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Review

The dynamic cytoskeletal-membrane interface during microparticle release

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ABSTRACT

Amongst various cellular phenomena, changes to the organisation and integrity of cytoskeletal proteins are required for vesiculation, the process of membrane microparticle (MP) shedding, to occur. As support for the biological significance of MP in regulating physiological and pathological processes increases, so does the demand for understanding the key mechanisms involved in their formation and release. This review summarises data investigating the cytoskeletal-membrane interface during vesiculation, focusing on the molecular signalling pathways that regulate actin dynamics in particular. A clear discrepancy in the literature, which is addressed in this assessment, is the distinction between mechanisms governing apoptotic bleb formation and MP release. This evaluation furthermore highlights the value of studying vesiculation from the perspective of the purified MP and intends to highlight the potential for cytoskeletal targeted therapies in modulating MP production.

KEYWORDS: microparticle, microvesicle, extracellular vesicle, vesiculation, cytoskeleton, actin, actin-signalling, microtubule

ABBREVIATIONS

ADF – Actin-depolymerising factor
BHK – Baby Hamster Kidney Cell
EC – Endothelial Cells
EM – Electron microscopy
HEK – Human Embryonic Kidney Cell
LIMK – LIM-kinase
LSC – Liver Stem Cell
MLC – Myosin light chain
MLCK – Myosin light chain kinase
Mϕ – Macrophage
Mo – Monocyte
MNC – Mononuclear Cell
MP – Microparticle
MSC – Mesenchymal Stem Cell
MT – Microtubule
MV – Microvesicle
PIP2 – Phosphatidylinositol 4,5-bisphosphate
PLT – Platelet
PMN – Polymorphonuclear Cells
PS – Phosphatidylserine
ROCK – Rho-kinase
RBC – Red Blood Cell
TNF – Tumour Necrosis Factor

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INTRODUCTION

Complex interactions and bidirectional regulation between the plasma membrane lipid bilayer and cytoskeleton control essential cellular functions including the maintenance of cell shape, motility, division, inter- and intracellular transport. The cytoskeleton is comprised of a harmonious self-assembling and disassembling system of integrated motor, regulatory and signalling proteins that form a dynamic scaffold through connections with three groups of filamentous structural polymers;

actin microfilaments, microtubules and intermediate filaments. Dissecting the functional role of any one cytoskeletal component in a cellular phenomenon is virtually impossible due to the close associations and tight interdependence between its constituents.

Vesiculation is the phenomenon by which conformational changes at the level of the plasma membrane, described as surface protrusions or membrane blebs, give rise to the release of submicron vesicles. All eukaryotic cells undergo this process of microparticle (MP) discharge, occurring at low levels in resting conditions and increasing following cellular activation and apoptosis. Their shedding requires sufficient membrane destabilisation achieved through modifications to enzymatic function, phospholipid asymmetry, membrane-bound protein integrity and cytoskeletal organisation (reviewed by [1-4]).

A number of debates cloud the field of microvesicle (MV) research including the appropriate methods used to detect, enumerate, analyse and classify shed vesicles (reviews by [5, 6]). Disparity of nomenclature also adds confusion upstream in understanding the mechanisms of MP formation with terms such as "membrane blebbing". The term has often been used to describe apoptotic bleb formation, which may result in either bleb retraction (no particle release) or the shedding of apoptotic bodies, however it also indiscriminately describes any spherical protrusions formed at the plasma membrane, irrespective of size. This review specifically refers to plasma membrane-derived vesicles termed hereon as MP, ranging in size from 100-1500 nm and sedimented by differential centrifugation at speeds ranging from 10,000-20,000 relative centrifugal force. These vesicles are distinct and should not be confused with smaller exosomes (< 100 nm) and much larger apoptotic bodies (1-5 μm).

MP cargo biologically active material from their cell of origin including surface receptors, phospholipids, signalling molecules and nuclear contents, allowing them to unconventionally regulate and exacerbate physiological processes. This double edged sword of MP being essential homeostatic regulators and pathological effectors highlights how fine a balance vesiculation is. Scott's syndrome is a prime example of the importance of MP shedding, as an inability for cells to release procoagulant platelet (PLT) MP detrimentally affects thrombus formation [7]. Furthermore it has been shown that vesiculation is essential for maintaining cell survival and prolonging apoptosis, with MP release by IL-1α and staurosporin activated endothelial cells (EC) preventing caspase-3 accumulation and subsequent cell rounding and detachment [8]. Conversely, elevated levels of circulating MP originating from various vascular cell types including PLT, red blood cells (RBC), leucocytes and EC are associated with many disease states. MP of endothelial origin alone have been implicated in acute coronary syndrome [9], multiple sclerosis [10], type 1 diabetes [11], cerebral malaria [12] and renal failure [13] and have been shown to be procoagulant [14], pro-inflammatory [15] and capable of impairing endothelial and vascular function [16]. MP of cancer cell origin are similarly shown to modulate drug resistance [17], tumour growth [18] and metastasis [19]. The study of MP is hence growing considerable interest as these membrane vesicles are novel biomarkers for disease severity and progression, and understanding the mechanisms of their formation may provide new avenues for therapeutic targeting in various fields including haematology, oncology and immunology.

