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Research review paper

Towards rationally designed biomanufacturing of therapeutic extracellular vesicles: impact of the bioproduction microenvironment

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ABSTRACT

Extracellular vesicles (EVs), including exosomes, microvesicles, and others, have emerged as potential therapeutics for a variety of applications. Pre-clinical reports of EV efficacy in treatment of non-healing wounds, myocardial infarction, osteoarthritis, traumatic brain injury, spinal cord injury, and many other injuries and diseases demonstrate the versatility of this nascent therapeutic modality. EVs have also been demonstrated to be effective in humans, and clinical trials are underway to further explore their potential. However, for EVs to become a new class of clinical therapeutics, issues related to translation must be addressed. For example, approaches originally developed for cell biomanufacturing, such as hollow fiber bioreactor culture, have been adapted for EV production, but limited knowledge of how the cell culture microenvironment specifically impacts EVs restricts the possibility for rational design and optimization of EV production and potency. In this review, we discuss current knowledge of this issue and delineate potential focus areas for future research towards enabling translation and widespread application of EV-based therapeutics.

1. Introduction

As natural carriers of bioactive cargo, extracellular vesicles (EVs) – especially microvesicles and exosomes – have emerged as potential therapeutics for a variety of applications. EVs have been shown to play roles in numerous physiological and pathological phenomena by imparting their bioactivity through various mechanisms, including signal transduction via protein or bioactive lipid ligand-mediated cell surface receptor activation (Bruno et al., 2009; Isakson et al., 2015), immune modulation by means of antigen presentation (Bobrie et al., 2011), and horizontal transfer of nucleic acids such as microRNAs (miRNAs) (Bovy et al., 2015; Valadi et al., 2007). Among many applications, regenerative or reparative effects of EVs have been observed in models of myocardial infarction (Lai et al., 2010), ischemia reperfusion injury (Chen et al., 2013), acute kidney injury (Bruno et al., 2012), skeletal muscle repair (Nakamura et al., 2015), and many others. EVs have also been utilized as biological drug carriers for delivery of therapeutic proteins (Sterzenbach et al., 2018), nucleic acids (Alvarez-Erviti et al., 2011; Lamichhane et al., 2015, 2016), and small molecules (Tian et al., 2014). Thus, interest in clinical translation of EV therapeutics is high.

However, significant barriers to translation of EV-based therapies remain. Noteworthy among these is the lack of a rationally designed scalable biomanufacturing process for EVs. Several methods have been reported to increase EV production, including cell stimulation in static culture by raising intracellular calcium (Constantinescu et al., 2010; Qu et al., 2009), inducing hypoxia (Kucharzewska et al., 2013), and serum starvation (Aharon et al., 2008). Additionally, dynamic culture systems such as hollow fiber bioreactors have been used to increase EV yield (Watson et al., 2016). Immortalization of mesenchymal stem cells (MSCs) also enabled enhanced EV production capability (Chen et al., 2011). Yet, in all of these cases, there is a lack of fundamental understanding of how changes in cellular function imposed by the culture

Abbreviations: EV, extracellular vesicle; miRNA, microRNA; 2D, two-dimensional; 3D, three-dimensional; ECM, extracellular matrix; KEC, kidney epithelial cell; CEC, corneal epithelial cell; RC, recombinant collagen; MSC, mesenchymal stem cells; ESC, embryonic stem cell; E1MYC16.3, MYC-immortalized ESC-derived MSC line; CMSC3A1, MYC-immortalized umbilical cord-derived MSC line; PC12, neuronal progenitor cells; MAPK, p38 mitogen activated protein kinase; SMC, smooth muscle cells; HUVEC, human umbilical vein endothelial cells; SMA, smooth muscle actin; EC, endothelial cell; VE-cad, vascular endothelial cadherin; vWF, von-Willebrand factor; FDA, US Food and Drug Administration

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system or environment specifically affect EVs. This knowledge gap hinders rational design of EV biomanufacturing strategies.

In this review, we describe current cell culture methods used for EV production and summarize what is known about how the biophysical culture microenvironment impacts cellular responses and, hence, EV production. We further discuss how cell culture parameters might impact EV biogenesis, cargo, and therapeutic function. Increased knowledge of these topics could ultimately spur further study of the mechanisms linking cellular responses with EV function towards enabling rationally designed EV biomanufacturing.

2. Current culture systems for EV production

Cell culture configurations for controlled production of EVs can be grouped into two-dimensional (2D) and three-dimensional (3D) approaches. 2D systems include conventional tissue culture polystyrene flasks for growth of EV-producing cells, which is typically carried out in EV-free media or media previously depleted of EVs by various methods, most commonly ultracentrifugation (Gudbergsson et al., 2016). In general, EV collection is carried out from cells in a sub-confluent state after 24-48 h of culture in collection media, however many papers do not report quantitative measures of cell confluence, seeding density, or collection time and frequency.

2.1. Media composition

It is important to note that, regardless of the culture system and source cells used, media composition plays a critical role in defining composition of generated EVs. The very use of EV-depleted media can impact cell growth (Eitan et al., 2015), function (Shelke et al., 2014), phenotype (Beninson and Fleshner, 2015), and differentiation potential (Aswad et al., 2016). Further, growth factors and other media additives can dramatically affect cell behavior in a wide variety of ways. Given the nearly innumerable possibilities, this review will not focus further on the effects of specific media components on EV generation. However, it is acknowledged that media selection - along with source cell selection, with the latter informing the former - will ultimately be a crucial component in the development of any next-generation EV biomanufacturing systems.

