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Exosomes for Repair, Regeneration and Rejuvenation

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Keywords: exosome, micro-vesicle, extra-cellular vesicle, cell therapy, tissue engineering, regenerative medicine, stem cell, progenitor cell, organogenesis, morphogen, manufacturing, quality control, regulatory

Abstract

Introduction: Application of regenerative medicine strategies for repair of organs/tissue impacted by chronic disease is an active subject for product development. Such methodologies emphasize the role of stem cells as the active biological ingredient. However, recent developments in elucidating mechanism of action of these therapies have focused on the role of paracrine, "action-at-a-distance" *modus operandi* in mediating the ability to catalyze regenerative outcomes without significant site-specific

engraftment. A salient component of this secreted regenerative milieu are exosomes: 40-100nm intraluminal vesicles that mediate transfer of proteins and nucleic acids across cellular boundaries.

Areas covered: Here, we synthesize recent studies from PubMed and Google Scholar highlighting how cell-based therapeutics and cosmeceutics are transitioning towards the secretome generally and exosomes specifically as a principal modulator of regenerative outcomes.

Expert Opinion: Exosomes contribute to organ development and mediate regenerative outcomes in injury and disease that recapitulate observed bioactivity of stem cell populations. Encapsulation of the active biological ingredients of regeneration within non-living exosome carriers may offer process, manufacturing and regulatory advantages over stem cell-based therapies.

1. Introduction

The arc of biopharmaceutical development over the past decades has been characterized by a movement away from the application of the cell as a mere manufacturing platform for medicinal proteins. Instead, there is increasing recognition of the cell itself as the active biological ingredient for catalyzing regeneration and repair of diseased tissue. Currently however, strategies for application of stem and progenitor cell populations for tissue engineering and regenerative medicine are being significantly influenced by a new mechanistic understanding. The activity of secreted cell-derived byproducts acting at a distance, rather than site-specific integration and directed differentiation, represents the principal mechanism of action by which these cell populations mediate regenerative outcomes [1, 2]. This *secretome* represents a regenerative milieu broadly composed of proteins, nucleic acids and membrane-bound vesicles of a variety of sizes. These may be capable of independently triggering regeneration and repair as well as mediating the *de novo* organogenesis of tissue engineered organs *ex vivo* [3, 4]. These observations signal a transitional return towards leveraging the cell as a medicinal factory with the secretome rather than the

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cell itself now representing the active biological ingredient [5]. Here, through synthesis of the salient recent literature, we attempt to capture this paradigm shift away from the manufacture and application of cells to cell-derived regenerative by-products such as exosomes and conditioned media. Specific examples documenting regeneration of heart, kidney, skin and the nervous system through application of exosome-based therapies will be examined. Although no industrial scale manufacturing pipeline specific to exosomes yet exists, we will leverage lessons learnt from cell-based systems to illustrate process development, scale-up, manufacturing, quality control, regulatory and intellectual property issues associated with exosome-based therapies (Figure 1).

2. Exosome Biology

Although extracellular particles described as "platelet dust" were observed in normal plasma in the first half of the 20th century [6, 7], a functional role for exosomes was first established in the form of MHC-II (Major Histocompatibility Class-II) presenting vesicles secreted by B-lymphocytes and capable of inducing a specific T-cell proliferative response [8]. While no rigorous and universally accepted definition has yet been established [9], the term *exosome* is generally understood to reference a specific class of lipid-membrane bound extra-cellular vesicle (EV) characterized by a diameter of 40-150nm and density of 1.09-1.18 g/ml (Figure 2). Additionally shown to be secreted from tumor cells [10], exosomes were first characterized by EM (Electron Microscope)-methodologies upon their release via invagination of endosomal membranes as multi-vesicular bodies during differentiation of erythrocyte progenitors [11]. Within the endosome compartment, exosomes and their payloads are released into the extracellular milieu upon fusion of the endosome with the plasma membrane. Exosomes participate in a variety of cellular activities and have been shown to be isolatable from multiple body fluids including saliva, urine, plasma, serum, breast milk and amniotic fluid, as well as from the conditioned media of cultured cells, with yields typically 0.5 μg exosomal protein per 10^6 cells over a 24 hour culture period [12]. However, from a regenerative medicine perspective, the salient function of exosomes is in inter-cell communication through transport of protein, mRNAs and micro-RNAs [13]. Such

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signaling typically takes place within an organism, as was shown by the ability of ESC sourced exosomes to reprogram murine hematopoietic progenitors towards acquisition of a more pluripotent, ESC-like, phenotype [14]. However, the nematode parasite *Heligmosmoides* has been shown to manipulate the innate immune response of its mouse host through secretion of exosomal particles, thereby establishing exosomes as a mechanism for inter-species transfer of RNA [15]. Other examples of immune modulation by exosomes include potent anti-tumor bioactivity observed in tumor bearing mice treated with dendritic cell-derived exosomes, which present MHC Class I and II glycoproteins [16]. Key protein markers that have been associated with exosomes include CD9, CD63, CD81, HSP70, HSP90 (see Figure 3), actin and annexin [17, 18]. A systematic review of exosome composition and functionality in broad range of biological fluids has recently been presented [19].

3. Regeneration leverages developmental signaling mechanisms: exosome-mediated transfer of morphogens

Organ regeneration technologies aim to restore the original structure and functionality of a diseased organ. In general, healing responses within mammals are characterized by fibrosis and scar tissue formation, not regeneration. Nevertheless, developing mammalian fetuses during the first trimester will typically present wound healing without fibrosis and scar tissue formation [20]. Additionally, compensatory hyperplasia of mammalian kidney or liver secondary to partial nephrectomy or hepatectomy, remodeling of epidermis or bone consequent to injury and regeneration of limb digit tips in humans and mice post-amputation are all examples of regenerative outcomes in adult mammals indicative of an innate regenerative potential within adult mammals (reviewed by Roy and Gatien [21]).

The mechanistic link between developmental and regenerative biology predicts that potential regenerative therapies may leverage or manipulate the fundamental signaling pathways governing cellular self-organization during embryonic organogenesis. For example, the highly convoluted nature of developing epithelia mandates the existence of an efficient mechanism for morphogen transport across the plasma membrane to

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establish the short and long range morphogen gradients central to assembly of the developing embryo. To this end, the observation that morphogens including *Wingless* and *Hedgehog* are closely associated with the plasma membrane, as opposed to freely diffusing across the cytosol, strongly suggests the existence of a membrane-based trans-cytotic vesicular mechanism for establishment of the morphogen gradient. Evidence from the developing *Drosophila* embryo demonstrates that the establishment of gradients of the morphogen *Wingless* during pattern formation of the imaginal disc epithelium occurs at least in part through membrane bound exosome-like particles called "argosomes" [22]. In *C.elegans*, an apical secretion pathway mediated by the membrane bound V0 sector of the vacuolar H+-ATPase controls secretion of Hedgehog-like proteins within exosomes [23]. Finally, the specification of left/right asymmetry in the developing mouse requires the exosome-mediated transport of the morphogens *Sonic Hedgehog* and retinoic acid in response to FGF (Fibroblast Growth Factor)-signaling [24]. Vertebrate *Sonic Hedgehog* has been reported to be secreted within two overlapping populations of exosomes, presenting distinctive accessory signaling proteins. Co-expression of integrins was required together with *Sonic Hedgehog* to activate certain *Sonic Hedgehog* target genes during differentiation of mouse ESCs (Embryonic Stem Cells), suggesting the existence of a mechanism for fine-tuning exosome-based morphogen gradients by presentation of distinctive sub-categories of morphogen presenting exosomes [25]. Finally, the *Xenopus* cleavage stage blastocoel is bridged by multiple arrays of parallel filopodia, that facilitate direct interaction between nonadjacent blastomeres; these filopodia in turn fragment into micro-vesicles (including exosomes) whose subsequent resorption identifies a specific mechanism for the potential transfer of morphogens across the developing embryo [26].