This review aims to outline the molecular mechanisms underlying membrane bleb formation which result in MP release, beginning with the basics of vesiculation and later focusing on the role of the cytoskeleton in modulating plasma membrane conformational changes. We intend to examine how literature specifically examining cytoskeletal biology in this phenomenon relates and connects to what is known at a broader cellular level, identifying potential areas of focus for further studies in this field. We next intend to collate and summarise molecules used to modulate MP formation and release, both *in vitro* and *in vivo*, with the aim of highlighting the potential use for cytoskeletal-directed therapeutics in targeting MP in various disease contexts.

Vesiculation basics - Calcium influx and a change in phospholipid symmetry

The shift towards lipid symmetry at the plasma membrane is a well-described event that occurs during vesiculation. Tight regulation by the

ATP-dependent aminophopholipid translocase [20] in combined efforts with floppase [21, 22], maintain phosphatidylserine (PS) and other anionic phospholipids including phosphatidylethanolamine, phosphatidylinositol and its derivatives (i.e. phosphatidylinositol 4,5-bisphosphate (PIP2)) in the membrane's inner leaflet during physiological conditions. Micromolar increases in cytosolic calcium, such as those observed after cellular activation, deplete the aminophopholipid translocase activity [23] and promote that of scramblase [24], resulting in an increased net movement of PS to the outer leaflet, a characteristic feature of this phenomenon. As this PS inversion is maintained after MP shedding, natural PS ligands including annexin V and lactadherin are commonly employed for MP detection [25-28], although cell-specific surface antigen markers are increasingly utilised following wide recognition that not all MP are PS positive/annexin V binding [29-31].

Concurrent to its effects in deregulating phospholipid asymmetry, further roles for calcium have been suggested in studies such as that by Williamson *et al.*, whereby the use of high K+ buffers could supress calcium induced MP biogenesis without correcting the induced phospholipid reorientation [24]. Calcium influx is shown to activate kinases, inhibit phosphatases and stimulate enzymes that act directly to affect the organisation of the actin cytoskeleton. The role of calcium is discussed further with the role of microfilaments in vesiculation.

Microfilaments in vesiculation

Of the filamentous cytoskeletal structures, actin microfilaments associate most closely with the cell's membrane in a dynamic bi-directional interplay, influencing cell shape and ultimately determining how it interacts with its environment. Through a myriad of physical interactions and under the tight control of various regulatory molecules, actin microfilaments affect the plasma membrane in a multitude of ways including: 1. at the leading edge of the cell where actin polymerisation in the form of filopodia, lamellipodia and pseudodopia drives locomotion, 2. at lateral junctions where cell-cell contact is maintained through connections with adhesion molecules, 3. at the ventral membrane where podosomes and invadopodia interact with and degrade the extracellular matrix, affecting the cell's metastatic potential and 4. at the surface of the cell where endocytosis and exocytosis are mediated. It is the precise unification of actin with specific actin binding proteins at discrete localisations within the cell which gives rise to this functional diversity amongst filament populations. The molecular mechanisms of the actin signalling pathways involved in the aforementioned membrane modulating events have been reviewed extensively [32-35], and will not be discussed at further length in this analysis.

Cytoskeletal-membrane anchoring and calcium signalling

Initial work examining the role of phospholipid asymmetry, calcium and the actin cytoskeleton in vesiculation occurred concurrently in numerous *in vitro* studies with spectrin identified early on as a protein of interest. Spectrin is found in both RBC and nucleated cells and associates at various sites with actin, ankyrin and phospholipids in order to anchor the membrane and maintain cell shape. In 1976, Allan *et al.* published that increased intracellular calcium levels following calcium ionophore treatment induced RBC vesicle release [36], whilst Lutz *et al.* published that vesicles spontaneously produced by sheep RBC ghosts displayed reduced levels of spectrin [37]. The same group the following year confirmed the formation of these 'spectrin-free' vesicles by human RBC, this time following ATP depletion [38]. The release of 'spectrin-free' vesicles could be inhibited with various K^+ channel and efflux blockers, whilst molecules used to prevent the proteolysis of ankyrin and band 4.1, which were devoid in purified vesicles, along with the accumulation of diacylglycerol and phosphatidate had no effect [39]. Oxidation of spectrin significantly correlated with vesiculation in stored RBC [40], with authors noting complementary studies in which storage resulted in a defect of the spectrin-protein 4.1 interaction [41]. This work paved the way for further research by Franck *et al.* in 1985, who in continuing to study spectrin-free vesicles, determined that dysregulated phospholipid asymmetry is favoured and maintained in areas of membrane-skeleton de-coupling [42]. In short, when these spectrin-membrane linkages are broken there is

translocation of the anionic phospholipids from the inner leaflet, leading to increased instability and protuberance formation at the plasma membrane.