2.2. 3D culture systems

3D culture systems for EV production consist primarily of scaffold-based and scaffold-free approaches, as well as bioreactors. 3D scaffolds are able to capture physiological aspects that are missing in conventional 2D systems, such as 3D tissue architecture, extracellular matrix (ECM) composition, and heterotypic cell-cell interactions (Ader and Tanaka, 2014). The enhanced biomimicry provided by 3D environments may confer a more physiologically relevant phenotype to cells compared to 2D systems and, accordingly, can be leveraged for production of EVs with more natural features (e.g. cargo content, lipid and protein content, etc.). For example, kidney epithelial cells (KECs) cultured in 3D ECM-like scaffolds undergo cystogenesis and produce microvesicles, a phenomenon not observed in 2D cultures (Guo et al., 2008). EV secretion in this system occurred at the abluminal side of the cyst, indicating that the 3D environment does not directly foster EV secretion but rather supports KEC cystogenesis, which in turn results in a polarized vesicular production and trafficking. Additionally, scaffold composition can affect both quantity and quality of the EVs secreted, as shown by Griffith and associates (Jangamreddy et al., 2018). Poly (ethylene glycol) scaffolds functionalized with collagen-like peptides (PEG-CLP) were seeded with corneal epithelial cells (CECs) and tested in vitro and in vivo for the regeneration of cornea defects. Comparisons between conventional 2D cultures and 3D scaffolds made of recombinant collagen (RC) were carried out. Interestingly, $CD9^+$ EVs localized differently in 2D (cell edges), in RC (cytoplasm) and in PEG-CLP (perinuclear region). Additionally, production of $Rab7^+$ EVs was markedly higher in PEG-CLP scaffolds compared to RC, a finding corroborated in animal models, where PEG-CLP constructs stimulated cell ingrowth and the secretion of vast quantities of EVs compared to other scaffolds.

Overall, these results support the hypothesis that scaffold composition can be leveraged to direct EV production and cargo content. Yet, despite the above-mentioned advantages over 2D culture systems, 3D systems also present some limitations, namely the more difficult retrieval of EVs produced within large non-porous scaffolds (e.g. hydrogels). In 2D culture, EVs are easily collected from the medium, however 3D scaffolds may partially retain EVs, requiring further processing (e.g. enzymatic digestion of the construct) that may have detrimental effects on EV integrity and bioactivity. Difficulties with reproducibility and scalability of 3D constructs must also be considered and depend largely on the fabrication method used. Emergent technologies such as 3D printing may ultimately enable reliable 3D scaffold production for industrial-scale EV generation.

2.3. Bioreactors

Following from their use for generation of clinical scale quantities of therapeutic cells, bioreactors are also being employed for large-scale EV production. In particular, hollow fiber bioreactor technology has been utilized effectively (Kim et al., 2017; Nold et al., 2013; Watson et al., 2016). In this setup, cells are seeded into cylindrical hollow fibers through which media is circulated. These fibers are bundled up within a tubular shell, resulting in a high surface area available for cell seeding. Accordingly, these bioreactors can house 3 orders of magnitude (billions vs. millions) more cells than the largest cell culture flask (T175) (Watson et al., 2016), and continuous flow of media through these reactors allows for the collection of roughly 4-fold more EVs than from a traditional 2D flask (Fig. 1). In fact, it has been estimated that to obtain the same amount of EVs in 20 mL media from one day using a hollow fiber bioreactor, it would take 53 T175 cell culture flasks and 800 mL of media (Watson et al., 2016). Other investigators produced EVs in a Biolevitator™, a commercially available bioreactor system where cells are cultured onto protein-coated magnetic particles within a vessel (Jarmalavičiūtė et al., 2015). Gas exchange and metabolite removal is provided by the continuous motion of the particles, which is directed by a magnet along the vertical axis, and by rotation of the culture vessel along the horizontal axis. Stem cells cultured in these conditions secreted exosomes that rescue human dopaminergic neurons from apoptosis, a phenomenon not observed with exosomes harvested in 2D cultures (Jarmalavičiūtė et al., 2015). Strikingly, microvesicles produced in the Biolevitator™ were ineffective and only exosomes had antiapoptotic effects on the dopaminergic neurons. While the overall yield in EV production was not indicated, this study clearly indicates that EVs produced by cells cultured in 3D systems under dynamic conditions are biologically active and have therapeutically relevant properties.

Beyond increasing the total number of EVs produced, the utilization of continuous flow bioreactors necessarily imposes conditions that might impact the identity and function of EVs. Specifically, while EV production may benefit from the enhanced nutrient exchange enabled within a bioreactor system, the presence of flow-derived shear stress may also act as a mechanomodulator of EV secretion and uptake, with unknown consequences. Indicative of this phenomenon, Watson et al. reported that EVs generated from hollow fiber bioreactor culture had a different size profile when compared to EVs from the same cells generated in 2D culture where both EV populations were collected using the same downstream process (Watson et al., 2016). Thus, future investigations should seek to assess the relevance of the mechanical perturbations brought on by flow on EV production and function, as has been done for cells in a variety of contexts (Santoro et al., 2015). Additionally, surface protein and intravesicular cargo content may be

