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SYMPOSIUM REVIEW

Emerging roles for vascular smooth muscle cell exosomes in calcification and coagulation

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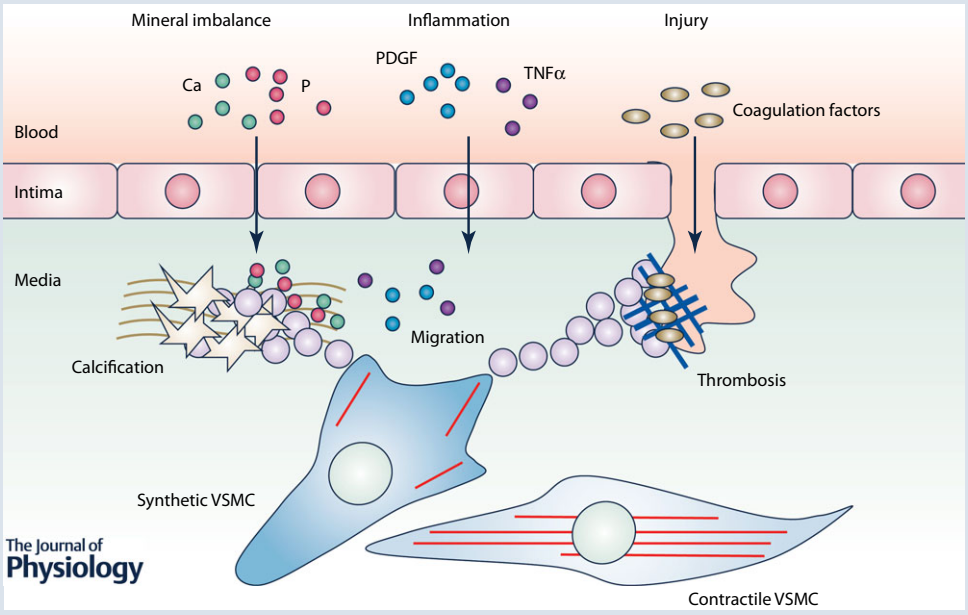

Abstract Vascular smooth muscle cell (VSMC) phenotypic conversion from a contractile to 'synthetic' state contributes to vascular pathologies including restenosis, atherosclerosis and vascular calcification. We have recently found that the secretion of exosomes is a feature of 'synthetic' VSMCs and that exosomes are novel players in vascular repair processes as well as pathological vascular thrombosis and calcification. Pro-inflammatory cytokines and growth factors as well as mineral imbalance stimulate exosome secretion by VSMCs, most likely by the activation of sphingomyelin phosphodiesterase 3 (SMPD3) and cytoskeletal remodelling. Calcium stress induces dramatic changes in VSMC exosome composition and accumulation of phosphatidylserine (PS), annexin A6 and matrix metalloproteinase-2, which converts exosomes

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into a nidus for calcification. In addition, by presenting PS, VSMC exosomes can also provide the catalytic surface for the activation of coagulation factors. Recent data showing that VSMC exosomes are loaded with proteins and miRNA regulating cell adhesion and migration highlight VSMC exosomes as potentially important communication messengers in vascular repair. Thus, the identification of signalling pathways regulating VSMC exosome secretion, including activation of SMPD3 and cytoskeletal rearrangements, opens up novel avenues for a deeper understanding of vascular remodelling processes.

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Abstract figure legend VSMC exosomes mediate vascular calcification and thrombosis. Mineral imbalance, inflammation or vascular injury trigger VSMC phenotypic modulation and exosome release. VSMC exosomes may be involved in repair processes but also can cause vascular calcification and thrombosis.

Abbreviations ECM, extracellular matrix; ESCRT, endosomal sorting complex required for transport; EV, extracellular vesicle; MGP, matrix Gla protein; MV, matrix vesicle; MVB, multivesicular body; oxLDL, oxidized phospholipid; PDGF, platelet-derived growth factor; PS, phosphatidyl serine; SM, smooth muscle; SMPD3, sphingomyelin phosphodiesterase 3; TNFα, tumour necrosis factor-α; TF, tissue factor; VSMC, vascular smooth muscle cell.