4. Selection and delivery of cargo

Exosomes also contain the protein TSG101 (see Figure 3), a component of the endosomal sorting complexes required for transport (ESCRT)-I, which regulates vesicular trafficking processes. The ESCRT machinery is made up of several cytosolic

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protein complexes, known as ESCRT-0, ESCRT-I, ESCRT-II, and ESCRT-III. Together with a number of accessory proteins, ESCRT enables a unique mode of membrane remodeling that results in membrane bending and budding away from the cytoplasm. In this regard, TSG101 binds to ubiquitinated cargo proteins and is required for the sorting of endocytic, ubiquitinated cargos into multi-vesicular bodies (MVBs). ESCRT mediated selection is not the only mechanism for getting protein cargo into exosomes: proteins may also be recruited into exosomes by virtue of their association with chaperones such as HSP70 and HSP90 [27].

The precise mechanism by which RNA species are selected for recruitment into exosomes is less well defined. One possible mechanism involves specific sequence motifs that may function as *cis*-acting elements for targeting RNAs to EV [28]. Another possibility involves specific post-transcriptional modifications such as 3' adenylation and uridylation that may serve to separate cellular small RNAs from secreted RNA populations packaged into exosomes [29]. The discovery that ESCRT-II is an RNA binding complex [30] suggests that it may also function to select RNA for incorporation into EVs. Finally, the observations that MVBs are sites of miRNA-loaded RISC (RNA-induced silencing complex) accumulation [31] and that exosome-like vesicles are considerably enriched in GW182 and AGO2 implicate functional roles of these proteins in RNA sorting to exosomes.

In order for exosomes to transfer their cargo (nucleic acid, protein), they must somehow be incorporated into the recipient cell. Fluorescently labeled lipophilic dye transferred from exosomes and incorporated into cultured cells has been used to show that exosomes are taken up by recipient cells but does not distinguish whether the incorporated exosomes are dissociated or degraded in the endosomal/lysosomal pathway [32]. A fusion event between exosomes and the cell membrane will transfer fluorescence from the labeled exosomes to the recipient cells. The molecular basis underlying the mechanism by which exosomes recognize, bind to and fuse with the intended target cells remains to be elucidated. However, minor differences in the

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exosomal tetraspanin-integrin complex translate into marked alterations in exosome target cell selection *in vitro* and *in vivo*, indicative of one potential mechanism of action [33]. Other targeting proteins may include galactin 5, galactin 9, integrins, MHC-II and ICAM1 [34]. Upon binding, exosomes remain attached to the plasma membrane or are internalized by endocytosis and subsequent fusion with the endosome or channeled to the lysosomal pathway for degradation. Such observations identify exosomes as vectors for information transfer between cells, and highlight a specific mechanism by which one cell population may manipulate another. To this end, stable modification of cell fate by exosomes has been observed in rodent models, where lung derived exosomes and micro-vesicles were shown to reprogram bone marrow cells towards a pulmonary phenotype *in vitro* and *in vivo* [35]. Similarly, exosomes sources from hepatic cells can promote acquisition of a hepatic phenotype in bone marrow cells [36]. In addition, exosomes derived from cancer cells can promote the development of tumorigenesis [37, 38]. Finally, in the brain, exosome mediated transfer of toxic protein aggregates including amyloid-β and prions may represent an important mechanism for the onset of pathology [39].

5. Exosomes, not cells, may be sufficient as cell-based therapies

A number of studies suggest that the cell itself may ultimately be superfluous in mediating observed regenerative bioactivity from cell-sourced therapeutic product candidates. To this end, therapeutic bioactivity associated with stem and progenitor cell populations can be recapitulated by conditioned media isolated from the culture, maintenance and expansion of those populations [40]. The conditioned media represents the complete regenerative milieu of cell-sourced secretomic and vesicular elements, including soluble proteins, growth factors, cytokines, nucleic acid and small molecules as well as micro-vesicles of varying size, composition and functionality present in suspension. The soluble secretomic component may be separated from the micro-vesicle fraction by centrifugation, filtration or polymer precipitation-based methodologies, reviewed by [41].

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(5.1) Regenerative bioactivity of conditioned media: Paracrine-based bioactivity constitutes an important if not sole component of MSC (Mesenchymal Stem Cell) therapeutic mechanism of action in addition to or instead of site specific engraftment and directed differentiation [42, 43]. It has been demonstrated that MSC-derived conditioned media is sufficient to significantly improve multiple biomarkers of renal pathophysiology in rodent models of chronic kidney disease [44]. Mechanistically, it is now understood that MSCs do not repair organ defects by differentiating into the desired tissue type, but rather function more in a regulatory role. This paradigm shift followed the demonstration that MSCs can inhibit apoptosis, stimulate angiogenesis, promote endogenous cell proliferation, and inhibit inflammation during tissue regeneration [45]. Such bioactivity is mediated through growth factor and cytokine secretion (paracrine effects) in addition to cell-cell interactions and has been directly leveraged in multiple models of regeneration. For example, the intramuscular injection of conditioned media derived from endothelial progenitor cells into a rat model of chronic hind-limb ischemia was shown to be as effective as transplantation of the endothelial progenitor cells themselves for promotion of angiogenesis [46]. Similar observations were made in a porcine acute myocardial infarction model, where functional equivalence between intracoronary delivery of endothelial progenitors and conditioned media derived from the same was demonstrated [47]. Endothelial cell-derived conditioned media has been shown to promote brain microvascular cell viability *in vitro* following ischemic insult in a manner contingent on the presence of both protein and lipidic elements [48]. In addition, conditioned media from MSCs was shown to resolve pulmonary inflammation in mouse models of lung injury in a manner similar to MSCs [49]. Finally, in a study of bone tissue engineering in a rat model, MSC-sourced conditioned media could actually outperform MSCs in regenerating bone [50]. Secretomic components of MSC-sourced conditioned media may also be leveraged for regenerative applications in orthopedics and cartilage repair and regeneration, including osteoarthritis and rheumatoid arthritis [51, 52].

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(5.2) Regenerative bioactivity of micro-vesicles, including exosomes: In a systematic review of the literature presenting preclinical animal data on the therapeutic potential of MSC-derived micro-vesicles including exosomes, all 13 reported studies indicated that treatment improved at least one clinically relevant parameter associated with organ functionality [53]. Purified exosomes from MSC-sourced conditioned media were first shown to decrease infarct size in mouse models of myocardial ischemia/reperfusion injury [54]. MSC-sourced micro-vesicles have also been shown to mediate reno-protective effects in rat models of acute kidney disease by promoting tubular epithelial cell proliferation and blocking the onset of apoptosis [55]. Examples of cell populations other than MSCs shown to secrete therapeutically bioactive exosomes include iPSCs (induced Pluripotent Stem Cells), where iPSC-derived exosomes have been demonstrated to protect against myocardial ischemia/reperfusion injury upon intra-myocardial injection into mouse ischemic myocardium prior to reperfusion [56]. Many of the regenerative properties previously credited to stem cells are being shown to be mediated through secreted exosomes, through mechanisms of action common to organogenesis in the developing embryo. If valid, innovative approaches to wound healing, tissue engineering, and regenerative medicine, whereby live cell therapies could be replaced with exosomes as an active biologic, would be enabled.

