The Role of Biomechanical Stress in Extracellular Vesicle Formation, Composition and Activity

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Abstract:
Extracellular vesicles (EVs) are cornerstones of intercellular communication with exciting fundamental, clinical, and more broadly biotechnological applications. However, variability in EV composition, which results from the culture conditions used to generate the EVs, poses significant fundamental and applied challenges and a hurdle for scalable bioprocessing. Thus, an understanding of the relationship between EV production (and for clinical applications, manufacturing) and EV composition is increasingly recognized as important and necessary. While chemical stimulation and culture conditions such as cell density are known to influence EV biology, the impact of biomechanical forces on the generation, properties, and biological activity of EVs remains poorly understood. Given the omnipresence of these forces in EV preparation and in biomanufacturing, expanding the understanding of their impact on EV composition—and thus, activity—is vital. Although several publications have examined EV preparation and bioprocessing and briefly discussed biomechanical stresses as variables of interest, this review represents the first comprehensive evaluation of the impact of such stresses on EV production, composition and biological activity. We review how EV biogenesis, cargo, efficacy, and uptake are uniquely affected by various types, magnitudes, and durations of biomechanical forces, identifying trends that emerge both generically and for individual cell types. We also describe implications for scalable bioprocessing, evaluating processes inherent in common EV production and isolation methods, and propose a path forward for rigorous EV quality control.

Keywords:
Extracellular vesicles, exosomes, microparticles, cellular communication, biomechanical force, shear, tension, compression, cyclic stretch, bioprocessing

Abbreviations:
ABCA1, ATP binding cassette transporter A1; ABP, actin binding protein; ADP, adenosine diphosphate; Ago2, Argonaute 2; Akt, protein kinase B; ALG-2, apoptosis-linked gene 2; ALIX, ALG-2-interacting protein X; ARF6, ADP-ribosylation factor 6; ASK1, apoptosis signal-regulating kinase 1; AT1R, angiotensin II type 1 receptor; ATF4, activating transcription factor 4; ATP, adenosine triphosphate; BAX, BCL-2-associated X protein; BCL, B-cell lymphoma 2; BEC, bronchial epithelial cell; CD, cluster of differentiation; C/EBP, cytosine-cytosine-adenosine-adenosine-thymidine-enhancer-binding protein; CHO, Chinese Hamster Ovary; CHOP, C/EBP homologous protein; DOK2, docking protein 2; ECMO, extracorporeal membrane oxygenation; eNOS, endothelial nitric oxide synthase; ER, endoplasmic reticulum; ERK, extracellular signal-regulated kinase; ESCRT, Endosomal Sorting Complex Required for Transport; EV, extracellular vesicle; EZH2, enhancer of zeste 2 polycomb repressive complex 2 subunit; GMP, good manufacturing practice; GPIb, glycoprotein Ib; GPIIb-IIIa, glycoprotein IIb-IIIa; GRK6, G protein-coupled receptor kinase; GRP78, glucose-regulating protein 78; GTP, guanosine triphosphate; HEK, human embryonic kidney; hnRNP, heterogeneous nuclear ribonucleoprotein; HSPC, hematopoietic stem and progenitor cell; HUVEC, human umbilical vein endothelial cells; ICAM-1, intercellular adhesion molecule 1; IGF2, insulin-like growth factor 2; IL, interleukin; KLF2, Krüppel-like Factor 2; KO, knockout; LAMP1, lysosomal-associated membrane protein 1; LC3, microtubule-associated protein light chain 3; lincRNA, long intergenic non-coding RNA; LVAD, left ventricular assist device; MAPK, mitogen-activated protein kinase; MGP, matrix Gla protein; miRNA, microRNA; Mk, megakaryocyte; M-MDSC, mononuclear myeloid-derived suppressor cell; MSC, mesenchymal stem cell; MVB, multivesicular body; NADPH, nicotinamide adenine dinucleotide phosphate; NF-κB, nuclear factor kappa-B; NO, nitric oxide; Notch2, neurogenic locus notch homolog protein 2; NOX4, NADPH oxidase 4; NPP1, nucleotide pyrophosphatase/phosphodiesterase-1; NTA, nanoparticle tracking analysis; NRP1, neuropilin 1; OMV, outer membrane vesicle; p53, tumor protein p53; PCR, polymerase

chain reaction; PD-L1, protein death-ligand 1; PECAM-1, platelet endothelial cell adhesion molecule 1; PI3K, phosphoinositide 3-kinase; PKC, protein kinase C; PLT, platelet; PS, phosphatidylserine; RAB, Ras-associated binding protein; RANKL, receptor activator of nuclear factor kappa-B ligand; RhoA, Ras homolog family member A; RNA, ribonucleic acid; RBP, RNA binding protein; ROCK, Rho-associated protein kinase; SAVR, surgical aortic valve replacement; sICAM-1, soluble intercellular adhesion molecule 1; siRNA, small interfering RNA; SIRT1, sirtuin 1; SkMC, skeletal muscle cell; SMC, smooth muscle cell; SNARE, soluble N-ethylmaleimide-sensitive factor attachment protein receptor; SOCS1, suppressor of cytokine signaling 1; Src, proto-oncogene tyrosine-protein kinase Src; TAZ, tafazzin; TF, tissue factor; TFAP2A, transcription factor activating enhancer binding protein 2 alpha; TGF, transforming growth factor; THBS1, thrombospondin-1; TIPA, transcription-independent p53-induced apoptosis; TNAP, tissue-nonspecific alkaline phosphatase; TRPV4, transient receptor potential vanilloid 4; TSG101, tumor susceptibility gene 101 (protein); UCHL1, ubiquitin C-terminal hydrolase L1; VCAM-1, vascular cell adhesion molecule 1; VEC, vascular endothelial cell; vWF, von Willebrand factor; WT, wild type; YAP, yes-associated protein

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1. Extracellular vesicles: exosomes, microparticles and their emerging clinical applications

Once maligned as the rubbish bins of the cell, extracellular vesicles (EVs) have recently been heralded as integral to intercellular communication (Hopkin, 2016; Ratajczak et al., 2006). Distinct from apoptotic bodies, these vesicles enclose nucleic acid, protein, and lipid cargo in a lipid bilayer membrane and are traditionally subdivided into two major categories: exosomes and microparticles (Ratajczak and Ratajczak, 2020). The smaller exosomes are produced via inward budding of multivesicular bodies (MVBs) and released into the extracellular space following the exocytosis of those same bodies. The larger microparticles, on the other hand, form directly from outward budding of the plasma membrane. Still, overlapping exosome/microparticle size distributions and challenges in biogenesis-based isolation have resulted in inconsistent sample categorization (Thery et al., 2018). This review therefore generously applies the term “EVs” in place of paper-specific nomenclature, except where exosome- or microparticle-enriched samples display different phenotypes. EVs bind—often specifically—to target cells and are subsequently internalized via endocytosis or membrane fusion, with successful cargo delivery commonly affecting cellular phenotypic change. The biogenesis, release, and uptake of EVs are illustrated in **Figure 1**.

The clinical implications of industrial-scale EV production are exciting and seemingly endless (Wiklander et al., 2019). Endogenous EV cargo is often capable of inducing specific desirable phenotypes in target cells. Exosomes from mesenchymal stem cells (MSCs) have been a prime focus, first used in humans as a successful therapy for graft-versus-host disease (Kordelas et al., 2014) and more recently evaluated as a treatment for severe COVID-19 (Sengupta et al., 2020). Megakaryocyte-derived microparticles, which uniquely target hematopoietic stem and progenitor cells (HSPCs) to promote *de novo* megakaryopoiesis, have been proposed as an alternative to platelet transfusions (Escobar et al., 2020; Jiang et al., 2014; Kao et al., 2022). EVs from endothelial progenitor cells have been shown to promote angiogenesis in endothelial cells (Deregibus et al., 2007), protect against lung and kidney injury (Cantaluppi et al., 2012; Wu et al., 2018), and ameliorate the effects of sepsis in murine models (Zhou et al., 2018). Absent significant endogenous cargo, however, EV membranes can still be harnessed as vehicles for drug delivery, transporting exogenous cargo to specific target cells while evading the immune system (Herrmann et al., 2021). In clinical settings, EVs can be used as a diagnostic tool: tumor-derived EVs, which promote tumor metastasis, could serve as valuable biomarkers for the progression of cancer (Becker et al., 2016). Even in biopharmaceutical manufacturing, EVs are omnipresent, facilitating massive and underappreciated intercellular communication among cultured Chinese Hamster Ovary (CHO) cells (Belliveau and Papoutsakis, 2022).

EV bioprocessing must enable the large-scale production of EVs with consistent purity, glycosylation patterns, and protein, lipid, and nucleic acid contents. Variation in these factors would likely impact EV efficacy, inhibiting the possibility of clinical applications for lack of material consistency. Although some progress has been made, the field still largely lacks rigorous correlations between specific bioprocessing procedures and EV quality control. Because biomechanical stresses are omnipresent in EV bioprocessing, an understanding of those stresses—both how they arise and their impact on EV biology—is essential. While several reviews (Colao et al., 2018; Grangier et al., 2021; Kao and Papoutsakis, 2019; Lee et al., 2021; Patel et al., 2018) have broadly discussed EV bioprocessing and briefly examined biomechanical stresses as relevant factors, this review represents the first comprehensive evaluation of the impact of biomechanical forces—such as those encountered during biomanufacturing and production generally—on EV generation, properties, and biological activity. It also examines the potential impact of biomechanical forces on EV separation, enrichment, and purification, as well as the ability of EVs to recognize target cells and deliver

cargo. The scope of this review is largely and necessarily confined to EVs from "natural"—as opposed to genetically engineered—cells, since research into the effects of biomechanical forces on the biology of EVs from genetically engineered cells is relatively scarce.

2. Biomechanical forces in defined flows and bioreactors

Cells and EVs are primarily exposed to and influenced by three types of mechanical forces—shear, tension, and compression—exerted by a variety of sources, both *in vivo* and *in vitro* (Shah et al., 2014; Wang and Li, 2010). Shear stress is defined as the parallel force on a "body" (here: cell or EV) induced by differing velocity of an adjacent body or fluid (Papaioannou and Stefanadis, 2005). Most often associated with the effects of fluid flow, the application of shear can be steady, pulsatile, or oscillatory (Shah et al., 2014). In pulsatile flow, the magnitude of shear varies regularly with time, while in oscillatory flow, the *direction* of shear varies with time (though the magnitude may also change) (Shah et al., 2014). Physiologically, shear stress—typically observed in blood vessels—is implicated in the biology of vascular endothelial cells (VECs), platelets (PLTs), and megakaryocytes (Mks) (Ballermann et al., 1998; Jiang et al., 2014; Shah et al., 2014). Interstitial fluid flow can likewise expose chondrocytes and osteocytes—in cartilage and bone, respectively—to shear stress (Wang and Li, 2010; Wittkowske et al., 2016). In contrast with shear stress, tensile and compressive stresses are exerted *perpendicularly* to their targets (Shah et al., 2014). However, compression acts in the direction of the target, imposing a "squeeze," while tension pulls away from the target, resulting in a "stretch" (Shah et al., 2014). Tension and compression are often paired, and—in the case of suspension cells—commonly result from "elongational" or "extensional" flows, which occur at flow field constrictions or in areas of accelerating/ decelerating flow (Chisti, 2001; Foster et al., 2021; Petry and Salzig, 2021). In physiological settings, tension is almost always cyclic; it applies mostly to fibroblasts in ligaments and tendons and is also important in bone and cartilage biology (Wada et al., 2017; Wang and Li, 2010). Frequently, tensile stress has been found to impact cellular phenotype more substantially than shear stress of the same magnitude (Foster et al., 2021; Gregoriades et al., 2000). Compression is widespread in the cartilage and is likewise an essential mediator of intercellular communication during proliferation, as cells are increasingly pressed together (Shah et al., 2014; Wada et al., 2017). Both tension and compression are applied to VECs and smooth muscle cells (SMCs) as a result of blood pressure, and to bronchial epithelial cells as a result of ventilation (Wada et al., 2017; Wang and Li, 2010). **Figure 2** illustrates the three basic biomechanical forces, methods for their lab-scale application, and their common manifestations in culture/bioreactors.

Generally, cells react differently to each of the three types of stresses. In fibroblasts, for instance, tension and compression of equal magnitude differentially regulate intracellular protein and RNA content (He et al., 2004). Erythrocyte membrane tension is on average far greater under shear than under tensile stress (Faghih and Sharp, 2018). VECs can likewise distinguish between shear and tension by virtue of plasma membrane characteristics such as lipid order, fluidity, cholesterol content, and receptor activation (Yamamoto and Ando, 2015).

2.1. Lab-scale isolation of defined biomechanical forces

Lab-scale experiments can effectively apply defined biomechanical forces. Microfluidic devices can isolate the impacts of shear via controlled, laminar flow over a stationary sample of adherent cells, avoiding complications at gas-liquid interfaces and any cell-cell or cell-to-hard surface collisions (Czermak et al., 2009; Papoutsakis, 1991). Various viscometers (e.g., cone-and-plate, cone-and cone, coaxial cylindrical) can likewise modulate shear while minimizing other mechanical stressors (Papoutsakis, 1991; Thomas, 1993). Isolation of tensile stress on adherent cells is often achieved via static or cyclic "stretch," which is induced by extending the length of (i.e., stretching) a flexible (silicone) substrate on which the cells are attached. This

stretching is accomplished by pulling a vacuum (e.g., the “Flexcell” system pictured in **Figure 2**) (Yu et al., 2016). The tension in such a system can be applied across any number of axes (e.g., uniaxial stretch, biaxial stretch, etc.) and is usually quantified by the “percent strain/elongation” (e.g., 5%, 10%, 15%) of the flexible substrate (Yu et al., 2016). Extracellular matrix stiffness also exerts tension on cells by promoting integrin clustering and subsequent cytoskeletal reinforcement (Paszek et al., 2005). There are notable parallels between this phenomenon and the tension exerted on cells as a result of microcarrier contact angle (Tsai et al., 2020). Elongational flow can be applied to suspension cells by use of microfluidic devices incorporating constrictions or cross-flow geometries (Foster et al., 2021). Optical tweezers have also been used to apply tension or compression on a single-cell basis (Foster et al., 2021). Even hydrostatic pressure and various osmotic treatments can be employed as models of tension and compression (Le Roux et al., 2019; Wang and Li, 2010).

2.2. Complex forces in cell culture bioreactors

The situation becomes murkier, however, for most practical applications, including agitated bioreactors employed in cell culture biomanufacturing. Mechanical stresses can no longer be individually isolated and analyzed because the use of impellers, baffles, probes, and direct gassing for oxygenation generate complex combinations of shear, tensile, and compressive forces (Cherry and Kwon, 1990; Czermak et al., 2009; Foster et al., 2021; Papoutsakis, 1991). Bubble entrainment and breakup at the bulk gas-liquid interface have been well-documented as inducing both shear and tension (Foster et al., 2021; Gregoriades et al., 2000; Papoutsakis, 1991), as have turbulent eddies (Cherry and Kwon, 1990; Faghih and Sharp, 2018; Papoutsakis, 1991; Petry and Salzig, 2021). The relative influence of such complex biomechanical forces in determining biological outcomes in cultures will vary with vessel geometry and culture properties, as discussed in more detail later in this review. As a further complication, complex biomechanical forces in agitated and aerated bioreactors of any scale are often categorized broadly as “shear stress,” confounding precise correlations between mechanical stressors and biological phenotypes (including EV generation and characteristics) (Chisti, 2001; Foster et al., 2021; Thomas, 1993).

Turbulent flow is the counterpart of laminar flow, and typically occurs in agitated systems such as stirred and aerated bioreactors. In turbulent flow, flow characteristics vary with time and space (Papoutsakis, 1991). In turbulent flows such as in bioreactors, energy is dissipated through a cascade of eddies which decrease in size and, after reaching some minimum length scale (where they are referred to as “Kolmogorov eddies”), are converted to heat (Thomas, 1993). These eddies begin imposing significant stress on cells (or on cells attached to small beads, known as microcarriers) when the eddy length scale—the size of the smallest eddies in the cascade—is less than or roughly equal to cell/microcarrier size (Croughan et al., 1987; Papoutsakis, 1991; Thomas, 1993). The size of Kolmogorov eddies in agitated bioreactors is typically higher than the size of mammalian cells (which, when in free suspension, and thus rounded, range in diameter from 7 to 15 μm), meaning that freely suspended cells in bioreactors do not experience damaging biomechanical forces from turbulent eddies (Kunas and Papoutsakis, 1990). This is in contrast to cells attached to microcarriers (which are much larger than cells, typically over 150 μm in diameter) (Cherry and Papoutsakis, 1988; Cherry and Papoutsakis, 1989; Papoutsakis, 1991; Thomas, 1993). Bubble breakup at the free liquid surface of agitated and aerated bioreactors is responsible for detrimental biomechanical effects on freely suspended cells (Kunas and Papoutsakis, 1990; Michaels et al., 1996; Papoutsakis, 1991).

The challenge in current EV research lies in isolating the impact of various stressors on EV biogenesis, composition, and uptake and subsequently applying these findings, in a meaningful

way, to scale up EV production. While some notable progress has been made in the former endeavor, the latter effort remains nearly nonexistent, complicated by the complexity of forces encountered in large-scale culture. If EVs are to have any significant industrial or clinical impact, however, correlations and empirical relationships between mechanical stress and EV product quantity/quality must be established, likely with computational aid. The difficulty of this endeavor is exacerbated by the fact that the susceptibility of cells to mechanical stress varies with cell type, age, and the culture environment (Chisti, 2001).

3. Impact of biomechanical forces on EV biogenesis and release

EV biogenesis and release is highly dependent on mechanical stress. However, the mechanisms underlying this dependence vary and almost always informed by the physiological relevance of the stress levels, which must be determined on a cell-by-cell basis. Indeed, some EV production processes—such as microparticle release from endothelial cells—are actually inhibited by higher shear. Below, we first summarize what is generally known about the mechanisms involved in EV biogenesis and release and then what is known about these processes under various conditions of biomechanical stress. The goal is to identify broad themes and to suggest avenues for future investigation.