Exosomes released from vascular smooth cells mediate calcification

Mature contractile vascular smooth muscle cells (VSMCs) are quiescent cells that play a key role in the conductive function of the vasculature by maintaining vessel wall integrity and regulating arterial tone. VSMC contractile function is mediated by a unique contractile apparatus consisting, in part, of smooth muscle (SM)-specific marker proteins such as α-SM actin, SM myosin heavy chain, h1-calponin, SM22α and smoothelin. Pathological cues such as ageing, oxidative stress, inflammation and mechanical injury induce phenotypic modulation of VSMCs leading to cell proliferation, migration and extracellular matrix (ECM) remodelling (Chamley-Campbell *et al.* 1979; Gomez & Owens, 2012). This phenotypic modulation contributes to a range of vascular pathologies including restenosis, atherosclerosis, thoracic aortic aneurysms and dissections, transplant vasculopathy, pulmonary hypertension and vascular calcification. Recent definitive *in vivo* lineage studies have established a dominant role for VSMC phenotypic modulation in atherosclerosis and vascular repair (Nemenoff *et al.* 2011; Robbins *et al.* 2013; Shankman *et al.* 2015). In addition, conditional knockout in postnatal mice of myocardin, the master regulator of SM-specific gene expression, induces profound loss of vascular SM contractile markers and extensive matrix remodelling leading to arterial aneurysms, aortic dissections, and rupture and death (Wang *et al.* 2003; Huang *et al.* 2015). The phenotypic plasticity of VSMCs is associated with expression of multiple markers including those from macrophages, mesenchymal stem cells, myofibrobalsts and osteoblasts (Shanahan *et al.* 1994; Rong *et al.* 2003; Shankman *et al.* 2015) and this is especially apparent within the atherosclerotic plaque. Importantly, the functionality of newly acquired phenotypes is limited and often maladaptive; take for example osteogenic VSMCs, which mediate vascular calcification, a pathology that has been intensively studied (Shanahan *et al.* 1994; Shankman *et al.* 2015; Vengrenyuk *et al.* 2015).

Recent advances have shown that vascular calcification is a tightly regulated process resembling bone mineralization (Shanahan *et al.* 2011) driven by VSMC osteogenic conversion and commonly observed in the aged population and patients with chronic kidney disease and type 2 diabetes. Deposition of insoluble calcium phosphate crystals reduces vascular compliance, promotes inflammation and stimulates VSMC death resulting in plaque biomechanical instability (Nadra *et al.* 2005; Ewence *et al.* 2008; Sage *et al.* 2011; Hutcheson *et al.* 2014*b*). A breakthrough in calcification studies came from the identification of small membrane-enclosed extracellular vesicles (EVs) found within the matrix and secreted by VSMCs (Tanimura *et al.* 1983; Reynolds *et al.* 2004) as well as infiltrating macrophages in atherosclerotic plaques (New *et al.* 2013). Similar EVs called matrix vesicles (MVs) were previously detected in the bone growth plate where they were postulated to form the nidus for mineralization (Anderson, 1995). In physiological conditions, MVs secreted by VSMCs are loaded with calcification inhibitors, such an endogenously expressed matrix Gla protein (MGP) and circulating fetuin-A (α2-Heremens–Schmid glycoprotein) (Reynolds *et al.* 2005). However, a prolonged mineral imbalance and/or inflammation induces depletion of MGP and fetuin-A in MV and enrichment with a protein–lipid complex consisting of phosphatidyl serine (PS) and annexin A6, which converts MV to the

nidus for calcification by providing mineral nucleation sites (Reynolds *et al.* 2005; Bobryshev *et al.* 2007; Shroff *et al.* 2008; Kapustin *et al.* 2011). We and others were able to observe calcifying MVs in the vasculature *in vitro* and *in vivo*, though it was not clear whether these were derived from apoptotic cells or formed in an intracellular compartment and budded from live cells. It is generally accepted that MVs secreted by bone-derived cells are budded from the plasma membrane, suggesting similar mechanisms could be implicated in MV production by VSMCs (Anderson, 1995).