The regenerative potential of exosomes may be modulated or tuned by prior exposure of the originating cell population to external stimuli. For example, inflammatory conditioning of human umbilical cord blood derived MSCs with IFN-γ (interferon-γ) results in MSCs less able than unconditioned MSCs to protect against acute ischemic renal injury *in vivo* [57]. Modulation of exosome bioactivity may also be achieved by the ectopic expression of therapeutically bioactive RNA or protein [58]. Both *ex vivo* and *in vitro* strategies may be employed to load therapeutically bioactive cargo molecules into exosomes [59]. Additional methodologies for exosome tuning may incorporate defined cell-biomaterial and 3D cell-cell interactions to modulate cargo loading and exosome biogenesis [60].

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CD34+ cell populations are known to promote neo-vascularization in preclinical studies and Phase I/II clinical trials. In *in vitro* and *in vivo* functional bioassays of angiogenesis, exosomes sourced from CD34+ cells were shown to recapitulate the effects of cells themselves, in some cases with increased potency relative to cells [61]. Additionally, treatment with MSC-sourced exosomes was shown to be capable of blocking activation of hypoxic signaling that triggers pulmonary inflammation and development of pulmonary hypertension in rodent models [62]. For skin, iPSC-MSC sourced exosomes, upon injection in and around the wound bed of rodent skin wounds, were found to significantly promote wound healing, collagen synthesis and revascularization of the wound site [63]; see illustrative example Figure 4. In proof of concept studies of myocardial infarct in the rat, exosomes sourced from cardiosphere-derived cells (CDCs), were shown to enhance cardiac functionality, decrease scar mass, increase viable tissue mass and infarct wall thickness relative to exosomes sourced from dermal fibroblasts or media controls. Importantly, injection of CDC-exosomes at 21 days post-infarct, a time-point with a well-established scarification profile, resulted in significant growth of new myocardial tissue as well as functional improvements consistent with a true regenerative outcome. miRNA profiling of CDC-sourced and fibroblast-sourced exosomes identified *mir-146a* as a potential active biological ingredient mediating observed functional activity. Aspects of CDC-exosome bioactivity could be recapitulated by treatment with *mir-146a* [64]. A summary of these studies is presented in Table 1.

(5.3) Exosomes in neurodegeneration and neuroregeneration

The potential of exosomes to mediate the transfer of neuropathogenic proteins between neuronal and glial cell populations may be counterbalanced by their development as therapeutic agents for the delivery of small molecules, proteins or nucleic acids for amelioration of inflammatory and degenerative diseases of the nervous system. Neurotoxic variants of key proteins including Aβ42 (amyloid beta 42), huntingtin, α-synuclein and certain prion proteins have been observed to transfer across cell populations via exosome-mediated inter-cellular communication [65]. It remains possible that the catalysis of disease is an inadvertent consequence of neurons ejecting

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toxic protein into the extracellular space via the multi-vesicular body/exosome pathway [66]. Conversely, exosome mediated secretion of myelin-associated glycoprotein (MAG) and stress-protective proteins by oligodendrocytes may provide trophic support and protection for neuronal cells [67]. Other proteins associated with neuroregeneration observed to be transferred between glial and neuronal cell populations following injury include galectin-3 [68] and AMPA (Alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) receptor subunits [69]. The presence of proteins and nucleic acid associated with neuroregeneration packaged into exosomes sourced from differentiating neuronal progenitor cells has been leveraged to promote the differentiation of MSCs into neuron-like cells. miRNA profiling of these exosomes confirmed the presence of miRNAs previously established to participate in neuronal differentiation [70]. Conversely, MSCs engineered to express certain miRNA elements were observed to promote neuronal differentiation of neural progenitor cells *in vitro* in an MSC-derived exosome dependent and cell contact independent manner [71].

The ability of exosomes to package small molecules, proteins or genetic material for transfer across the Blood Brain Barrier (BBB) in a low immunogenicity package is a critical advantage. Encapsulation of the anti-inflammatory small molecule curcumin into exosomes was found to increase bioavailability of curcumin *in vitro* and *in vivo* [72]. Intranasal administration of curcumin carrying exosomes into three independent mouse models of neuroinflammatory disease provides proof-of-concept for a clinically relevant exosome-based product prototype. Following lipopolysaccharide (LPS)-induced inflammation in mice, curcumin carrying exosomes reduced activated inflammatory microglial cells within 2 hours of delivery. In a mouse model of myelin oligodendrocyte glycoprotein–induced experimental allergic encephalomyelitis, mice treated with curcumin carrying exosomes for 31 days had significantly reduced disease outcomes relative to controls treated with unloaded exosomes. Finally, in a mouse glioblastoma model, mice treated with exosome-encapsulated STAT3 inhibitor significantly delayed tumor growth and extended mouse survival over controls treated with unloaded exosomes [73]. In other rodent models of stroke, intravenous administration of MSC-

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sourced exosomes was shown to improve functional recovery while promoting neurite remodeling, neurogenesis and angiogenesis [74-76]. This observed neuroregenerative effect has been linked to the exosome-mediated transfer of certain key microRNAs such as *miR-133b* [77].

(5.4) Identification of exosome-based mechanistic pathways of regenerative bioactivity: As discussed previously, a number of methodological approaches may be leveraged to dissect the specific contributions of the soluble, paracrine component of conditioned media as opposed to the suspended, insoluble, micro-vesicle fraction, including exosomes, to the mechanism(s) of action of observed, clinically active, cell-based therapies. The soluble secretomic component may be separated from the micro-vesicle fraction by centrifugation, filtration or polymer precipitation-based methodologies, reviewed by [41]. As a specific example of how such techniques may be applied, Selected Renal Cells (SRCs) represent a heterogenous, tubular epithelial cell enriched renal cell population currently under evaluation in Phase I/II clinical trials of chronic kidney disease patients [78]. Studies to identify key mechanistic pathways leveraged by SRCs have highlighted attenuation of NFκB (Nuclear Factor κB) and PAI-1 (Plasminogen Activator Inhibitor-1) signalling pathways *in vivo* with concomitant promotion of host tubular cell expansion [78]. *In vitro*, SRC-derived conditioned media attenuated TNFα (Tumor Necrosis Factor-α)-induced NFκB response, TGFβ (Transforming Growth Factor-β) mediated PAI-1 response, and increased expression of transcripts associated with cell cycle regulation [78]. Observed bioactive responses were from vesicle and non-vesicle associated factors, including specific miRNAs. This was shown by the use of ultracentrifugation techniques to isolate soluble, paracrine factors from vesicular elements in suspension. The latter could be further size-fractionated by systematic passage through membranes with differential molecular weight cutoffs ranging from 5-100 kDa [78].