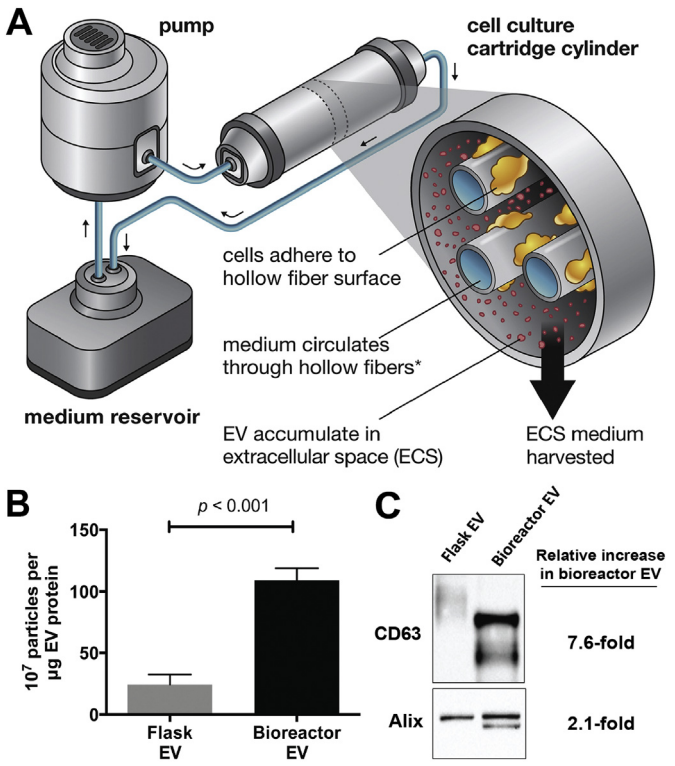

Fig. 1. Hollow fiber bioreactor and 3D culture impact on EV production and bioactivity. (A) Schematic of the hollow fiber bioreactor system. (B) HEK293 cells cultured in a hollow fiber bioreactor produced ~4-fold more EVs than cells cultured in conventional tissue culture flasks. Data presented as mean ± SEM and statistical significance was compared by ANOVA. (B) Western blot analysis showed 7.6-fold and 2.1-fold increase in EV-associated markers of EVs (20 µg) from hollow-fiber bioreactor compared to tissue culture flask. Additional bands observed for CD63 and Alix in Bioreactor EVs is hypothesized to be due to differential glycosylation pattern and phosphorylation, respectively. Adapted via open access from Watson et al. (2016).

reduced with increased EV production in bioreactors on a per vesicle basis, possibly leading to reduced therapeutic potency via reduced cell uptake or other mechanisms. This phenomenon was observed for the rapid production of EV-like vesicles by cavitation. Using a nitrogen cavitation chamber, neutrophils suspended in media were able to produce 16-fold more vesicles compared to 2D culture (Gao et al., 2017). The vesicles produced by cavitation contained more integrin B_2 (a plasma membrane marker of activated neutrophils) and less nucleic acids and proteins commonly found in exosomes, suggesting the force of the nitrogen gas against the cell membrane caused removal of parts of the plasma membrane and formation of vacant vesicles. While not completely analogous to bioreactor culture, this study reinforces the idea that increased production of EVs could have negative consequences on therapeutic potency and functionality if EV composition is not conserved.

3. Potential impact of cellular microenvironment on EVs

Tremendous progress has been made in EV production methods in only a short time, however there are still no rationally-designed approaches that have been specifically developed for EV generation. Currently, rational design of EV production is limited by a lack of fundamental knowledge of the relationships between specific production parameters and EV generation. While these parameters have scarcely been explicitly studied, it is clear that parental cell identity impacts EV cargo and function, suggesting that parameters that influence cells also affect EVs. One cell type for which environmental effects on cell function have been extensively characterized is mesenchymal stem/stromal cells (MSCs), which are responsive to culture substrate properties, extracellular matrix topography and rigidity, and various biochemical and mechanical cues (Fig. 2). Below, we discuss effects of the cell culture environment on MSCs and how such effects might modify MSC EVs, which are of significant clinical interest for a variety of therapeutic applications (Merino-González et al., 2016).

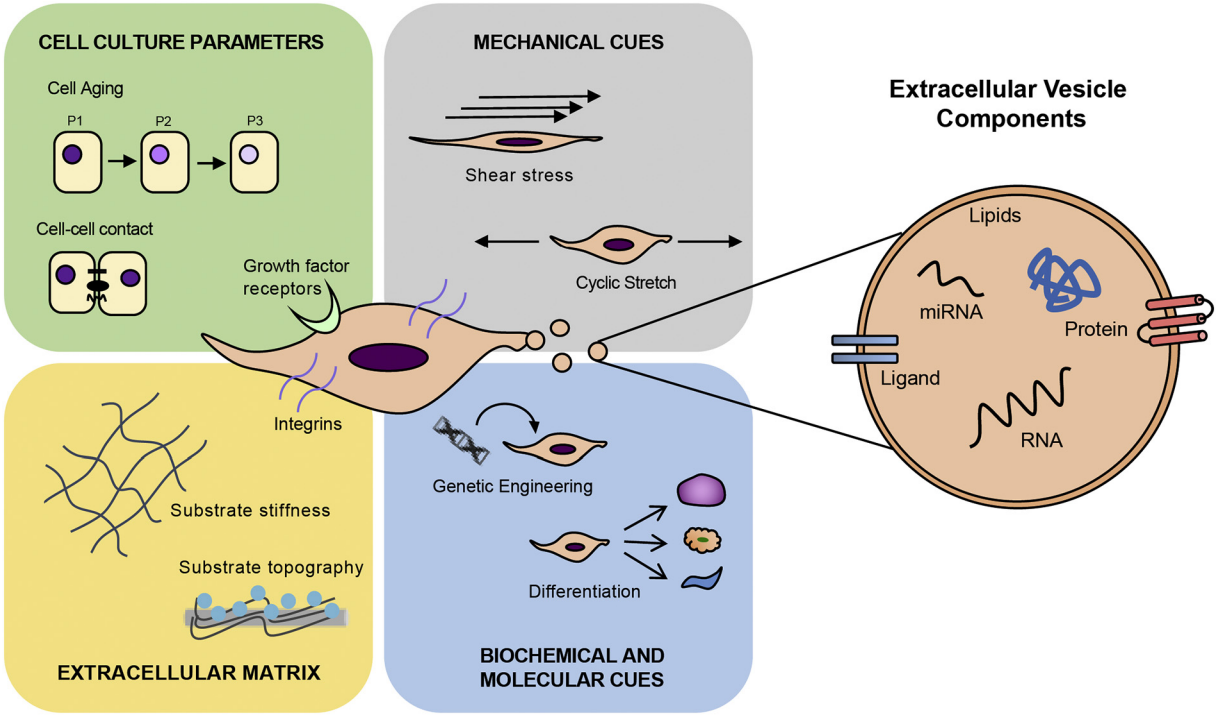

Fig. 2. Schematic representations of factors influencing MSC-derived EV production and cargo composition.

