STEM CELLS AS TARGETED DRUG DELIVERY VEHICLES

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STEM CELLS AS TARGETED DRUG DELIVERY VEHICLES

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Table of Contents

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EDITORIAL
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Editorial: Stem Cells as Targeted Drug Delivery Vehicles

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Editorial on the Research Topic

Stem Cells as Targeted Drug Delivery Vehicles

The therapeutic benefits of many stem cell-based therapies are now widely believed to be mediated through the secretion of paracrine factors, such as cytokines, chemokines, growth factors, and extracellular vesicles (EVs). The future of stem cell-based therapies may well lie in our ability to manipulate these factors. As stem cells are sensitive to their microenvironment, the components of their secretome may be manipulated by altering their culture conditions. This themed issue comprises narrative reviews and original research articles on the emerging therapeutic use of stem cells and EVs, along with novel bioengineering and manufacturing technologies that leverage these paracrine outputs.

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STEM CELLS AS DRUG FACTORIES FOR THERAPEUTIC FACTORS

The therapeutic benefit of mesenchymal stem/stromal cell (MSC)-based therapies has been attributed to their pleiotropic effects, mainly through their secretome including EVs, which are nanovesicles secreted by all cells to facilitate intercellular communication. A significant hurdle to widespread use of EVs lies in the ability to manufacture them at scale. Zavala et al. describe a method for efficient enrichment of MSC-derived EVs by encapsulating the MSCs in semi-permeable cellulose beads. By creating capsules of a pre-specified pore size, the authors were able to selectively control the release of particles less than <200 nm in diameter. This was achieved while maintaining the MSCs in 3D culture and producing EVs with characteristics comparable to those derived from standard 2D MSC cultures. This method also retained the *in vitro* biological properties of MSCs, including angiogenesis, immunosuppression and stimulation of neuritic outgrowth (Zavala et al.).

BIOENGINEERING CELLULAR MICROENVIRONMENT TO MODIFY PARACRINE FUNCTION

The microenvironment is a critical contributor to the paracrine function of MSCs. Brooks et al. compared *ex vivo* immunomagnetic bead-sorted adipose tissue-derived mesenchymal stromal cells (ASCs) against culture-expanded ASCs. They reported significant changes to global gene expression during the first 3 days of culture where *ex vivo*-sorted ASCs more readily differentiated into mesenchymal lineages. The levels of paracrine molecules were also significantly different (Brooks et al.), suggesting that even a brief culture period could affect ASC characteristics.

NEXT GENERATION SCALABLE MANUFACTURING OF STEM CELLS

A major bottleneck in the industrialisation of stem cell manufacturing includes the scalable expansion of stem cells while maintaining stem cell phenotype and fidelity of potency. Cherian et al. summarised the current commercial manufacturing solutions for MSCs and their impact on bioactivity and their secretome. The contribution of substrate stiffness, surface topography and extracellular matrix components to microenvironmental cues for MSCs expansion were discussed (Cherian et al.).

NANOPARTICLES IN REGENERATIVE MEDICINE

While MSC-derived EVs show great potential for therapeutic applications, their uptake mechanisms remain poorly understood. Huang et al. describes an MSC-EV uptake mechanism involving common endocytosis in monocytes and keratinocytes, mediated by heparan sulfate proteoglycans on cell surfaces. Osteogenic, chondrogenic, and adipogenic EVs induced significant increases in the expression levels of respective lineage-specific marker genes in recipient cells and these effects were verified *in vivo* using a mouse subcutaneous implantation model (Huang et al.). These findings show the opportunity to modulate EV cargo and direct tissue-specific regeneration using EVs from differentiated MSCs.

Riau et al. discusses the challenges of conventional EV administration and highlights key techniques in fabricating novel sustained delivery systems for EVs in their mini review. Biodegradable hydrogels used to encapsulate EVs prevent premature clearance and allow a more concentrated EV dose at the target site. This EV-hydrogel system has been used to stimulate skin regeneration, angiogenesis, cardiac regeneration and wound healing (Riau et al.).