Exosomes are formed through both ESCRT-dependent and ESCRT-independent pathways, where ESCRT stands for Endosomal Sorting Complex Required for Transport (van Niel et al., 2018). In the ESCRT-dependent pathway, ubiquitinated MVB membrane cargo congregates in tetraspanin-rich “microdomains” on the MVB membrane (van Niel et al., 2018). The ESCRT-0, ESCRT-I, and ESCRT-II complexes subsequently recruit cytosolic cargo, RNA, and associated binding proteins (van Niel et al., 2018). Finally, ESCRT-III serves to “pinch off” the microdomains, which become intraluminal vesicles via “inward budding” of the MVB membrane (van Niel et al., 2018). The ESCRT-independent pathway is similar but distinct: both membrane and cytosolic cargo congregate on the MVB membrane, likely due to regulatory behavior of tetraspanins (especially CD9, CD63, and CD81); the subsequent conversion of sphingomyelin to ceramide results in negative membrane curvature and promotes the “pinching off” of intraluminal vesicles (Anand et al., 2019; Trajkovic et al., 2008; van Niel et al., 2018). Nevertheless, ESCRT-III is once again required for inward budding of the MVB membrane (van Niel et al., 2018). During both types of exosome biogenesis, interactions between the proteins syndecan and syntenin serve to recruit ALIX, which works in tandem with fellow endosomal protein TSG101 to assist ESCRT function (Anand et al., 2019; Baietti et al., 2012; van Niel et al., 2018). Once intraluminal vesicles have been created, their parent MVBs may be transported to lysosomes (often, by way of autophagosomes) for degradation; the MVBs that escape this fate (likely due to their enrichment in cholesterol) bind to the plasma membrane via actin, SNARE proteins, and synaptotagmin family members, subsequently releasing intraluminal vesicles as exosomes (Mathieu et al., 2019; van Niel et al., 2018). Distinct RAB GTPases drive the intracellular transport of MVBs along microtubules, variously directing said MVBs to either lysosomes or the plasma membrane (Mathieu et al., 2019; van Niel et al., 2018).

Unlike exosomes, microparticle biogenesis is initiated by the congregation of membrane cargo and ceramide in tetraspanin-rich microdomains on the *plasma* membrane (Raposo and Stoorvogel, 2013; van Niel et al., 2018). Much as in the case of ESCRT-dependent exosome biogenesis, ESCRT complexes serve to recruit microparticle cargo from the cytoplasm (van Niel et al., 2018). Again, as before, ESCRT-III plays a particularly vital role in the outward blebbing by which microparticles are eventually released (van Niel et al., 2018). This blebbing is characterized by an influx of Ca^{2+} ions and the protein ARF6 (van Niel et al., 2018). Subsequent actin- and myosin-driven contractions of the plasma membrane accompany disruption of

phosphatidylserine (PS) equilibrium, which—through a process known as “lipid flipping”—spurs local reorganization of the actin cytoskeleton (van Niel et al., 2018).

3.1. Mesenchymal stem cells

The substantial impact of mechanical stress on MSC biology has been well-established. For instance, cyclic stretch of 10% promotes MSC chondrogenesis, while relatively high shear stress—in the range of to 5 to 25 dyn/cm^2—will induce osteoblast differentiation (Kang et al., 2022; Vining and Mooney, 2017). However, despite relatively widespread research into MSC EVs, the relationship between mechanical agitation and MSC EV production is only just emerging. To date, controlled *in vitro* experiments have found low (0.001 and 0.0001 dyn/cm^2) shear stress (Kang et al., 2022), physiological (8%) cyclic stretch (Xiao et al., 2021), and (1.5 g/cm^2) compression to promote MSC EV production. For the noted shear stresses, EV yield (i.e., overall EV quantity) increased by 6.5- and 6.7-fold after 24 h (Kang et al., 2022); the stretch-induced increase in EVs was not quantified (Xiao et al., 2021) and the compressive stress resulted in a 4-fold increase in EV yield after 24 h, with similar results observed *in vivo* using a murine model (Huang et al., 2022). MSC EV production was also boosted by nearly 4-fold in the 48 h following brief application of a custom microfluidic device designed to gently “squeeze” the cells (Hao et al., 2023). These effects of mechanical stress have also been confirmed in more complex spheroid (Cha et al., 2018; Min Lim et al., 2022), scaffold-based (Guo et al., 2021), and microcarrier-based (Adlerz et al., 2019; de Almeida Fuzeta et al., 2020; Gazeau et al., 2020; Haraszti et al., 2018) cultures, but often cannot be directly attributed to shear, tension, or compression, as differences between control and experimental culture architecture create numerous confounding variables. In one study of spheroid culture, however, the impact of culture architecture *was* controlled, and EV production under complex mechanical stress increased by as much as 9-fold (Cha et al., 2018). Indeed, among common mechanical stresses, only 20% static stretch (imposed for 72 h via the Flexcell system) failed to appreciably increase MSC EV yield (Yu, Wenting et al., 2021). Comparable results have been observed for MSCs grown on stiff hydrogel substrates, which exert continual (not cyclic) tension; MSCs in these conditions produced 2- and 10-fold *more* EVs than MSCs grown on soft hydrogel substrates and rigid plastic substrates, respectively (Lenzini et al., 2021). This was the result of tension-induced development of dense actin “mesh” which served to stabilize the cytoskeleton and inhibited MVB transport to the plasma membrane; such transport is required for MVB exocytosis and subsequent exosome release (Lenzini et al., 2021). It appears that this phenomenon may be unique to the static/continual application of tension on parent MSCs, because, as noted, MSC EV release is increased under cyclic stretch.

Notably, expected EV production sometimes differs remarkably between MSC subtypes. For instance, shear stress is far more effective at promoting EV production in MSCs derived from dental pulp than in those derived from adipose tissue (Guo et al., 2021). Under average shear of 0.5 to 5 dyn/cm^2, EV production by dental pulp-derived MSCs increased by 24-fold after 48 h, while EVs production by adipose tissue increased by only 2-fold in the same conditions. In fact, for the latter MSC subtype, EV release appeared to decrease under higher shear (5 to 30 dyn/cm^2), suggesting the existence of cell-specific optimal shear levels for EV production, beyond which additional mechanical stress negatively impacts EV yield (Guo et al., 2021).

The reasons for mechanical stress-induced increases in MSC EV generation need additional investigation. Low shear stress (0.001 to 0.0001 dyn/cm^2) has been linked to plasma membrane ion channel activation, with a subsequent influx of intracellular Ca^{2+} promoting EV release (Kang et al., 2022). Moreover, the impact of Ca^{2+} is likely mediated by upregulation of proteins related to cytoskeleton remodeling, including β-catenin, phosphorylated extracellular signal-regulated kinases 1 and 2 (ERK 1/2), and calpain (Kang et al., 2022). Involvement of caspase-3 was

explicitly ruled out (Kang et al., 2022), a largely predictable outcome at lower shear stresses due to the absence of shear-induced apoptosis (in which caspase-3 is implicated). The accumulation of the Yes-Associated Protein (YAP) in MSC nuclei was additionally found to be required for increased MSC EV production under shear (Guo et al., 2021). YAP likely mediates the EV release process via activation of the canonical Wnt signaling pathway, which is itself responsible for the accumulation of β-catenin in the cytoplasm (Guo et al., 2021). MSC EV release under compression is dependent on RAB27b (but not RAB27a) (Huang et al., 2022), while EV production by the aforementioned "squeezed" MSCs is thought to depend on plasma membrane permeabilization and the corresponding formation of transient nanopores through which Ca^{2+} can enter the cell(s) (Hao et al., 2023).

3.2. Fibroblasts

The impact of mechanical stress on fibroblast EV biogenesis and release—not extensively studied to this point—varies widely as a function of cell source and stimulation method, perhaps a testament to the diversity of fibroblast types. Fibroblast-like periodontal ligament cells released roughly 30-fold more EVs under 20% cyclic stretch (at 0.17 Hz for 24 h) than under static conditions, but EV yield from both gingival and dental pulp fibroblasts remained unchanged, possibly because these latter two cell types do not experience cyclic stretching *in vivo* (Wang, Z. et al., 2019). Similarly, aortic fibroblasts exposed to slightly less acute cyclic stretch of 12% (at 1 Hz for 18 h) experienced only a 1.3-fold boost in EV production (Akerman et al., 2019). Static stretch of 15% also increased EV production—by 2.5-fold after 24 h—in cultures of hallux valgus (bunion) fibroblasts (Xie et al., 2020). Interestingly, however, periodontal ligament fibroblasts exposed to 1 g/cm^2 *compression* (for 4 h) displayed a 2-fold *decrease* in EV release relative to unstressed control cultures (Zhao et al., 2021), though a separate study observed a 1.3-fold *increase* in EV yield from the same fibroblasts following 24 h exposure to the same stress (Zhang et al., 2022). Whether this discrepancy is attributable to the nature of the compressive force, the unique characteristics of the parent fibroblasts in question, or variation in experimental methods (e.g., EV isolation and quantification) remains unclear. In every case, the mechanisms informing fibroblast EV production under mechanical stress have not been investigated. Moreover, published research is limited in scope, examining only exosome-enriched EV samples. The impact of mechanical stress on the release of larger fibroblast EVs is therefore unknown.

3.3. Bone and cartilage tissue

Bone and cartilage are highly responsive to mechanical loading. Bone integrity depends on physical exercise, with loss of bone tissue occurring in response to underuse and/or microgravity environments (Morrell et al., 2018). While tension and compression remain unevaluated for their role in EV production by bone and cartilage tissue, shear stress promotes EV production in both tissue types (Liu, Q. et al., 2022; Morrell et al., 2018). Shear stress of 35 dyn/cm^2 applied in two 10 min intervals boosted EV production by 22-fold in osteocyte-like MLO-Y4 cells, a result of transient intracellular influxes of Ca^{2+} that trigger simultaneous actomyosin contractions (Morrell et al., 2018). Similarly, cultures of rat condylar chondrocytes exposed to shear stresses of 4, 8, and 16 dyn/cm^2 produced 1.3, 1.7, and 2.5 times as many EVs as control cultures (Liu, Q. et al., 2022). Additional study into the impact of tension and compression on EV production by bone and cartilage tissue must be a priority.

3.4. Bronchial epithelial cells

The bronchial epithelium is exposed to cyclic tension and compression during natural ventilation; this stress reaches pathological levels during mechanical ventilation (Wang et al., 2022) and asthmatic episodes, with constricted airways amplifying compressive forces in the latter case (Mitchel et al., 2016; Mwase et al., 2022; Park et al., 2012). The impacts of these

stresses on EV production by bronchial epithelial cells (BECs) is not yet well-understood. In the most comprehensive study to date, BEC EV yield was increased by 2-fold following 10% cyclic stretch (at 0.67 Hz for 24 h), but remained unchanged following 5% static stretch over the same period (Najrana et al., 2020). Both of the aforementioned stretch protocols were developed to mimic the *in vivo* forces exerted during fetal lung development (Najrana et al., 2020). BEC EV production was likewise boosted by nearly 2-fold under even higher (supraphysiological) levels of cyclic tension (i.e., 20% cyclic stretch at 1 Hz for 24 h), a direct result of endoplasmic reticulum (ER) stress mediated by apoptosis signal-regulating kinase 1 (ASK1) (Tang et al., 2022). A similar increase in BEC EV yield—incurred via the same mechanism—was observed *in vivo* for a murine model of mechanical ventilation-induced pulmonary fibrosis (Tang et al., 2022). BEC EV yield also appeared to increase following the application of supraphysiological levels of compression (at 30 cm H_2O for 3 h), but this increase was not quantified (Mwase et al., 2022).

3.5. Endothelial cells

The impact of shear stress on vascular endothelial cells (VECs) has been well-reviewed (Feng, S. et al., 2022). Unlike most other cell types, cultured VECs have repeatedly yielded *more* microparticle-enriched EVs as shear magnitude decreases (Kim et al., 2015; Ramkhelawon et al., 2008; Vion et al., 2013b). For physiological levels of shear stress between 1.5 and 2 dyn/cm^2, VEC-derived EV production was consistently about 1.3-fold *lower*—relative to static conditions—after 24 h (Ramkhelawon et al., 2008; Vion et al., 2013b). Under supraphysiological shear levels of 15 to 20 dyn/cm^2, EV production dropped even further: by 3- to 4-fold (relative to static conditions) after 24 to 36 h (Kim et al., 2015; Ramkhelawon et al., 2008; Vion et al., 2013b). These findings have been confirmed *in vivo* for low and oscillatory shear, and manifest most substantially in atheroprone or otherwise diseased individuals (Boulanger et al., 2007; Jenkins et al., 2013; Kim et al., 2015; Navasiolava et al., 2010; Silva et al., 2021; Tremblay et al., 2017; Vion et al., 2013b). Still, this makes physiological sense, as higher shear in blood vessels is atheroprotective, while lesser, oscillatory, or retrograde shear is associated with the turbulent flow patterns caused by vessel blockage and atherosclerosis (Rautou et al., 2011). Whereas excess shear is an unwelcome stressor for most cell types, VECs—normally exposed to high shear *in vivo*—may experience static (i.e., subphysiological) conditions as “more stressful” and release EVs as a result. Indeed, endothelial microparticles produced via various chemical stressors transport apoptotic agent caspase-3 away from parent cells, preserving local viability of the endothelium; a similar role has been suggested for microparticles forged under shear (Abid Hussein et al., 2007; Rautou et al., 2011). However, one recent study found VECs to produce roughly thrice as many EVs after 24 h exposed to both supraphysiological (20 dyn/cm^2) and turbulent oscillatory (±5 dyn/cm^2) shear stress (Chung et al., 2022). HUVEC-like cells seeded on microcarriers have also produced increasing EV yields with increased rotational agitation, though the yields were correlated with Kolmogorov eddy length scale, not shear stress (Gazeau et al., 2020). Moreover, even under very low average and maximum shear rates (0.015 dyn/cm^2 and 1.3 dyn/cm^2, respectively), VECs seeded on scaffolds in flow-perfusion bioreactors were found to boost EV production by 100- and 10,000-fold after 24 and 36 h, respectively (though much of this yield increase may be due to shear-independent factors such as improved nutrient availability, oxygenation, and waste removal) (Patel et al., 2019). The observed discrepancy in VEC EV production as a function of shear stress may be due to the recent ascent of nanoparticle tracking analysis (NTA) as the primary method of EV quantification. The earlier papers described above all employed flow cytometry, which is limited in its ability to count exosome-scale particles (Kim et al., 2015; Ramkhelawon et al., 2008; Vion et al., 2013b). Moreover, the differential centrifugation techniques in these papers preclude any significant inclusion of exosome-scale particles, which generally require ultracentrifugation at 100,000 g to precipitate. This suggests distinct and possibly opposing roles for endothelial exosomes and

microparticles in the presence of shear stress, with higher exosome levels under physiological conditions and higher microparticle levels following exposure to subphysiological magnitudes (or absence) of shear. Still, more investigation is necessary, and oscillatory shear, despite its pathogenic associations, appears to promote exosome and microparticle formation alike (though the content of EVs produced under oscillatory shear is distinct, as discussed in section 4.5) (Chung et al., 2022). Low CD63 (an exosome marker) levels in VEC-derived EV samples from the flow-perfusion bioreactors suggest that the release of uniquely small microparticles (i.e., plasma membrane-derived particles), rather than exosomes, may be responsible for the NTA-detected uptick in EV biogenesis under shear (Patel et al., 2019).

In the lungs, tensile and compressive forces imposed by ventilation may be more influential than shear in modulating VEC phenotypes. Even absent ventilation, blood pressure imposes significant tensile stress on VEC cells in a cyclic manner. Three papers have quantified the impact of cyclic stretching on microparticle-enriched EV production from VECs (Jia et al., 2017; Letsiou et al., 2015; Vion et al., 2013a). Supraphysiological levels of cyclic stretching (i.e., 15% to 18%) have increased in EV production by 2- to 3-fold after 4 to 6 h, 1.5- to 4-fold after 24 h, and up to 13-fold after 48 h (Jia et al., 2017; Letsiou et al., 2015; Vion et al., 2013a). To the contrary, physiological levels of cyclic stretching (i.e., 5%) have not appreciably increased EV yields relative to static conditions (Vion et al., 2013a), though one paper notes an increase of roughly 2-fold that remains consistent from 4 to 24 h (Letsiou et al., 2015). These trends have been confirmed in murine models (Letsiou et al., 2015). Notably, only microparticle-enriched samples have been evaluated in the context of cyclic stretch; the impact of cyclic stretch on VEC exosome production remains unknown.

Mechanisms underlying mechanical stress-induced variation in VEC microparticle release have been proposed. Static conditions induce apoptotic processes in VECs, and subsequent microparticle release is dependent on caspase activation (Vion et al., 2013b). Under physiological levels of shear stress, however, microparticle release is caspase-independent, mediated instead by the activity of ERK 1/2 and Rho kinase (ROCK) (Vion et al., 2013b). Mitochondrial dysfunction and ABCA1-mediated phosphatidylserine (PS) exposure serve as additional mechanisms promoting microparticle release in these conditions (Kim et al., 2015; Vion et al., 2013b). Under high levels of shear, activation of sirtuin 1 (SIRT1) raises nitric oxide (NO) levels; the joint action of SIRT1 and NO serves to increase mitochondrial biogenesis in VECs, and NO additionally attenuates ABCA1 expression (Kim et al., 2015; Vion et al., 2013b). Notably, calpain activity is not associated with VEC microparticle release in any instance (Vion et al., 2013b). To date, no explanation has been offered regarding the increase in exosome-scale EVs derived from VECs under shear. The increase in VEC microparticles under cyclic stretch, meanwhile, is mediated by non-apoptotic caspase action, but not ROCK or calpain activity (Vion et al., 2013a). ER stress has also been implicated in this phenomenon (Jia et al., 2017).

3.6. Smooth muscle cells

Smooth muscle cells (SMCs) in the blood vessels, protected from shear by the endothelial lining, are nonetheless exposed to significant tensile stress imposed by blood pressure. SMC EV production as a function of tension is not well-characterized. In one study, murine aortic SMCs exposed to supraphysiological (18%) cyclic stretch (at 1 Hz) experienced 2- and 3-fold increases in microparticle-enriched EV yield after 24 and 48 h, respectively (Jia et al., 2017). This stretch-mediated increase in EV production was further found to depend on ER stress, and, in particular, the up-regulation of ER stress proteins GRP78, ATF4, and CHOP (Jia et al., 2017). A second study, however, observed no change in the production of exosome-enriched EVs

following 12% cyclic stretch (at 1 Hz) for 24 h (Akerman et al., 2019). The reason for this discrepancy is unclear, but may involve differences in mechanical loading or EV isolation.