3.1. Cell culture parameters

3.1.1. Cell density

EVs are typically harvested from cell cultures at 60–90% confluence (Gudbergsson et al., 2016), although confluence estimates may vary between individual researchers. Interestingly, previous studies have linked low seeding densities with rapid proliferation of MSCs as well as the highest percentage of multipotent cells compared to higher culture densities (Colter et al., 2000; Sekiya et al., 2002). Furthermore, cells experience contact inhibition at higher densities, which triggers confluent cells to enter quiescence (Lieberman, 1981). The variance in density has also been shown to impact growth kinetics (Colter et al., 2000). In one study, Ho et al. (2011) found that MSCs reached cellular senescence more rapidly in 100% confluent culture, and the doubling time was significantly prolonged (Ho et al., 2011). They also showed that this process was independent of both telomere shortening and p53 activation. Instead, the group showed that the decrease in cell proliferation was due to the observed upregulation of cell cycle regulating protein, p16(INK4a), through Ras pathway (Ho et al., 2011).

Taken together, these studies suggest that cell culture parameters such as cell density can impact not only cell fate but also cause genetic changes, which may influence their secreted paracrine factors such as EVs. Recent work by our group using MSCs showed up to 100-fold decrease in EV production per cell at a higher density of 10,000 cells/cm^2 compared to cells seeded at 100 cells/cm^2; although no significant change in bioactivity of the EVs from low and high density cultures was observed as assessed in an endothelial cell gap closure assay (Patel et al., 2017) (Fig. 3). In a different study by Llorente et al. (2013), dense prostate cancer cells, which have decreased cholesterol metabolism, secreted EVs enriched in cholesterol (Llorente et al., 2013).

These studies further suggest that the production rate and composition of EVs are highly dependent upon their parent cells. While these results begin to establish the importance of cell culture parameters on EV production, better understanding of the molecular mechanisms of the observed phenomena is needed in order to inform rational design of therapeutic MSC EV production. For example, the observed decrease in EV production at higher densities may be due to the well-established phenomenon of contact inhibition. Alternatively, limited direct cell-cell contact in less confluent culture may increase demand for indirect cell-cell communication, such as that mediated by EVs, therefore leading to increased production of EVs per cell. It is likely that the impact of cell density on EV production as well as bioactivity is multifaceted. Thus, more work needs to be carried out to elucidate this mechanism towards establishing a scalable manufacturing pathway for therapeutic EVs.

3.1.2. Cell aging/passage

MSCs are found in bone marrow (BM) at a low frequency (Wexler et al., 2003), requiring high expansion in vitro to obtain relevant quantities for cell therapy as well as EV production. However, researchers have reported the association of long-term in vitro cell expansion with alterations in genes involved in the cell cycle, protein ubiquitination, and apoptosis (Izadpanah et al., 2008). Such changes can affect MSC differentiation potential; Liu et al. report that aged MSCs demonstrate biased differentiation to adipocytes at the cost of osteoblasts (Liu et al., 2015). These changes can be attributed to loss of cell pluripotency as well as senescence during in vitro culture (Bonab et al., 2006; Izadpanah et al., 2008). The mechanisms of aging of MSCs include telomere attrition, DNA damage, and changes in microenvironmental factors with long-term MSC expansion, among others (Liu et al., 2015). This is corroborated in another study by Bonab et al., which showed that mean telomere length decreased by > 15% in MSCs as early as the 9th passage. Furthermore, population doubling time decreased 5-fold by the 10th passage (Bonab et al., 2006).

These results suggest that MSCs enter senescence and begin losing their plasticity and genetic stability rapidly, which may be reflected in their EV cargo as demonstrated by our group in a recent study where we observed a significant decrease in endothelial gap closure bioactivity of MSC-derived EVs with increasing cell passage (Patel et al., 2017) (Fig. 3). However, further investigations are warranted to establish a mechanistic link between the bioactivity phenomenon and the