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(5.5): Specific advantages of using exosomes instead of conditioned media for regenerative therapy:

Several clear advantages of exosome-based therapeutics over cell-based therapeutics have been identified. These include the following:

1) *Encapsulation* of therapeutically relevant molecules (protein and nucleic acid) means that the active biological ingredients (ABI) are protected from degradation, unlike the cytokines, growth factors and nucleic acid that are present as soluble factors in conditioned media. Such factors are rapidly degraded [20].

2) From a *manufacturing* perspective, the durability of exosomes as highlighted in (1) above means that large amounts of exosomes may be derived simply by extended culture of a producer cell line. This is not the case for soluble elements of conditioned media which are subject to continued degradation while in extended culture.

3) Again from a *manufacturing* perspective, exosomes may be stored in a much smaller volume compared to conditioned medium. This significantly impacts the cost of goods associated with the product candidate.

These observations notwithstanding, delivery of combinations of growth factors and or cell-derived micro-vesicles do not recapitulate the sustained, physiologically relevant expression of these regenerative molecules from living cells. In circumstances where the delivery of regenerative factors as discrete boluses in single or even multi-dose units is inadequate to achieve clinical relevance, the application of living cells may be unavoidable. Nevertheless, micro-vesicles in general and exosomes more specifically may represent an ideal, generally non-cytotoxic and well-tolerated “off-the-shelf” regenerative therapy, delivering most of the potential of cell-based therapies while considerably streamlining process development and manufacturing.

6. Advantages of Exosomes over Cells as Therapeutic Agents

Exosome-based therapeutics have very clear advantages over their cell-based counterparts. Application of exosomes resolves several safety considerations

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associated with transplantation of living, proliferative cell populations. Using naturally occurring secreted vesicles such as exosomes might allow overcoming toxicity or immunogenicity associated with other developed carrying agents like liposomes or nanoparticles [79]. Compared with cells, MSC-sourced exosomes are more stable and storable, have no risk of aneuploidy, a lower possibility of immune rejection following *in vivo* allogeneic administration, and may provide an alternative therapy for various diseases [80]. In short, MSC-sourced exosomes share the immune-privileged properties of their origin cells. Like MSCs, their utility as a therapeutic is vastly expanded over non-immune-privileged cell types since MSC-sourced exosomes may be applied allogeneically. This greatly facilitates the development of allogeneic therapies that can be stored and used directly “off the shelf”. It is likely that exosomes derived from antigen presenting cell populations will induce an immune response. For example, exosomes derived from glioblastoma cultures trigger an immune response in mice and are recognized by sera from glioblastoma patients. These observations underlie the potential application of exosome-based vaccines for cancer immunotherapy [81]. Exosomes also present less of a health and safety risk for such adverse events as tumor or emboli formation. These are often major concerns for cellular therapies, since exosomes are both non-viable and much smaller in size compared to live cells. Unlike cellular therapeutics, exosomes may be evaluated for safety, dosage and potency in a manner analogous to conventional pharmaceutical agents. Finally, exosomes may be stored without application of potentially toxic cryopreservative agents for up to 6 months at -20 without loss of product potency [82; 41]. Clinical application of a stem cell-derived exosome product prototype has been reported [83]. Increasing dosage of MSC-exosomes into a patient presenting with severe therapy-refractory cutaneous and intestinal graft-versus-host disease grade IV was well tolerated and led to a significant and sustainable amelioration of symptoms. In the USA, the fact that no clinical trial for exosome-based therapy has been approved by FDA merely reflects the fact that this is a much more recent product concept with a clinical development pipeline about 5-10 years behind that of stem cell therapies. Given that multiple clinical trials involving MSCs have been approved by FDA, it is perfectly reasonable to expect approval of conditioned media or conditioned media-derived biologics sourced from MSCs or other

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stem cell populations. To this end, a number of clinical trials have been reported globally investigating the clinical potential of exosome-based therapeutics [8-88].

Although the *composition of matter* of exosome-based products is indeed complex, this has never been a reason in and of itself for FDA to refuse approval of a regenerative product. For example, amniotic fluid (AF) and platelet rich plasma (PRP) is routinely used as a regenerative therapy for multiple applications in wound healing and orthopedics [89]. The composition of matter of AF and PRP is highly complex, and includes numerous growth factors and exosomes that remain poorly characterized. Nevertheless, *minimally manipulated* AF and PRP are accepted by FDA for clinical use in humans under section 21CFR, subsections 1270, 1271 dealing with HCT/Ps (Human Cells, Tissues, and Cellular and Tissue-Based Products) and Section 361 of the Public Health Services Act. Finally, exosomes sourced from dendritic cells are in clinical trials for immunotherapy of certain cancers [90-93].

7. Exosomes as vectors for repair: skin

The skin is frequently injured by acute and chronic wounds, such as diabetic skin ulcerations or extensive burns. In a recent study, exosomes from iPSC-MSCs were found to exert beneficial effects on granulation tissue formation and angiogenesis, which are two critical phases of the wound-healing process [63]. In addition, exosomes from these cells facilitated a significant therapeutic effect during cutaneous wound healing, supporting the notion that exosomes may be used as therapeutic tools in wound healing. Mechanistically, WNT4 delivered by exosomes appears to be the key mediator in this type of skin healing and repair [94], see illustrative example Figure 4. Keloids represent the most extreme example of cutaneous scarring as a pathological response to wound healing. Enhanced STAT3 expression and phosphorylation has been observed in keloid scar tissue and in cultured keloid fibroblasts [95]. This type of scarring has an overabundance of collagen deposition, contributing to its lack of softening, flattening, and remodeling over time. *In vitro* inhibition of STAT3

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phosphorylation has been shown to contribute to the loss of collagen production in these cells. This raises the interesting possibility that inhibitors of STAT3 phosphorylation may be useful in prospectively treating burn wounds *in vivo* to reduce keloid scar formation. Indeed, treatment with mouse exosomes or exosomes derived from MSCs isolated from human umbilical cord stroma completely abrogated STAT3 phosphorylation due to hypoxia [96].

8. Exosomes as Cosmeceuticals; vectors of rejuvenation

Cosmeceuticals are cosmetic products with biologically active ingredients purporting to have medical or drug-like benefits. The "cosmeceutical" label applies only to products applied topically, such as creams, lotions and ointments. Liposomes are well-known vesicular cosmetic delivery systems [97]. For example, liposomes may potentially be used to deliver avobenzone (a sunscreen) and arbutin (a skin whitening agent) in a differential manner such that the sunscreen is retained at the skin surface while the whitening agent is delivered further into the dermal strata [98]. Nebulized liposomes have also been evaluated for delivery of vitamin K1 into the skin [99]. Their topical application offers several advantages including increased moisturization, restoring action, biodegradability, biocompatibility and extended and slow dermal release. Their similar structure to biological membranes allows penetration into the epidermal barrier [100]. Given the structural similarities between liposomes and exosomes, it seems reasonable to expect that exosomes will find their place in the cosmeceutical industry much like liposomes and other cell-derived products have. Although initially potentially cost-prohibitive due to the relative tediousness in isolating significant quantities of exosomes (see below), profitability may be achieved by clever marketing as a "regenerative" product of boutique interest to a niche market of high-net value consumers. While it is expected that exosomes sourced from immune-privileged cell populations like MSC will *not* trigger risks such as skin rashes or related immune responses, this must be confirmed with the appropriate preclinical animal models.

