● PERSPECTIVE

Matrix bound vesicles and miRNA cargoes are bioactive factors within extracellular matrix bioscaffolds

Injury to central nervous system (CNS) tissues in adult mammals often leads to neuronal loss, scarring, and permanently lost neurologic functions, and this default healing response is increasingly linked to a pro-inflammatory innate immune response. Extracellular matrix (ECM) technology can reduce inflammation, while increasing functional tissue remodeling in various tissues and organs, including the CNS. However, ECM bioscaffold delivery and the rapid ECM degradation rate *in vivo* have complicated advancing ECM technology in the CNS. The recent discovery of bioactive matrix bound vesicles (MBV) within ECM bioscaffolds represents a potentially significant vertical advance in our understanding of how ECM bioscaffolds modulate the default healing response in various tissues and organs. MBV enable new approaches to delivering ECM derived bioactive components in a minimally invasive manner, and open new areas of investigation into how the ECM derived MBV regulate tissue and organ organization during development, normal function, aging, and after injury or disease. This perspective provides an overview of MBV, initial studies on macrophages, neuroblastoma cells, and primary neurons, and discusses the potential diagnostic and therapeutic potentials of MBV in CNS applications.

ECM bioscaffolds are widely used pre-clinically and clinically to positively modulate the innate immune response to reduce scarring and promote functional tissue remodeling (Dziki et al., 2017). ECM is the protein and glycosaminoglycan matrix in tissues and organs that provides mechanical and biochemical support, facilitates cellular communication, and directs cellular phenotype and function. ECM bioscaffolds derived by decellularizing healthy xenogeneic tissues can promote constructive tissue remodeling and functional recovery, over scarring, in diverse tissues, including esophagus, lower urinary tract, and musculotendinous tissues, among others. Recent pre-clinical studies suggest ECM bioscaffolds can also promote positive tissue remodeling in the nervous system (Ren et al., 2015), in part, by positively regulating the innate immune response (Tukmachev et al., 2016). However, the bioactive factors within ECM bioscaffolds are not well understood. Thus, the recent discovery of bioactive MBV in virtually all ECM bioscaffolds analyzed and the ability of purified MBV to regulate diverse cellular phenotypes suggests that MBV are critical bioactive factors within ECM bioscaffolds (Huleihel et al., 2016). This holds particular promise for CNS applications in which ECM delivery is complicated by the necessity for minimally invasive approaches (Massensini et al., 2015).

MBV appear to represent a distinct class of extracellular vesicles (EV) localized to the collagen fibrillary network within the ECM. MBV were originally discovered due to the presence of non-degradable, non-coding nucleic acids, specifically miRNAs, within various ECM bioscaffolds (Huleihel et al., 2016). Huleihel et al. (2016) showed MBV are a protective source of miRNAs in all laboratory and commercially produced ECM bioscaffolds tested, including ECM bioscaffolds from muscle, intestine, urinary bladder, and even CNS tissues, suggesting MBV are ubiquitous ECM components positioned to play a role in ECM organization and function. MBV readily bind to collagen fibrils and evidence suggests the localization of MBV to tightly packed collagen fibrils protect MBV from detergent-mediated degradation during ECM decellularization. In agreement, MBV are small, nanoscale vesicles ranging in size from about 10 to 300 nm. This size places MBV in the range of exosomes, which are secreted from virtually all cell types and found in all bodily fluids. However, MBV differ from exosomes not only in their localization but also in their general lack of typical extra-vesicular exosome markers, like the tetraspanins CD9, CD63, and CD81 (Huleihel et al., 2016), suggesting MBV originate from a unique, ECM-specific, cellular origin.

MBV can recapitulate effects of the ECM bioscaffolds from which they were derived and these effects appear to depend on MBV miRNA cargoes (Huleihel et al., 2017a). Like other EV, MBV carry unique and complex cargoes, including lipids, proteins, cytokines, carbohydrates, and other small molecules (**Figure 1**), capable of activating a variety of extra- and intracellular signaling pathways. As with other EVs, initial studies indicate the cellular responses to MBV depend on both extra-vesicular proteins and the intra-vesicular miRNA cargoes (Faust et al., 2017). Interestingly, miRNA cargoes in MBV, derived from different tissues, are enriched in highly conserved miRNAs involved in cell cycle regulation and differentiation (Faust et al., 2017; Huleihel et al., 2017b), suggesting MBV and their cargoes may play a fundamental role in ECM mediated tissue and organ development, organization, and function, possibly by regulating cellular phenotypes in a site appropriate manner. Moreover, several miRNAs highly enriched in MBVs are known to regulate neuronal signaling pathways underlying neuronal differentiation and neurite growth, including miRNAs-30b, -125b, and -133b (van der Merwe and Steketee, 2017).