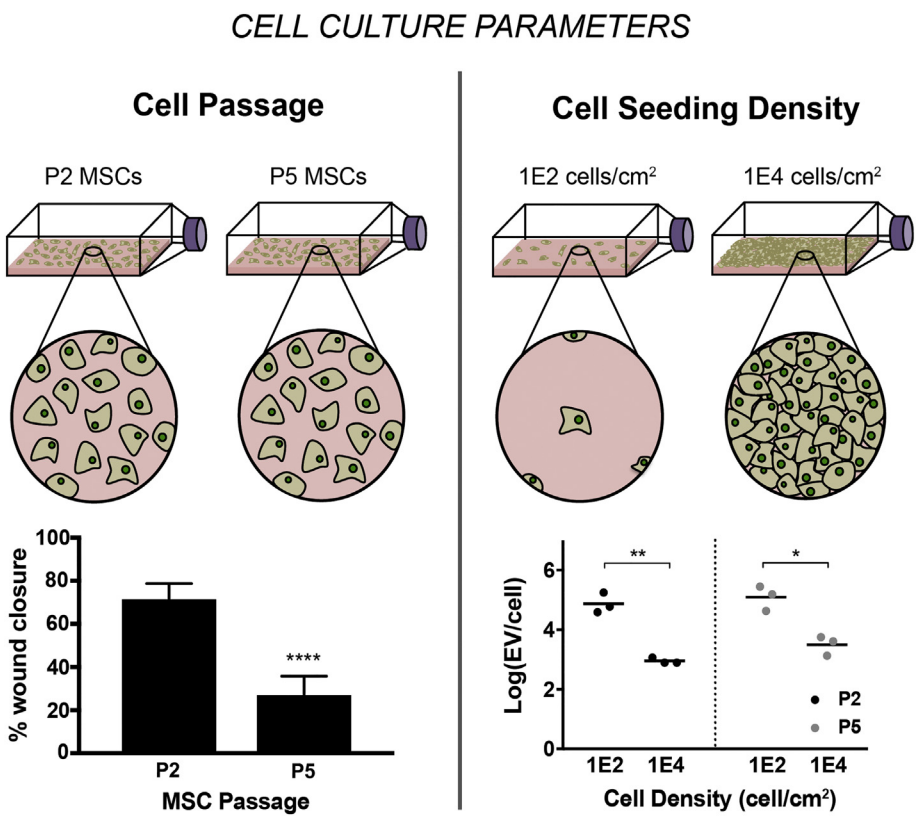

Fig. 3. Impact of MSC passage and seeding density on EV production and vascular bioactivity. (A) EVs were isolated from MSCs at P2, P3, P4, and P5 were seeded at 1E2, 5E2, 1E3, 1E4 cells/cm^2. For MSCs at all passages, increase in EV production is observed per cell with decreasing seeding densities. Statistical significance was calculated using two-way ANOVA with Tukey's multiple comparison test (* $p < .05$, ** $p < .01$). Vascular bioactivity of EVs from different densities (B) and different passages (C) was analyzed using a gap closure assay with endothelial cells. (B) No significant difference in gap closure was observed for EVs from MSCs seeded at 1E2 and 1E4 cells/cm^2. (C) Bioactivity of EVs significantly diminishes with increasing passage. Data are representative of three independent experiments with three replicates each ($n = 3$). Statistical significance was calculated using two-way ANOVA with Tukey's multiple comparison test (* p < .05, ** p < .01, **** $p < .0001$). Figure reprinted from Patel et al. (2017) via open access.

influence of long-term expansion of MSCs on their EVs. To begin understanding this connection, changes in EV cargo composition as well as molecular changes in cells treated with these EVs should be identified by applying unbiased approaches where possible (e.g. proteomics, transcriptomics). A detailed assessment of potential mechanism should follow via specific knockdown and overexpression experiments of identified molecular targets to corroborate their roles in EV-mediated bioactivity with increasing MSC passage.

3.2. Biochemical and molecular cues

3.2.1. Cell Immortalization

To overcome the limited lifespan of MSCs and loss of MSC-derived EV bioactivity with increasing passage, Chen and colleagues investigated the effect of immortalizing MSCs on EV production and cardioprotective phenotype (Chen et al., 2011). The authors immortalized an embryonic stem cell (ESC)-derived MSC line (E1MYC16.3) and an umbilical cord-derived MSC line (CMSC3A1). Upon immortalization, both lines of MSCs demonstrated higher proliferative capacity and increased growth rate compared to their primary counterpart; however, cord-derived MSCs lost the ability to undergo adipogenic differentiation and had reduced adherence to tissue culture plastic, which is uncharacteristic of MSCs. Cord-derived MSCs also presented more disparity in gene regulation after MYC-immortalization than ESC-derived MSCs. The authors observed a general increase in EV production post-immortalization of ESC-derived MSCs, albeit for cord-derived MSCs, MYC-immortalization significantly reduced EV production. EVs from both cell lines were positive for exosomal markers CD9 and C81, but MYC protein was undetected. Evidently, EVs from both immortalized cell lines exhibited cardioprotective and therapeutic phenotype against myocardial ischemia/reperfusion injury. While this transformation reportedly produced EVs with preserved therapeutic potential, the process of cell immortalization required lentiviral transfection, which rendered a change in MSC phenotype to a non-MSC classification that could be reflected in the EV population over long-term in vitro expansion.

3.2.2. Cell differentiation

Stem cell differentiation is a highly complex process requiring substantial changes in genetic regulation of cells, which are likely to be reflected in their EVs. In a recent study by Wang et al. (2018), the authors isolated EVs from conditioned media of MSCs at different stages of osteogenic differentiation and evaluated their potential to stimulate osteogenic differentiation of homotypic cells (Wang et al., 2018). Interestingly, their results suggest that homotypic cells treated with EVs from only late-stage differentiation showed increased ALP activity and extracellular matrix (ECM) mineralization, indicative of their osteogenic specification (Fig. 4). Further, microarray-based miRNA analysis revealed variance in miRNA composition of EVs in a stage-dependent manner, with higher levels of miRNAs favoring osteogenic induction present in late-stage EVs compared to early-stage EVs. Other researchers have reported similar findings related to neuronal differentiation, where EVs derived from neuronal progenitor cells (PC12) at a late stage of differentiation induced neuronal differentiation of MSCs through miRNA transfer (Takeda and Xu, 2015). While the specific roles of miRNAs in EV function remain a subject of active investigation, these studies reinforce the concepts that parent cell phenotype dictates EV function and that molecular composition of parent cell is reflected in their EV population.

3.3. Extracellular matrix

3.3.1. Substrate topography

Topography of the cell substrate or extracellular matrix has been shown to govern several aspects of MSC behavior, including migration, proliferation, and differentiation. Abagnale et al. (2015) systematically

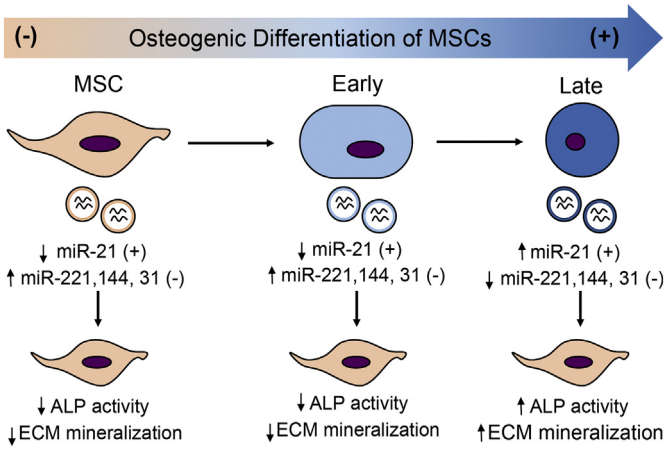

Fig. 4. Schematic describing that EVs from varying stages of differentiating MSCs induce osteogenic differentiation of MSCs differently. Osteogenic differentiation was induced in MSCs using osteogenic differentiation media. EVs were isolated from conditioned media collected at different stages after media change, namely, early and late stages. Exosomes from MSCs in growth media were used as control. Using an array-based method, expression of miRNA associated with osteogenic differentiation of MSCs was measured. ALP activity, ECM calcium, and phosphate was measured to evaluate osteogenic differentiation of MSCs after EV treatment. Adapted from data presented in Wang et al. (2018) via open access.