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Although data demonstrating the impact of stem cell or cell-sourced products in modulating the biologic and biophysical properties of aging skin does not in itself prove that exosomes may be useful or relevant in these applications, such a role may reasonably be extrapolated based on regenerative outcomes associated with exosomes in other systems. To this end, cell-derived secretomic extracts have demonstrated value as cosmeceuticals for rejuvenation of aging skin as well as for the promotion of hair growth. Evidence for a direct impact of stem cell-derived secretomic factors in promoting skin rejuvenation is provided by randomized, investigator blinded "split-face" studies where cell derived secretomic extracts are delivered by micro-needle to one half of a subject's face. A control, mock procedure using just the micro-needle is applied to the other half. In such studies, a statistically significant improvement in skin pigmentation, wrinkling and roughness was noted in the presence of the cell-derived secretome [101, 102]. Similarly, the intradermal injection of GCSF (Granulocyte Colony Stimulating Factor)-mobilized PBMCs (Peripheral Blood Mononuclear Cells) from young pig could rejuvenate cheek skin of aged pigs as shown by increased levels of collagen, elastin, hyaluronic acid and CD44, involucrin, integrin as well as increases in proliferative capacity in the basal layer [103]. In mouse model of wrinkling created by UV-B irradiation of hairless mice, wrinkling, dermal thickness and collagen content were all improved by injection of adipose-derived stem cells. *In vitro* studies implicated secretomic factors sourced from the adipose stem cells as potentially important in mediating their anti-aging properties [104]. Conditioned media derived from human dermal fibroblasts were shown to ameliorate the UV-A induced up-regulation of MMP1 (Matrix Metalloproteinase 1) and associated down-regulation of collagen and TIMP1 (Tissue Inhibitor of Metalloproteinase 1) transcripts as well as promoting migration and inhibiting apoptosis *in vitro* [105]. Regenerative cycling of hair waves has been studied in mouse skin. In this model, such cycling slows down with increasing age; however, this behavior is non-cell autonomous, such that transplantation of aged mouse skin into a young host rescues regenerative cycling, thus implicating secretomic factors as inductive for hair follicle regeneration [106]. Conditioned media from adipose stem cells, upon intradermal injection into alopecia patients using a "split-scalp" study design, has been reported to significantly promote hair growth [107]. Finally, the observation that

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miR-214,acting through the WNT pathway, regulates both skin morphogenesis and hair follicle development, opens the possibility of leveraging discrete populations of defined microRNAs delivered through exosomes for triggering the regeneration of hair [108]. These observations notwithstanding, cosmeceutical products simply are not subject to the same degree of regulatory scrutiny and oversight as therapeutic products are. This provides an alternative pathway for exosome-based products to reach the marketplace that is largely independent of any requirement to demonstrate product stability or potency, beyond a simple demonstration of product biosafety.

9. Potential risks associated with exosome-based therapies

The role of exosomes in mediating horizontal transfer of genetic information within and even across species boundaries [13, 15] raises the potential of risk associated with the uncontrolled transfer of genetic information between cell populations. Rigorous, genome-wide definition of all miRNA and other genetic elements incorporated within candidate exosome therapeutics is therefore a prerequisite for clinical application. In addition, it is now understood that cancer cells leverage exosome-mediated communication pathways to signal to cancerous and non-cancerous cells in the local environment, potentially catalyzing transformation of the latter [109]. Exosomes sourced from breast cancer cells of increasing metastatic potential secrete exosomes with proportionately greater potential to induce cell migration in in vitro cell migration assays [110]. Furthermore, tumor supportive miRNA and other bioactive factors have been shown to be present in MSC secretome [111]. The identification of specific biomarkers associated with cancer such as claudin-4 which is increased in patients presenting with ovarian carcinoma [112] may assist in risk mitigation of producer cell lines.

Exosomes can modulate the immune response through transport and presentation of key antigens. For example, expression of FAS ligand and TRAIL (TNF-related apoptosis-inducing ligand) in human placental-sourced exosomes can trigger apoptosis in activated PBMCs in a dose-dependent manner [113]. Although it has been

demonstrated that B-lymphocyte sourced exosomes present MHC Class-II antigen [8], the T-cell stimulatory activity of free exosomes is significantly less than that of the producer cell line [114] and free exosomes present significantly lowered ability to activate naïve T-cells in vitro [115], suggesting that potency assays for exosome immunogenicity *in vitro* may not adequately predict behavior *in vivo*. Taken together, these observations indicate that immune-privileged producer cell lines may be a prerequisite for clinical-grade exosome production.

Another aspect of the evaluation of potential toxicities associated with administration of exosome-based therapies is bio-distribution- understanding the dynamics of exosome bio-distribution post-delivery is key to ameliorating risk associated with the uncontrolled localization of exosomes at sites other than the intended target site. To this end, bioluminescence analysis of intravenously delivered, luciferase labelled exosomes in mice showed strong localization to spleen, liver, lung, kidney with detection also possible in brain, heart and muscle within 30 minutes of injection, prior to spiking in the urine at 60 minutes post-delivery [116]. A clear understanding of the relationships between delivery site, dosage and bio-distribution and clearing dynamics is essential for ensuring product biosafety.

10. Scalable Production of Exosomes

Pre-clinically, the use of MSC-derived exosomes is strongly associated with improved organ function following injury and may be useful for inhibiting tumor growth [53]. Exosomes have already been tested as a cancer vaccine in the clinic [91, 92, 117]. These studies were limited to particles produced during short-term *ex vivo* culture of autologous dendritic cells. While limited in scope, this work is significant because the exosomes were deemed safe in the small clinical trials conducted [117]. As with any biologic, scalable production of the active ingredient must be achieved to have relevance as a readily available and commercially feasible therapeutic. Unfortunately, the process by which these exosomes were manufactured for these studies provides

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little guidance for large-scale cGMP (current Good Manufacturing Practice) manufacturing of exosomes needed for more comprehensive clinical trials. In addition, hundreds of micrograms to milligram quantities of exosomes may be needed to treat many patients in a clinical trial. Senescence of the cells from which exosomes are being manufactured represents an intrinsic limitation on final absolute amounts. Loss of actively growing cells will most certainly effect exosome production, which in turn would jeopardize trial outcomes. One approach to address the growth arrest/ senescence issue is cell immortalization. Indeed, *MYC* transformation may represent a practical strategy in ensuring an infinite supply of cells for production of exosomes in the milligram range as a therapeutic agent [118]. In addition, the increased proliferative rate of cells should reduce time for cell production, thus reducing production costs.

Another hurdle to overcome is how to culture a sufficient number of cells to produce enough conditioned medium from which milligram quantities of exosomes may be isolated. Creation of exosomes is straightforward-exosomes are isolatable from the conditioned media of most cell populations. As discussed earlier, producer cell populations may be selectively tuned to promote the overexpression of certain proteins within the exosome fraction. Alternatively, genetic manipulation of the producer cell miRNAome can modulate the expression of clinically relevant miRNA in the resulting exosome product [119]. Broadly, methodologies for the isolation of exosomes from conditioned media are based on ultracentrifugation, ultrafiltration or polymer-mediated precipitation. The latter, while most straightforward and amenable to rapid isolation of exosomes from small volumes of material, is not appropriate for large scale process manufacture or for clinical application owing to the presence of the precipitating polymer within the final exosome pellet [120]. Methodologies based on ultracentrifugation are currently most typically applied to the preparation of exosomes from larger volumes of conditioned media. Here, a preliminary spin of <10K *g* is used to remove larger vesicular materials, cellular debris etc. from the conditioned media prior to centrifugation of the crude exosome fraction at up to 100K *g*. Although amenable to the preparation of large scale amounts of exosome, ultracentrifugation is a time-consuming option, usually

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requiring spin times of at least 10-12 hours. The forced filtration of conditioned media through membranes of variable molecular weight cut-off may also be applied to exosome isolation, as described in [78]. Perhaps the technology most relevant to process manufacture of exosomes is immuno-affinity purification of exosomes from conditioned media with antibodies targeting exosome-specific surface markers (CD81/CD9/CD63) that are conjugated to magnetic beads or other matrix. Combinations of these methodologies may also be applied, for example, ultracentrifugation with an added filtration or immuno-affinity step to achieve both scale and purity. For additional details, please see [41].