3.7. Platelets and megakaryocytes

Supraphysiological levels of shear stress (variously >60 or ≥100 dyn/cm^2) (Haga et al., 2003; Holme et al., 1997) have been found to significantly boost human platelet EV production *in vitro* (Chen et al., 2010; Haga et al., 2003; Holme et al., 1997; Leytin et al., 2004; Miyazaki et al., 1996; Reininger et al., 2006; Roka-Moiia et al., 2021; Sakariassen et al., 1998). Where quantified (using flow cytometry), EV yields under shear stress of 100 to 108 dyn/cm^2 have increased by between 3.1- and 14.5-fold after 1 to 5 min (relative to yields in static conditions) (Chen et al., 2010; Haga et al., 2003; Miyazaki et al., 1996; Reininger et al., 2006). One study further suggests that EV undercounting may disguise even higher "true" EV yields—of up to 55-fold—in these conditions, as platelets present in the samples may "mask" the presence of EVs during flow cytometry (Reininger et al., 2006). On the other hand, lower, physiological shear levels (1.4 to 60 dyn/cm^2) (Haga et al., 2003; Holme et al., 1997) have repeatedly failed to significantly elevate EV production, though most studies nonetheless note small production increases of between 1.1- and 2.7-fold after 1 to 5 min, relative to EV production in static conditions (Chen et al., 2010; Haga et al., 2003; Holme et al., 1997; Leytin et al., 2004; Miyazaki et al., 1996; Reininger et al., 2006; Roka-Moiia et al., 2021; Sakariassen et al., 1998; Shai et al., 2012). These conclusions are supported by a significant body of research examining shear-induced platelet EV production *in vivo*. With noted exceptions (Rigamonti et al., 2020), increases in circulating platelet-derived EVs have been observed in response to a variety of physical exercises, including strenuous treadmill (Maruyama et al., 2012) and cycling exercise (Chaar et al., 2011; Wilhelm et al., 2016) and moderate cycling exercise (Sossdorf et al., 2010, 2011). Whether such EV increases can be primarily attributed to increased shear in the blood vessels is disputed, however (Wilhelm et al., 2017). Platelets sampled from subjects engaged in strenuous physical exertion (graded exercise on a bicycle ergometer until exhaustion) and subsequently sheared *in vitro* have also demonstrated increased capacity for EV production under supraphysiological (but not physiological) shear levels (Chen et al., 2010). Elevated platelet EV production has also been confirmed *in vitro* and *in vivo* for cardiopulmonary bypass systems—such as left ventricular assist devices (LVADs) and extracorporeal membrane oxygenation (ECMO)—that induce supraphysiological levels of shear or other mechanical stress (Diehl et al., 2010; Meyer et al., 2020; Sun et al., 2022). Interestingly, platelets exposed to an artificial stenosis produced EVs more efficiently (i.e., produced more EVs per unit time) than platelets exposed to constant shear of the same magnitude exerted by the stenosis (Holme et al., 1997; Sakariassen et al., 1998). This suggests that sudden changes in the magnitude of shear may be more important in platelet EV biology than the shear levels themselves. Notably, however, all of the platelet research described to this point was conducted exclusively on microparticle-enriched EV samples. The role of shear in modulating platelet-derived exosome release remains unclear. Only one study has evaluated the role of shear in exosome-scale EV production, reporting that platelet exosome yield is unaffected by variation in shear stress induced by surgical aortic valve replacement (SAVR) (Weber et al., 2020). This stands in significant contrast to the stress-induced production of larger EVs, and requires controlled *in vitro* validation, especially as the shear in question was broadly defined and, by nature, transiently applied.

The mechanisms by which supraphysiological levels of shear promote platelet EV release have been extensively investigated. In particular, the binding of platelet glycoprotein Ib (GPIb) to von Willebrand factor (vWF) is significantly implicated in this process (Goto et al., 2003; Miyazaki et al., 1996; Pontiggia et al., 2006; Reininger et al., 2006). Under high shear, platelet membranes form "tethers" following their GPIb-mediated binding to immobilized or platelet-expressed vWF

(Reininger et al., 2006). These tethers act as a mechanism by which the shear-exposed platelets maintain contact with bound vWF, even as they are increasingly dragged downstream (Reininger et al., 2006). Eventually, the tethers detach from their parent platelets, fragmenting into numerous EVs that remain bound—at least temporarily—to vWF (Reininger et al., 2006). This process is mediated by an influx of intracellular Ca^{2+}, which activates platelet calpain, itself required for the degradation of actin binding protein (ABP) (Miyazaki et al., 1996). Binding of platelet glycoprotein IIb-IIIa (GPIIb-IIIa) to vWF—a process that further strengthens the bonds established by the adhesion of GPIb to vWF—has likewise been suggested to have a minor (Goto et al., 2003; Miyazaki et al., 1996; Pontiggia et al., 2006) or even significant (Haga et al., 2003) role in shear-induced platelet EV release, though data conflict (Reininger et al., 2006). For longer shearing timeframes (i.e., >2 min), EV production may operate independently from the binding of GPIb and vWF, and be mediated instead by activation of protein kinase C (PKC) (Miyazaki et al., 1996). In other words, PKC seemingly works to extend—but not initiate—platelet EV release (Miyazaki et al., 1996). EV release under supraphysiological levels of shear has additionally been shown to depend on stress-induced increases in cytokine levels (i.e., IL-6, erythropoietin, and thrombopoietin levels) (Nomura et al., 2000).

Importantly, the bulk of the aforementioned literature concerning platelets was published prior to 2009, when the field lacked differentiation between platelet- and Mk-derived EVs (Flaumenhaft et al., 2009; Stone et al., 2022). In 2009, a large fraction of so-called “platelet-derived EVs” were recognized as originating from Mks (Flaumenhaft et al., 2009). Renewed research into the distinctions between platelet and Mk EV production under mechanical stress must therefore be a priority. Mks, themselves platelet progenitors, are exposed to shear stress as they protrude through the endothelial lining of sinusoidal blood vessels and subsequently encounter blood flow (Junt et al., 2007). Mks have experienced a boost in EV production—by 47-fold—following 2 h of exposure to physiological (2.5 dyn/cm^2) shear (Jiang et al., 2014). Shorter exposure (for 0.5 h) was associated with lesser production (a 24- to 27-fold increase over static conditions). This shear-induced Mk EV release is dependent on a non-lethal subset of apoptotic processes mediated by activation of caspase-9 and caspase-3 (Jiang et al., 2014; Luff et al., 2018). The binding of p53 to BAX is up-regulated in Mks under shear, and subsequent cytochrome c release from the mitochondria—likely caused by insertion of BAX into the mitochondrial membrane—activates the caspase cascade (Luff et al., 2018). As with platelets, mechanical stress-related Mk EV research has been conducted almost exclusively on microparticle-enriched samples; relatively little is known about exosome-scale EVs derived from Mks. Moreover, tensile and compressive stress have not been investigated for any potential role in EV release from platelets/Mks, though these stressors are not commonly experienced by such cell types (with the exception of Mks migrating through the endothelial lining of sinusoidal blood vessels). Additionally, given the propensity of suspension cells—such as platelets and Mks—to rotate in response to shear, we remind the reader that shear stress will be experienced differently by suspension and adherent cells.

3.8. Skeletal muscle cells

Although data are somewhat sparse, skeletal muscle cells (SkMCs) *in vitro* have thus far demonstrated increased EV production *only* in response to tension. This is likely due to the significant involvement of tension in skeletal muscle function—and the relative physiological irrelevance of other forms of mechanical stress, such as shear. Although mild shear stress (6 dyn/cm^2) (Takafuji et al., 2021) and general mechanical overload (induced by electric pulse stimulation) (Vechetti et al., 2021) demonstrated no impact on SkMC EV yield *in vitro*, 25% cyclic stretch (at 1 Hz for 48 h) resulted in an 11-fold increase in EV yield (Guo et al., 2021). This increase is dependent on YAP, which accumulates in the nuclei of SkMCs in response to mechanical stretch, likely activating the canonical Wnt signaling pathway (Guo et al., 2021). In

another study, sustained, low-strain cyclic stretch (of 15% at 1 Hz for 24 h) and brief, high-strain cyclic stretch (of 12-22% at 1 Hz for 24 h, with 50 min of rest for every 10 min stretching) boosted SkMC EV yield by 2.5- and 4.2-fold, respectively (Mullen et al., 2022). More complex forms of mechanical stress—resulting from physical exercise in humans (Rigamonti et al., 2020) and synergist ablation in a murine model (Vechetti et al., 2021)—have also promoted SkMC EV generation *in vivo*. We note that the described research involving SkMC EVs has focused exclusively on exosomes or exosome-enriched samples; the interrelationships between mechanical stress and SkMC microparticle production have yet to be investigated.

3.9. Tumor cells

Relative to healthy surrounding tissue, the tumor microenvironment is characterized by high levels of mechanical stress, primarily incurred by elevated interstitial fluid flow rates and increased hydrostatic and compression-induced pressure (Feng, T. et al., 2022; Stylianopoulos et al., 2013). By nature, tumor growth serves to continually modify interstitial fluid flow, creating blockages and increasing pressure in the surrounding microenvironment (Koomullil et al., 2021). This phenomenon renders tumor-derived EV production rates uniquely time-dependent, with higher such rates likely to occur in late-stage tumors (Koomullil et al., 2021). Additionally, circulating tumor cells encounter significant mechanical stress during transport through the vasculature, with membrane deformation likely common in narrow capillaries (Wan et al., 2022). Without exception, application of shear, tension, and compression on tumor cells has served to increase exosome-scale EV production (Koomullil et al., 2021; Wang, K. et al., 2019; Wang, Y. et al., 2020). Shear of 10 dyn/cm^2 applied for 1 h resulted in a 2-fold increase in HeLa cell-derived EVs and a 2.5-fold increase in EVs from MDA-MB-231 (human triple negative breast cancer) cells (Wang, K. et al., 2019). Uniaxial cyclic stretch of 10% (applied at 0.3 Hz for 48 h) had a similar impact on exosome production, with EV production in MCF-7 (human estrogen receptor positive breast cancer), MDA-MB-231, and 4T1.2 (murine triple negative breast cancer) cells increasing by 1.2-fold (insignificant), 6-fold, and between 1.9- and 2.4-fold, respectively (Koomullil et al., 2021; Wang, Y. et al., 2020). Compression of HeLa cells has likewise promoted EV release, with a peak production increase of 4.7-fold observed following the application of 0.23 kPa press stress (1 h) (Wang, K. et al., 2019). Increased substrate stiffness, which induces tension on parent cells, has also been well-established as increasing tumor EV yields from breast (Patwardhan et al., 2021; Wu et al., 2023), liver (Wu et al., 2023), pancreatic (Wu et al., 2023), and prostate cancer (Liu, Z. et al., 2022) cell lines. Where quantified, tumor EV yields in stiff substrates have increased by 3- to 7-fold relative to EV yields from soft substrates (Liu, Z. et al., 2022; Wu et al., 2023). Similar findings have been observed for cancer-associated fibroblasts grown on stiff substrates (Xiao et al., 2022). More complex mechanical forces induced by turbulent agitation in a bioreactor have also been correlated with increased EV production in HeLa cells (Grangier et al., 2020). Notably, malignant parent cells have demonstrated higher EV yields than non-malignant cells in response to similar substrate stiffness, suggesting oncogenic mutations may uniquely increase cellular sensitivity to biomechanical force (Wu et al., 2023). The mechanism underlying increased EV production in tumor cells under mechanical stress is not well understood, except in the case of substrate stiffness, where increased EV yields have been linked to phosphorylation of focal adhesion kinases and subsequent activation of Akt, which boosts GTP-RAB8 levels. YAP/TAZ action also plays a key role in increased breast cancer EV production in stiff substrates. Additionally, EV production by MDA-MB-231 and 4T1.2 cells under long-term (48 h) cyclic stretch can be explained almost entirely by increased cellular proliferation, suggesting EV release in these conditions remains constant on a per-cell basis (Wang, Y. et al., 2020). Mechanical stress may also modulate the size of secreted EVs. In one case, the tension-mediated action of myosin II served to decrease the average size of exosome-enriched EVs derived from Ewing's sarcoma cells (Villasante et al., 2016), though this phenomenon was not observed in other models that

increased stiffness of the extracellular matrix in order to induce tension (Liu, Z. et al., 2022; Wu et al., 2023). In contrast with most other cell types, tumor cells have been well-characterized with respect to exosome production under mechanical stress; after all, almost all the research described above involves exosomes or exosome-enriched samples. However, the role of mechanical stress in the biogenesis/release of larger (microparticle-scale) EVs remains to be investigated.

3.10. Other mammalian cells

The impact of mechanical stress on EV generation has been briefly examined in several additional cell types. For instance, human urinary podocytes have been found to increase EV production by 3.8-fold following the application of 10% cyclic stretch (at 0.5 Hz and for 24 h) (Burger et al., 2014). This magnitude of cyclic stretch mimics pathological levels of intraglomerular stress experienced *in vivo* (Burger et al., 2014). EV yields from HEK293T cells overexpressing angiotensin II type 1 receptor (AT1R) were increased by 2-fold following osmotic stretch (i.e., application of 143 mOsm/kg hypotonicity for 30 min) (Pironti et al., 2015). This research is particularly notable due to the genetic engineering of the parent cells. Similarly, a murine model of cardiac pressure overload—induced by surgical transverse aortic constriction—boosted serum EV yields by 2-fold after 1 day and 3-fold after 7 days; most such EVs were derived from cardiomyocytes (Pironti et al., 2015). Neonatal rat cardiomyocyte EV yield also increased by 2-fold following 24 h application of 20% static stretch *in vitro* (Yuan et al., 2018). However, for Schwann cells—the glial cells of the peripheral nervous system—no EV yield differentials were observed following the application of complex mechanical force (induced by the magnetic stimulation of co-cultured nanoparticles) (Xia et al., 2020).

Publications relating biomechanical stress to the biogenesis of leukocyte-derived EVs are limited. In one study of note, stirred-tank bioreactors increased EV yields from THP-1 (immortalized human monocyte) cells by 10-fold (Grangier et al., 2020). In another instance, a novel seesaw-motion bioreactor boosted daily EV production from natural killer cells by almost 2-fold relative to static culture (Wu et al., 2022). Beyond these two cases, however, the impact of mechanical stress on the biogenesis of leukocyte- and erythrocyte-derived EVs has only been examined in the context of physical exercise. In such studies, mechanical stress cannot be effectively isolated as an independent variable and results understandably conflict (Chaar et al., 2011; Sossdorf et al., 2011). However, atomic force microscopy has indicated an increase in EV-like particles on the membranes of erythrocytes cultured on tilting shakers, a phenomenon possibly linked to faster cellular aging under sustained biomechanical stress (Dinarelli et al., 2022).

3.11. Non-mammalian cells

Though clinical relevance and unique susceptibility to mechanical stress (Chalmers and Ma, 2015; Thomas, 1993) have made mammalian cells the prime targets of research involving stress-induced EV production, EVs derived from non-mammalian sources will likely exhibit characteristics similar to those observed in their mammalian counterparts. For instance, preliminary research suggests mechanical stress serves to increase ciliary EV production in *Caenorhabditis elegans* (Wang, J. et al., 2020). The yield of outer membrane vesicle (OMV) derived from *Acinetobacter baumannii* bacteria is likewise boosted by more than 5-fold following agitation with a high speed dispersator (Li et al., 2020). OMVs produced under these stress conditions were nearly 1.5 times larger, less polydisperse, and more negatively charged, and possessed distinct proteomic profiles and increased (by 3-fold) lipopolysaccharide content (Li et al., 2020).

3.12. General themes and trends

Clearly, the reasons for the summarized correlations between mechanical stress and EV biogenesis are complex, depending heavily on both parent cell type and the nature of the applied stress. Nevertheless, recurring themes have begun to emerge. In the majority of cases, EV release increases following exposure of parent cells to mechanical stress. However, we have found five exceptions to this rule. The first occurs when the applied stress is not reflective of pathological conditions experienced *in vivo* by the cell type in question. For instance, microparticle production is not increased in VECs under high shear, since shear is required for normal VEC physiology. The second exception occurs when the stress is not physiologically relevant. For instance, shear stress applied to SkMCs and cyclic stretch applied to both gingival and dental pulp fibroblasts fails to appreciably alter EV release, as the mechanical stresses in question are not natively experienced by the respective cell types. The third exception occurs when EV yields "plateau" (and in some cases, subsequently decrease) at high stresses or after long periods of stress exposure. For several samples, including adipose-derived MSCs under shear (Guo et al., 2021) and HeLa tumor cells under compression (Wang, K. et al., 2019), EV yields began decreasing when exposed to the highest tested mechanical stress levels, suggesting the existence of "optimal" stress levels beyond which maximum EV yields diminish. Similarly, periodontal ligament cell EV production plateaued after 36 h of cyclic stretch, suggesting cells' EV production machinery may adapt to chronic mechanical stress (Wang, Z. et al., 2019). The fourth exception occurs when the applied mechanical stress results in high levels of apoptosis, preventing the active release of EVs; such an exception has been proposed as the reason for decreased fibroblast EV production under compression (Zhao et al., 2021). Finally, and perhaps most importantly, the fifth exception occurs when exosomes yields are unaffected by mechanical stress, even as microparticle yields (which may not be measured) increase. This phenomenon has been observed in exosomes from platelets (Weber et al., 2020) and SMCs (Akerman et al., 2019).

Given differential exosome and microparticle yields in parent cells exposed to mechanical stress, it grows increasingly prudent to analyze the production of both EV subtypes in a single study. Still, to date, such research is rare. Indeed, among many cell types, the entire relevant body of literature has been limited to an evaluation of *either* exosome or microparticle production (but not both). For instance, studies of stress-induced changes in MSC, fibroblast, and tumor cell EV production are limited almost exclusively to exosomes, while the impact of mechanical stress on platelet and Mk EVs has primarily been studied in the context of microparticles. In addition, many cell types—including erythrocytes and leukocytes—have largely been ignored in the context of mechanical stress-induced EV production.