The hypothesis that MBV play a fundamental role in regulating cellular behavior is supported by initial functional studies on diverse cellular populations that mediate the default healing response in the CNS: bone marrow derived macrophages, neuroblastoma cells, and primary CNS neurons. Macrophage phenotype is a critical determinant of the healing response to injury in tissues throughout the body, including the CNS. After injury, a pro-inflammatory innate immune response promotes scarring and leads to permanently lost neurologic function, and this pro-inflammatory response is due in large part to M1-like activated resident microglia and infiltrating macrophages. Once polarized, microglia and macrophages release cytokines and other inflammatory factors that activate and recruit additional immune cells,

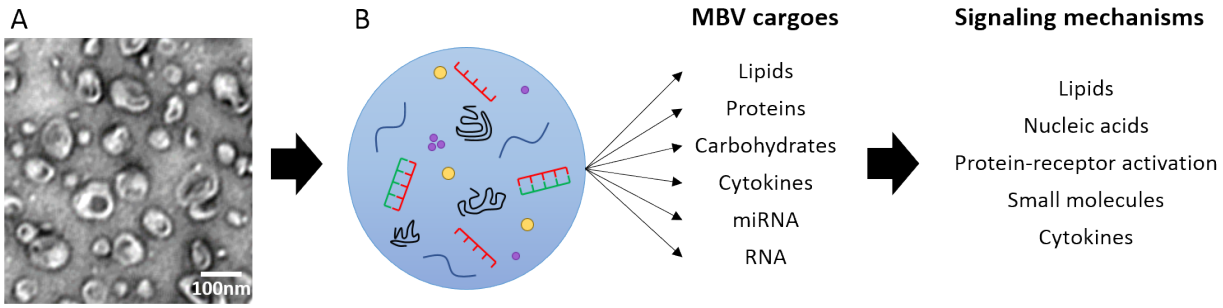

Figure 1 MBV carry diverse cargoes that can signal *via* multiple mechanisms.
(A) Transmission electron microscopy (TEM) image of MBV derived from urinary bladder extracellular matrix (UBM). Bar: 100 nm. (B) MBV with cargoes and potential signaling mechanisms. MBV contain bioactive cargoes that are transported to cells, tissues, and organs locally and systemically, where they interact through various potential signaling mechanisms to affect cellular behavior. MBV: Matrix bound vesicles.

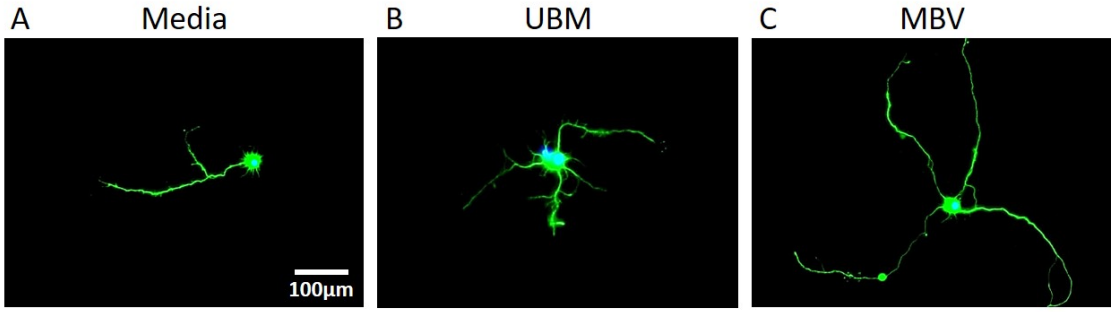

Figure 2 MBV derived from UBM induce significantly longer neurite extensions in CNS neurons compared to media and UBM conditions (Faust et al., 2017).
Similar to previous studies, MBV increase neurite growth in RGCs. Images show RGC neurite growth after 3 days *in vitro* in media only (A), UBM (B), and MBV purified from UBM (C). Bar: 100 µm. RGC: Retinal ganglion cell; UBM: urinary bladder extracellular matrix; MBV: matrix bound vesicles; CNS: central nervous system.

including astrocytes, to the injury site that in turn facilitate neuronal cell death (Liddelow et al., 2017). ECM bioscaffolds derived from proregenerative tissues, like either urinary bladder matrix (UBM) or small intestine mucosa (SIS), can modulate macrophages to promote an anti-inflammatory, M2-like phenotype that supports constructive tissue remodeling in both non-CNS and CNS tissues. Recent studies showed that miRNA cargoes delivered by MBV purified from UBM and SIS ECM appear to play a significant role in mediating the effects of both ECM bioscaffolds on macrophage phenotype (Huleihel et al., 2017a). UBM and SIS ECM are enriched in miRNAs 125b-5p, 143-3p, and 145-5p, and inhibiting these miRNAs induces a gene and protein expression profile in macrophages that is consistent with a pro-inflammatory phenotype suggesting the MBV associated miRNA cargoes mediate, at least in part, the anti-inflammatory effects of both ECM bioscaffolds on macrophage phenotype.