Despite a role for spectrin in vesiculation being evident, what remains widely debated is the physiological relevance of direct interactions between the phospholipids and cytoskeletal proteins. Manno *et al*. propose that it is the interaction between spectrin and PS in RBC which allows membranes to endure high levels of sheer stress without undergoing fragmentation [43], a suggestion supported with further experimental data [44]. A vast amount of research however disputes the specificity of this interaction, as well as the strength of the binding affinity between phospholipid head groups and domains in spectrin and other actin-associated proteins such as ankyrin, band 4.1 and filamin [45-47].

An interaction that is clear in vesiculation is the strong regulatory role phospholipids themselves, specifically PIP2, mediate at this membrane interface (Figure 1). PIP2 facilitates the dissociation of actin with the capping and severing proteins profilin, plectin, gelsolin and actin-depolymerising factor (ADF)/cofilin, generating an increase in the abundance of actin monomers available for polymerisation [48-52]. Chlorpromazine, an inhibitor of RBC vesiculation, is shown to significantly inhibit the dephosphorylation of PIP2 to PI [53]. O'Connell *et al*. confirmed the connection between PIP2 facilitated membrane stability and vesiculation, demonstrating that increased incorporation of PIP2 into PLT membranes significantly reduces the ability for activated PLT to release increased MP levels [54]. A review by Montoro-Garcia describes the detailed mechanisms of PIP2 in vesiculation [4]. Briefly, PIP2 binds gelsolin (and other actin-associated proteins) rendering it incapable of performing its actin severing activity [50]. Cellular activation, resulting in increased calcium levels, drives PIP2 hydrolysis into diacyl-glycerol and IP3, further favouring elevated cytosolic calcium. Gelsolin is directly activated by calcium and may be further activated following proteolytic cleavage by calpain and caspase (described below), and has been implicated in PLT MP formation and apoptotic membrane blebbing [55, 56]. Calpain further facilitates MP formation by acting on PIP kinase to reduce PIP2 levels and increase membrane destabilisation [57].

Calpain is a calcium-dependent cysteine protease dispersed throughout the cell's cytosol, and is translocated to the plasma membrane when sufficient intracellular calcium concentrations are obtained from both intra- and extra-cellular sources [58]. Fox *et al.* first described calcium's activity via calpain in PLT vesiculation where they set out to determine if PLT had a similar membrane skeleton to that described in RBC. Calpain activating compounds dibucaine and calcium ionophore, and later described physiological agonists thrombin and collagen, induced hydrolysis of actin binding protein and talin, ultimately causing dissociation of cytoskeletal-membrane linkages and significantly increasing MP release [59, 60]. MP levels following thrombin/collagen activation were returned to basal following treatment with MDL (calpain inhibitor III), calpeptin, prostaglandin E and E-64-d; each acting to inhibit calpain. Further cytoskeletal substrates of this proteolytic enzyme determined to be affected in MP biogenesis are those incorporated into protein adhesion complexes at the membrane including talin, α-actinin, integrin, filamin and myosin heavy chain, along with gelsolin (Figure 1) [55, 61-64]. Pharmacological calpain inhibition stabilises vinculin in protein adhesion complexes at the cell periphery, prevents the translocation of other actin associated proteins such as α-actinin to focal adhesion sites and inhibits stress fibre formation [65, 66].

The acto-myosin contractile system

In conjunction with the breakdown of connections that tether the cytoskeleton and membrane, the formation of stress fibres in adherent cells occurs synonymously with MP release (see Figure 1). Stress fibres are contractile structures required for motility, cell adhesion and morphogenesis that are comprised of multiple actin filaments, associated linking proteins and myosin motors. Fishkind *et al*. first proposed a role for the actin-myosin contractile system in membrane blebbing when a high abundance of cells injected with the catalytically active myosin light chain kinase (MLCK) exhibited obvious surface protrusions during cytokinesis [67]. In this context, blebbing refers to the formation of membrane protrusions