The reasons for EV production (or the lack thereof) under mechanical stress are numerous and vary on a cell-by-cell basis, though common trends nonetheless emerge. For very high stress levels, unique, non-apoptotic caspase action appears responsible for the bulk of EV release (Hill et al., 2022; Jiang et al., 2014; Vion et al., 2013a; Vion et al., 2013b). Both ROCK-dependent and ROCK-independent pathways have been proposed as an intermediate mechanism (Hill et al., 2022). Mitochondrial dysfunction appears to wield significant influence in this process; for both megakaryocytic and endothelial cells, said dysfunction—induced by p53 and the absence of sirtuin 1 (SIRT1), respectively—is correlated with pathological levels of shear (which differ with cell type) and linked to a decrease in microparticle-enriched EV samples (Kim et al., 2015; Luff et al., 2018). In cases of less severe mechanical stress, alternative pathways have been proposed. An influx in intracellular Ca^{2+} levels, for instance, is required for stress-induced EV production by MSCs (Kang et al., 2022), osteocytes (Morrell et al., 2018), and platelets (Miyazaki et al., 1996). As noted, high Ca^{2+} levels serve to activate various proteins involved in cytoskeletal remodeling, including PKC and calpain (Kang et al., 2022; Miyazaki et al., 1996), and even induce actomyosin contractions along the plasma membrane, with notable examples

in blood cells and osteocytes (Morrell et al., 2018; Zwaal and Schroit, 1997). Though not yet directly linked to increased (exosome-scale) EV production, intracellular Ca^{2+} levels are also elevated in VECs under shear stress, likely a result of ATP release at the caveolae (Yamamoto et al., 2011). High Ca^{2+} levels additionally promote PS externalization—an important step in membrane budding—via simultaneous activation of scramblase and inhibition of translocase/floppase coordination (Zwaal and Schroit, 1997). Exosomes in particular may also be released from the locations of Ca^{2+} channels in the plasma membrane, since ESCRT machinery linked to MVBs often migrates to the channels, complexing with proteins ALIX, syntenin, and TSG101 as a means of repairing the membrane (Ambattu et al., 2020; Jimenez et al., 2014; Scheffer et al., 2014). Endoplasmic reticulum (ER) stress has also been linked with increased EV production by SMCs, VECs, and BECs exposed to supraphysiological cyclic stretch (Jia et al., 2017; Tang et al., 2022). There is likely significant interplay between the distinct mechanisms discussed above. For instance, excess Ca^{2+} induces mitochondrial dysfunction independently of the noted p53/SIRT1 pathways (Smith et al., 2012), and the cytochrome c released as a result may bind to the ER, causing the release even more cytosolic Ca^{2+} and introducing a positive feedback loop (Boehning et al., 2003). Shear-induced ATP release from the mitochondria can trigger an additional influx of extracellular Ca^{2+} into the cell (Yamamoto et al., 2018).

YAP was heavily implicated in up-regulated EV release by MSCs and SkMCs under shear and cyclic stretch, respectively (Guo et al., 2021), and by breast cancer cells grown on stiff substrates (Patwardhan et al., 2021). Such findings are of particular note due to the widespread applicability of the observed YAP-dependent mechanism. The application of external force to cell membranes has been observed to stretch the pores of the nuclear membrane and subsequently increase nuclear import of YAP (Elosegui-Artola et al., 2017), suggesting that YAP mediation of EV release in response to biomechanical stress proceeds via transcriptional regulation.

Simple membrane mechanics, not yet discussed in detail, may also weigh heavily on EV release under stress. For any cell type, increased exosome release during the application of tensile stress (e.g., cyclic stretch) may be attributed to accelerated exocytosis, which serves to ease plasma membrane tension by extending the membrane surface (Apodaca, 2002; Le Roux et al., 2019). Of course, the opposite is likely true for microparticles: membrane budding is synonymous with membrane loss, which serves to increase tension. Absent other factors, membrane mechanics would therefore suggest that microparticle release decreases under tension and increases under compression. Supporting this conclusion is the finding that actin polymerization induced by cytoskeletal tension has been linked to decreased microparticle release (Flaumenhaft et al., 2009; Headland et al., 2014; Mo et al., 2018). **Figure 3** illustrates the various mechanisms informing differential EV biogenesis and release in response to biomechanical stress.

For any cell type, extremely high levels of mechanical stress force the plasma membrane to rupture entirely, resulting in free-floating membrane fragments. Often intentionally produced via the extrusion of cells through microchannels, these fragments spontaneously reassemble into empty vessels resembling EVs (Jo, Wonju et al., 2014; Jo, W. et al., 2014; Wen et al., 2022). However, the unique biogenesis of these vessels, which relies on “brute force” and entails the absence of usual cargo-sorting mechanisms, suggests they ought not be classified as EVs for the purposes of this discussion. Similarly, we forgo an analysis of acoustic stimulation, which, while somewhat “mechanical” in nature, is not particularly relevant to EV behavior *in vivo* or during biomanufacturing. Acoustic stimulation has been found to boost exosome production (via

Ca^{2+}-mediated ESCRT pathway activation) (Ambattu et al., 2020) and promote nanoparticle and macromolecule uptake (Ramesan et al., 2018) in a variety of cell types.

4. Impact of biomechanical forces on EV cargo and composition

Both the magnitude and duration of biomechanical force influence EV cargo loading by parent cells. As a result, EVs produced under such force possess different cargo—and, as a result, promote different cellular phenotypes—than their statically-derived siblings. While miRNAs and surface/intracellular proteins have received the most attention for their role in modulating EV bioactivity, any EV cargo—from lipids to additional nucleic acids—may have a role to play. EV cargo loading is modulated by a bevy of distinct mechanisms (Chen et al., 2021; Corrado et al., 2021; Fabbiano et al., 2020; Groot and Lee, 2020; Mir and Goettsch, 2020). Below, we summarize first the known mechanisms underlying EV cargo loading (by parent cells) and then the published research on biomechanical stress-mediated cargo loading, identifying overarching themes and directions for future inquiry.

While numerous individual proteins involved in EV loading have been identified, the full, complex cascade of involved mechanisms is not well understood (Groot and Lee, 2020). Protein sorting in exosomes usually begins with ubiquitination (or similar post-translational modification) of MVB membrane proteins; subsequent loading occurs via action of the ESCRT complexes and associated proteins, such as ALIX and TSG101 (Chen et al., 2021). Tetraspanins (especially CD9, CD63, and CD81) and lipid rafts also have roles in this protein loading process, and notably serve as dominant protein loading mechanisms during ESCRT-independent exosome biogenesis (Chen et al., 2021). Though less is known about protein loading into microparticles, similar mechanisms—with confirmed involvement of ESCRT complexes—have been proposed (Mir and Goettsch, 2020; van Niel et al., 2018). For both exosomes and microparticles, RNA loading is mediated by a large number of RNA binding proteins (RBPs), of which notable examples include Argonaute 2 (Ago2) and several heterogeneous nuclear ribonucleoproteins (hnRNPs) (Chen et al., 2021; Corrado et al., 2021; Fabbiano et al., 2020; Groot and Lee, 2020). Recent work has also hinted at the possible involvement of ESCRT-associated proteins (including ALIX), tetraspanins and lipid rafts (Corrado et al., 2021). Very little is known about the processes involved in the loading of lipid cargo (Chen et al., 2021).

4.1. Mesenchymal stem cells

With few exceptions, the application of mechanical stress to MSCs promotes the release of EVs with “therapeutic” (clinically desirable) cargo and bioactivity. Total MSC EV protein levels increased significantly under low (0.001 and 0.0001 dyn/cm^2) shear (by 6.7- and 6.5-fold after 24 h), 10% static stretch (after 24 h), and complex agitation of MSC spheroid culture (by 5.6-fold after 7 days), with the two noted shear conditions resulting in the up-regulation of CD105 and CD44 (traditional MSC markers) and down-regulation of CD81, CD63, and CD9 (traditional EV markers) (Cha et al., 2018; Kang et al., 2022; Vangapandu et al., 2019). To the contrary, MSC EVs produced under 1.5 g/cm^2 compression (24 h) were enriched in CD81 and CD63, as well as in annexin A3, annexin A6, and endocytic/phagocytic proteins generally (Huang et al., 2022). Proteomic analysis of EVs from shear-exposed, dental pulp-derived MSCs found similarly differential expression of nearly half the loaded proteins (relative to control EVs) after 48 h (Guo et al., 2021). Despite its apparent lack of influence over EV yield, static stretch was also found to significantly impact miRNA loading in MSC EVs (Vangapandu et al., 2019; Yu, Wenting et al., 2021). Under 10% static stretch (24 h), total tRNA levels increased, while total miRNA and lincRNA levels—as well as levels of individual miRNAs responsible for inhibiting wound healing—decreased (Vangapandu et al., 2019). Under slightly increased 20% static stretch (72 h), 565 types of EV-loaded miRNA were differentially expressed, with 16 and 9 miRNAs up- and down-regulated, respectively, by a substantial margin (i.e., more than 2-fold) (Yu, Wenting et al.,

2021). Relative to MSC EVs derived from (low-tension) soft hydrogel substrates, EVs from stiff substrates were loaded with similar levels of mRNA, miRNA, and mitochondrial DNA associated with alleviation of lung injury; similar levels of ribosomal RNA were also observed (Lenzini et al., 2021). An increased EV size distribution was noted following production under 10% static stretch (24 h), a trend perhaps related to the corresponding increase in EV protein cargo (Vangapandu et al., 2019), while EV samples produced via the "squeezing" action of a custom microfluidic device included a relatively higher proportion of small (i.e., 40-80 nm) specimens (Hao et al., 2023). No size differences were observed in EVs derived from soft and stiff substrates, however (Lenzini et al., 2021).

The desirable bioactivity of MSC EVs—particularly as it relates to bone and neuron health—is nearly always increased as a result of mechanical stress-induced EV biogenesis, even as the identities of the specific cargo responsible for the EV bioactivity remain unknown. For instance, MSC EVs produced under 8% cyclic stretch (48 h) inhibited RANKL-mediated osteoclast differentiation by attenuating the nuclear factor kappa-B (NF-κB) signaling pathway, and were uniquely capable of reversing osteoporosis in a murine model (Xiao et al., 2021). Likewise, EVs produced under 20% static stretch demonstrated faster initial uptake by target MSCs and subsequently increased the osteogenic differentiation of these MSCs both *in vitro* and *in vivo* (Yu, Wenting et al., 2021). Separately, MSC exosomes from agitated, microcarrier-based culture were more efficiently taken up by neurons and were uniquely capable of rescuing them from 6-hydroxydopamine-induced apoptosis (notably, this latter behavior is not observed in MSC microparticles produced in agitated cultures) (Haraszti et al., 2018; Jarmalavičiūtė et al., 2015). Similarly, MSC EVs released under physiological shear promoted relatively more robust axonal sprouting in primary rat neurons (Guo et al., 2021). Under 10% static stretch, MSCs released EVs with improved ability to promote fibroblast migration in a scratch wound assay (Vangapandu et al., 2019). Interestingly, MSC EVs derived under 1.5 g/cm^2 compression promoted higher levels of osteoclast differentiation in target murine macrophages, likely via annexin A3-mediated EV uptake and subsequent ERK activation (Huang et al., 2022). Although this phenotype is associated with clinical undesirability, it is not unexpected; compression—as opposed to shear and tension—is a well-known promoter of osteoclast activity in bone tissue (Wang et al., 2018). We have found only three studies in which MSC EVs derived under mechanical stress do not possess enhanced bioactivity: EVs produced under very low shear (0.001 and 0.0001 dyn/cm^2) were not significantly more effective than their statically-derived counterparts in reducing cisplatin-induced acute kidney injury *in vitro* and *in vivo* (Kang et al., 2022), while EVs produced via "squeezing" in a custom microfluidic device were likewise comparable to control EVs in their capacity to promote corneal epithelial wound healing (Hao et al., 2023). Likewise, EVs produced using soft and stiff hydrogel substrates were similarly efficacious in alleviating acute lung injury (Lenzini et al., 2021).

4.2. Fibroblasts

Fibroblast EVs play a vital role in inflammation and wound healing, the nature of which is determined by mechanical stress-induced cargo loading. Levels of transforming growth factor β1 (TGF-β1)—a fibrosis promoter—were elevated in EVs from hallux valgus fibroblasts under 15% static stretch (Xie et al., 2020). Moreover, these EVs displayed significant differential expression of 24 miRNAs; miRNAs linked to increased pathological angiogenesis (e.g., miR-1246) were up-regulated, while miRNAs associated with inhibition of angiogenesis (e.g., miR-133a-3p and miR-203-3p) were down-regulated (Xie et al., 2020). More efficiently taken up by target VECs, these stretch-derived hallux valgus EVs were likely responsible for increased proliferation, migration, and angiogenesis of the VECs *in vitro*, probably via ERK 1/2 activation mediated by the described miRNA cargo (Xie et al., 2020). The miRNA and lincRNA profiles of

other fibroblast EVs exposed to static stretch were similarly distinct (Blum et al., 2018). Interestingly, EVs derived from aortic fibroblasts under 12% cyclic stretch were enriched in miR-133a, suggesting these EVs may prevent pathological cardiovascular remodeling (Akerman et al., 2019). Whether this particular enrichment is due to cell type or the nature of the applied stress is unclear, though cell type is clearly a factor in differential fibroblast responses to mechanical stress. Even among ostensibly similar dermal fibroblasts, for instance, tension-derived EVs from high-scarring individuals possess different characteristics than similar EVs from low-scarring individuals (Blum et al., 2018). EVs from compressed (1 g/cm^2) periodontal ligament fibroblasts are differentially loaded with at least eight cytokines, including immune regulator sICAM-1 (Zhang et al., 2022), and are additionally enriched in YAP (Zhao et al., 2021). Notably, while compressed fibroblasts are enriched in both activated and inactivated (phosphorylated) YAP, they selectively load activated YAP into EVs (Zhao et al., 2021). As a result, the EVs loaded under compression affect higher levels of inflammatory M1 polarization in target THP-1 macrophages (Zhao et al., 2021). However, periodontal ligament cell EVs produced under 20% cyclic stretch work to suppress the activation of inflammasomes in macrophages via inhibition of the NF-κB pathway (this phenotype was not evaluated in EVs produced under static conditions) (Wang, Z. et al., 2019).

4.3. Bone and cartilage tissue

Mechanical loading has a significant impact on the balance of osteogenesis (bone formation) and osteoclastogenesis (bone resorption), with shear stress and cyclic stretch promoting bone formation and compression inhibiting it (Wang et al., 2018). Similarly, EVs produced by bone tissue under shear stress or cyclic stretch exhibit osteogenic phenotypes, while compressive stress results in EVs with osteoclastic potential. Specifically, EVs from osteocyte-like MLO-Y4 cells under 8% cyclic stretch were more effective in promoting the miR-181b-5p-mediated proliferation and osteogenic differentiation of periodontal ligament stem cells (Lv et al., 2020). Levels of miR-181b-5p were 5-fold higher in EVs from stretched culture, and, in total, 206 miRNAs were differentially expressed between the experimental and control EVs (Lv et al., 2020). Additionally, higher levels of proteins associated with the regulation of bone metabolism—RANKL, sclerostin, and osteoprotegerin—were observed in EV samples from MLO-Y4 cells exposed to 35 dyn/cm^2 shear stress, though levels of enrichment in individual EVs is unclear, as EVs were not quantified (Morrell et al., 2018). Under 1 g/cm^2 compression, however, osteoclast-derived EVs were relatively more effective at inhibiting the osteogenesis of target periodontal ligament stem cells, a likely result of their enrichment in miR-133a-3p, miR-203a-3p, miR-106a-5p, and miR-331-3p, which down-regulate bone formation, and their lack of miR-223-5p and miR-181-5p, which promote bone remodeling (Wang et al., 2021).

In the only study to date on chondrocyte-derived EVs produced under shear stress, shear of 16 dyn/cm^2 resulted in EVs with increased levels of tissue-nonspecific alkaline phosphatase (TNAP) and nucleotide pyrophosphatase/phosphodiesterase-1 (NPP1) and decreased levels of matrix Gla protein (MGP) (Liu, Q. et al., 2022). TNAP and NPP1 catalyze reactions necessary for the formation of calcified nodules, while MGP decreases mineralization (Liu, Q. et al., 2022). Therefore, as expected, EVs produced under shear promoted far higher levels of calcification in target chondrocytes than control EVs (Liu, Q. et al., 2022). This finding was confirmed *in vivo* for rats with temporomandibular joint osteoarthritis, which involves aberrant mechanical stress akin to the aforementioned shear stress applied *in vitro* (Liu, Q. et al., 2022). Similarly, chondrocyte-derived EVs produced under cyclic tension (i.e., 10% cyclic stretch at 1 Hz for 24 h) promoted more extensive osteogenic differentiation of chondrocytes, likely due to relatively higher levels of pro-osteogenic cargo (such as miR-199a-5p) and lower levels of anti-osteogenic cargo (such as miR-186-5p and miR-339-5p) (Shi et al., 2022). However, cyclic tension has also been linked to EV samples with greater capacity for miR-9-5p delivery to osteoblasts, whether by higher

overall EV production or enhanced miR-9-5p loading (Li, B. et al., 2023). Since osteoblast differentiation is inhibited by miR-9-5p (Li, B. et al., 2023), we suggest that the bioactivity of EVs derived from chondrocytes under cyclic tension is somewhat complex, with potential for both pro- and anti-osteogenic phenotypes.

4.4. Bronchial epithelial cells

The nature of BEC EV cargo is heavily influenced by mechanical stress, but the details of this relationship require additional investigation. Following supraphysiological levels of compression (at 30 cm H_2O), BEC EV samples were enriched in tissue factor (Mitchel et al., 2016; Park et al., 2012) and tenascin C (Mwase et al., 2022), both of which inform the pathogenesis of asthma. Whether the aforementioned proteins were up-regulated in individual EVs is unclear, however, as protein levels were not normalized to EV counts. Surprisingly, BEC EVs produced under supraphysiological levels of cyclic stretch (20% cyclic stretch at 0.5 Hz for 6 h) exerted an anti-inflammatory phenotype, more effectively promoting M2 macrophage polarization via the miR-21a-5p-mediated downregulation of the Notch2/SOCS1 signaling axis (Wang et al., 2022). Levels of miR-21a-5p were increased by almost 150-fold in EVs from the stretched BECs (Wang et al., 2022). However, a second study reported significantly higher levels of fibrosis-related mRNA cargo in BEC EVs produced under similar levels of cyclic tension (20% cyclic stretch at 1 Hz for 24 h) (Tang et al., 2022). As a result, these EVs were more effective at activating lung fibroblasts *in vitro*; similar bioactivity was observed for BEC EVs produced *in vivo* using a murine model of mechanical ventilation-induced pulmonary fibrosis (Tang et al., 2022). The BEC EV miRNA profiles produced under physiological (10% cyclic and 5% static) stretch levels were similarly diverse, with 9 miRNAs differentially loaded under cyclic stretch and 33 miRNAs differentially loaded under static stretch (Najrana et al., 2020). In both cases, these miRNAs were often implicated in lung development, proliferation, and cell cycle regulation (Najrana et al., 2020). Taken together, the current literature therefore suggests a pathology-inducing role for BEC EVs produced under compression, but a more complex function—both anti-inflammatory and pro-fibrotic—for similar EVs produced under supraphysiological levels of cyclic stretch. Meanwhile, the impact of physiological levels of cyclic stretch on EV cargo and bioactivity needs further investigation. We expect to dramatically refine these conclusions as new research comes to light.