On a similar note, Shukla et al. summarises the clinical applications of adipose tissue including contemporary attempts to enrich ADSCs within the fat graft, harnessing paracrine effects of the ADSC secretome, and the most recent iteration—ADSC-derived EVs. Components of the ADSC secretome have been shown to promote wound healing and neuro-regeneration, ameliorate renal diseases, and for cardiac protection (Shukla et al.).

Golinelli et al. discusses the potential of MSC-based anti-cancer strategies. Due to the ability of MSCs to engraft into malignant tissues and their immune-privileged status, the authors postulate that MSCs may be ideal vehicles for the delivery of anti-cancer agents. Their review summarises the two major strategies in using MSCs to target cancer. Firstly, the non-genetic modification of MSCs by loading chemotherapeutic agents (via nanoparticles and/or EVs) for targeted delivery at tumor sites, and secondly, the genetic modification of MSCs to induce the expression of anticancer proteins, oncolytic viruses or suicide genes. The potential for MSC-based therapies in oncology lies in the combination of tumour-targeting approaches to improve MSC homing (Golinelli et al.).

In a tumor microenvironment, cancer stem cell (CSC)-EVs mediate cell-to-cell communication to support and promote tumorigenesis, where alterations to parent cells will also alter EV secretion. The opinion paper by Al-Sowayan et al. discusses the increased awareness of CSCs and highlights their possible role in promoting cancer progression by facilitating metastasis. Studies that target CSCs and inhibition of CSC-EV release and/or uptake may be an impetus for anti-cancer drug development (Al-Sowayan et al.).

NOVEL BIOMATERIALS FOR STEM CELLS DELIVERY

Mukherjee et al. investigated the use of tissue-engineered constructs for pelvic organ prolapse repair, on the basis that biomimetic and biodegradable nanofiber meshes mimicking natural ECM would yield superior vaginal constructs by reducing the foreign body response. The implanted constructs resulted in significantly increased expression of genes associated with ECM regulation, cell adhesion, angiogenesis, and immune response compared to the nanomesh alone. The combination of biomaterials and endometrial MSCs reduced acute inflammation and showed the hallmarks of successful implantation (Mukherjee et al.).

The breadth of articles covered within this Research Topic demonstrate the diversity in stem cell and EV research, as well as the significant challenges for clinical translation. The development of stem cells as drug delivery vehicles is rapidly progressing and the translational effort will require collaboration between multidisciplinary experts (stem cell biologists, biomaterial scientists, bioengineers, regulatory experts, healthcare professionals) involved in all development stages of stem cell-derived products including discovery research, manufacturing, preclinical and clinical trials.

AUTHOR CONTRIBUTIONS

GK and RL wrote this article. JF and CS have made a direct and intellectual contribution to the work. All authors have approved the article for publication.

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MINI REVIEW
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Sustained Delivery System for Stem Cell-Derived Exosomes

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Recent literature has ascribed that the paracrine action of stem cells is mediated by exosomes. Exosomes are nano-sized extracellular vesicles (30 to 100 nm) of endocytic origin that play important roles in intercellular communication. They have the ability to deliver various therapeutic effects, e.g., skin regeneration or cardiac function recovery, when applied topically or injected systemically. However, injection of exosomes has been shown to result in rapid clearance from blood circulation and accumulation of the exosomes in the liver, spleen, lung, and gastrointestinal tract can be found as early as 2 h after injection. Topical administration of exosomes on the skin or ocular surface would suffer the same fate due to rapid fluid turnover (sweat or tears). Biodegradable or highly porous hydrogels have been utilized to load exosomes and to deliver a sustained therapeutic effect. They can also prevent the exosomes from being cleared prematurely and allow the delivery of a more localized and concentrated exosome dosage by placing the hydrogel directly at or in the proximity of the target site. In this mini-review, we elaborate on the challenges of conventional exosome administration and highlight the solution to the shortcomings in the form of exosome-incorporated hydrogels. Different techniques to encapsulate exosomes and examples of hydrogels that have been used to create sustained delivery systems of exosomes are also discussed.

