyperplasia may be at least partially
21 related to differences in the experimental settings between the studies. Limited
22 mechanistic investigations along with the absence of comprehensive
23 characterisation of the EV populations used in the experiments are limitations for
24 these studies. The findings from a recent studies comparing different commonly
25 used EV isolation methods, suggests that the EV yield along with the depletion of
26 lipoprotein particles and protein contaminants are highly dependent on the method
27 used to isolate EVs [94,95]. The purity of EV preparations is an important factor that
28 can significantly affect both downstream biochemical characterisation of EVs and
29 also their potential effects [139,140]. Therefore, given that not only the
30 characteristics but also the content of EVs may be influenced by different cell culture
31 conditions and isolation techniques, findings reported by different studies need to be
32 carefully interpreted [141,142].

EC injury initiates the release of EC-derived EVs [143,144] and in this context, EC-derived EVs have also been shown to mediate endothelial regeneration and repair in experimental *in vivo* models of intimal hyperplasia [128]. Furthermore, there is now substantial evidence to suggest that ECs could also influence VSMC function through a paracrine pathway [129,145,146]. EVs are key transport vehicles for distinct functional miRNAs [147] meaning that their effect on recipient cells is highly dependent on their specific miRNA expression pattern which may essentially be influenced by the status of the cells that release them [128,148].

A number of miRNAs have been found to be highly expressed in ECs including miR-126, miR-146, miR-197, and miR-625 where miR-126 has been reported as the most highly enriched miRNA involved in the regulation of EC migration, cytoskeleton, reorganization, capillary network stability and cell survival [149]. Additionally, miR-126 has also been demonstrated to exert anti-inflammatory effects by directly inhibiting the expression of vascular cell adhesion molecule-1 (VCAM-1), which prevents leukocyte adhesion to ECs [150][151]. As a critical modulator of different aspects of EC biology and ultimately angiogenic signalling and vascular integrity, miR-126 represents a potentially exciting therapeutic target in the context of vascular remodelling post vascular injury. A recent study investigating the role of miR-126 in the setting of VGD demonstrated its vasculoprotective effects *in vitro* and *in vivo* [152]. In this study, miR-126 has been shown to promote EC migration and proliferation by targeting sprouty-related protein-1 (SPRED-1) and phosphatidylinositol-3-kinase regulatory subunit 2 (PIK3R2) which resulted in increased re-endothelisation and ultimately reduced neointimal formation *in vivo* [152]. Similarly, in the context of arterial restenosis, the implantation of miR-126 nanoparticles-conjugated stent significantly reduced the development of neointimal formation *in vivo* by potentially stimulating ECs proliferation and migration while inhibiting the proliferation and migration of VSMC via the regulation of SPRED1 and insulin receptor substrate-1 (IRS-1) respectively [103].

Interestingly, miR-126 is not only one of the most abundant miRNAs in EC but it is also highly expressed in EC-derived EVs [128,129,149]. It has been suggested that EC-derived EV-mediated delivery of functional miR-126-3p to target endothelial and VSMC leads to increased EC regeneration and inhibition of VSMC migration,