MBV derived from UBM can also regulate differentiation and axon growth in neuroblastoma and primary CNS neurons. In neuroblastoma cells, UBM MBV increased neuronal differentiation and neurite extension at a faster rate than the UBM ECM (Huleihel et al., 2016). In primary CNS neuron cultures, MBV increased axon growth specifically, without altering survival or dendrite growth (Faust et al., 2017). MBV also increased retinal ganglion cell axon growth compared to the UBM ECM from which they were derived and media controls (**Figure 2**). Moreover, similar to macrophages (Huleihel et al., 2017b), increased axon growth appears to depend on MBV miRNAs. UBM derived MBV are internalized by virtually all neurons within minutes and delivered labeled miRNA cargoes to the cytoplasm (Faust et al., 2017), and in our preliminary experiments increased axon growth is differentially regulated by pre-transfecting MBV with sequence specific antagomirs against miRNAs-30b, -125b, and 133b.

Can cell or tissue specific MBV be used to prevent scarring and promote functional tissue remodeling in the CNS (van der Merwe and Steketee, 2016)? ECM has been shown to prevent scarring in numerous tissues throughout the body, but it is unknown whether MBV recapitulate these ECM effects *in vivo* as they do *in vitro*. Injury to the CNS results in permanently lost neurologic function, partly due to the innate immune response after injury, which results in a persistent inflammatory response and astrogliosis, eventually leading to scar tissue deposition and cessation of neurologic function. Thus, multifunctional biologics that can both modulate the inflammatory innate immune response and increase CNS axon growth are attractive approaches to treat CNS injury and promote functional recovery. MBV enable the combination of CNS growth promotion and macrophage polarization, indicating MBV may be used *in vivo* to alter the default healing response and promote functional remodeling after CNS injury. MBV may offer a superior alternative to whole ECM since MBV are easy to introduce *in vivo*, thereby offering a minimally invasive therapy.

MBV can be used as biomarkers to assess tissue health and disease state. Exosomal EV cargoes change with trauma and

disease and have successfully been used as biomarkers for numerous diseases. However, the effects of injury and disease on MBV cargoes remains unknown. ECM bioactivity changes with regard to source tissue injury and disease state. Therefore, MBV cargoes and surface markers are hypothesized to also change after injury and potentially act to potentiate injuries and disease. For instance, ECM isolated from different aged tissues have different effects on cellular responses *in vivo* and *in vitro* (Sicari et al., 2012), logically suggesting MBV and their cargoes may differ based on source tissue age, and future studies will focus on determining how MBV composition changes throughout cellular development and after injury.

MBV are remarkably resistant to degradation and even lyophilization, therefore MBV may provide a new metric for analyzing cadaver tissues and bones. Though further work is necessary to confirm the localization and function of MBV *in vivo*, the presence of MBV within the ECM supports the notion that MBV are optimally positioned to play a role in regulating tissue and organ architecture during normal function, aging, and after injury. Thus, analysis of MBV cargoes could provide minimally invasive diagnostic approaches for analyzing injury severity or disease progression.

Conclusion: Regenerative medicine therapies are increasingly investigating *in vivo* tissue reconstruction strategies to promote functional recovery after injury. These strategies utilize the intrinsic ability of cells to organize and differentiate into functional tissues after injury by promoting site appropriate remodeling *in vivo* instead of *ex vivo* tissue engineering and re-implantation. The concept that the secreted EVs may provide a safer, more tunable, and easily deliverable platform for modulating the default healing response in the CNS has recently been discussed (van der Merwe and Steketee, 2016). MBV are a natural component of the ECM in healthy tissues, have been shown to be non-cytotoxic, can promote CNS neurite growth, promote an anti-inflammatory macrophage phenotype, and can easily be administered *in vivo*, therefore making MBV an attractive option to promote neuroprotection and constructive remodeling in the CNS after injury.

Finally, compared to other extracellular vesicles derived from undifferentiated clonal cell lines *in vitro,* MBV are likely more relevant to positive tissue remodeling since MBV are derived from healthy, pro-regenerative adult tissues.

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