Keywords: exosomes, sustained delivery, hydrogel, stem cell, biomaterials, regenerative medicine

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INTRODUCTION

The discovery of the therapeutic potential of stem cells has been one of the most exciting advancements in the field of biomedicine (Trounson and McDonald, 2015). Decades of research in stem cell biology has significantly improved our understanding of mechanistic pathways that stem cells take in tissue repair and regeneration (Körbling and Estrov, 2003; Sanchez Alvarado and Yamanaka, 2014). Pluripotent and multipotent stem cells are known for their self-renewal capacity and ability to transform into multiple cell types. For this reason, they have the ability to replace tissue loss in degenerative conditions, injuries, or due to aging. Despite the overwhelming potential, there are drawbacks associated with the direct application of stem cells on the tissue damage site. Transplanted stem cells may undergo uncontrolled proliferation forming unwanted tissue mass resembling primitive tissue structures (Erdo et al., 2003; Volarevic et al., 2018). Tumorigenesis and mutagenesis have regularly been a safety issue to be taken into consideration in stem cell therapy (Cairns, 2002; Erdo et al., 2003; Volarevic et al., 2018). Reports have also shown that many pluripotent stem cells, including induced pluripotent stem cells (iPSCs), are able to induce teratoma formation in a much faster rate than embryonic stem cells. This further raises the question regarding the safety of stem cell therapies (Gutierrez-Aranda et al., 2010; Zhang et al., 2011). Another common complication

of allogeneic stem cell transplantation is graft-versus-host disease (GVHD). Although the risk can be minimized by donor-recipient matching, patients are still required to undergo long-term administration of immunosuppressive drugs. There is also a possibility of infection of these cells by contaminating bacteria, viruses or fungi that can transmit diseases to the recipients, particularly to patients receiving hematopoietic stem cell transplantation (Marr et al., 2004; Pascutti et al., 2016; Cho et al., 2018). In addition, improper handling methods, storage, and transportation can be detrimental to stem cell quality; potentially affecting the success rate of the treatment (Herberts et al., 2011).

There is substantial evidence that stem cells exert their therapeutic effect *via* secretion of soluble factors, as well as the production of exosomes (Lai et al., 2010; Raposo and Stoorvogel, 2013; Lin and Du, 2018). Exosomes are nano-sized vesicles (30 to 100 nm) of endocytic origin that play a pivotal role in intercellular communication (Raposo and Stoorvogel, 2013). The exosomes are released by every cell into extracellular environment. Their therapeutic effect takes place when they are internalized or in some cases, attached on the cell surface, and the effect typically depends on the content they carry, which includes DNA, proteins, mRNA, lipids, and miRNA (Raposo and Stoorvogel, 2013; Colombo et al., 2014). The content may vary depending on the physiological and pathological state of the cells from which the exosomes originate (Colombo et al., 2014; Schorey and Harding, 2016).

The use of exosomes in patients has several potential advantages: (i) Their use avoids the transfer of cells, which may have immunogenic molecules and even mutated or damaged DNA. The cell-free nature of exosomes makes it more favorable to regulatory bodies; (ii) The exosomes are small and can readily circulate through any organ, whereas cells are too large to circulate easily through capillaries and many do not get beyond the first pass capillary bed (Verweij et al., 2019); (iii) As exosomes are native to the body, their surface has inherent biochemical properties that are similar to cells, hence, they are able to avoid phagocytosis, fuse with cell membranes, and also bypass lysosomal engulfment (Xu et al., 2018a). The fact that exosomes are a natural product of the body results in a low immune response (EL Andaloussi et al., 2013); (iv) Exosomes have unique homing characteristic due to unique membrane proteins and lipids that bind to specific receptors on the recipient cell surface (Quah and O'Neill, 2005). However, delivering a therapeutic dosage of exosomes to the target cells, particularly *via* systemic injection, is not always as straightforward as it seems and has its challenges. This mini-review highlights those challenges and the solution to the shortcomings in the form of exosome encapsulation in biodegradable or highly porous hydrogels. Strategies to encapsulate soft nanoparticles, such as exosomes, and examples of materials that have been used for sustained delivery of stem cell-derived exosomes are also discussed.