compared in vitro differentiation of MSCs on a variety of patterned polyimide surfaces (Abagnale et al., 2015). Differences in substrate grooves and ridges affected spreading and orientation of MSCs and guided them towards specific lineages. For example, 15 μm ridges increased adipogenic differentiation whereas 2 μm ridges enhanced osteogenic differentiation. Notably, nano-patterns with a periodicity of 650 nm increased differentiation towards both osteogenic and adipogenic lineages (Abagnale et al., 2015).

Other studies have focused on elucidating changes in gene expression profiles due to surface patterning. Dalby et al. (2008) compared the genetic profile of MSCs cultured on osteogenic nanoscale topographies to cells treated with dexamethasone, a corticosteroid routinely used to stimulate bone formation from MSCs. The topographies were shown to activate integrin-mediated actin cytoskeleton signaling and p38 mitogen-activated protein kinase (MAPK) signaling to manipulate MSC cytoskeleton towards osteogenic differentiation (Dalby et al., 2007, 2008). Lee et al. observed that etched microgrooves on titanium triggered differential expression of genes involved in cell adhesion, migration, and cytoskeletal reorganization in MSCs. They also showed that microgrooves resulted in increased type I collagen production, osteoblast differentiation, and activation of TGF-β signaling and MAPK signaling (Lee et al., 2012). In a different study, Kurpinski et al. (2006) used a micropatterned strip to align MSCs along the direction of uniaxial strain, which resulted in increased expression of calponin-1, a marker for smooth muscle cells (SMCs). However, perpendicular alignment of the cells caused no changes in gene expression of calponin-1 (Kurpinski et al., 2006). Thus, surface topography alone does not govern cellular differentiation but rather triggers a signaling pathway that elicits specific cell responses when synergizing with corresponding biochemical stimuli.

Despite numerous studies describing the effects of substrate topography on MSC fate, additional work needs be done to elucidate the resulting impact on MSC EVs. Tauro et al. (2013) showed that a single LIM1863 colon-cancer cell carcinoma organoid with basolateral and apical topography produced two distinct population of EVs. The basolateral and apical surfaces of the cells exocytose A33- and EpCAM-rich EVs, respectively, each with a different cargo composition (Tauro et al., 2013) (Fig. 5). This study illustrates the impact of the mechanical