From a cGMP standpoint, cell culturing in a closed system is preferred. One approach may be the use of hollow-fiber cell bioreactors, as a cGMP-compliant closed culture system, for culturing large numbers of cells to produce large quantities of exosomes. A bioreactor approach should also abolish the need to continually passage cells during a production run, alleviating the need for huge numbers of plastic tissue culture vessels while reducing medium volume. In the long term, use of bioreactors has the potential to increase efficiency of exosome production while simultaneously reducing cost-of-goods. Culture of placental derived MSCs in a hollow fiber bioreactor is a useful guide for starting to address the scalable production of exosomes [121]. Preliminary results have shown the bioreactor yield is in milligrams, approximately 10-fold greater than cultures grown in T-flasks and cell factories, while simultaneously resulting in a higher concentration/mL conditioned medium [121]. Finally, an additional factor for consideration is that any therapeutically relevant bioactivity may be a function of an exosome-mediated secretory milieu that is by definition heterogeneous and not necessarily associated with any single molecule or medicinal agent. As a precedent, a heterogeneous population of renal cells has been developed as a cell-based therapeutic for chronic kidney disease- no single, definable cell population is understood to mediate observed regenerative outcomes [122, 123]. It is likely that exosome-based therapeutics and cosmeceutics catalyze their bioactivity as a function of their difficult to define, heterogeneous nature as admixtures of medicinal agents.

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11. Regulatory requirements for manufacturing and quality control

The regulatory requirements placed upon the biotechnology industry for production of medicinal products are quite demanding. Manufacturing of exosomes for therapeutic applications needs to take place in a tightly controlled and qualified setting. Quality systems must be in place to control the manufacturing environment, validation of equipment, material and operational controls. Process controls and validation are critical to meeting regulatory agency standards for product approval. For therapeutic development, it is anticipated that exosomes will fall under the purview of the Center for Biologics Evaluation and Research (CBER) -vaccines, blood, and biologics- of the FDA [124] This Center reviews a wide range of products such as vaccines, blood and blood components, allergenics, somatic cells, gene therapy, tissues, and recombinant therapeutic proteins. Such agents can be composed of sugars, proteins, or nucleic acids or complex combinations of these substances (exosomes fall into this category), or may be living entities such as cells and tissues. These agents are isolated from a variety of natural sources - human, animal, or microorganism - and may be produced by biotechnology methods and other cutting-edge methodologies.

Below is a potential example, based on our experiences in developing several cell therapeutic and tissue engineered products, of a flow-diagram for development of exosomes as a therapeutic illustrating what the FDA might look for in a manufacturing scheme (Figure 5). At left – cells are isolated, cultured, expanded, and exosomes isolated from conditioned medium. This schematic assumes that cells will be extracted from a specific tissue type for use in exosome isolation; for cells already isolated, the steps will begin at the cell expansion stage. The quality tasks, which FDA is most interested in during the manufacturing process, are in boxes at right. Notice that they are heavily focused on testing for contamination by micro-organisms, cell number, and cell viability during multiple steps of the process. Testing of the final product, the exosomes, also includes testing for micro-organism contamination. In addition, the exosomes must be characterized, which will include the determination of

physicochemical properties, biological activity, immunochemical properties, purity and impurities. This is necessary to establish the safety and efficacy profile of the product.

12. Synthetic exosomes and exosome mimetics

Exosomes by their nature represent a heterogeneous, incompletely characterized biologic product. In addition, it remains to be established whether comparable lots of exosome preparations are routinely and consistently isolatable at large scale. Together with the somewhat tedious and time consuming nature of the exosome isolation and manufacturing process [125], see also above, these factors have triggered attempts to design and synthesize exosome-like particles or exosome mimetics that could potentially be made at much larger scale. Such particles have a potentially significant advantage in being fully definable at the lipidomic, proteomic and transcriptomic levels [126]. In a separate example, ESC derived nano-vesicles that mimic exosomes have been created by extruding living ESCs through micro-filters and shown to promote proliferation of primary murine skin fibroblasts [127]. Such nano-vesicles are of course not fully definable in the manner that a truly synthetic exosome would be. Other methodologies currently under development include exosomes as vectors for microRNAs, siRNAs or other defined protein cargo [128].

13. Exosomes as biomarkers for disease and regeneration

Finally, the presence of exosomes in multiple body secretions and fluids may be leveraged as a mechanism to monitor disease phenotypes or regenerative outcomes associated with a therapy. For example, the presence of certain microRNA biomarkers in urine sourced exosomes may be leveraged to evaluate development of renal fibrosis. Conversely, the presence of exosomes expressing CD133 or other stem and progenitor cell proteins may be an indicator of regenerative activity within the kidney. Molecular assays have been proposed to facilitate the rapid assessment of renal regeneration associated with application of cell-based therapies [123]. However, the ability to monitor

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such outcomes merely by measurement of certain defined urinary exosomes would represent a significant improvement [128].

14. Expert Opinion

Our understanding of the biological significance of exosomes has matured considerably since their initial characterization and dismissal as platelet associated dust to our current appreciation that exosome-mediated transfer of proteins and nucleic acid represents a central and universal mechanism of cell-cell communication at a distance. Exosomes are isolatable from multiple species and from most if not all biological fluids examined to date. Exosomes have already been leveraged clinically as an agent for vaccination. However, from a regenerative medicine perspective, the ability of exosomes to mediate regenerative and reparative responses typically associated with stem and progenitor cell bioactivity is most relevant. Such regenerative bioactivity may be directly related to the role of exosomes as agents of morphogenesis during embryonic development, pattern formation and organogenesis. Evidence from multiple experimental systems is implicating the secretome in general and vesicular components such as exosomes in particular as principal mechanistic agents that catalyze the observed regenerative bioactivity of cell-based products. Specific examples of such regenerative potential include observed regenerative outcomes from MSC-sourced exosomes in multiple diseased or injured organ systems including kidney, heart and skin as well as the ability of exosomes to reprogram targeted cell populations towards acquisition of a cellular phenotype associated with that of the donor cell population. A parallel emphasis on product development is transitioning to increasingly focus on exosome-based therapies over cell-based therapies. Exosomes may present considerable potential advantages over cells for manufacturing, storage, handling, product shelf life and their potential as a ready to go biologic. This is a direct reflection of the fact that processing, transport and storage of non-living biologics will always be more cost-effective than delivery of cell-based therapeutics from a product development perspective. Therefore, exosome-based therapies have the potential to mature as a new class of regenerative therapeutic biologicals. Globally, at least one clinical trial of a