CHALLENGES IN EXOSOME DELIVERY

The intended biological effects of exosomes can only be produced as a result of internalization by target cells *via* an endocytic pathway (Mulcahy et al., 2014). The ability to prolong the half-life of exosomes at the target site is crucial in order to achieve the therapeutic dosage of the exosomes. Studies have shown that direct intravenous, intraperitoneal or subcutaneous injection of exosomes results in rapid clearance from the blood circulation and accumulation in the liver, spleen, lung, and gastrointestinal tract (Takahashi et al., 2013; Smyth et al., 2015). Regardless of the delivery route and cell source, the majority of systemically injected exosomes are rapidly taken up by macrophages in the reticuloendothelial system to be ejected from the body (Wiklander et al., 2015; Charoenviriyakul et al., 2017). The half-life of topically applied exosomes, e.g., on skin or ocular surface, may even be shorter due to the rapid clearance of vesicles by fluid (sweat or tears) and exposure to external elements. Topical application of drugs on the ocular surface has always resulted in low bioavailability due to the presence of epithelial tight junctions and rapid tear turnover (Agrahari et al., 2016).

Another issue that further advocates the need for a sustained delivery system of exosomes is the difficulty in producing the vesicles not only in a large quantity, but also in high purity and consistent quality (Yamashita et al., 2018). The large scale production for clinical studies and commercialization can become expensive (Taylor and Shah, 2015). The typical yield of an exosome isolation can be less than 1 μg of exosomal protein from 1 ml of culture medium (Yamashita et al., 2016; Charoenviriyakul et al., 2017), whereas the therapeutic dose of exosomes is usually in the range of 10–100 μg of protein in mouse model (Willis et al., 2017). In humans, the effective dose could be an order of magnitude or more to compensate for the rapid clearance of exosomes from the body. Biodegradable or porous hydrogels can be used to load a relatively low amount of exosomes, but still be able to produce the intended therapeutic effect and sustain the effect over a period of time, because hydrogels can prevent the encapsulated exosomes from being cleared prematurely (Liu et al., 2018). In addition, they also allow the delivery of a more localized and concentrated dosage by placing the exosome-loaded hydrogel directly at or in the proximity of the target site.

EXOSOME ENCAPSULATION STRATEGIES

Hydrogels have been extensively used to create drug delivery systems with desirable therapeutic effects (Caló and Khutoryanskiy, 2015). They are crosslinked, three-dimensional hydrophilic polymer networks that form matrices with high water content (Peppas et al., 2006). The polymers commonly used to prepare the hydrogels are from natural (e.g., collagen, gelatin, chitosan, hyaluronic acid or alginate) or synthetic (e.g., poly(ethylene glycol) (PEG), poly(lactic-co-glycolic acid) (PLGA) or poly(hydroxyethyl methacrylate (pHEMA)) origins or the combination of both (Peppas et al., 2006). Hydrogels typically have tunable physical properties that can be taken advantage of to customize the degradation rate of the matrices to release the entrapped exosomes. They also have similarities to the native extracellular matrix (ECM), excellent biocompatibility, and malleable (Annabi et al., 2014). With these combined characteristics, hydrogels are an excellent candidate to encapsulate exosomes.

Encapsulation of exosomes into the hydrogel matrix can be performed in three ways:

1. Exosomes can be incorporated by mixing with the polymers, followed by addition of crosslinkers to gel the composite (**Figure 1A**). An example of this method would be the composite hydrogel created by Qin and coworkers (Qin et al., 2016). They utilized HyStem®-HP hydrogel, which is a composite substrate containing thiolated hyaluronic acid, thiolated heparin and thiolated gelatin (Ghosh et al., 2005), to incorporate bone marrow stem cell (BMSC)-derived exosomes. The thiolated polymers and exosomes were crosslinked/gelated with the addition of poly(ethylene glycol) diacrylate (PEGDA).
2. Exosomes can also be physically incorporated after the polymerization of hydrogel (**Figure 1B**). This incorporation technique is sometimes known as "breathing" method (Thomas et al., 2009). The "breathing" typically consists of placing the swollen hydrogel into a solvent to remove the entrapped water, followed by soaking the hydrogel in an aqueous solution containing the exosomes that causes the hydrogel to swell and "breath in" the exosomes. The technique requires hydrogels with pores larger than the exosomes, such as the chitosan/silk