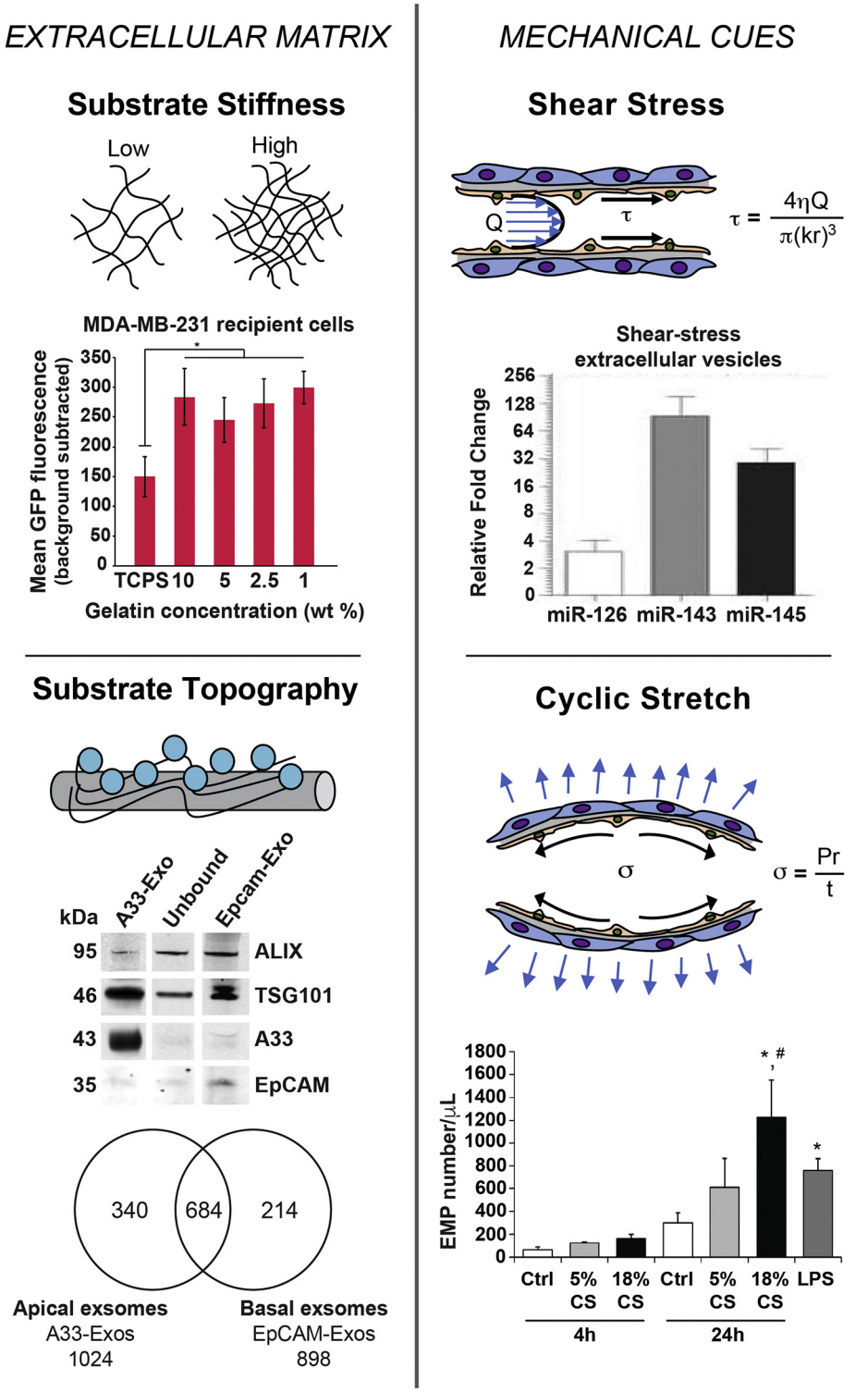

Fig. 5. ECM characteristics and mechanical stimulation of cells can dictate EV production, composition, and uptake profiles. In the top left figure, breast cancer cell lines MCF-7 and MDA-MB-231 were seeded on gelatin substrate with varying stiffness. MDA-MB-231 cells seeded on gelatin matrix with different stiffness were treated with CD63-GFP-labled EVs. EV uptake was monitored following 2 h of treatment through live-cell imaging. Seeding cell on softer gelatin matrices compared to tissue culture polystyrene (TCPS), resulted in significantly increased EV uptake for both cell lines. Adapted from Tauro et al., (2013) via open access. Bottom left figure shows western blot analysis of exosomes (10 μg) derived from LIM1863 cells from apical (EpCAM-Exos) or basolateral (A33-Exos) surfaces, which were enriched in EpCAM and A33, respectively. Both sources were also positive for exosomal markers TSG101 and Alix. Venn diagram shows differences in protein expression between EpCAM-Exos and A33-Exos. In total, 340 and 214 proteins were specifically found in A33- and EpCAM-Exos, respectively. In comparison, 684 proteins were present in both exosome sources. Adapted from Stranford et al., (2017) with permission. For the top right figure, EVs were isolated from HUVECs with or without exposure to shear-stress of 20 dynes/cm^2 for 3 days. RNA analysis revealed that EVs derived from HUVECs under shear stress were highly enriched in miR-143/145, which are known to modulate SMC phenotype. Adapted from Hergenreider et al., (2012) with permission. For the bottom right figure, confluent lung-ECs were subjected to 5% CS or 18% CS for 4 and 24 h or to LPS (1 μg/mL) for 24 h. Lung-EC-derived microparticles (EMPs) (0.1–1 μm) were isolated from conditioned media and quantified by flow cytometry. Significant increase in EMP production is observed after 24 h exposure to 18% CS and LPS compared to the static control. Reprinted from Letsiou et al., (2015) with permission.

environment on the production of bioactive EVs and thus the importance of its consideration when formulating a rational design for EV biomanufacturing.

3.3.2. Substrate stiffness

MSC fate can be governed by the rigidity of the cell substrate (Engler et al., 2017). For example MSCs plated on soft substrates (0.1–1 kPa) exhibited a neuronal phenotype whereas on stiff substrates (25–40 kPa physiological stiffness of collagenous bone) MSCs differentiated into an osteogenic lineage. On intermediate stiffness substrates (11–30 kPa) mimicking muscle substratum MSCs displayed a myogenic phenotype. Further the regulation of MSC specification by matrix stiffness was shown to involve non-muscle myosin (Engler et al., 2004). This observation was supported in a study by Zemel et al. (2010) who demonstrated that non-muscle myosin alignment was dependent on matrix rigidity implying mechanical coupling between external environment and internal cytoskeletal organization (Zemel et al., 2010).

The influence of ECM stiffness on MSC specification has also been assessed in 3D culture systems. In a 3D alginate hydrogel with integrin-binding RGD peptides, the regulation of cell fate through stiffness

coincided with observations in 2D cultures. MSCs committed to an osteogenic phenotype at intermediate (11–30 kPa) elasticity and adipogenic lineage in softer (2.5–5 kPa) hydrogels (Engler et al., 2017). However, 3D culture significantly altered cell morphology and proliferation rate compared to 2D culture. MSCs cultured in spheroids have been shown to be more spherical inside and elongated outside with an overall reduction of cytoskeletal molecules and the constituted ECM. Small, rounded MSCs are prone to differentiate into adipogenic lineage, whereas large, extended MSCs tend to differentiate into osteogenic lineages (Cesarz and Tamama, 2016). Thus, differences in dimensionality of MSC culture systems can alter cellular morphology, which is a key characteristic used to determine cellular phenotypes and fates of MSCs. Furthermore, the ECM formed by MSCs in 3D spheroid culture has a very low rigidity of < 0.1 kPa, whereas the tissue culture plastic used in 2D culture has an elastic modulus in the GPa range. As described above, changes in substrate stiffness contribute to alterations of gene expression and cell phenotype (Cesarz and Tamama, 2016), which could dictate EV production and function.

Although the impact of matrix stiffness on EV production has yet to be sufficiently investigated, de Jong et al. (2012) showed that EVs have the ability to contribute to ECM stiffness. Specifically, EVs from hypoxic endothelial cells showed increased abundances of the ECM components fibronectin, collagen-4 and -12 subunits, and LOXL2 (de Jong et al., 2012). Similar trends could be possible for EVs obtained from cells cultured on substrates with varying stiffness. IN a different study, Stranford et al. (2017) recently conducted a systematic analysis of stiffness influencing EV uptake. Their findings showed a significant increase in EV uptake by breast cancer cell lines MDA-MB-231 and MCF-7 when seeded on softer gelatin matrix compared to stiff tissue culture polystyrene (Stranford et al., 2017) (Fig. 5). Thus, the substrate stiffness that a cell experiences can affect both EV secretion and uptake, which could profoundly impact net EV production. To better understand the impact of stiffness on EV generation and cargo composition, a methodical evaluation needs to be carried out.