proliferation and neointimal formation after vascular injury (Figure 4) [128,129]. In their study investigating the effects of EC-derived EVs, Jansen *et al.* found that EC-derived EVs containing miR-126-3p promoted EC migration and proliferation *in vitro* and increased re-endothelialization *in vivo* by actively regulating SPRED1 [128]. A similar protective effect of miR-126-3p delivered through EC-derived EVs was also described in VSMC. A possible underlying mechanism was proposed involving EV-mediated transfer of miR-126-3p from EC to VSMCs resulting in inhibition of *LRP6* and *β-catenin* and a consequent reduction of VSMC migration and proliferation *in vitro*, and reduced neointimal formation *in vivo* [129]. Conversely, another group suggested that reduced activity of LRP6 in *LRP6^{R611C}* mice led to a pro-proliferative VSMC phenotype and aortic medial hyperplasia [153]. Although there are also clinical studies supporting the protective role of circulating EV-derived miR-126 against coronary restenosis and major cardiovascular events in patients with CAD [129,154], contradictory findings have been previously reported as independently of EV transfer miR-126 stimulates VSMC proliferation leading to neointimal formation [155]. While there is strong evidence to support the crucial role of EVs containing miR-126 in vascular protection, the potential influence of other miRNAs and/or bioactive molecules contained in the same EVs cannot be excluded and more comprehensive characterisation of the specific EV population is required to fully understand their physiological and pathophysiological functions in vascular biology. For example, it has been suggested that larger EVs (<1µm), and not exosomes, are responsible for the beneficial effects of miR-126 in the regulation of intimal hyperplasia [128,129,154]; however, in none of these studies were the EV populations comprehensively characterised by size, concentration and detection of specific protein markers to facilitate a clear conclusion regarding the type of transporting vesicle. Additionally, ultracentrifugation and electron microscopy are commonly used to isolate and confirm the presence of EVs respectively in order to compare the expression of different miRNAs between EVs of larger size (<1 µm) and exosomes (~100 nm). However, neither ultracentrifugation as a method of EV isolation can ensure the specific pelleting of exosomes only, nor can electron microscopy clearly discriminate exosomes from other non-vesicular nanoparticles of similar sizes which may limit the extent to which any inferences can be made [94]. Given that different EV types cannot be distinguished solely based on size [79], any

assumptions about the type of EVs responsible for the observed effects might be somewhat speculative.

In addition to miRNAs, EC-derived EVs are also known to successfully deliver biologically active proteins to recipient VSMCs, thus controlling their function/behaviour [146,156]. In response to inflammatory stimuli, ECs become activated and start to release EVs with altered molecular content ultimately affecting the protective effects that ECs exert on VSMCs (Figure 4) [146]. A well characterised population of small EVs (exosomes <150 nm), secreted by ECs in response to CD137 activation, is found to contain reduced levels of ten-eleven translocation 2 (TET2) [146], a DNA demethylase known to regulate VSMC plasticity after vascular injury [157]. Such changes in EV cargo in turn result in increased VSMC proliferation and migration as well as intimal hyperplasia after arterial injury (Figure 4) [146].

3.3. Vascular smooth muscle cell-derived EVs

The population of EVs secreted by quiescent, contractile VSMCs appear to be fundamentally different in comparison to the EVs secreted by synthetic, activated VSMCs [158] further emphasising the effect that the phenotypical status of the secreting cells may have on the characteristics of secreted EVs. Comelli *et al.* comprehensively characterised and compared microvesicles and exosomes secreted by both quiescent and activated VSMCs [158]. Interestingly, they found that microvesicles, or larger EVs, were associated with predominantly cytoplasmic or organelle-related and functionally housekeeping proteins, potentially owing to the unselective budding mechanism of EV formation. Conversely, exosomes (or smaller EVs), which are produced by specific mechanisms, were associated with more nuclear-related proteins that may be important for transcriptional regulation in exosome target cells [158]. Based on findings in this study, it seems that the exosomes secreted by VSMCs may potentially play a more significant role in the regulation of vascular remodelling processes, including intimal hyperplasia, compared to microvesicles. Further supporting this idea, exosomal EVs did not appear to express the mitochondrial marker called Prohibitin, which was used as an additional confirmation step in determining the type of isolated EVs [158]. This observation was consistent with previously suggested absence of mitochondrial markers in exosomes [159]. Abnormal PDGF signalling after vessel injury is

1 implicated in vascular remodelling and greatly contributes to different vascular
 2 pathologies including restenosis and pulmonary hypertension [36,160]. A recent
 3 study by Heo *et al.* proposed that PDGF stimulation of VSMC results in the
 4 generation of EVs with altered miRNA expression profile. The absence of miR-1246,
 5 miR-182, and miR-486 in VSMC-derived EVs in response to PDGF stimulation was
 6 associated with an increase in EC migration [161]. Although it is well-known that
 7 activated VSMCs greatly contribute to many vascular pathologies, the role of VSMC-
 8 derived EVs has not yet been properly investigated in the development of intimal
 9 hyperplasia after vascular injury. Indeed, research on VSMC-derived EVs has
 10 primarily focused on vascular calcification and diabetes [162].