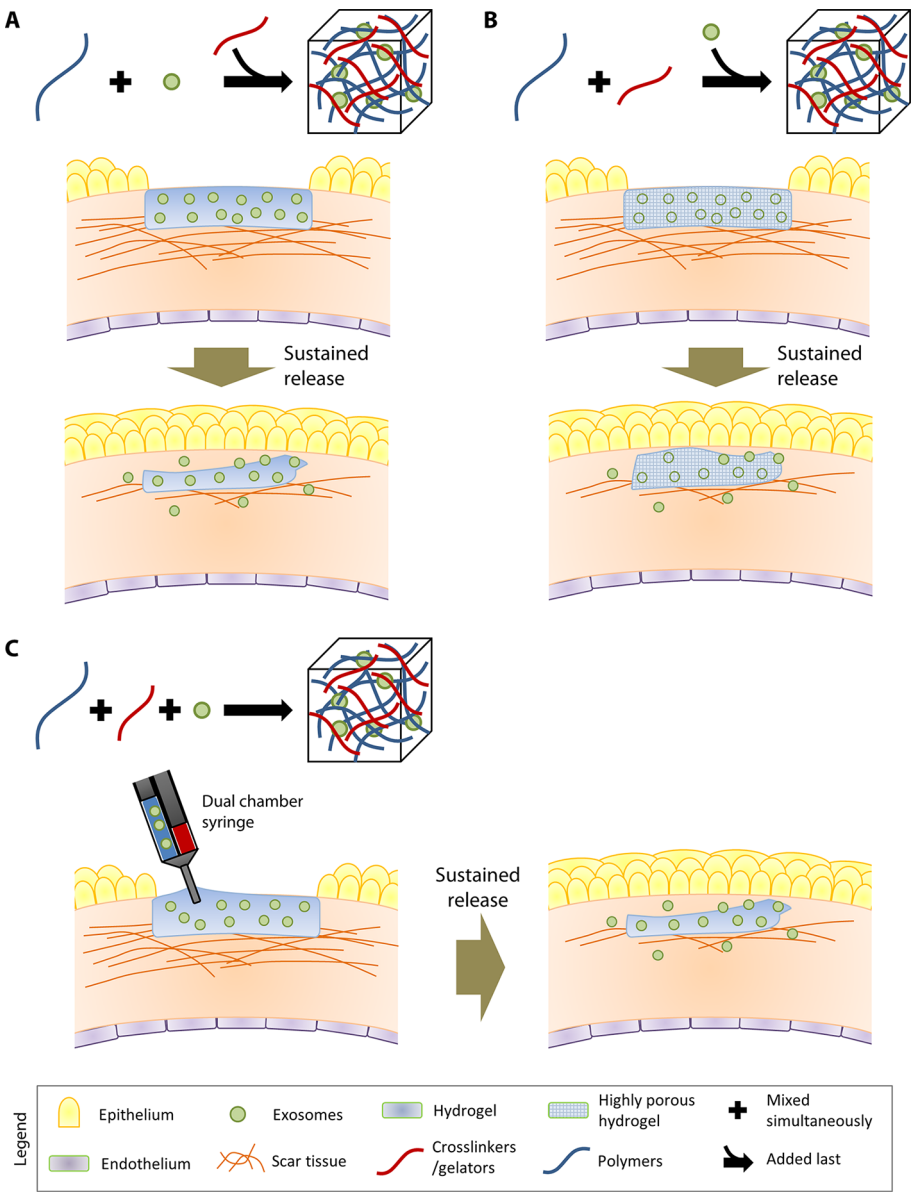

FIGURE 1 | Three different methods to incorporate exosomes in hydrogels. In the illustrations, the therapeutic application of the exosome-loaded hydrogels is exemplified by their role in reducing scar tissue in the cornea. **(A)** The first strategy involves incorporation of exosomes by mixing with the polymers, followed by addition of crosslinkers to gel the composite. **(B)** The second strategy involves physically incorporation of exosomes after the gelation of the hydrogel. **(C)** The third strategy involves mixing the polymers, exosomes, and crosslinkers simultaneously. In situ gelation can be achieved by injecting the three hydrogel components using a dual-chamber syringe directly at the target site.

fibroin hydrogel sponge that was used by Xu and coworkers to encapsulate platelet-rich plasma exosomes (Xu et al., 2018b). The exosomes that are weakly attached to the matrices would be able to leach through the large pores.

3. It is possible to incorporate exosomes by mixing them with the polymers in solution form and crosslinkers simultaneously (**Figure 1C**). This technique would enable an *in situ* gelation, allowing direct injection of the hydrogel components (exosomes + polymers in solultion form + crosslinkers) at the target site. An example of the technique was carried out by Wang and colleagues, where they encapsulated adipose-derived mesenchymal stem cells (MSCs) in a polypeptide hydrogel made of Pluronic F127, oxidative hyaluronic acid, and poly-ε-L-lysine (Wang et al., 2019).

SUSTAINED DELIVERY SYSTEM FOR STEM CELL-DERIVED EXOSOMES

Research into encapsulating exosomes in a hydrogel is still in its infancy; hence, there are currently only a limited number of studies in the literature. **Table 1** summarizes the studies that have described the use of hydrogel to encapsulate exosomes and the duration of release achieved by each type of material.