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MSC-sourced exosomes for improvement of β-cell mass in type 1 diabetes patients has been reported [83-88]. We anticipate many more studies will be initiated in the next 1-5 years [83-88]. In addition, exosome-based cosmeceuticals may see development as a boutique "regenerative" product in the near future. However, from the point of view of the biotechnology entrepreneurial community, a number of key scientific, process manufacture and business development questions remain to be resolved. Mechanistically, identification of the specific proteins or nucleic acids being transported by exosomes that mediate observed regenerative outcomes will be a primary focus of continued research. Alternatively, if regeneration is a function of a heterogeneous composite of multiple bioactive exosome sub-populations, a clear demonstration of this will also be of significance. In parallel, further clarification of the role of exosomes in mediating development of disease conditions such as cancer and neurodegenerative disorders will be required. Investment activity into new companies developing exosome-based therapeutics is contingent upon a clear intellectual property landscape securing such technologies into defendable portfolios. To this end, the principal intellectual property claims surrounding exosomes and their applications for regenerative therapies remain to be resolved. From a process development and manufacturing perspective, a commonly agreed upon framework for the establishment of exosome identity/composition, purity and potency in reliable and reproducibly quantifiable manner remains to be established. In addition, recapitulation of exosome bioactivity by synthetic, exosome-like particles will considerably simplify manufacturing by facilitating a more robust definition of product identity. The regenerative potential of exosomes may be also modulated or tuned by prior exposure of the originating cell population to external stimuli. Further research is needed to identify preconditioning methodologies best suited to achieve a desired regenerative outcome. Finally, the ability to obtain exosomes sourced from non-stem cell populations that can also catalyze clinically relevant regenerative outcomes will considerably simplify manufacturing regimens by removing the requirements to maintain and monitor populations of stem and progenitor cells in their undifferentiated, proliferative condition.

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Declaration of interest

This work has been funded by ZenBio, Inc. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

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Article highlights

1) Exosomes participate in key mechanistic pathways in development, organogenesis, wound healing and regeneration in adults by mediating inter-cell communication of key developmental morphogens and other signaling elements.

2) Exosomes can reprogram target cells towards acquisition of characteristics associated with the donor cell, including differentiated or stem cell-like phenotypes.

3) Regenerative bioactivity associated with stem and progenitor cell populations can be recapitulated by conditioned media isolated from the culture, maintenance and expansion of those populations. At least part of this bioactivity is specific to micro-vesicles, including exosomes.

4) Purified exosomes have been demonstrated to have clinically relevant therapeutic bioactivity across multiple *in vitro* and *in vivo* models.

5) Compared with cells, exosomes are more stable and storable, have no risk of aneuploidy, a lower possibility of immune rejection following *in vivo* allogeneic administration, and may provide an alternative therapy for various diseases.

6) Secretomic products including exosomes are being developed as cosmeceuticals.

7) Methodologies for the industrial scale manufacture of exosome-based therapeutics and the associated regulatory and quality control infrastructure remain generally undefined.

8) Tunable exosomes, synthetic exosomes and exosome mimetics, as well as exosomes engineered to overexpress or knockdown signaling pathways associated with disease pathology represent the next generation of exosome-based product candidates to be developed.

Figure legends

Figure 1: Exosomes for repair and regeneration. Regeneration leverages mechanisms of organogenesis.

(A) Exosome mediated morphogen gradients are one such mechanism of action active in the developing embryo. In this illustrative example, exosome gradients are instructive in establishment of axial symmetry in the developing embryo. Similar instructive signaling mediates exosome mechanism of action in regeneration.

(B) Although exosomes may be isolated from any cell type or bodily secretion, in this example, exosomes are being sourced from MSC-like cell populations derived from adipose or bone marrow (pelvis).

(C, D) Close-up illustration of cells showing genesis of exosomes through invagination of endosomal membranes and ultimate secretion by fusion with plasma membrane.

(E) Manufacture of a clinically relevant dose will involve cell expansion in bioreactors and may include tuning or modulation of specific exosome sub-populations carrying defined payloads as illustrated below: in (F). Importantly, exosomes may be sourced allogeneically as a storable, immune-privileged, “off-the-shelf” product that can be delivered to a broad patient population.

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(G) Examples of organs potentially treatable with exosome-based therapeutics as suggested by preclinical data include the brain, heart, kidneys, and skin.

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Figure 2: Particle size determination using NanoSight's Nanoparticle Analysis Instrument, and their proprietary nanoparticle Tracking Analysis software, V2.3. Samples derived from adipose mesenchymal stem cell lot #ZB0029 stored at 4°C were vortexed for 1 min prior to a 1:10^6 dilution in DPBS.

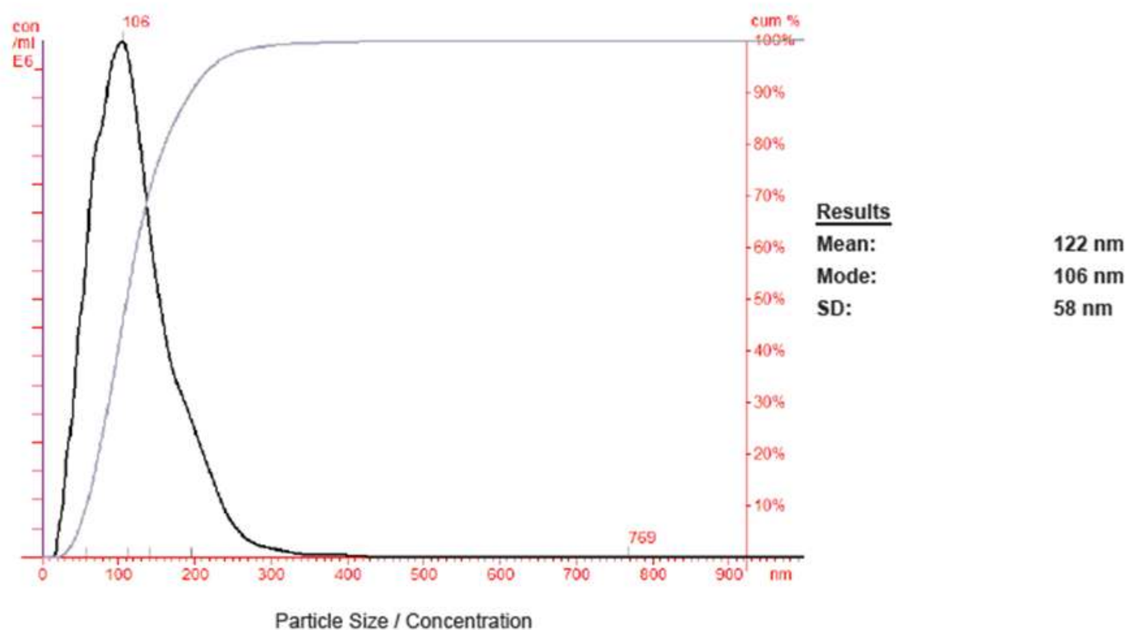

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Figure 3: Western blot analysis of exosomes (20ug) from ZB002 (passage 4; lanes 2) and ZB003 (passage 5, lanes 3). Cell lysate from ZB002 (passage 4, lanes 1) was used as a control. Approximate molecular weights are as follows: CD63- 53 kDa, Hsp70- 70 kDa, TSG101 – 47 kDa. ZB002 and ZB003 are independent lots of adipose-derived mesenchymal stem cells.