3.4. Mechanical cues

3.4.1. Fluid shear stress

Physiologically, MSCs present in the bone marrow and in circulation are exposed to shear stresses imposed by interstitial fluid and blood, respectively (Coughlin and Niebur, 2012). In culture, Castillo and Jacobs (2010) showed that shear stress in the range of 0.5 to 2.0 Pa increases MSC proliferation (Castillo and Jacobs, 2010). In another study by Rubin et al. (2007), similar shear stress decreased adipogenic differentiation in MSCs (Rubin et al., 2007), which was corroborated by Ruiz and Chen (2008) who showed that when MSCs seeded in a collagen matrix are subjected to stress, those experiencing low shear stress in the center of the construct differentiated into adipocytes, while those experiencing higher stresses on the surface of the construct differentiated into osteoblasts (Ruiz and Chen, 2008).

Although a number of studies have determined the influence of shear stress on MSCs, their impact on secretion of EVs is yet to be fully understood. As described earlier, Watson et al. (2016) showed that HEK293 cells cultured in a hollow fiber bioreactor with slow media circulation produced 40-fold more EVs to be used for drug delivery as compared to the conventional static cell culture (Fig. 1B). Apart from the rate of EV production, cargo loading has also been shown to altered when source cells are exposed to shear stress. Hergenreider et al. (2012) showed that exposure of human umbilical vein endothelial cells (HUVECs) to shear stress of 20 dynes/cm^2 for 72 h increased miR-143/145 in HUVEC EVs by 30-fold compared to only 10-fold in the cells, suggesting targeted transcription of miRNAs specifically for EV-mediated release and eventual transfer to SMCs to regulate their function (Hergenreider et al., 2012) (Fig. 5). Although researchers are beginning to investigate the impact of mechanical cues such as shear stress on EV production and cargo loading, further analysis with multiple shear stresses over different time periods need to be conducted to fully decipher the impact of shear stress on MSC EV production and bioactivity.

3.4.2. Cyclic stretch

The effect of cyclic strain has been intensively researched in cardiovascular cells, especially cardiomyocytes and SMCs, as they are physiologically exposed to periodic stretch. However, the effect of cyclic stretch on MSCs has only recently been explored. Park et al. (2004) demonstrated that cyclic equiaxial strain in MSCs downregulates smooth muscle actin (SMA) and SM-22a, markers of SMC differentiation, whereas cyclic uniaxial strain transiently increases the expression of these markers intermittently. This transient expression correlated with cell reorganization in the perpendicular direction, relative to the direction of stretch. Thus, uniaxial strain may be used to guide MSC differentiation into SMCs (Park et al., 2004). However, more frequently, researchers have investigated the impact of cyclic stretch in combination with shear stress. For example, O'Cearbhaill et al. (2008) showed that under pulsatile pressure, radial distention of 5%, and a shear stress of 10 dyn/cm^2 (1 Pa), MSCs exhibit similar mechanosensitive responses to those of endothelial cells (ECs) (O'Cearbhaill et al., 2008), reorienting along the direction of flow and displaying EC-like morphology. Interestingly, genetic profiling of these MSCs revealed greater expression of SMC-associated markers SMA and calponin (O'Cearbhaill et al., 2008). In another study, however, application of 5% circumferential stretch for 4 days following 2.5 dyn/cm^2 shear stress for 1 day resulted in increased MSC expression of EC marker proteins vascular endothelial cadherin (VE-cad) and von Willebrand factor (vWF), with SMA and calponin being undetectable. These contradicting results demand further investigation focused on mechanisms by which mechanical forces impact MSC fate. Regardless, it is clear that these factors significantly impact MSC phenotype and have the ability to alter MSC EV characteristics and thus should be further evaluated.

Although the focus of this article is on understanding how microenvironmental factors affect EVs derived especially from MSCs, it widely applicable to EVs derived from any cell type. Several studies have shown therapeutic potential of EVs derived from different vascular cells including ECs and SMCs towards therapeutic vascularization. Significant efforts have been made to understand the physiological processes by which EVs mediate intercellular communication between ECs and SMCs (Hergenreider et al., 2012; van Balkom et al., 2013). Moreover, the impact of fluid shear stress and cyclic strain on ECs in mature blood vessels has been widely studied and has been summarized by Hsieh et al. (2014), reiterating the importance of careful consideration of microenvironmental factors for generating therapeutic EVs from any given cell type. Interestingly, in a model of acute lung injury, Letsiou et al. (2015) showed that exposing endothelial cells to 18% cyclic stretch results in ~4-fold increase in EC-derived EVs compared to static cells, each with differing protein composition (Letsiou et al., 2015) (Fig. 5).

4. Conclusions

In this review, we have outlined various factors that could impact EV biogenesis and function. The work done in this area so far supports the importance of careful consideration of the parameters discussed above on EV production. However, in-depth analyses identifying mechanistic links between these factors and EV phenotype still need to be conducted. Particularly, alterations in EV-associated lipids, proteins, and/or nucleic acid cargo and their downstream targets in the recipient cells should be assessed alongside the phenotypic changes imparted by EVs in recipient cells. A complete understanding of the multitude of ways in which these factors impact EV production and specific cargo components would inform rational design of a large scale biomanufacturing platform that could yield not only increased production, but also increased potency of therapeutic EVs. Although beyond the scope of this review, it is important to note that many factors in the

downstream isolation and characterization processes of EVs can also significantly impact the outcome of EV production, as discussed in numerous reports (Gudbergsson et al., 2016; Lai et al., 2016; Tauro et al., 2012). Thus, continuous efforts towards establishing standardized protocols are paramount. Finally, a special focus on choosing appropriate cell lines and cell donors as well as establishing guidelines for consistent production of therapeutic EVs will also be necessary to overcome regulatory issues facing cell-based therapies. Cooperative efforts between researchers and the US Food and Drug Administration (FDA) will be crucial to ensure successful establishment of good manufacturing practice-grade production of therapeutic EVs.

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