Figure 4. Schematic diagram for the role of EV signalling in neointimal formation. EVs secreted by various cells within the vessel wall contribute to a heterogeneous population of EVs which appears to play a key role in the development of neointimal thickening. In this diagram, three potential EV signalling mechanisms have been illustrated. EC-derived EVs, the most widely studied population of EVs in the context of neointimal formation, contain different bioactive cargo molecules which once transferred to target recipient cells have the ability to control vessel remodelling processes including cell migration and proliferation.

3.4. EVs derived from other cells within the vascular wall

EV-mediated crosstalk under pathological conditions has also been demonstrated between VSMCs and other cells within the vascular wall including macrophages, adventitial fibroblasts (AFs) and mesenchymal stem cells (MSCs) [163–165]. Although it is quite well understood that inflammatory cells, such as macrophages, contribute to the development of neointimal formation both in the early stage after vessel injury by promoting neointimal hyperplasia and also in the late stage of accelerated atherosclerosis [6], the different interaction and communication mechanisms between inflammatory and vascular cells remain to be fully investigated. However, studies focusing on vesicle-based intercellular communication between macrophages and VSMCs are still rare. Only recently, EV-mediated communication between macrophages and VSMCs has been demonstrated for the first time [163]. Wang *et al.* proposed that M1 macrophage-derived EVs could enhance neointimal hyperplasia post vascular injury and this effect appeared to be dependent on EV-mediated delivery of miR-222 from M1 macrophages to VSMCs. A suggested underlying mechanism for the observed increased VSMC proliferation and migration *in vitro* involved miR-222 targeting *CDKN1B* (cyclin dependent kinase inhibitor 1B) and *CDKN1C* (cyclin dependent kinase inhibitor 1C) which resulted in reduced CDKN1B and CDKN1C protein

1 expression ultimately promoting cell cycle progression and cell proliferation [163].
2 Additionally, in the context of atherogenesis, a relevant pathological process
3 observed in the later stages of neointimal formation, Zhu *et al.* demonstrated that
4 EV-mediated delivery of miR-21-3p from nicotine-stimulated macrophages to VSMCs
5 could promote the development of atherosclerosis by directly targeting phosphatase
6 and tensin homologue (PTEN) leading to increased VSMC migration and
7 proliferation [166].

8
9 EV-mediated communication between AF and VSMCs in vascular remodelling in the
10 context of hypertension has also been studied, where EVs have been found to exert
11 their effects by delivering different types of bioactive molecules including angiotensin
12 converting enzyme (ACE) and miR-155-5p to recipient VSMCs [164,167]. EVs
13 secreted by AFs derived from spontaneously hypertensive rats (SHR) have been
14 shown to contain more ACE than AFs from Wistar Kyoto (WKY) control rats and
15 furthermore EV-mediated delivery of ACE from SHR AFs to VSMCs resulted in
16 increased angiotensin receptor 1 (AT₁R) activity followed by an increase in VSMC
17 migration [164]. Consistent with these findings, AFs have been reported to modulate
18 VSMC behaviour via EV-mediated transfer of both miR-155-5p and ACE, where both
19 increased ACE and reduced miR155-5p in EVs from SHR AFs contribute to
20 augmented proliferation of SHR VSMCs [167]. Additionally, the EVs from WKY AFs
21 were found to exert an inhibitory effect on SHR VSMC proliferation, an effect
22 mediated by EV transfer of miR155-5p which suppressed vascular ACE expression
23 [167]. The isolated EV populations in these studies were well characterised providing
24 considerable evidence supporting exosome-mediated effects [163,164,166,167].
25 Another recent study, investigating the communication between MSCs and VSMCs
26 revealed that inhibition of Myosin 1E expression due to EV-mediated transfer of
27 miR-125b from MSCs to VSMCs resulted in suppressed VSMC proliferation and
28 migration *in vitro* and ultimately inhibited neointima formation *in vivo* [165]. Such
29 findings demonstrate the significance of EV-mediated communication between
30 different cells, thus, highlighting the need for more in-depth research on this subject
31 in order to better understand the possible underlying mechanisms of vascular
32 remodelling processes and to fully elucidate the therapeutic potential of EVs in
33 different vascular pathologies, including, intimal hyperplasia/neointimal formation.