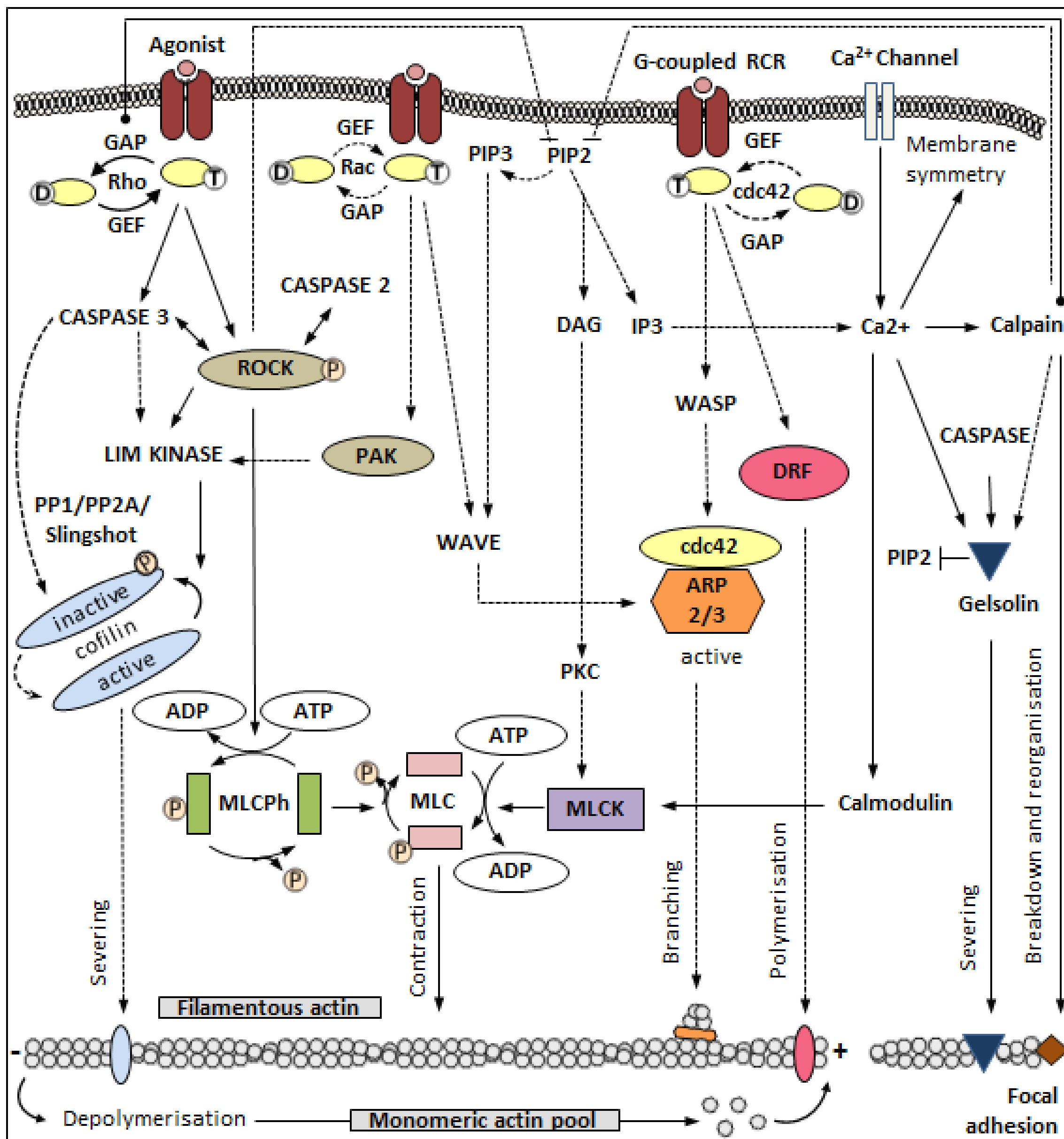

Figure 1. Molecular mechanisms regulating actin signalling in vesiculation. G-coupled receptor (RCR) activation results in an increase in cytosolic calcium, from both the endoplasmic reticulum and external sources, inhibiting the aminophospholipid translocase and promoting scramblase activity to disrupt phospholipid asymmetry. Simultaneously the calcium spike activates calpains to degrade focal adhesion proteins at sites of membrane-cytoskeletal interaction, and furthermore activates the actin-associated protein gelsolin to promote filament severing. PIP2, which binds gelsolin to inhibit its severing function is in turn inhibited by calcium's activation of calpain. Calcium further promotes calmodulin's effects on MLCK, which in turn actively phosphorylates myosin light chain (MLC), specifically myosin II, inducing stress fibre contraction. Rho-dependent activation of capase 3 and ROCK complement this via MLCPh phosphorylation and deactivation. Additional filament destabilisation occurs through ROCK- and caspase-induced cofilin phospho-cycling. In summary, cellular activation facilitates vesiculation by promoting phospholipid symmetry, increasing tension within the cell through stress fibre contraction and subsequently destabilising the membrane at sites of cytoskeletal-membrane tethering. Recent work by our group suggests that these sites of membrane vesiculation are not random, but lie directly above particulate accumulations of actin and various associated proteins. Known vesiculation mechanisms are represented as solid lines, with interlinked pathways represented as broken lines.