To identify the origin of calcifying VSMC MVs we used the potent calcification inhibitor fetuin-A as a tracer. Fetuin-A is not expressed by VSMCs yet is efficiently loaded into MVs (Reynolds *et al.* 2005). We found that alexa488-labelled fetuin-A is rapidly taken up by human VSMCs and delivered to early and late endosomal compartments (Kapustin *et al.* 2015). From here a subset of late endosomal compartment, multivesicular bodies (MVBs), is involved in the production of small, ∼100 nm, extracellular vesicles or exosomes which are generated by the inverted budding of the MVB limiting membrane into the MVB lumen. MVBs are transported to the cell periphery where the fusion of the MVB limiting membrane and plasma membrane releases intraluminal exosomes into the extracellular matrix (Harding *et al.* 1983; Raposo & Stoorvogel, 2013). We observed colocalization of fetuin-A positive intracellular organelles and a MVB marker in VSMCs indicating that fetuin-A is delivered to MVBs and recycled via the exosomal pathway. Further studies identified an enrichment of exosomal protein markers, tetraspanins CD9 and CD63, as well as the presence of the endosomal sorting complex required for transport (ESCRT) machinery, Tsg101, in VSMC MVs, isolated by differential ultracentrifugation. A comparison of the proteomic composition revealed high levels of similarity between VSMC-derived MVs and exosomes secreted by other cells. The size and morphological appearance of VSMC-derived vesicles detected with nanoparticle tracking analysis and electron microscopy also were indicative of exosome characteristics (Kapustin *et al.* 2015). Sphingomyelin phosphodiesterase 3 (SMPD3) has been implicated in exosome biogenesis so we next tested whether it played a role in VSMC calcification. We found that inhibition of SMPD3 blocks exosome secretion and VSMC calcification (Trajkovic *et al.* 2008; Kapustin *et al.* 2015). Moreover, elevated extracellular calcium, a known cause of calcification, induces SMPD3 expression and exosome production (Kapustin *et al.* 2015). Interestingly, these data are in agreement with the phenotype of *fragilitas ossium* (*fro/fro*) mice with a *SMPD3* gene deletion that inactivates enzyme activity. These mice are characterized by an osteogenesis imperfecta phenotype with significant impairment in growth plate structure, short-limbed dwarfism and undermineralised bones and teeth (Aubin *et al.* 2005; Khavandgar *et al.* 2011). Importantly, loss of SMPD3 activity in osteoblasts also dramatically reduced their mineralization capacity *in vitro* confirming the local role of SMDP3 during bone development (Khavandgar *et al.* 2011). However, further work is required to determine whether this is via a similar MV/exosome pathway as shown for VSMCs. The exact intracellular origin of macrophage-derived EVs that contribute to vascular calcification also remains unknown.

At the molecular level calcium phosphate crystal formation is triggered in osteoblast-derived MVs by nucleation sites consisting of PS and annexin A5 (Genge *et al.* 2007). Annexins were originally isolated from avian growth plate cartilage-derived MVs as Ca^{2+}-dependent membrane-binding proteins and were later identified as crucial MV nucleation core components due to their capacity to bind calcium in conjunction with PS (Genge *et al.* 1989; Wu *et al.* 1993). It has also been suggested that annexin A5 can form a voltage-gated calcium channel (Rojas *et al.* 1990). However, this notion remains controversial as annexins lack transmembrane domains and calcium transport activity was not directly detected in native MVs (Gerke *et al.* 2005). Treatment of chondrocytes with osteogenic conditions *in vitro* results in elevated cytosolic calcium levels followed by accumulation of annexins A2, A5 and A6 in calcifying MVs (Kirsch *et al.* 1997; Wang & Kirsch, 2002). These data are in agreement with elevated cytosolic calcium detected in chondrocytes in the growth plate hypertrophic zone colocalizing with increased levels of calcifying MVs just before the onset of mineralization (Buckwalter *et al.* 1987; Iannotti & Brighton, 1989). Apart from annexins, accumulation of alkaline phosphatase (AP) in calcifying chondrocyte-derived MVs has been observed in a number of studies and these data directly implicate osteogenic transcription factors in the regulation of MV composition, though the exact links are still unclear (Anderson, 1995; Kirsch *et al.* 1997; Wang & Kirsch, 2002). Currently the cellular origin of these chondrocyte-derived MVs is unknown, however, the evidence that they are regulated by SMPD3 and comparative proteomics suggests that at least a subset may be of exosomal origin, but this requires further detailed investigation (Aubin *et al.* 2005; Kapustin *et al.* 2015).