4.5. Endothelial cells

The impact of mechanical stress on VEC EV cargo has been studied extensively. In general, the cargo of VEC EVs exacerbates the pathology induced by the stress (or lack thereof) to which the parent cells were exposed. Expression of adhesion molecules ICAM-1 and VCAM-1 and exposure of PS were down-regulated on VEC EVs produced under physiological levels of shear, suggesting that subphysiological (or absent) flow promotes the formation of EVs with abnormally enhanced thrombotic properties (Ramkhelawon et al., 2008). Physiological (high) shear also results in significant, selective up-regulation of miR-143 and miR-145 cargo via action of the flow-sensitive protein Krüppel-like Factor 2 (KLF2) (Hergenreider et al., 2012; Jae et al., 2015). VEC EV enrichment in miR-143 under shear has additionally been shown to depend on RAB7a and RAB27b (but not RAB27a) (Jae et al., 2015). Examination of EV cargo selected by KLF2-transduced parent cells suggests that physiological shear will additionally promote the down-regulation of inflammation-associated miR-155 (He, S. et al., 2018). Notably, EVs forged under physiological shear reduced atherosclerotic lesion formation in mouse aortas and were more effective at preventing SMC de-differentiation (Hergenreider et al., 2012), confirming the known role of the miR-143/145 cluster in ameliorating pathological phenotypes in SMCs (Deng et al., 2015). EVs from KLF2-transduced cells, meanwhile, decreased proinflammatory M1 macrophage markers and increased anti-inflammatory M2 macrophage markers both in vivo and in vitro (He, S. et al., 2018).

Recent miRNA sequencing suggests exosome-enriched EVs derived from VECs also experience dramatically different miRNA cargo sorting following exposure to physiological laminar or subphysiological oscillatory shear (Chung et al., 2022). Both miRNA sequencing and subsequent PCR confirmed the up-regulation of 9 miRNAs (including miR-92a-3p, expressed at a particularly high level) and down-regulation of 4 miRNAs (including miR-143-3p and miR-145-5p) under oscillatory shear (Chung et al., 2022). In general, the differentially-expressed miRNAs were related to genes involving vascularization/angiogenesis, cell migration, and inflammatory responses (Chung et al., 2022). Indeed, the EVs formed under oscillatory shear were more effective in promoting angiogenesis, HUVEC apoptosis, and expression of pro-inflammatory adhesion molecules (ICAM-1, VCAM-1, and E-selectin) and genes *in vivo* and *in vitro*. It seems that exosome-enriched EVs promote the same phenotypes as the shear stress under which they were derived; for instance, EVs produced under oscillatory shear *abolish* shear-induced activation of phosphoinositide 3-kinase (PI3K), protein kinase B (Akt), endothelial nitric oxide synthase (eNOS), and ERK 1/2, while EVs produced under physiological shear uniquely activate these same molecules, even in static culture conditions (Chung et al., 2022). Similar bioactivity has been observed in exosomes derived from endothelial progenitor cells exposed to (3.5 dyn/cm^2) oscillatory shear stress (Li, L. et al., 2023). Specifically, these exosomes were found to inhibit angiogenesis of endothelial progenitor cells *in vitro* and *in vivo*, promoting endothelial-to-mesenchymal transition and accelerating pathological vascular remodeling via delivery of circular RNA (circ-1199) cargo (the levels of which were enriched relative to those in exosomes from static culture) (Li, L. et al., 2023).

Numerous proteomic analyses have been performed on microparticle-enriched EVs derived from VECs exposed to physiological (i.e., 5%) and supraphysiological (i.e., 15% to 18%) levels of cyclic stretch, with EVs from each condition possessing highly unique protein cargo profiles (Letsiou et al., 2015; Zhuang et al., 2020a; Zhuang et al., 2020b). Up-regulated EV proteins under supraphysiological levels of stretch included antioxidants and cytoskeletal linkers—including moesin, an actin-binding linker—as well as proteins involved in neutrophil transmigration, actin cytoskeleton regulation, and VEC barrier function (Letsiou et al., 2015). Proteins involved in acute lung injury (such as UCHL1) and associated with cardiovascular, inflammatory, and lung diseases (such as glutathione S-transferase P1) were also unique to EVs produced in this condition (Letsiou et al., 2015). CD151—necessary for Src activation in VECs and subsequent inhibition of VEC apoptosis—was notably down-regulated in EVs from highly-stretched culture (Zhuang et al., 2020a). Moreover, relative to EVs produced under physiological stretch, both statically-derived EVs and EVs from highly-stretched culture appear to be enriched in adhesion molecules ICAM-1 and VCAM-1 (Zhuang et al., 2020a; Zhuang et al., 2020b). We also point out that no efforts have been made to document differences in RNA loading among VEC EVs produced under cyclic stretch. In one unique study, the impact of traumatic brain injury on brain microvascular endothelial cells was modeled with a one-time (non-cyclic) biaxial stretch—lasting 1 s—of either 12% or 22% (Andrews et al., 2016). EV samples from stretch-exposed cells were similarly enriched in ICAM-1, PECAM-1, and occludin (a tight junction protein); however, it remains unclear whether this enrichment was the result of higher total EV yields or protein up-regulation on a per-EV basis (Andrews et al., 2016). Nevertheless, these findings suggest a role for EVs in trafficking disassembled tight junction proteins and various inflammatory molecules away from the brain following traumatic injury (Andrews et al., 2016).

The behavior of VEC microparticles produced under cyclic stretch reflects their contents. VEC microparticles derived under physiological stretch decrease inflammatory leukocyte-VEC adhesion—a result of decreased ICAM-1 enrichment—relative to microparticles produced in

static culture (Zhuang et al., 2020b). On the other hand, VEC microparticles derived from highly-stretched parent cells promote anoikis ("to remove from home;" refers to programmed cell death induced upon cell detachment from the extracellular matrix) and apoptosis in target VECs via Src inhibition (Jia et al., 2017; Zhuang et al., 2020a). These microparticles are about 2.3-fold as efficient (compared to particles from physiologically-stretched parent cells) at binding to target VECs, likely due to their relative enrichment in adhesion molecules ICAM-1 and VCAM-1 (Zhuang et al., 2020a). However, they simultaneously increase Src activation in target SMCs, suggesting that the mechanism for Src stimulation may be largely dependent on cell type (Zhuang et al., 2020a). VEC microparticles produced under supraphysiological levels of stretch have also been reported to uniquely promote lung inflammation and injury in mice, probably as a result of mitogen-activated protein kinase (MAPK) activation (Letsiou et al., 2015). Notably, nothing is known about exosomes derived from cyclically-stretched VECs.

4.6. Smooth muscle cells

As noted previously, the SMCs comprising the blood vessels experience significant tensile stress as a result of blood pressure, yet are only exposed to shear in cases where VEC damage allows direct contact with blood flow. SMC EVs derived under conditions of supraphysiological tension (15-18% cyclic stretch) promote abnormal phenotypes in target VECs (Jia et al., 2017; Wang, L. et al., 2017). Specifically, these EV samples are enriched (by 5-fold) in miR-27a, which down-regulates G protein-coupled receptor kinase (GRK6) and promotes excessive VEC proliferation (Wang, L. et al., 2017). Notably, EVs produced under physiological tension (5% cyclic stretch) did not display these characteristics. SMC EVs derived under supraphysiological levels of tension have also been shown to promote the expression of inflammatory molecules (VCAM-1, ICAM-1, IL-6, and IL-1β) on target VEC cells and induce subsequent anoikis (Jia et al., 2017). Similarly, shear stress—which only affects SMCs *in vivo* following disruption of the VEC barrier—has been found to promote the formation of uniquely procoagulant EVs from SMCs (Stampfuss et al., 2006). Specifically, increasing shear stress on parent SMCs from 0.1 to 15 dyn/cm^2 resulted in a log-linear increase in tissue factor (TF) enrichment and corresponding procoagulant activity in EV samples (Stampfuss et al., 2006). Clearly, non-physiological levels of mechanical stress promote the formation of SMC EVs with cargo capable of producing various related pathologies in target cells. It should additionally be noted that all of the described research was performed on microparticle-enriched samples. The impact of mechanical stress on SMC-derived exosomes remains unknown.

4.7. Platelets and megakaryocytes

As with VEC EV cargo, the cargo of platelet EVs exacerbates the pathology induced by the shear stress (or lack thereof) to which the parent cells were exposed. Supraphysiological levels of shear promote *in vitro* formation of uniquely procoagulant platelet EVs, though increased shear is correlated with increased procoagulant behavior even in physiological shear regimes (Chen et al., 2010; Reininger et al., 2006). Increased procoagulant activity of platelet MPs has likewise been observed *in vivo* following moderate physical exercise (90 min. on a bicycle ergometer) (Sossdorf et al., 2010, 2011). Moreover, platelets sampled from individuals engaged in strenuous physical exertion (graded exercise on a bicycle ergometer until exhaustion) and subsequently exposed to supraphysiological levels of shear *in vitro* possessed more procoagulant properties than platelets from resting individuals (Chen et al., 2010). This phenomenon is likely driven by a variety of selectively-loaded EV cargo. Shear stress promotes the formation of platelet EVs enriched in both coagulation factors—including factor V/Va (FV/Va), factor VIII (FVIII), and tissue factor (TF)—and exposed PS (the negative charge of which promotes the binding of coagulation factors) (Chen et al., 2010). Interestingly, however, EVs derived *in vitro* under supraphysiological levels of shear also display increased annexin V binding (relative to EVs produced via calcium ionophore) (Nomura et al., 2000), a generally

anticoagulant phenomenon perhaps mediated by the presence of excess PS binding sites. Platelets may also use EVs as a vehicle in which to dispose of excess adhesion molecules responsible for pathological aggregation under excess shear stress. Levels of GPIb-IX-V, GPIIb-IIIa, and P-selectin on the surfaces of platelet EVs were all up-regulated in a shear-dependent manner for continuous *in vitro* stress of up to 70 dyn/cm^2 (Roka-Moiia et al., 2021). Levels of GPIb-IX-V and GPIIb-IIIa in parent platelets were simultaneously down-regulated, and concentrations of all three molecules were higher on EV surfaces than on parent platelet surfaces, suggesting selective loading (Roka-Moiia et al., 2021). The shear-induced clustering of adhesion molecules in lipid rafts is suggested as a possible mechanism for such loading (Roka-Moiia et al., 2021). Notably, pulsatile shear was relatively ineffective in enriching platelet EVs in the aforementioned adhesion molecules; a minor increase in the per-EV level of GPIIb-IIIa was observed, but failed to increase with shear magnitude (Roka-Moiia et al., 2021). A comparative proteomic analysis of platelet EVs produced *in vitro* via physiological shear stress or thrombin stimulation found 26 differentially-expressed proteins, 15 of which were involved in platelet activation and 21 of which were involved in cellular assembly, organization, and morphology (Shai et al., 2012). The shear-induced up-regulation of integrin α6 and down-regulation of DOK2—both pro-angiogenic proteins that modulate platelet activation and endothelial cell function—were confirmed via western blotting (Shai et al., 2012).

Almost nothing is known about mechanical stress-induced variation in Mk EV cargo, although the ability of Mk-derived microparticles from static cultures to promote megakaryopoiesis is dependent on transcription-independent p53-induced apoptosis (TIPA) in parent Mks, a process which is notably up-regulated in Mks under shear (Luff et al., 2018). Given that the described Mk microparticle bioactivity (the promotion of megakaryopoiesis) is largely informed by the action of miR-486-5p and miR-22-3p (Kao et al., 2022), shear stress may prove a powerful tool with which to regulate the miRNA cargo of Mk EVs. The impact of tension and compression on platelet/Mk EV cargo has not been evaluated, likely due to the relative physiological irrelevance of such stresses.

4.8. Skeletal muscle cells

SkMC-derived EVs produced under mechanical stress are enriched in cargo that promotes "healthy" phenotypes *in vivo*, likely reflecting the protective effects of physical exercise on overall health. Levels of 18 different miRNAs are increased by at least 1.25-fold in SkMC EVs produced under 6 dyn/cm^2 shear, with increases in miR-155-5p and miR-196a-5p likely informing an anti-osteoclastic phenotype (Takafuji et al., 2021). Specifically, EVs produced under shear more effectively suppressed RANKL-mediated osteoclast formation and mitochondrial biogenesis (Takafuji et al., 2021). Similarly, EVs from SkMCs exposed to brief, high-strain cyclic stretch (of 12-22% at 1 Hz for 24 h, with 50 min of rest for every 10 min stretching) had reduced levels of 35 different miRNAs relative to EVs from low-strain or static cultures (Mullen et al., 2022). No change in total protein content among EV samples was observed in either of the noted studies (Mullen et al., 2022; Takafuji et al., 2021). SkMC EVs produced under 25% cyclic stretch (at 1 Hz for 48 h) were particularly enriched in miR-1 (by 12-fold after 24 h), with correspondingly diminished miR-1 levels observed in parent SkMCs (Vechetti et al., 2021). This result was confirmed *in vivo* following resistance exercise in humans and synergist ablation in a murine model (Vechetti et al., 2021). These miR-1-enriched EVs were preferentially taken up by epididymal white adipose tissue, promoting lipolysis by targeting *TFAP2A*, a repressor of β-adrenergic receptor expression (Vechetti et al., 2021). The preferential uptake (by the adipose tissue) of EVs produced under cyclic stretch is likely due to surface receptor changes on either the EVs or the target cells (Vechetti et al., 2021). Stress-induced SkMC EVs may likewise be instrumental in informing myoblast phenotypes, as EVs produced under sustained, low-strain cyclic stretch (of 15% at 1 Hz for 24 h) were relatively

more effective at promoting myoblast proliferation and possessed a unique ability to mediate myogenic differentiation (Mullen et al., 2022).

4.9. Tumor cells

In general, higher levels of mechanical stress tend to bolster the carcinogenic and immunosuppressive functions of tumor cell EVs. This suggests that elevated mechanical stress associated with tumor growth (Stylianopoulos et al., 2013) may be a required trigger for the EV-mediated survival and metastasis of cancer. Under 10 dyn/cm^2 shear stress, HeLa and MDA-MB-231 cells load EVs with high levels of autophagosomes and autophagic vesicles as a means of preventing autophagic tumor cell death (Wang, K. et al., 2019). Notably, this EV loading is independent of autophagosome levels, occurring only when high shear promotes an influx of intracellular Ca^{2+} and the subsequent fusion of autophagic vesicles with MVBs (the organelles responsible for exosome biogenesis) (Wang, K. et al., 2019). Lysosome marker LAMP1 and autophagosome marker LC3-II are up-regulated by 15- and 3-fold, respectively, in EVs produced under shear (Wang, K. et al., 2019). Moreover, insulin-like growth factor 2 (IGF2) is up-regulated in EV samples derived from HepG2 (human liver cancer) cells under low (1.4 dyn/cm^2) shear, and is responsible for an increase in PI3K/Akt-mediated activation of cancer-associated fibroblasts (Feng, T. et al., 2022).

Tumor-derived EVs produced under tension have similarly pronounced roles in carcinogenesis and immunosuppression. 4T1.2 cells under uniaxial 10% cyclic stretch produced EV samples relatively enriched in protein death-ligand 1 (PD-L1) and depleted of CD54; both proteins are associated with immunosuppression/evasion and metastasis (Wang, Y. et al., 2020). The PD-L1 enrichment appears to be more significant than the CD54 depletion, however, as the EVs produced under tension promoted more aggressive tumor growth in murine models, and were more efficiently taken up by immunosuppressive M-MDSCs (myeloid-derived suppressor cells) and recruited macrophages (though the reason for this is not investigated) (Wang, Y. et al., 2020). MCF-7 (but not MDA-MB-231 or 4T1.2) cells under the same cyclic tension produced EV samples higher in CD63 but lower in CD81; in this context, the function of these proteins is unclear, but may be related to cargo loading generally (Wang, Y. et al., 2020). EVs from Hepa1-6 (murine liver cancer) cells grown on stiff (as opposed to soft) substrates promoted more tumor growth among target Hepa1-6 cells *in vivo*, likely via Notch signaling (Wu et al., 2023). Similarly, EVs from LNCaP (prostate cancer) cells grown on stiff substrates were differentially enriched in miRNAs implicated in cancer progression, and, likely as a result, were relatively more effective in promoting the *in vitro* migration of LNCaP cells (Liu, Z. et al., 2022). MCF-7 and MDA-MB-231 (breast cancer) EVs grown on stiff hydrogel substrates were loaded with relatively higher levels of the protein thrombospondin-1 (THBS1, associated with cancer metastasis) (Patwardhan et al., 2021), while Ewing's sarcoma (SK-N-MC) EVs grown in a more complex tumor model with a similarly stiff extracellular matrix were loaded with relatively higher levels of mRNA implicated in EZH2-mediated tumor growth and cancer progression (Villasante et al., 2016). Given the continuous variation of mechanical stress levels in the tumor microenvironment (Koomullil et al., 2021), we suggest that the identity of EV cargo, like EV production, is a uniquely time-dependent variable in the context of tumor cells *in vivo*. We also note, once again, that although tumor-derived exosomes are well-studied, relatively little is known about the loading of tumor microparticles under mechanical stress.

4.10. Other mammalian cells

As was the case with mechanical stress-induced changes in EV biogenesis, stress-induced differences in EV cargo loading have been observed among a variety of additional, less-studied cell types. EVs released by both AT1R-expressing HEK293T cells under osmotic stretch and mice with induced cardiac pressure overload were enriched in AT1R cargo (by roughly 18-fold

and over 100-fold, respectively) relative to EVs from unstressed HEK293T cells and control mice (Pironti et al., 2015). As a result of this enhanced AT1R loading—mediated by β-arrestin 2—EVs from stressed cells/mice increased ERK 1/2 phosphorylation in WT HEK293T cells and reconstituted blood pressure in AT1R KO mice (Pironti et al., 2015). This instance of biomechanical force-mediated cargo loading by cells engineered to overexpress said cargo is unique in the literature and invites future investigation into the yet-unknown impacts of genetic engineering on EV biology under biomechanical force. In another murine model of cardiac pressure overload, serum samples from afflicted mice contained higher levels of miR-378, a fibrosis inhibitor (Yuan et al., 2018). This suggests that supraphysiological levels of biomechanical stress promote the formation of cardiomyocyte-derived EVs with higher levels of anti-fibrotic cargo, though this finding may also be a result of higher EV yields overall, as miR-378 levels were not characterized on a per-EV basis (Yuan et al., 2018). *In vitro*, however, opposing factors were also at play: relative to EVs from static culture, neonatal rat cardiomyocyte EVs exposed to 24 h of biaxial, 15% cyclic stretch were larger and contained more than twice as much miR-494-3p, a known *promoter* of fibrosis (Tang et al., 2023). As a result, these EVs were *more* effective in activating cardiac fibroblasts, a process mediated by the miR-494-3p-induced inhibition of PTEN and subsequent activation of Akt, Smad, and ERK signaling pathways (Tang et al., 2023). In another study, Schwann cells co-cultured with magnetically-stimulated nanoparticles produced EVs with relatively higher miRNA levels (as a fraction of the total RNA cargo) (Xia et al., 2020). Of the 20 miRNAs differentially expressed between EVs from stimulated and unstimulated cultures, the five most up-regulated miRNAs in the former EVs were all predicted to target the neuropilin 1 gene (*NRP1*) (Xia et al., 2020). Indeed, this action was verified for the most significantly up-regulated miRNA—miR-23b-3p—and it was further determined that the EVs derived from stimulated culture enhanced axonal elongation *in vitro* and axonal regeneration *in vivo* due largely to the miR-23b-3p-mediated inhibition of *NRP1* (Xia et al., 2020).