where it is not clearly stated if shedding results in vesicles that meet our 'MP' criteria. It was later shown in apoptotic and stimulated cells that phosphorylation of myosin light chain (MLC), specifically non-muscle myosin II, by both MLCK and Rho through activation of Rho Kinase (ROCK), induces membrane blebbing [68, 69]. Betapudi *et al.* recently confirmed this pathway for endothelial MP shedding, where pharmacological inhibition of MLCK, non-muscle myosin II and ROCK with ML-7, Blebbistatin and Y27632, respectively, significantly reduced anti-β_2-glycoprotein I induced vesiculation [70]. They further established that specific siRNA targeting both ROCKI and II isoforms could reduce MP release.

Involvement of RhoA, the upstream regulator of ROCK, was established by Sapet *et al.* who showed that thrombin-induced endothelial MP release could be significantly inhibited by both pharmacological inhibition with fluvastatin targeting CoA reductase and transfection with the defective-inactive ΔDN19RhoA plasmid [71]. The involvement of Rac, Cdc42 and Ras was investigated by Li *et al.*, who identified that expression of activated forms of these small G proteins had no effect on vesicle formation, whilst the over expression of activated Rho increased it [72]. It should however be noted that this quantification was performed by immunofluorescence microscopy of cells and not by widely accepted methods such as flow cytometry.

Functional activation of both ROCKI and II isoforms occurs following cleavage at their C-terminus [73-75]. In membrane blebbing, caspase-3 cleavage was found to be essential during ROCKI-mediated apoptotic bleb formation, whilst a study examining thrombin-induced endothelial MP implicated ROCKII and caspase-2 activity [71, 76, 77]. However, the precise sequence of signalling activation remains unclear as RhoA may activate both caspase-3 and ROCKI, which in turn have been shown capable of activating one another [78, 79]. ROCK dually activates MLC through direct phosphorylation and inhibition of MLC phosphatase [80-82]. Once phosphorylated, the MLC head binds actin and the myosin ATPase generates force resulting in actin stress fibre contraction. Inhibitors of Rho, ROCK, MLCK, myosin ATPase, such as those listed above, successfully reduce membrane blebbing and MP shedding in numerous *in vitro* and *in vivo* settings (see Table 1 for full list of effective blebbing inhibitors).

In addition to the previously mentioned roles of calcium and calpain, both are closely linked to various steps of the Rho/ROCK signalling pathway. For example whilst ROCK activity is caspase-dependent, MLCK activity is dependent on calcium and calmodulin, with some suggestion that this double pronged approach is required to achieve and maintain sufficient MLC phosphorylation [68]. A unique balance between calpain and the RhoGTPase regulator, RhoGDI-1 was established by Larsen *et al.* in the context of membrane blebbing [83]. RhoGDI-1 prevents GDP dissociation from Rho maintaining it in an inactive state. Calpain knock-out mouse embryonic fibroblasts with a markedly reduced ability to form membrane blebs displayed a two-fold reduction of RhoGDI-1, which in turn became 2-fold over-expressed when calpain was genetically rescued. Balancing acts such as these may exist as a way for cells to maintain vesicle formation and shedding.

The aforementioned work of Li *et al.* examining RhoA- and ROCK-dependent MV shedding further implicated LIM-kinase (LIMK) and the cyclic phosphorylation and dephosphorylation of cofilin in this pathway. Cofilin, a member of the ADF/cofilin family, is an actin binding protein that drives actin depolymerisation by increasing actin monomer dissociation from the pointed end of the filament [84]. Cofilin is active in a dephosphorylated form, a state enhanced by phosphatases such as PP1, PP2A and Slingshot [85, 86], and inactivated following phosphorylation with enzymes including LIMK 1 [87, 88]. MV release could be enhanced by ectopically expressing wild-type LIMK in Hela cells, and significantly reduced by expression of the LIMK D460N mutant [72]. Furthermore, cells transfected with the S3A dominant-active cofilin did not form MV on their surface. Similarly, Tomiyoshi *et al.* found that LIMK 1 activation was essential for apoptotic membrane blebbing in Jurkat T cells, in a capase 3-dependent manner [89]. Data obtained in a study of membrane blebbing in epidermoid cancer cells and normal fibroblasts however opposes this role for inactive cofilin, with the S3A dominant