Secretion of calcifying exosomes by VSMCs is also driven by pathological changes in cytosolic calcium homeostasis that triggers dramatic changes in exosome composition including enrichment with nucleating PS/AnxA6 complexes, loss of MGP and appearance of amorphous calcium phosphate (Kapustin *et al.* 2011, 2015). The role of AP in exosome-mediated VSMC calcification is not definitive as no or modest changes in AP activity in EVs isolated from the media of bovine or human aortic VSMCs after short-term treatment in calcifying conditions have been reported (Chen *et al.* 2008; Kapustin *et al.* 2011). On the contrary, MVs isolated

from the media of coronary artery VSMCs after long-term treatment in osteogenic media or MVs obtained from bovine VSMC matrix by collagenase digestion were enriched with AP (Chen *et al.* 2008; Hutcheson *et al.* 2014*a*). These data suggest that osteogenic conditions may affect the VSMC EV repertoire and/or composition in a cell and context-specific manner and the exact role of osteogenic transcription factors in the production of calcifying EVs by VSMCs is yet to be determined. Recent studies showing that BMP2-activated Runx2 upregulates SMPD3 expression in C2C12 myoblasts and chondrocytes directly links the exosome biogenesis machinery with osteogenic master genes and further studies are required to clarify this link (Chae *et al.* 2009; Kakoi *et al.* 2014).

To summarize, VSMC exosomes are novel players in vascular calcification and their composition rapidly changes in response to environmental stresses (e.g. calcifying conditions) and alterations in exosomal lipid and protein composition can either facilitate or ameliorate the calcification process.

Are VSMC exosomes a novel trigger of vascular coagulation?

A role for EVs in the activation of coagulation pathways was originally observed for circulating platelet-derived EVs (Wolf, 1967; Sims *et al.* 1988). It has been hypothesized that tumour cell-derived EVs also contribute to high levels of vascular thrombotic events in cancer patients (Gardiner *et al.* 2015), and recently the levels of circulating EVs associated with tissue factor (TF), an important activator of extrinsic coagulation pathways, was linked to the increased risk of venous thromboembolism in cancer patients (Tesselaar *et al.* 2007). Vascular thrombosis due to atherosclerotic plaque rupture is the main cause of vascular occlusion events including myocardial infarction, unstable angina, stroke and sudden cardiac death. Exposure of TF, which is expressed by VSMCs, activated monocytes and endothelial cells, upon plaque rupture initiates the extrinsic coagulation pathway with TF forming a complex with factors VII and VIIa and thus activating factors IX and X (Nemerson, 1988; Wilcox *et al.* 1989; Toschi *et al.* 1997; Crawley *et al.* 2000; Mann, 2011). Vitamin K-dependent factor X in turn binds to PS exposed on the membrane on the activated platelets or apoptotic VSMCs, macrophages or endothelial cells and forms the membrane-associated prothrombinase complex, consisting of activated protease factor Xa, co-factor Va and calcium (Krishnaswamy *et al.* 1992; Walker & Krishnaswamy, 1994; Bombeli *et al.* 1997; Flynn *et al.* 1997; Mallat *et al.* 1999). Prothrombinase converts prothrombin to thrombin, which catalyses fibrin clot formation and platelet activation and also stimulates protease-activated receptor-dependent signalling pathways regulating VSMC contraction, proliferation and migration (McNamara *et al.* 1993; Seasholtz *et al.* 1999; Patterson *et al.* 2001; Mann, 2011; Sevigny *et al.* 2011).