4.11. General themes and trends

Clearly, type and quantity of mechanical stress are inextricably linked with the quality and quantity of EV cargo. In nearly every case, significant differences exist in the RNA and proteomic profiles of EVs produced under mechanical stress, even as EV size and morphology remain generally consistent. **Figure 4** illustrates the nature of these differences—and their clinical implications—in four cell types. In some cases, stress-induced EV production promotes the loading of cargo that exerts pro-survival phenotypes in target cells (e.g., EVs from mechanically-stressed MSCs promote desirable phenotypes in a variety of target cells, including neurons and fibroblasts; EVs from mechanically-stressed VECs promote positive outcomes; EVs from mechanically-stressed tumors drive subsequent tumor growth) or in the broader organism (e.g., EVs from mechanically-stressed skeletal muscle trigger bone growth and adipose tissue lipolysis). However, in other instances, significant mechanical stress serves to induce pathological outcomes (e.g., EVs from mechanically-stressed platelets and SMCs possess procoagulant and inflammatory properties). The mechanism by which mechanical stress influences this loading is somewhat unclear, but depends to a large extent on the physiological relevance of the stress in question. That is, with some notable exceptions, “healthy” cells exposed to physiological levels of mechanical stress load EVs with pro-survival cargo, which, upon delivery to target cells, assists in maintaining a “healthy” homeostasis. Meanwhile, “unhealthy” cells exposed to non-physiological (i.e., pathological) levels of mechanical stress selectively load EVs with inflammatory, apoptotic, or otherwise damaging cargo, perhaps intending to rid themselves of dangerous proteins and RNAs. However, uptake of these harmful EVs by target cells establishes a pathological phenotype as the new local homeostasis. Still, exceptions to this rule exist; for instance, BEC EVs produced under supraphysiological cyclic stretch exerted an anti-inflammatory phenotype in target macrophages

(Wang et al., 2022). As in the prior section of this review, investigations into EV cargo are limited by bodies of literature that exclusively examine either exosomes or microparticles (but not both) from a given cell type. We expect that in many cases, exosomes and microparticles from the same cell type may possess distinct cargo loading processes, perhaps even affecting opposing phenotypes in target cells as a means of maintaining homeostasis in response to stressful conditions. Nevertheless, this topic has strangely not yet been evaluated in the context of mechanical stimulation. Moreover, the diverse impact of mechanical stress on EV cargo suggests that common metrics for EV quantity—such as total protein content or surface receptor concentration—are inappropriate for comparing EV yields from static and agitated cultures. Finally, we again note that the impact of mechanical stress on EV cargo remains to be investigated in a wide variety of cell types, including erythrocytes, leukocytes, and, notably, megakaryocytes.

Notably, there is evidence that some EVs can ameliorate the impacts of mechanical stress on various non-parent cell types. For instance, exosome-enriched EVs from human MSCs were shown to attenuate cyclic stretch-induced oxidative stress and apoptosis in chondrocytes via miR-100-5p-mediated down-regulation of NADPH oxidase 4 (NOX4) (Li et al., 2021). In a second study, similar EV samples from murine MSCs reduced compression-induced apoptosis of nucleus pulposus cells (which generate energy through anaerobic glycolysis), alleviating mitochondrial damage and inhibiting oxidative stress (Hu et al., 2021). Adipose-derived EVs have likewise demonstrated a protective effect on murine pulmonary endothelial cells exposed to supraphysiological cyclic stretch, lowering intracellular Ca^{2+} levels by inhibiting transient receptor potential vanilloid 4 (TRPV4) (Yu et al., 2020). In each of the three described studies, the EVs in question were derived from static cultures; effects of similar EVs from mechanically-stressed cultures have not been investigated. Collectively, such findings complicate EV biomanufacturing. After all, if certain EVs serve to modulate cellular sensitivity to mechanical stress, we suspect that local EV concentration—a time-dependent variable—informs subsequent EV biogenesis and loading in stressed cultures. In other words, the response of an EV parent cell to mechanical stress may be modulated by the quantity and cargo of already-released EVs near the cell surface. This transient nature of EV quality is illustrated in **Figure 5**.

5. Impact of biomechanical forces on EV uptake and cargo delivery

The impact of biomechanical force on EV uptake and delivery, a subject of relatively less study, remains just as integral to a full understanding of EV behavior. EV uptake occurs in three steps: collision, adhesion, and internalization. First, a cell-EV collision must provide the necessary membrane contact for subsequent binding. Thereafter, cell-EV adhesion proceeds via the coupling of appropriate membrane proteins. Finally, endocytosis (for smaller EVs) or membrane fusion (for larger EVs) facilitates EV internalization. Both *in vivo* and *in vitro*, biomechanical forces influence each of these three steps. **Figure 6** summarizes the impacts of biomechanical force on EV uptake by target cells.

5.1. Impact of biomechanical forces on cell-EV collision and adhesion

By its nature, biomechanical force-induced fluid mixing will increase cell-EV collision probability. However, the strong flow inherent in many forms of shear will also naturally decrease the residence time of EVs or other particles on or near the target cell membrane, inhibiting adhesion and preventing uptake (Chen, Y.Y. et al., 2020; Karami et al., 2022). Moreover, as discussed previously, EV biogenesis is nearly always accelerated under mechanical stress. Often, such increased biogenesis correlates with increased delivery to other cells in culture or *in vivo* by increasing EV concentration and facilitating faster EV motion, thereby enhancing the likelihood of cell-EV contact (Koomullil et al., 2021). Specific cellular protein receptors required for EV

binding may also be up- or down-regulated by mechanical stress of any kind on a case-by-case basis, subsequently mediating cell-EV adhesion (Chappell et al., 1998; Letsiou et al., 2015).

5.2. Impact of biomechanical forces on EV internalization

The same processes responsible for small EV (i.e., exosome) release and uptake—namely, exocytosis and endocytosis—also help mediate mechanical stress on the plasma membrane. Importantly, these processes are also a major mechanism by which biomechanical forces regulate EV internalization. Membrane fusion—which facilitates the internalization of predominantly larger EVs—may serve a similar function, though published research in this regard in scarce.

During the application of tensile stress, exocytosis and endocytosis are often up-regulated and down-regulated, respectively, as a means of easing membrane tension (Apodaca, 2002; Le Roux et al., 2019). In particular, tension blocks the polymerization of clathrin (Kaksonen and Roux, 2018) and results in the compensatory disassembly of caveolae (Sinha et al., 2011). Both clathrin- and caveolin-mediated endocytosis are heavily implicated in (primarily small) EV internalization (Ratajczak and Ratajczak, 2020). In some cases, however, endocytotic rates may increase during tension to compensate for excess exocytosis (Apodaca, 2002). This allows an equilibrium membrane tension to be sustained and may make endocytosis relatively more common in cells that have been well-adapted to cyclic stretch or other tensile force (as opposed to naïve cells). Similarly, endocytosis rates generally increase under compression, as the plasma membrane suddenly develops an excess of invaginations which must be reduced (Apodaca, 2002; Le Roux et al., 2019). Mechanical force broadly can also trigger rearrangement of actin in the cytoskeleton, recruiting it to form stress fibers as a means to stabilize the cell in response to chronic stress (Bhowmick et al., 2012; Han et al., 2012). Such a phenomenon results in lessened actin availability for modulation of plasma membrane deformation during endocytosis (Bhowmick et al., 2012; Han et al., 2012).

Shear stress increases the disorder and fluidity of the plasma membrane, though its generalized impact on exocytosis and/or endocytosis is less apparent (Le Roux et al., 2019). Actin recruitment to stress fibers will again down-regulate endocytosis (Bhowmick et al., 2012; Han et al., 2012). In one study of HUVECs and HepG2 cells, however, shear stress was shown to enhance caveolin-dependent (but not clathrin-dependent) endocytosis (He, Z. et al., 2018). In another study, clathrin- and dynamin-dependent endocytosis *were* enhanced in kidney proximal tubule cells exposed to shear (though caveolin-dependent endocytosis does not occur in this cell type) (Raghavan et al., 2014).

Cell cycle phase—itself modulated by mechanical stress—may also play an unappreciated role in the internalization of bound EVs. Generally speaking, endocytosis is almost completely inhibited during early mitosis (Fielding and Royle, 2013). In one study, however, prostate cancer cells in the G_2/M phase of the cell cycle were relatively *more* likely to take up their own EVs (Lazaro-Ibanez et al., 2017). Given that tumor cell cycle arrest in those same phases has been observed following shear application *in vitro* (Chang et al., 2008; Lee et al., 2018), shear-mediated cell cycle regulation presents as an additional possible mechanism governing EV internalization, though the impacts of shear on cycle phase distribution differ with cell type (Lakhotia et al., 1992; Lin et al., 2000).

The role of the membrane fusion internalization pathway—and its susceptibility to mechanical stress—is not well-understood. Given the importance of this particular internalization pathway to EV-induced phenotypic change in target cells (Ratajczak and Ratajczak, 2020), this is a topic in need of immediate attention. The ability of fused EVs to extend the plasma membrane suggests

that membrane fusion, unlike endocytosis, may be up-regulated under tension as a mechanism of stress alleviation.

5.3. Examples from published research

The multitude of factors described above suggests that the specific effects of biomechanical force on EV uptake are deeply situation-dependent. Numerous studies relating cyclic stretch to protein or synthetic nanoparticle uptake have been decidedly inconclusive, with uptake increasing (Doryab et al., 2021; Doryab et al., 2020; Hu and Liu, 2015; Huh et al., 2010; Rouse et al., 2008; Yu, W. et al., 2021), decreasing (Freese et al., 2014; Tsai et al., 2022), or remaining unchanged (Freese et al., 2017; Schmitz et al., 2019) depending on the nature and concentration of the proteins/nanoparticles, the type of implicated target cells, and the characteristics of the cyclic stretch. The synthesis of existing literature relating protein and synthetic nanoparticle uptake under shear is just as inconclusive (Godoy-Gallardo et al., 2015); shear has been regularly found to both promote (Han et al., 2015; He, Z. et al., 2018; Lawler et al., 2009; Raghavan et al., 2014; Samuel et al., 2012) and inhibit (Bhowmick et al., 2012; Chen, Y.Y. et al., 2020; Lin et al., 2010; Nguyen et al., 2009) uptake of proteins and nanoparticles. In at least one case involving endothelial cells, lower shear magnitude promoted protein endocytosis (relative to static conditions) and higher such magnitude *inhibited* it (Ueda et al., 2004). Increases in protein/nanoparticle uptake induced by shear exposure may also be reversed following the cessation of stress (Raghavan et al., 2014), a phenomenon similarly observed for tension induced by cyclic stretch (Hu and Liu, 2015).

The only EV-specific research on EV delivery under tension and/or compression involves the adhesion of platelet microparticles to vascular smooth muscle cells, which is increased under supraphysiological levels (15%) of cyclic stretch relative to physiological levels (5%) of cyclic stretch (Chen, Y. et al., 2020). We have also found two studies examining EV binding and/or uptake under conditions of shear stress, both of which concern VECs (Gomez et al., 2020; Qin et al., 2022). Specifically, both adhesion of neutrophil-derived EVs (Gomez et al., 2020) and uptake of erythrocyte-derived EVs (Qin et al., 2022) were found to increase under conditions of low and/or oscillatory (i.e., atheroprone) shear. Such flow promotes an increase in oxidative stress (Qin et al., 2022), which in turn serves to up-regulate various surface receptors—such as VCAM-1, ICAM-1, and E-selectin—on the target cells (Chappell et al., 1998). In particular, ICAM-1 likely serves to bind with CD18 present on neutrophil EVs (Gomez et al., 2020). Expression of VCAM-1 and ICAM-1 on VEC EVs was likewise up-regulated under oscillatory shear (Chung et al., 2022), demonstrating that EV characteristics—including those modulated by flow—may also impact EV uptake, though a discussion of such characteristics is better suited to the previous section of this review. Still, one other example of note involves exosomes derived from MSCs and passively loaded with siRNA via simple co-incubation (1 h at 37°C). When produced in agitated microcarrier cultures, these exosomes were significantly more likely to be taken up by target neurons (Haraszti et al., 2018). Similar phenomena have been described in other publications (Huang et al., 2022; Vechetti et al., 2021; Wang, Y. et al., 2020; Xie et al., 2020). Proteins enriched in EVs produced under biomechanical force may promote EV binding to target cell ligands, facilitate endocytosis, or simplify intracellular processing. In general, EV characteristics, discussed in detail previously, go hand-in-hand with cellular characteristics in determining the frequency and nature of EV uptake, though a lack of EV-specific research renders the impact of mechanical stress on EV uptake an area ripe for future study. Regarding EV delivery specifically, the only conclusion currently available is that non-physiological (i.e., pathological) levels of mechanical stress increase EV uptake, though such a finding incorporates only three studies and two cell types (Chen, Y. et al., 2020; Gomez et al., 2020; Qin et al., 2022).

6. Do biomechanical forces affect and complicate EV isolation and purification?

The role of biomechanical forces in impacting EV characteristics and biological efficacy does not cease at harvest. Most EV collection and isolation methods involve some form of biomechanical stress applied to EVs. The extent of the effects on EVs will largely depend on the nature of the surrounding medium and the properties of the cell/EV type(s) in question. Both theoretical and experimental work *in vivo* has demonstrated an increase in EV counts resulting from the shear of needle-based sampling on red blood cells (Stukelj et al., 2017). Ultracentrifugation, a standard lab-scale isolation technique, has likewise been shown to deform and even fragment existing EVs, creating significant new, distinct EV populations as a result (Božič et al., 2020; Stukelj et al., 2017; Sustar et al., 2011). Cells and EVs experience very high levels of shear due to friction with tube walls during centrifugation and ultracentrifugation, a phenomenon exacerbated by the higher viscosities observed at low temperatures (Božič et al., 2020; Stukelj et al., 2017; Sustar et al., 2011). In fact, current work suggests that the *majority* of EVs isolated from blood using common protocols may be generated during harvest and centrifugation via shear-induced stimulation of cells and fragmentation of EVs (Stukelj et al., 2017; Sustar et al., 2011). At very high speeds (100,000 g) and for long centrifugation times (8 h), cargo may even leak through EV membranes (Božič et al., 2020). Although the impact of biomechanical force applied during “dead-end” ultrafiltration has not been evaluated in the context of EV production, the involvement of significant shear stress—intensified by membrane fouling—nonetheless gives cause for concern (Gagnon et al., 2020; Li et al., 2019). Tangential flow filtration, a uniquely scalable isolation method with relatively high EV yields (Busatto et al., 2018; Kim et al., 2021; McNamara et al., 2018), also involves significant biomechanical stresses (Vickroy et al., 2007; Wang, S. et al., 2017), though the impact of such stresses on EV properties and biological efficacy have not been investigated. Indeed, among current scalable EV isolation techniques, only EV precipitation (e.g., with polyethylene glycol) and chromatography appear to avoid significant mechanical stressors that could damage cell/EV membranes or alter EV quantity and quality (Gagnon et al., 2020; Li et al., 2019; Staubach et al., 2021). It should be noted that much of the aforementioned research (Božič et al., 2020; Stukelj et al., 2017; Sustar et al., 2011) has been conducted exclusively on blood samples and the EVs therein; future work must expand the scope of the findings to different cell types. In the studies above, as in many, “shear” may be used as an umbrella term under which non-shear forces are unwittingly included, but not mentioned. Contributions of extensional flow to the overall mechanical force exerted by needle-based sampling may be particularly important, due to rapid contraction of the flow field (Foster et al., 2021). Likewise, during ultracentrifugation, hefty compressive forces on EV pellets are likely partly determinative of EV quantities and properties.

Excess debris resulting from biomechanical forces during EV generation and isolation may also pose a significant issue for EV sample purity. Several studies have suggested that cellular debris levels are correlated with increased mechanical stresses during EV preparation, though whether this debris release is intentional or not on the part of the parent cells is unclear (Piffoux et al., 2017). The risk of co-isolating debris and EVs is therefore a significant concern, especially for more forceful, extrusion-based production methods (Piffoux et al., 2017). In agitated bioreactors, the presence of debris in EV samples has not yet been evaluated, except in the context of protein contamination, where data conflict (de Almeida Fuzeta et al., 2020; Haraszti et al., 2018).

7. Considerations for the scale-up of EV production

Given the high EV doses required for clinical applications (Gupta et al., 2021), scaled EV production will almost certainly require mechanical agitation to attain clinical relevance (Colao et al., 2018; Gobin et al., 2021; Grangier et al., 2021; Paganini et al., 2019; Staubach et al., 2021). As EV technology remains in its infancy from a commercial perspective, however, few scale-up

studies have been published. Production schemes employing Good Manufacturing Practices (GMPs) have been developed at the lab scale, but either rely entirely on static culture or fail to consider the role of mechanical agitation as a significant driver of EV quantity and quality (Andriolo et al., 2018; Gobin et al., 2021; Mendt et al., 2018; Watson et al., 2018). To date, lab-scale research into EV production in stirred/aerated tank bioreactors has been limited.

7.1. EV production in stirred/aerated tank bioreactors

Thanks to a tremendous accumulation of empirical knowledge regarding design and scale-up (Chalmers and Ma, 2015), stirred/aerated tank bioreactors are likely the most practical and preferred option for industrial-scale EV manufacturing. For suspension cells or adherent cells bound to microcarriers, culture in these tanks offers large-scale production capacity and a relatively homogenous culture environment with respect to nutrient, oxygen, and pH gradients (Tsai et al., 2020). However, these reactors are in fact quite heterogeneous where mechanical stress distribution is concerned. Turbulence predominates, meaning Kolmogorov eddies induce constant, random variation in flow (Papoutsakis, 1991). Where microcarriers are present, these eddies—even large eddies produced under relatively mild stirring or sparging—and microcarrier collisions combine to induce significant cell damage (Cherry and Papoutsakis, 1988; Cherry and Papoutsakis, 1989; Chisti, 2001; Croughan et al., 1987; Papoutsakis, 1991). For smaller suspension cells, however, cell damage is most often the result of biomechanical stress imposed by bubble breakup at the bulk gas-liquid interface (Chisti, 2001; Kunas and Papoutsakis, 1990; Papoutsakis, 1991). For a given bioreactor, it seems likely that the phenomena governing cell damage will also have the most significant impact on EV biology, though this hypothesis remains to be investigated. Importantly, however, we expect that even mild, sublethal mechanical stressors will influence EV biology to some degree, given the extent to which such stressors have been shown to modify cellular metabolism, intracellular protein and nucleic acid levels, cell cycle phase distribution, specific productivity, and mAb glycosylation, even as cells remain “undamaged” with respect to viability and proliferative capacity (Chalmers and Ma, 2015; Chisti, 2001; Papoutsakis, 1991). Even microcarrier curvature—which dictates the shape of cells’ projected area—may impact EV production, with cells adhered to smaller microcarriers experiencing greater mechanical force (Tsai et al., 2020).