active cofilin alternatively found to increase bleb formation [90]. Active cofilin was colocalised with the ARP2/3 and F-actin accumulations at sites of bleb formation, whilst inactive cofilin was more centrally localised within the cell. They suggest that as PP2A is also activated following caspase-3 cleavage [91] there is an increase in phosphate turnover leading to a cyclic "on/off" activation of cofilin that would recycle cortical actin and maintain protrusion conformation at the membrane surface. Our group has similarly observed accumulations of actin, precisely perinuclear particulate structures composed of both β- and γ-actin isoforms, underneath sites of membrane protrusions by correlative light and scanning electron microscopy [92]. This work indicated that their apical association at the plasma membrane of EC was directly related to the Rho-kinase-dependent formation of basal β-actin stress fibres.

Intermediate filaments and microtubules in vesiculation

Increasing in scale are intermediate filaments (10 nm diameter) and microtubules (25 nm diameter), which together oppose the compression generated by actin contraction in order to maintain cell structure and serve as organisers of cytoplasmic contents. Intermediate filaments provide mechanical force through interactions with the plasma membrane at lateral and basal boundaries, whilst microtubules regulate a number of cellular processes including mitotic division and intracellular transport. Perhaps for their comparatively reduced physical interaction with the apical membrane relative to actin, their influence and roles in MP release have been overlooked.

Of the few studies explicitly examining the role of microtubules (MT) in vesiculation, a study of megakaryocyte MP utilising the MT depolymerising agent nocodazole to inhibit PLT formation, found the drug to have no effect on the numbers of CD41+ MP detected by flow cytometry [93]. Whilst this may be true for megakaryocytes, the literature suggests that MT disassembly would conversely facilitate MP release. Rho activation, as previously described, regulates MP release via actin-myosin stress fibre formation and focal adhesion re-organisation. It has been shown that Rho activation can be achieved following MT depolymerisation after the release of the MT-associated nucleotide exchanger, GEF-H1 [94-97], and that MT destabilisation via the P38 MAPK pathway induces endothelial actin stress fibre formation following activation with TNF [98], a well-described agonist of endothelial vesiculation [14]. MT additionally regulate focal adhesion turnover and dynamics in adherent cells, acting upstream of calpain [65]. Further supporting this is a study by Freikman *et al.* comparing the MT assembly enhancer paclitaxel and inhibitor colchicine in relation to PS exposure and shedding. They found that while both drugs increase PS exposure on the surface of developing erythroid precursor cells, the MT inhibitor alone increased the amount of PS shed, as measured by flow cytometry following annexin V labelling of culture supernatants [99]. Although this evidence is circumstantial and the precise role for MT in this phenomenon is still to be determined, these data are highly suggestive of a role for MT in MP formation through its ability to influence stress fibre formation, calpain activity, focal adhesion integrity and phospholipid movement at the plasma membrane.

The MP cytoskeleton

Can it guide us in understanding fine mechanisms of vesiculation?

It is increasingly evident that analysis of purified MP themselves can provide valuable insight into the mechanisms both upstream and downstream of their release. As previously described, many of the first papers investigating the role of the actin cytoskeleton in vesiculation studied purified vesicle composition by gel electrophoresis, identifying the absence of membrane-skeletal polypeptides spectrin, ankyrin and band 4.1 [36, 38, 100]. Our group recently observed stark differences in the expression of focal adhesion proteins in MP relative to their mother cell. Whilst the levels of talin decreased, the expression of vinculin relative to GAPDH significantly increased in MP [92]. Degradation of these proteins was evident in MP samples, confirming published work of other groups examining their proteolysis [61]. Similarly to these gel electrophoresis experiments, proteomics approaches have the potential to provide insight into which proteins are implicated in and essential

for vesiculation. The unbiased profiling of the MP proteome is increasingly studied in various disease contexts and from an array of sources including human plasma, PLT, EC, monocytes (Mo), RBC and mouse thymocytes [101-106]. Each of these studies acknowledge the high abundance of structural proteins associated with the cytoskeleton, in particular actin signalling pathways, including actin itself, tubulin, myosin heavy chain, filamin-A, talin, tropomyosin α3 and 4 chains, F-actin capping proteins, ARP2/3, cofilin, vimentin, gelsolin and various integrins. Other commonly identified classification groups include chaperones, enzymes as well as proteins associated with signalling. Although the precise constituents vary amongst the differentially sourced MP, the wealth of cytoskeletal-related molecules, and in particular actin-associated proteins, highlights their relevance to the formation and shedding of MP.

Can it participate in regulating MP function and interactions?