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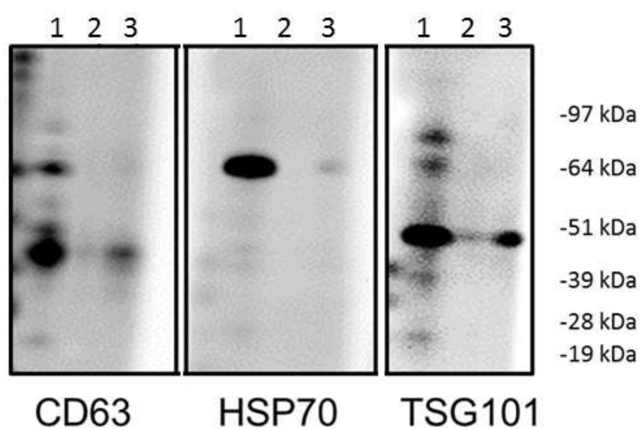

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Figure 4: Skin wound healing model in rodent. Illustrative example. Top panel. 2cm diameter complete removal of dermis from dorsal surface of white Lewis rat. One wound treated with 25ug of human placental MSC-sourced exosomes delivered by direct injection in 100ul of PBS, other treated with an equivalent volume of PBS as control. Only one dose was delivered and the experiment was terminated 2 weeks post-injury. Bottom panel. 2 weeks post-injury, both wounds have healed substantially, but wound treated with human placental MSC-sourced exosomes (red circle) has healed substantially faster.

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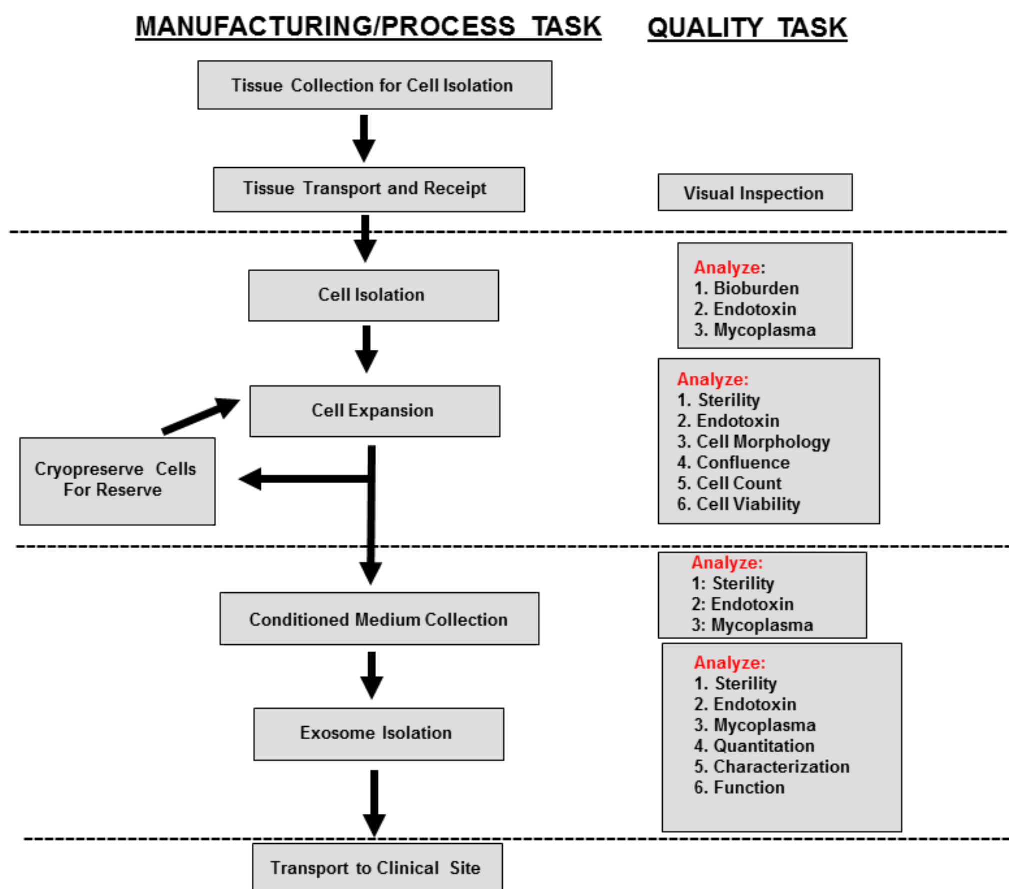
Figure 5: Flowchart of manufacturing and quality-control strategy for exosome-based therapies. At left – cells are isolated, cultured, expanded, and exosomes isolated from conditioned medium. This schematic assumes that cells will be extracted from a specific tissue type for use in exosome isolation; for cells already isolated, the steps will begin at the cell expansion stage. The quality tasks, which FDA is most interested in during the manufacturing process, are in boxes at right. Notice that they are heavily focused on testing for contamination by micro-organisms, cell number, and cell viability during multiple steps of the process. Testing of the final product, the exosomes, also includes testing for micro-organism contamination. In addition, the exosomes must be characterized, which will include the determination of physicochemical properties, biological activity, immunochemical properties, purity and impurities. This is necessary to establish the safety and efficacy profile of the product.



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Study reference	**Cell source**	**Conditioned medium?**	**Isolated exosome?**	**Test platform**	**Outcome**
[59, 37]	MSC	Yes	N/A	*In vivo* rodent model of chronic kidney disease	Significant improvement of multiple biomarkers of renal disease
[50, 28]	Endothelial progenitor	Yes	N/A	*In vivo* rodent model, chronic hind limb ischemia	Significant improvement of capillary density, muscular viability
[30]	MSC	Yes	N/A	*In vivo* mouse model, acute lung injury	Amelioration of pulmonary inflammation
[31]	MSC	Yes	N/A	*In vivo* rat calvarial bone defect model	Significant improvement in bone regeneration
[36]	MSC	N/A	Yes	*In vivo* mouse model, myocardial injury	Reduction in infarct size
[32]	MSC	N/A	Yes	*In vivo* rat model, chronic kidney disease	Promotion of tubular epithelial cell proliferation, blocking onset of apoptosis
[39]	iPSC	N/A	Yes	*In vivo* mouse myocardial ischemia/reperfusion injury	Protection of myocardium from reperfusion induced ischemic injury
[44]	CD34+	N/A	Yes	*In vivo* rodent corneal angiogenesis assay	Significant pro-angiogenic activity
[45]	MSC	N/A	Yes	*In vivo* rodent model, pulmonary	Blocking activation of

				hypertension (PH)	hypoxic signaling prior to onset of PH
[46]	iPSC	N/A	Yes	*In vivo* rodent skin wound healing model	Significant promotion of wound healing, collagen synthesis and revascularization of wound site
[47]	CDC	N/A	Yes	*In vivo* rodent myocardial infarct model	Enhancement of cardiac function, decreased scarification
[48, 49]	MSC	N/A	Yes	*In vivo* rodent models of stroke/brain injury	Functional recovery: neurite remodeling, neurogenesis, angiogenesis

Table 1: Regenerative bioactivity of conditioned medium and exosomes. MSC: Mesenchymal Stem Cell, iPSC: induced Pluripotent Stem Cell, CDC: Cardiosphere Derived Cell, N/A: Not Applicable

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