To date, lab-scale EV production in stirred/aerated tanks has been demonstrated for both suspension cells—notably, CHO (Keysberg et al., 2021), THP-1, HeLa, and Raji cells (Grangier et al., 2020)—and microcarrier-bound adherent cells, including human HUVECs (Gazeau et al., 2020) and MSCs (Adlerz et al., 2019; de Almeida Fuzeta et al., 2020; Gazeau et al., 2020; Haraszti et al., 2018; Jarmalavičiūtė et al., 2015) and murine MSCs (Berger et al., 2021; Gazeau et al., 2020; Pinto et al., 2021). Relative to static cultures, EV yield in these production schemes has increased by as much as 20-fold (for a 48 h culture of microcarrier-bound MSCs) (Haraszti et al., 2018) and as little as 3-fold (for a 48 h culture of microcarrier-bound MSCs agitated with a gentle “Vertical-Wheel” impeller) (de Almeida Fuzeta et al., 2020). Bioreactor-derived EVs also possessed consistently distinct proteomic profiles, though their relative purity varied between studies (de Almeida Fuzeta et al., 2020; Haraszti et al., 2018). In two instances, bioreactor-derived EVs demonstrated desirable bioactivity (with respect to target cells) not observed in EVs from static cultures (Haraszti et al., 2018; Jarmalavičiūtė et al., 2015). Similar results have been observed in other types of bioreactors that impose biomechanical force. For instance, a seesaw-motion bioreactor yielded a nearly 2-fold increase in daily EV production from natural killer cells without impacting EV morphology, structure, or bioactivity (though differences in protein cargo were noted) (Wu et al., 2022). Various flow-perfusion bioreactors have also demonstrated dramatically increased EV yields for a variety of cell types, even after controlling for the architecture of cell scaffolds (Guo et al., 2021; Patel et al., 2019). It remains difficult to fully attribute the aforementioned instances of differential EV biology to mechanical

stress, as growing cells on microcarriers or other various scaffolds can independently alter—and often improve—various EV production metrics, even in static conditions (Cha et al., 2018; Kim et al., 2018; Patel et al., 2019; Yang et al., 2020; Zhang et al., 2017). Still, the innumerable lab-scale studies cited throughout this review attest to the crucial contributions of culture agitation to EV quantity and quality.

7.2. Mixing characteristics for scalable EV production

Some research into microcarrier-based EV production has begun using the Kolmogorov eddy length scale as a characteristic value with which to describe, compare, and scale stirred/aerated-tank cultures (Berger et al., 2021; Gazeau et al., 2020; Pinto et al., 2021). EV production by HUVECs and human/murine MSCs seeded on ~200 μm microcarriers was found to accelerate markedly for eddy length scales below 50 μm, with the most substantial increases beginning consistently in the 30-40 μm range (Gazeau et al., 2020). Recently, this technique has also been applied to suspension cells: EV production by monocytic cell line THP-1 underwent a 10-fold increase after 72 h of agitation with an eddy length scale less than 30 μm (Grangier et al., 2020). No product quality differential was noted, though testing was not comprehensive in this regard. Given the lack of a full paper in this instance, it remains unclear how researchers accounted for the significant stress imposed by bubble entrainment and breakup, which is far more impactful than eddies for suspension cells in a stirred/aerated tank (Kunas and Papoutsakis, 1990; Papoutsakis, 1991).

In most of the aforementioned publications, the role of mechanical agitation, while noted, has not been rigorously documented or optimized. Rather, most studies simply confirm that biomechanical force broadly has boosted EV yields relative to static cultures, with no mention of the impact of particular subcategories of stress (e.g., shear, tension, or compression). Of paramount importance for industrial- and clinical-scale EV production, then, is the establishment of rigorous correlations—applicable even in turbulence—between mechanical agitation and the quality and quantity of the resulting EVs. As noted, the Kolmogorov eddy length scale is gaining attention as a characteristic parameter with which to define EV production across various cell types and reactor geometries (Berger et al., 2021; Gazeau et al., 2020; Grangier et al., 2020). Many other possible such quantities—including average or maximum shear rate (Chisti, 2001; Garcia-Briones and Chalmers, 1994), impeller speed (Chisti, 2001; Garcia-Briones and Chalmers, 1994), integrated shear factor (Chisti, 2001; Croughan et al., 1987; Garcia-Briones and Chalmers, 1994), turbulent collision severity (Cherry and Papoutsakis, 1989), frequency of bubble production or rupture (Czermak et al., 2009; Garcia-Briones and Chalmers, 1994), and mean energy dissipation rate (mathematically dependent on the Kolmogorov eddy length scale) (Chalmers and Ma, 2015; Czermak et al., 2009; Garcia-Briones and Chalmers, 1994) remain unexplored. More complex parameters have also been proposed (Garcia-Briones and Chalmers, 1994). Employing many of these quantities could be problematic, however. For instance, mean energy dissipation rate does not always scale with shear stress, possibly explaining why shear effects on cells are less pronounced in industrial-scale operations (Czermak et al., 2009; Henzler and Kauling, 1993; Nienow, 2006). Use of the Kolmogorov eddy length scale also breaks down at scales of production beyond ~1 to 2 L, given the uncertainty in estimating the mean energy dissipation rate (Garcia-Briones and Chalmers, 1994; Gregoriades et al., 2000). Values that depend on reactor geometry—and not intrinsic characteristics of the flow—are likewise ineffective, a conclusion that implicates impeller speed and the integrated shear factor (Garcia-Briones and Chalmers, 1994). All the proposed parameters are independent of cell type, which we have highlighted previously as a crucial variable in EV production. Any chosen parameter must also describe the impact of transient mechanical stress application; after all, cells in most bioreactors are not exposed to “average” forces, but local, temporary extremes, which may differently impact EV biology (Garcia-Briones and Chalmers,

1994; Gregoriades et al., 2000; Holme et al., 1997; Roka-Moiia et al., 2021; Sakariassen et al., 1998). Currently, and with all this in mind, it appears unlikely that any common characteristic value will be sufficient to account for the effect of mechanical stress on cellular phenotype across all bioreactor geometries and/or cell types (Freiberger et al., 2022). Thus, any broad correlation of EV quantity/quality with generalized flow characteristics may be doomed to the realm of academic curiosity. Absent precise scale-up on a reactor-by-reactor basis, tailored to each individual cell type of interest, computational fluid dynamics will surely be required for industrial EV production (Freiberger et al., 2022; Gregoriades et al., 2000).

7.3. Shear sensitivity and EV product quality

Complicating the situation is the concept of "shear sensitivity," in which previous exposure to shear, culture age, and changes in pH can all modulate the cellular response to mechanical stress (Petersen et al., 1988)—and with it, EV biogenesis and uptake. The nature of the factors informing shear sensitivity suggests that process control for EV manufacturing must account for time-dependent variation in the impact of agitation (illustrated previously in **Figure 5**). The ramifications of shear protectants such as Pluronic F-68—commonplace in industry but unexplored in the context of EV production—are another topic requiring investigation (Chalmers and Ma, 2015; Chisti, 2001; Thomas, 1993). Specifically, the reduced plasma membrane fluidity induced by many shear protectants (Czermak et al., 2009) may interfere with the exocytosis, membrane blebbing, and endocytosis required for exosome release, microparticle release, and EV uptake, respectively.

8. Future directions

The intersection of biomechanical stress and EV biology offers multiple avenues for continued exploration. In this context, numerous parent cell varieties—including leukocytes and erythrocytes—remain almost entirely unexplored, while in other cases, either exosomes (e.g., from MSCs, fibroblasts, and tumors) or microparticles (e.g., from platelets) have been examined exclusively, without regard for the other EV subtype. Detailed investigation into said subtypes is particularly important given the degree to which mechanical stress may differently mediate their loading and release, a phenomenon most apparent in VECs. Additionally, despite basic characterization of EV cargo loading under mechanical stress, the cellular mechanisms implicated in said loading are almost entirely unexplored. The impact of mechanical stress on EV uptake is likewise poorly understood, with most of the relevant existing literature failing to fully differentiate between EV collision with, adhesion to, and internalization by the target cell(s). In this respect, internalization via membrane fusion is in particular need of study. Nevertheless, the massive, ongoing exchange of EVs *in vitro* and *in vivo* can rapidly and continually alter cellular phenotypes, a phenomenon we suggest is substantially influenced by mechanical stress. In sum, although the eventual relevance of this research involves EV production in scalable, complex bioreactors, there remains an urgent need for fundamental research into the distinct impacts of isolated shear/tensile/compressive forces on EV biology.

EV glycosylation is now recognized as a key factor influencing EV biogenesis, cargo loading, uptake, and bioactivity (Harada et al., 2021; Macedo-da-Silva et al., 2021; Martins et al., 2021; Williams et al., 2018). While no published data have yet linked biomechanical force on parent cells to differential glycosylation in EVs, such force is a known mediator of glycosylation in a variety of cell types, including platelets (Nowak et al., 2012) and CHO cells (Senger and Karim, 2003). Indeed, EV isolation method has already been linked to differential EV protein glycosylation, though the mechanisms underlying this phenomenon are unclear (Freitas et al., 2019). With this in mind, we hypothesize that the impact of biomechanical stress on EV protein glycosylation is substantial and just as deserving of future study as stress-induced variation in

protein and miRNA cargo. Heterogeneous glycosylation in EV samples—whether a result of biomechanical force or other factors—will hinder clinical implementation of EV technology.

We also note a significant dearth of research into the impacts of biomechanical forces on EVs from genetically engineered cells. Although Pironti et al. observed increased loading (into EVs) of overexpressed AT1R cargo following the application of high osmotic stress to HEK293T cells (Pironti et al., 2015), whether such a phenomenon would be similarly observed for other overexpressed biomolecules or in other cells in unclear. Genetic engineering that specifically targets EV biogenesis, cargo loading, and uptake pathways is particularly intriguing: such methods may provide a path to modulate the impact of biomechanical force on EV biology and thereby remedy some of the quality control issues inherent in EV biomanufacturing, though no specific research yet supports this hypothesis.

As fundamental research continues to expand our basic knowledge, it is increasingly desirable to transfer our findings to the complex flows in laboratory and industrial-scale bioreactors, where individual forces cannot be easily isolated outside of computational fluid dynamics. EV quantity and quality must be correlated with reactor-independent flow characteristics on a cell-by-cell basis. At the same time, the impact of common media additives—such as shear protectants—and processing steps—such as EV isolation—on EV biology must be rigorously monitored. Notably, we suggest that any quality control efforts for EV production must additionally account for the time-dependent nature of cellular responses to mechanical stress (illustrated previously in **Figure 5**). That is, as cultures grow and age and EV exchange proceeds, cells may become more or less sensitive to stress and respond by altering their EV output, creating a time-dependent differential in EV product quality. With respect to EVs, however, research on these topics is almost nonexistent. Nevertheless, as the field of EV research expands and breaches the gates of clinical relevance, we ignore the impacts of mechanical stress on EV biology at our own peril.

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Will Thompson: conceptualization, writing - original draft, writing - review & editing. **Eleftherios Terry Papoutsakis**: conceptualization, funding acquisition, writing - review & editing, supervision.

Declaration of competing interest

The authors declare no conflicts of interest.

Data availability

No data were produced for the preparation of this manuscript.

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Figures:

Figure 1. The biology of extracellular vesicles. Exosomes (small EVs, generally 30-100 nm) are formed when intraluminal vesicles produced via the inward budding of multivesicular body (MVB) membranes are released into the extracellular space following MVB exocytosis. Microparticles (large EVs, generally 100-1,000 nm) form as a result of the direct outward budding of the plasma membrane. In both EV types, cargo is selectively loaded. EVs bind with target cells and are taken up via either membrane fusion or endocytosis.

Figure 2. Basic biomechanical forces, their applications, and their manifestations in bioreactors. Cells and EVs experience biomechanical forces as shear, tension, compression, or some combination thereof. Lab-scale application of each individual type of force can involve a variety of techniques and equipment. In most bioreactors, the individual effects of these forces combine to create complex biomechanical stimulation.

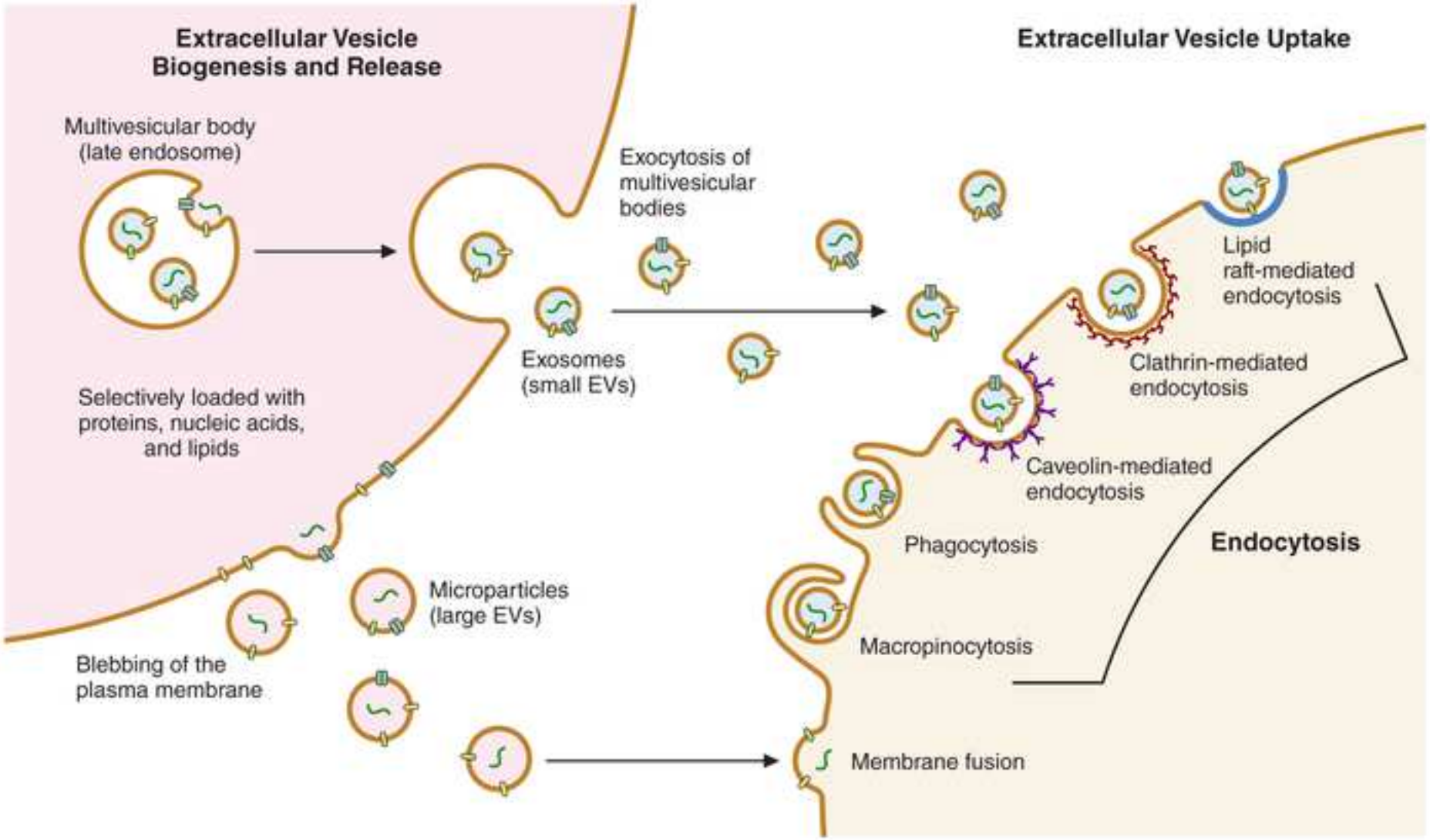
Figure 3. Mechanisms for the impact of biomechanical force on EV biogenesis. Mechanisms for EV biogenesis under biomechanical stress are numerous. Non-apoptotic caspase action—initiated by several pathways and mediated by mitochondrial dysfunction—is one mechanism of note. Alternatively, influxes in intracellular Ca^{2+} levels may activate PKC and/or calpain, promote PS externalization, and trigger actomyosin contractions on the plasma membrane. Additional EV release can occur during ESCRT-mediated repair of Ca^{2+} membrane channels. Mitochondrial dysfunction may further exacerbate the flood of Ca^{2+} via the binding of cytochrome c to the ER and/or release of extracellular ATP. Various integrin signaling pathways can independently mediate EV biogenesis via PKC, ERK, or RhoA/ROCK activity. In some cases, stress-induced nuclear import of YAP may result in transcriptional regulation of EV biogenesis. Shear can directly sever protrusions on the plasma membrane (as in the case of platelet EVs), while tension and compression may upregulate MVB exocytosis and plasma membrane blebbing, respectively.

Figure 4. EV cargo loading *in vivo* for selected cases of aberrant biomechanical stress. Most instances of biomechanical stress *in vivo* affect multiple cell types at once. EV cargo loading is subsequently altered in each cell type, resulting in several diverse responses to the original stimuli that influence an overall phenotype.