MP released from the same cell type, however following different stimulating conditions, are often described to display varying functional properties. The cytoskeletal profile of MP released from various stimuli has been examined in a few studies utilising electron microscopy (EM), Western blots and proteomic methods (listed above), with no significant differences observed between activation conditions. Bernimoulin *et al.* reported β-actin, α-actinin and ARP2/3 were common amongst MP from lipopolysaccharide and soluble P-selectin chimer-activated Mo [104], with the cytoskeleton in these vesicles, as seen by freeze-fracture EM, forming a 3-dimensional scaffold most likely functioning to maintain structural stability. Our group similarly reported that the expression of β-actin, γ-actin, talin, vinculin and GAPDH was unchanged in endothelial MP produced following A23187, TRAP-6, tumour necrosis factor (TNF), interferon-γ and lipopolysaccharide activation [92]. In addition, we have observed that endothelial MP are also comprised of cofilin, ARP3 and cortactin as determined by Western blot (Latham, unpublished data). Whilst we consistently witness no difference in the overall expression of these proteins between varying stimuli, transmission EM data have suggested there is a substantial degree of heterogeneity in MP size, density and internal composition (Latham, unpublished data). It remains to be investigated whether cytoskeletal-associated proteins participate in/regulate MP content packaging and internal organisation within a single MP population.

A proteomic study comparing varying size, rather than stimuli, of pooled plasma MP, demonstrates clear differences in the number of proteins and functional output of MP from different size ranges [107]. MP fractions were separated into four size ranges on a Sepharose column, with average diameters of 130 and 260 nm, with the two smallest groups enriched for membrane and cytoskeletal-associated proteins along with adhesion proteins, chemokines and growth factors. These groups elicited negative effects on PLT function, inhibiting thrombus formation, whilst the larger fractions did not, suggesting there may be a link between cytoskeletal content and functional output.

There is some data to suggest that cytoskeletal proteins may assist or effect MP function. Collier *et al.*, showed that suppression of filamin-A significantly affected the incorporation of tissue factor into MP from breast cancer cell origin [108]. Tissue factor regulates the extrinsic coagulation pathway and its expression together with PS on the MP surface gives them their pro-coagulant capabilities. Filamin-A binds the cytoplasmic domain of tissue factor inducing its phosphorylation and enhanced incorporation into the MP membrane [109]. Filamin-A did not modulate MP size or numbers and was hence shown not to be an essential regulator of vesiculation itself. The exposure of vimentin, a type III intermediate filament, on the surface of PLT MP is thought to promote their pro-coagulant function [110]. In this case exposure of vimentin on the surface of thrombin-activated PLT and their MP enabled them to bind vitronectin along with active type 1 plasminogen activator inhibitor, thus inhibiting fibrinolysis. Whilst further experimentation is required to make this functional link, the authors identify cases where the exposure of vimentin and other intermediate filaments has facilitated haemostasis and inflammation.

Cytoskeletal-targeted therapies as MP modulators

The biological relevance of MP in facilitating and promoting disease pathogenesis is increasingly

demonstrated, with researchers foreseeing a role for them in the clinic as novel biomarkers and adjunctive therapies (see the following reviews [111-113]). Table 1, a collation of the pharmacological compounds effective in inhibiting MP formation *in vitro and in vivo*, highlights the predominant mechanisms regulating vesiculation. It is clear that their actions are to 1) prevent cellular activation through receptor antagonisation, 2) stabilise plasma membrane asymmetry, 3) minimize cytosolic calcium influx, 4) block signalling pathways, many of which in turn regulate actin signalling and stress fibre formation and 5) appease the effects of oxidative stress (not discussed further in this review). The therapeutic targeting of these mechanisms has been successfully utilised in a variety of clinical settings in which MP are known regulators. Extensive reviews have previously investigated the targeting of receptor blockers, calcium channel blockers, statins, Rho/ROCK signalling and the actin cytoskeleton in hypertension, cardiovascular disease and cancer [114-117].

Successful treatments thus far employed to minimise patient MP numbers have employed the use of statins, receptor antagonists and antioxidants in cases of hypertension, hyperlipidaemia, diabetes, metabolic syndrome and connective tissue disease (see Table 2). Statins, through their blocking activity on CoA reductase, in turn block the downstream translocation and activation of the Rho/ROCK pathway, an essential regulator of actin signalling as described above. Separate studies found both simvastatin and pitavastatin to be most effective in reducing MP numbers when used in combination with other receptor

Table 1. *In vitro/vivo* vesiculation inhibitors.