We reported earlier the presence of externalized PS on VSMC exosomes, which can provide the catalytic surface for binding and activation of coagulation cascade factors (Kapustin *et al.* 2011). Although VSMCs are one of the main sources of TF in the vasculature, it is unclear if TF is secreted associated with VSMC exosomes. Interestingly, almost all TF activity in the atherosclerotic plaque has been associated with EVs released by apoptotic or activated cells (Mallat *et al.* 1999). Similar to circulating EVs, vascular EVs are enriched with TF and PS and are highly procoagulant (Mallat *et al.* 1999; Leroyer *et al.* 2007). Thrombogenic EVs in the plaque have a heterogeneous origin and EVs from leukocytes, erythrocytes, VSMCs and endothelial cells, but not platelets, have been detected in the plaque (Leroyer *et al.* 2007). Prominently, plaque-derived EVs are more thrombogenic than plasma-derived EVs and this is most likely due to the presence of TF-enriched EVs secreted by apoptotic VSMCs (Leroyer *et al.* 2007). Plasma membrane TF is thought to be packed into the EVs budding from the plasma membrane upon cell activation or cell apoptosis (Mallat *et al.* 1999; Del Conde *et al.* 2005; Ettelaie *et al.* 2014; Gardiner *et al.* 2015). However, immunocytochemical analysis of intracellular TF distribution in fibroblasts, VSMCs and monocytes revealed that TF resides both on the plasma membrane and within intracellular organelles, including endosomes (Schecter *et al.* 1997; Egorina *et al.* 2005; Mandal *et al.* 2006). Moreover, Mulder *et al.* (1996) utilized immunogold-labelling to locate TF in intracellular lysosome-like structures that exhibited high morphological resemblance to MVBs with intraluminal exosomes. Thus these data suggest that TF can be loaded into exosomes originating from VSMC MVBs. In agreement with this hypothesis, Mark Taubman's group and others showed that TF is secreted by VSMCs in small (less than 200 nm) vesicles and this secretion is tightly regulated by platelet-derived growth factor (PDGF) and tumour necrosis factor-α (TNFα) (Schecter *et al.* 1997, 2000; Llorente-Cortes *et al.* 2004). These data correlate well with our recent observation showing that VSMCs secrete exosomes with an average size of $\sim$150 nm and their production is stimulated by PDGF and TNFα (Kapustin *et al.* 2015). Thus, it is likely that TF can be released by non-apoptotic VSMCs on exosomes, which may then contribute to vascular thrombosis events.

Exosome secretion by VSMCs is modulated by phenotypic transition

Exosome secretion is a tightly regulated process that begins at the plasma membrane upon oligomerization or clustering of signalling transmembrane proteins such as

the heparin sulphate proteoglycan syndecan or sortilin (Fang *et al.* 2007; Baietti *et al.* 2012; Wilson *et al.* 2014). Upon endocytosis these proteins are delivered to early and late endosomes where they are sorted for either degradation or exosomal recycling. Protein sorting and intraluminal vesicle biogenesis at MVBs is orchestrated by ESCRT components including Alix, Tsg101, HRS and STAM1, and protein post-translational modifications such as ubiquitination or glycosylation can be involved in protein targeting (Buschow *et al.* 2005; Baietti *et al.* 2012; Colombo *et al.* 2013). Following MVB trafficking to the cellular periphery, subsequent docking to the plasma membrane is regulated by the Rab family of small GTPases and Rab11, Rab27a and Rab27b in particular (Savina *et al.* 2005; Ostrowski *et al.* 2010; Baietti *et al.* 2012). Lipids have also been implicated in exosome biogenesis and it has been shown that SMPD3 generates ceramide required for inward budding of the MVB membrane (Trajkovic *et al.* 2008). In addition, alterations in cytosolic calcium also stimulate exosome secretion, probably by regulating calcium-dependent fusion events or by activation of calcium-dependent calpains, proteases involved in the remodelling of the cortical cytoskeleton required for plasma membrane dynamics (Savina *et al.* 2005; Mellgren *et al.* 2007).