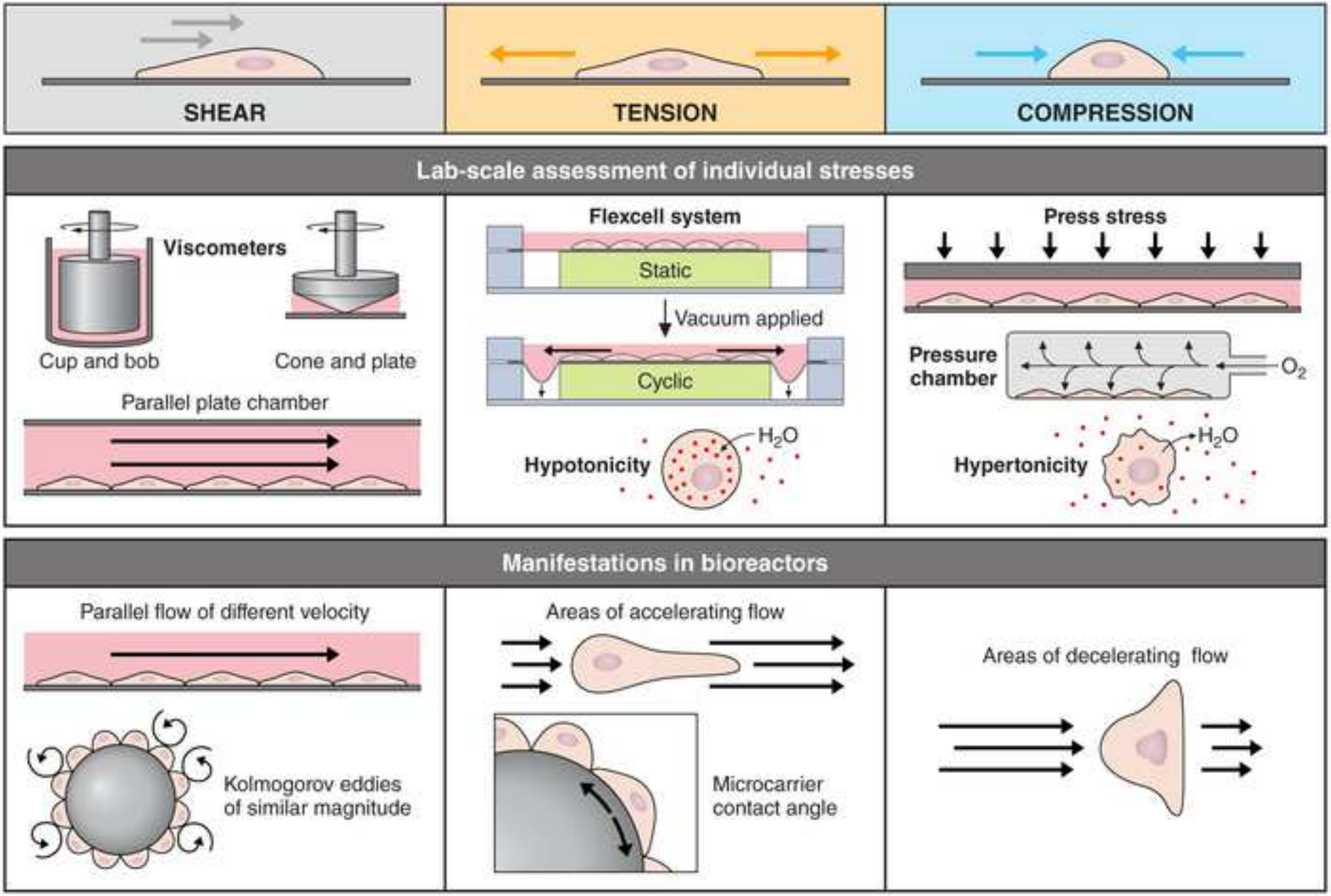
Figure 5. EV sample quality is transient and varies with biomechanical stress. As levels of cellular sensitivity to stress vary with time, the characteristics of released EVs will fluctuate. Subsequent uptake of these EVs by nearby cells may further mediate cell and EV phenotypes, complicating EV quality control during bioprocessing.

Figure 6. The impact of biomechanical force on EV uptake by target cells. EV uptake by target cells occurs in three steps: (I) cell-EV collision, (II) cell-EV adhesion, and (III) EV internalization/uptake. Biomechanical forces will influence all three steps, with particularly notable influence on endocytosis (or the lack thereof). For the variety of reasons identified, endocytosis appears to be upregulated by compression, downregulated by tension, and variably regulated by shear.

Figure



Figure



Figure

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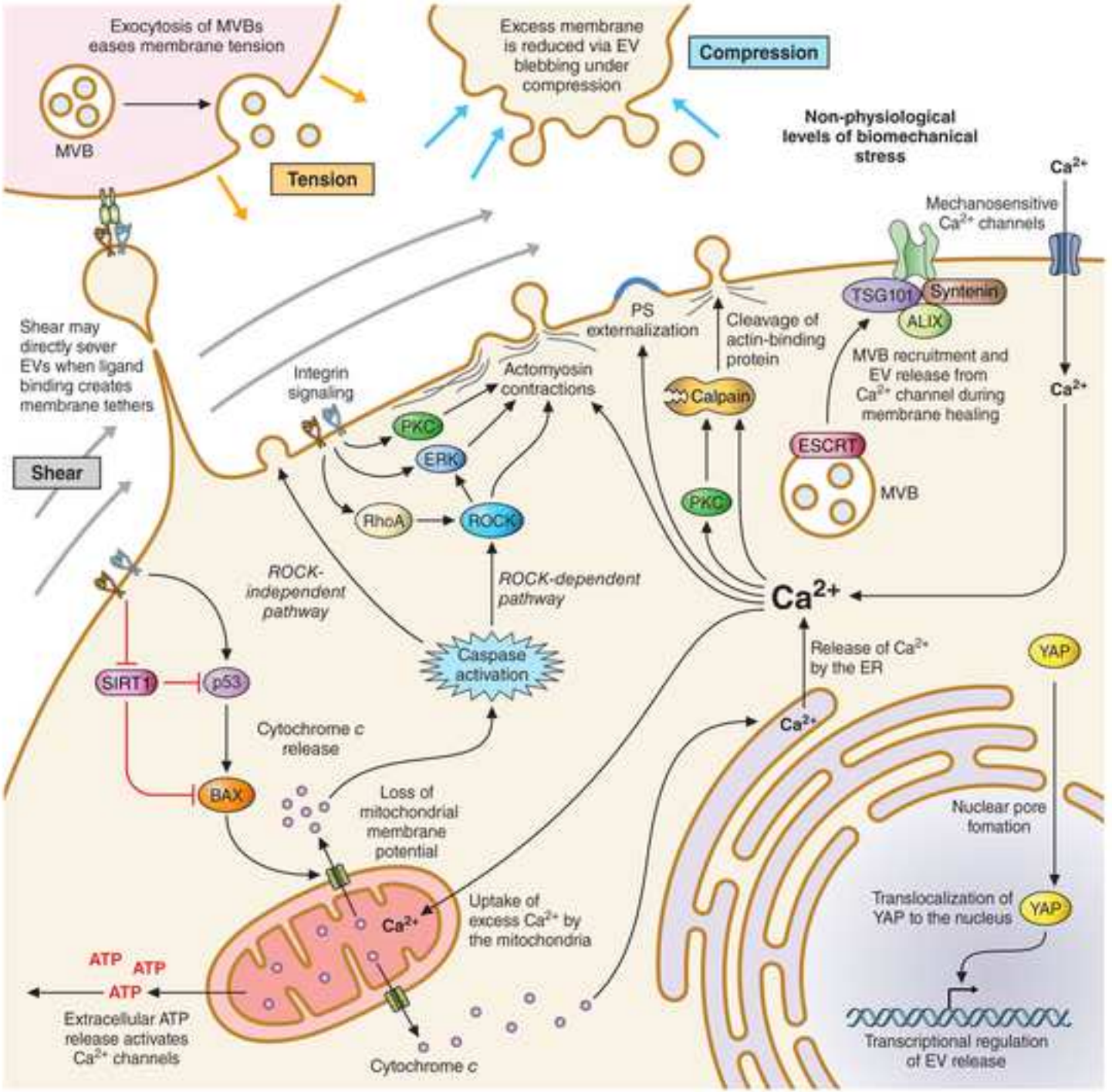
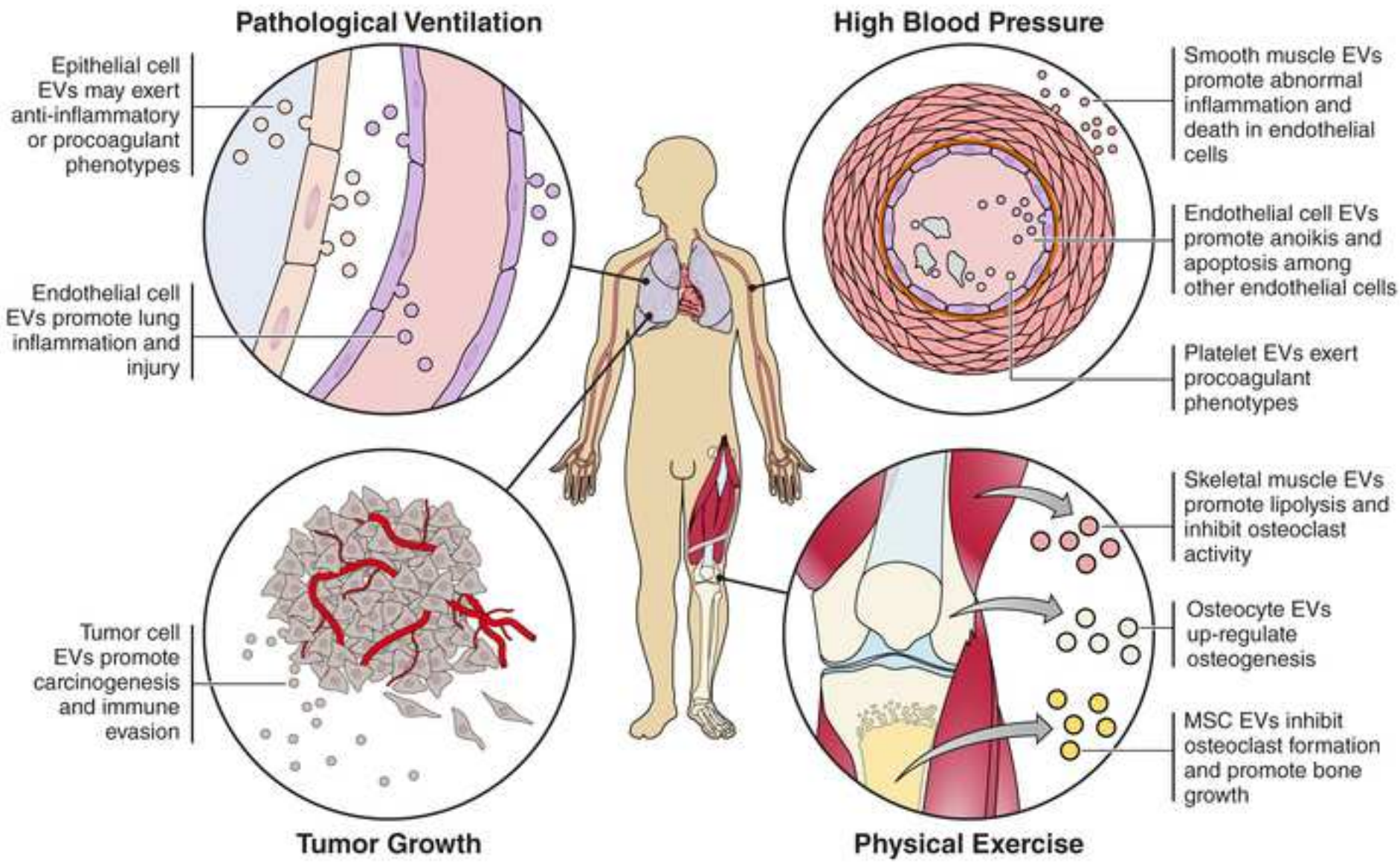
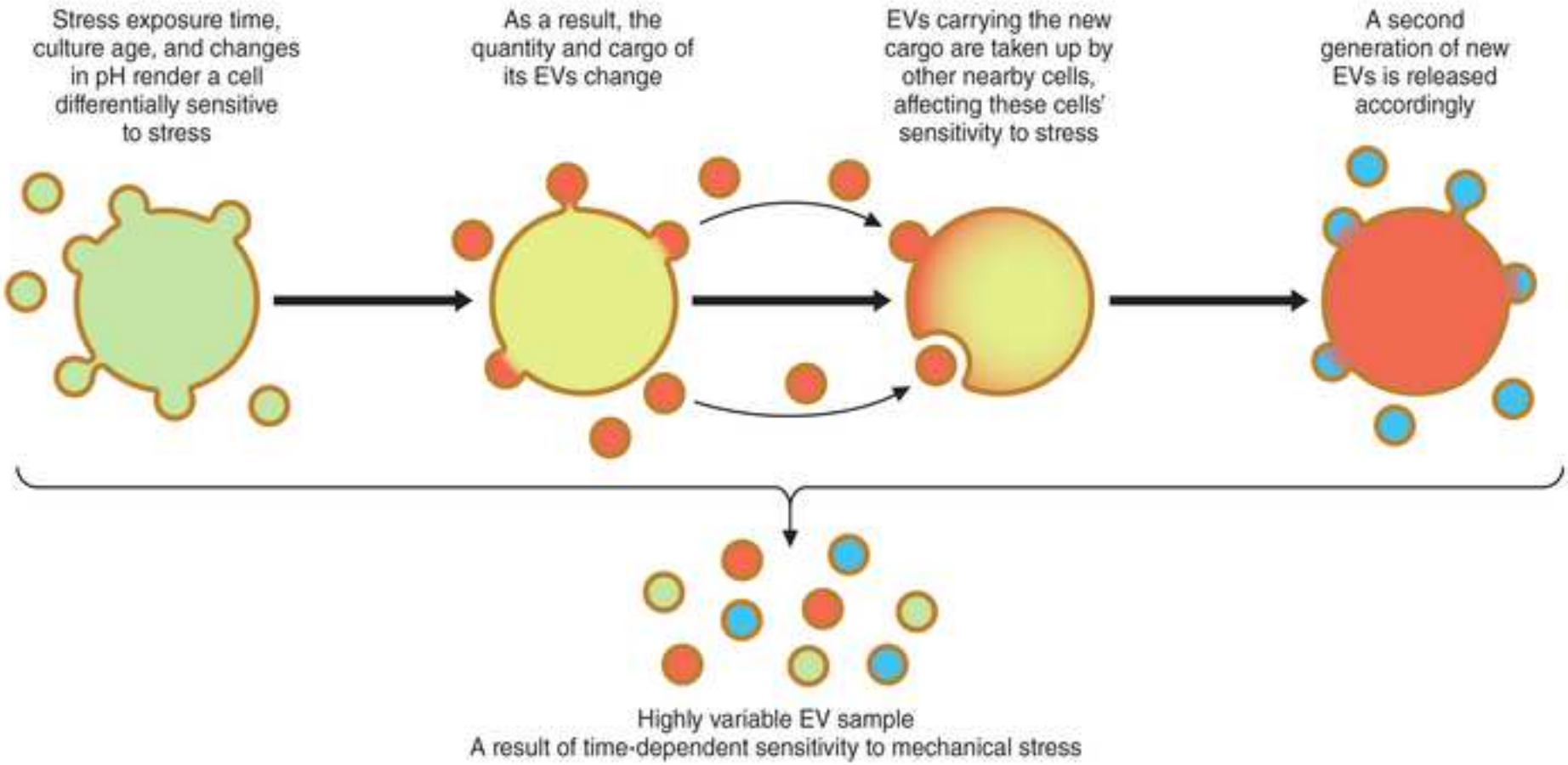


Figure

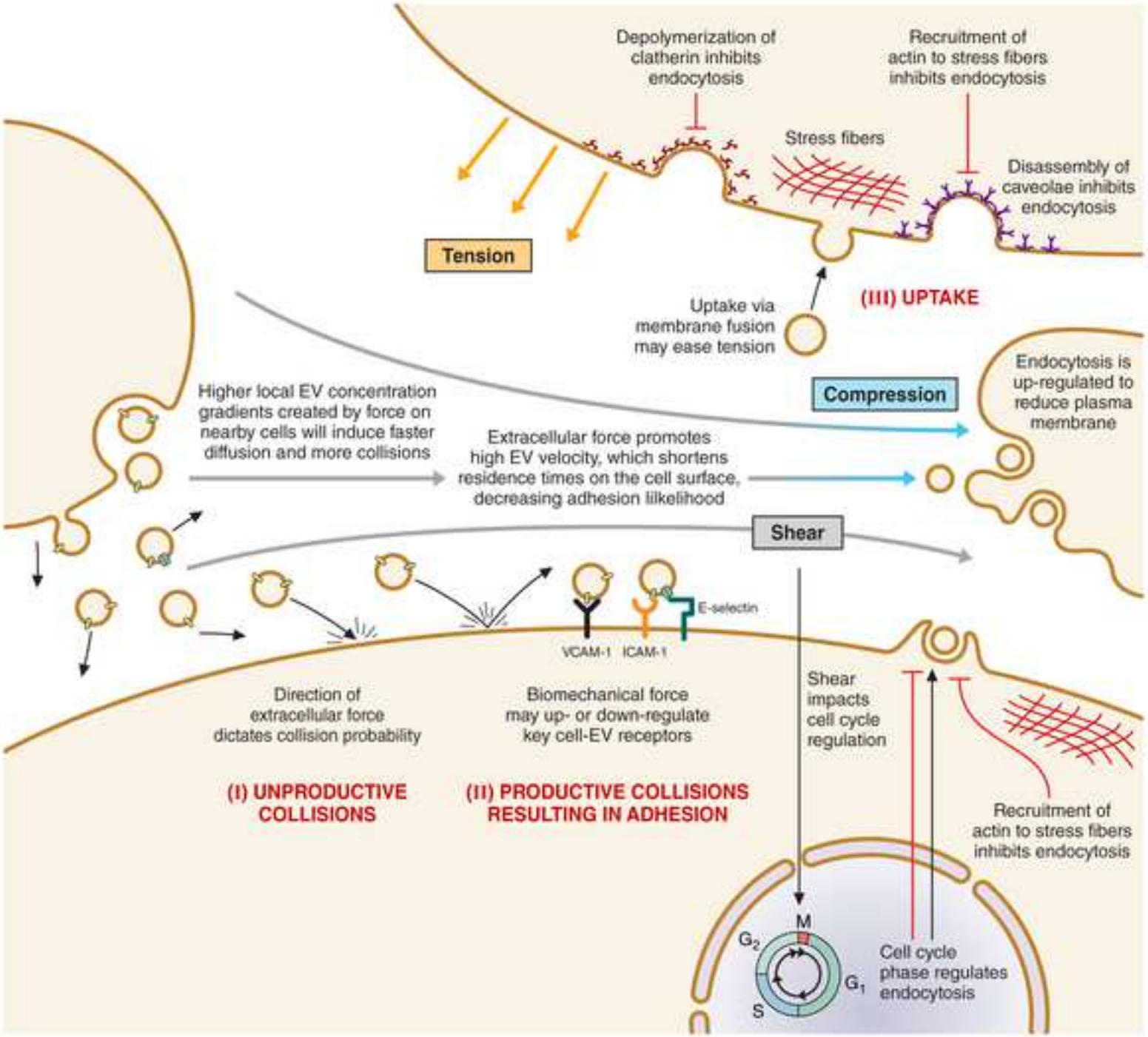


Figure



Figure

Click here to access/download;Figure;Figure 6 (COLOR Reproduction).tif



CRediT authorship contribution statement

Will Thompson: conceptualization, writing - original draft, writing - review & editing. **Eleftherios Terry Papoutsakis**: conceptualization, funding acquisition, writing - review & editing, supervision.