4. Lipid components in EV cargo

Lipid molecules are not only important for the biogenesis and release of EVs but also for their functional effects [168][169]. It has been found that EVs contain several bioactive lipids as well as enzymes involved in the synthesis of those bioactive lipids [169][170] that may affect the behaviour of recipient cells. Unlike the protein and RNA cargo, the lipid content of EVs has been poorly investigated. Interestingly, the EV lipid composition does not completely resemble that of the releasing cells [169]. EVs have been reported to carry and exchange between cells various GTP-activatable phospholipases and lipid mediators including arachidonic acid (AA) and derivatives such as prostaglandins [171] suggesting the active involvement of EVs in lipid-mediated intracellular signalling processes. In the context of vascular injury, AA has been shown to be potentially involved in vascular remodelling processes by stimulating VSMCs [172] and EC motility [173], though the exact molecular mechanisms have not been properly investigated. Secreted phospholipase A₂ (sPLA₂) is a lipolytic enzyme that catalyses the hydrolysis of membrane phospholipids to release free fatty acids and lysophospholipids like AA and lysophosphatidic acid (LPA) [174]. In their study, Rizzo *et al.* reported that inhibition of sPLA₂ catalytic activity significantly reduced EC migration while AA and other lipid mediators enhanced EC migration [173]. Consistent with these findings, both EC adhesion and migration seem to be enhanced by low concentrations of AA in a non-receptor specific manner, effects which may be mediated by AA metabolites [175]. These effects of AA on ECs may be particularly important in the re-endothelisation stage post vascular injury as previously described EVs are known to influence EC behaviour.

Both cyclooxygenase and thromboxane synthase detected in platelet-derived EVs were found to be capable of metabolising AA into thromboxane A₂ leading to platelet activation and aggregation [176]. However, when platelets were treated with sPLA₂, neither thromboxane A₂ production nor platelet aggregation was increased suggesting a unique function for platelet-derived EVs [176] potentially owing to their ability to produce potent eicosanoids. Thromboxane A₂ has also been shown to promote VSMC migration and proliferation through activation of the yes-associated protein (YAP) / transcriptional co-activator with PDZ-binding motif (TAZ) pathway

[177] suggesting its potential involvement in crucial vascular remodelling processes. Interestingly, the AA produced in response to treatment of platelet-derived EVs with a sPLA₂ can also influence EC function in a paracrine manner [176]. In this case, a counteracting pathway seem to be activated where an increased expression of cyclooxygenase 2 in ECs was observed along with an increased formation of prostacyclin with the AA in platelet EVs potentially serving as a substrate for the induced enzyme [176]. Prostacyclin has been shown to exert a variety of vasculoprotective effects including inhibition of EC apoptosis and VSMC proliferation [178][179]. The protective effects of prostacyclin have been demonstrated in the context of vascular injury since mice lacking prostacyclin receptor exhibited enhanced neointimal formation and VSMC proliferation [179]. Given that both prostacyclin and thromboxane A₂ have been implicated in EV signalling mechanisms and vascular remodelling processes it is of great importance to more comprehensively study the role of the lipid component of EVs in different vascular pathologies including neointimal formation.

These findings indicate that EVs containing bioactive lipid molecules could potentially play a significant role in the regulation of vascular remodelling processes post vascular injury. Unfortunately, the role of bioactive lipid molecules in EVs has not been yet thoroughly investigated and there are significant gaps in knowledge. It is essential to understand the mechanisms involved not only in the biogenesis and release of different types of EVs but also their specific function associated with their unique cargo including miRNAs, protein and lipid molecules.

5. Conclusion

Despite recent advancements in coronary interventions, continuous vessel remodelling due to neointimal formation remains one of the main causes of VGF and arterial restenosis post-stent implantation. There is now emerging evidence supporting the involvement of EVs in the development of neointimal formation post vascular injury, however, recent studies primarily focused on investigating the role of EC-derived EVs. As various different cell types are involved in the process of pathological neointimal growth, it is important that the role of EVs derived from other

participating cells are comprehensively investigated including EVs derived from platelets, VSMCs, fibroblasts, macrophages and other immune cells. Better understanding of the intercellular communication mechanisms remains of great importance in order to develop therapeutic strategies to preserve vascular health. The therapeutic potential of EVs in neointimal formation post vascular injury has been investigated to some extent, however, the specific involvement of distinct EV types remains to be fully elucidated.

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