Target		Approach	MP Origin	Ref.
Phospholipid organisation	Phosphatidylserine externalisation	Annexin V	PLT, EC, Mo, Plasma	[25, 118]
		Pantethine		
	Membrane fluidity	ABCA1 KO	PLT, Mϕ, Plasma, BHK, EC	[119-121]
		ABCA1 mutation A937V		
		Protein Kinase A INH		
		Wheat Germ Aglutinin		
		Citicholine		
	Scramblase	R5421	RBC	[122]
	Lipid rafts	Nystatin	EC	[123]
		Methyl-b-cyclodextrin		
	PIP2	Chlorpromazine	RBC	[53]
	PI3K	LY294002	PLT	[124]
Calcium influx and signalling	Cytosolic Ca2+	EGTA	PLT, MNC	[125-127]
		Verapamil	MNC	[127]
	Calmodulin	W-7	MNC	[127]
	Extracellular K+	Quinine	RBC	[39, 128]
		Iso-osmotic KCl	RBC	[24, 39, 128]
		Nigericin		
		Gamicidin		
	Calpain	Calpeptin	PLT, RBC, Plasma, PMN	[25, 60, 125, 128-131]
		MDL		
		EST (E-64-d)		
		Thiosulfinates		
		Calpastatin (over exp.)		
		PD150606		

Table 1 continued..

Rho/ROCK signalling	RhoA	DDN19RhoA plasmid	EC	[71]
	CoA reductase	Fluvastatin	EC	[71, 132]
	ROCK	Y27632	EC, HEK, plasma	[70, 71, 92, 123, 133, 134]
		Fasudil		
		ROCK I and II siRNA		
Caspase activity	Pan Caspase	z-VAD-FMK	EC	[71, 135, 136]
	Caspase-2	z-VDVAD-FMK	EC	
	Caspase-6	z-VEID-FMK	EC	
Cytoskeletal, associated and motor proteins	Actin polymerisation	Cytochalasin B	MSC, LSC, PMN	[137, 138]
		Jasplakinolide		
	MLCK	siRNA	EC	[70]
		ML-7		
	Myosin II	Blebbistatin	EC	[70]
	Microtubule	Paclitaxel	RBC	[99]
	Dynamin	Dynasore	HEK	[133]
Other signalling molecules	P38 MAPK	SB-239063AN	EC	[139, 140]
		Clopidogrel		
		Losartan		
		Lovastatin		
		Mesoglycan		
	ERK	PD98059	Mo, Mϕ	[126]
	src Kinase	PP1	PLT	[141]
		PP2		
Receptor activation	PPAR-γ	GW9662	Mo, Mϕ	[126]
	Angiotensin II Type I receptor	Irbesartan	EC	[123, 139]
		Losartan		
	Integrins	Abciximab	PLT	[141]
		Tirofiban		
Oxidation	NADPH oxidase	Apocynin	EC	[123]
	Superoxide	Superoxide dismutase	PMN	[131]

Table 2. Clinical applications targeting MP release.

Therapy	Known target	Clinical setting	MP origin	Ref.
Benzafibrate	PPAR-pan	Connective tissue disease and secondary hyperlipidaemia	PLT	[145]
Vitamin C	Oxidation	Diabetic and dyslipidaemic patients	EC, PLT	[146]
Simvastatin* + Lorstan	Lorstan antagonises the angiotensin II receptor	Hypertension and Type 2 Diabetes	EC, Mo, PLT	[142]
Pitavastatin* + Eicosapentaenoic Acid	EPA is an anti-inflammatory drug	Hyperlipidaemic diabetic patients	PLT	[144]
Aspirin	COX-1	Activated with ADP after donation	PLT	[124]
Clopidogrel	ADP receptor P2Y12			

*Statins are hypolipidaemic, targeting cholesterol through CoA Reductase.

antagonists and anti-inflammatory drugs [142-144]. Figure 1 is an attempt to illustrate how these mechanisms are closely interconnected and often dependent on one another, requiring therapeutic approaches to be multi-factorial and target these in combination rather than separately.

CONCLUSIONS

This review has collated research examining the known mechanisms of vesiculation and highlighted the role for the cytoskeleton, in particular actin signalling, in facilitating the formation and release of MP. The literature shows that vesiculation is multi-factorial, whereby cellular activation induces a multitude of signalling cascades leading to the dysregulation of phospholipid asymmetry, disruption of cytoskeletal-membrane bonds and ultimate destabilisation of the plasma membrane. With the precise packaging and function of MP making it appear increasingly unlikely that membrane shedding is spontaneous, further examination of the cell and MP together using unbiased approaches is essential if we are to make headway in better understanding the mechanisms at hand. Knowledge of precise vesiculation mechanisms is essential if we are to better bridge the translational gap between experimental/clinical observation and therapeutic application.

CONFLICT OF INTEREST STATEMENT

The authors declare there are no conflicts of interest.

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