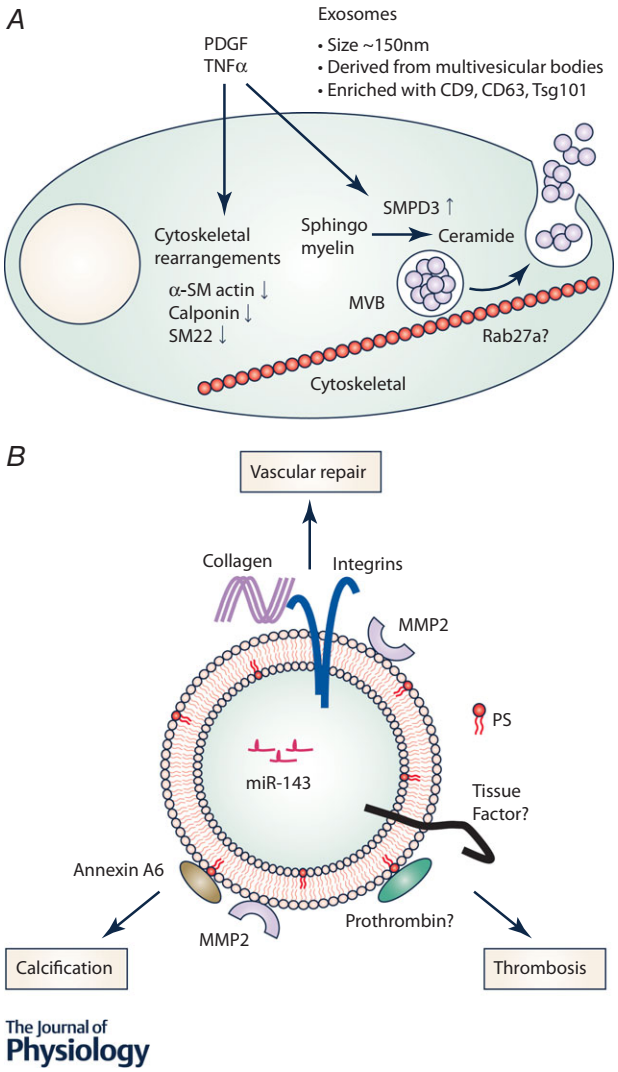
Environmental factors inducing VSMC phenotypic modulation include oxidized phospholipids (oxLDLs), growth factors PDGF-BB and PDGF-DD and proinflammatory cytokines such as TNFα or IL1β (Pidkovka *et al.* 2007; Deaton *et al.* 2009; Thomas *et al.* 2009; Gomez & Owens, 2012). These factors modulate expression and activity of the myocardin family of transcriptional coactivators for serum response factor (SRF) driving expression of contractile VSMC proteins (Gomez & Owens, 2012). Similar environmental factors, including oxLDLs, TNFα and oxidative stress stimulate SMPD3 activity and proliferation of VSMCs and fibroblasts (Auge *et al.* 1999; Tellier *et al.* 2007; Cinq-Frais *et al.* 2015). Given the role of the SMPD3 pathway in exosome biogenesis, we tested if SMPD3 is involved in exosome secretion by VSMCs and found that inhibition of SMPD3 reduced exosome production (Trajkovic *et al.* 2008; Kapustin *et al.* 2015). Importantly, PDGF-BB treatment reduced expression of VSMC contractile markers and increased exosome secretion whilst TGFβ1 induced VSMC marker expression and nearly abolished exosome secretion (Kapustin *et al.* 2015). Thus, there is an inverse correlation between VSMC phenotypic marker expression and exosome secretion, most likely driven by the SMPD3-dependent pathway. Changes in cell phenotype, namely the epithelial-to-mesenchymal transition, were previously linked to elevated exosome-like vesicle secretion though it is not clear what the links between phenotypic changes and exosome production are (Garnier *et al.* 2012). Interestingly, VSMC phenotype transition is driven by cytoskeleton remodelling and single mutations in the contractile proteins ACTA2 or MYH11 are enough to cause the dilatation of the thoracic aorta and aortic dissections (Guo *et al.* 2007; Kuang *et al.* 2012). Actin cytoskeletal proteins are also downstream targets of Rab27A and Rab27B, which play crucial roles in exosome secretion by regulating MVB trafficking (Seabra & Coudrier, 2004; Ostrowski *et al.* 2010). These data suggest the cytoskeletal changes upon VSMC phenotypic modulation might enable MVB trafficking and exosome secretion, but further studies are required to fully establish the spatiotemporal links between exosome production, VSMC phenotypic switching and cytoskeleton remodelling.

Perspectives and conclusions

Exosomes are novel intercellular communication messengers acting both in a paracrine and an autocrine manner by delivering biologically active proteins, lipids and RNA species (Raposo & Stoorvogel, 2013). In the cardiovascular system exosomes are secreted by endothelial cells, VSMCs, monocytes and cardiac progenitor cells and they act as stimulators of endothelial cell migration, proliferation and cell survival (Vrijsen *et al.* 2010; Li *et al.* 2013; van Balkom *et al.* 2013; Deng *et al.* 2015; Kapustin *et al.* 2015). VSMC phenotypic modulation is a hallmark of vascular repair and remodelling processes and elevated exosome secretion by 'synthetic' VSMCs, upon activation of SMPD3 and cytoskeletal rearrangements, highlights a potentially important physiological role for VSMC exosomes in vascular repair (Fig. 1*A* and *B*). On one side VSMC exosomes are enriched with miR-143 and proteins regulating cell adhesion and migration and can be involved in the regulation of cell proliferation and migration in an autocrine and paracrine manner with overall exosome effects an integer of activation of multiple signalling pathways by different exosome cargos (Deng *et al.* 2015; Kapustin *et al.* 2015). On the other hand, VSMC exosomes can contribute to pathological processes such as vascular calcification when the balance between calcification activators and inhibitors is lost (Kapustin *et al.* 2011, 2015). The exposure of PS on VSMC exosomes along with VSMC TF expression also implicates exosomes in the activation of coagulation cascades (Wilcox *et al.* 1989; Schecter *et al.* 2000; Leroyer *et al.* 2007; Kapustin *et al.* 2011) with further characterization required to determine the exact role of VSMC exosomes in vascular thrombosis. Importantly, environmental cues may define the composition and secretion levels of exosomes by VSMCs thus modulating their functional role and proposed contribution to vascular repair processes. Current developments in this area are limited by a number of unknowns including (1) the exact mechanisms of exosome cargo loading and (2) a lack of gold-standard

markers for different EV populations. Indeed, multiple organelle proteins are detected in exosome preparations isolated from different cell types and it is not clear whether these are loaded in the exosome or secreted as independent entities that are then co-purified with exosomes during analytical purification. In addition, different catabolic membrane trafficking processes, such has exocytosis and authophagy, can cross-talk with each other increasing the level of complexity and the difficulty in determining EV origins and function.

Although the spatiotemporal and mechanistic relationships between pathological factors, phenotypic VSMC conversion and activation of exosome biogenesis are still unknown, the modulation of exosome biogenesis may be a novel therapeutic approach to facilitate improvement in vascular repair. Inhibition of exosome production or introduction of specifically designed cargos targeted to exosomes might prevent exosomal nucleation core formation and/or binding of coagulating factors thus blocking excessive thrombosis and unwanted calcification. However, these approaches will have to be carefully considered to ensure that the physiological roles of exosomes in vascular repair are not compromised. Finally, EVs are also promising novel diagnostic makers and their use to assess cardiovascular risk is an emerging field (Loyer *et al.* 2014).



The Journal of Physiology

Figure 1. Functional roles for VSMC exosomes
A, cytokines and growth factors induce phenotypic conversion of VSMCs accompanied by cytoskeletal remodelling and SMPD3 activation. These changes enable budding of exosomes in MVBs and MVB trafficking and docking to the plasma membrane. Exosomes are released in the extracellular space upon the fusion of MVBs and the plasma membrane. *B*, exosome composition defines the role of exosomes in vascular repair. Extracellular matrix proteins, integrins and miRNA stimulate vascular repair by stimulating cell adhesion and migration. Externalized PS forms the nidus for calcification by forming a complex with annexin A6. Vascular calcification is also facilitated by matrix degradation catalysed by matrix metalloproteinase-2 (MMP2). Exosomal PS can also bind coagulation factors and may stimulate vascular thrombosis activated by tissue factor expressed by VSMCs.

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Additional information

Competing interests

None of the authors has any conflicts of interests.

Authors contribution

A.N.K.: conception, manuscript writing (sections 1–4). C.M.S.: manuscript writing and editing (sections 1–4). Both authors have approved the final version of the manuscript and agree to be accountable for all aspects of the work. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

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