Qin et al. were the first to describe the idea of encapsulation of exosomes in a hydrogel (Qin et al., 2016). In an effort to stimulate skin regeneration in diabetic rats with chronic skin wounds, Guo and colleagues loaded exosomes isolated from platelet-rich plasma (PRP) in sodium alginate hydrogel and observed a 96-h exosome release (Guo et al., 2017). In a separate study by Tao et al., also with the intention to accelerate skin wound healing, they loaded exosomes derived from miR-125-3p-overexpressing synovium MSCs in chitosan hydrogel (Tao et al., 2017). Patching of the exosome-loaded hydrogel over the skin wound resulted in significantly more rapid healing and more new vessel formation compared to controls (untreated skin and skin treated with the blank hydrogel). Entrapping platelet-rich plasma exosomes in chitosan/silk sponge resulted in close to 20% faster skin wound healing than untreated skin wound of diabetic rats after 15 days (Xu et al., 2018b).

The above studies have only shown a rather short particle release period (under 1 week). For certain clinical applications, such as treatment of myocardial infarction, the ability to deliver the exosomes for a longer period of time might be more practical in order to avoid repeated implantation of newly loaded hydrogel within a short period of time in target sites that are challenging to access. By encapsulating exosomes in collagen type I Gelfoam® mesh, Liu et al. observed a 21-day release of exosomes isolated from cardiomyocyte-derived IPSCs (Liu et al., 2018). The cardiomyocyte-derived IPSCs were shown to be enriched in miRNAs that were beneficial in reducing infarct size, hypertrophy, and apoptosis in a rat model of acute heart infarction. Chen and et al. loaded exosomes isolated from bone marrow-derived endothelial progenitor cells in an injectable hyaluronic acid hydrogel that was modified with adamantane and β-cyclodextrin, and observe a linear particle release profile over 21 days (Chen et al., 2018). The exosome-loaded hydrogel resulted in better recovery of cardiac functions at 4 weeks after the onset of myocardial infarction *in vivo*, compared to the rats treated with free exosomes. By adding β-glycerophosphate in chitosan solution, it enabled *in situ* gelation of chitosan hydrogel loaded with placenta MSC-derived exosomes (Zhang et al., 2018). Although the release duration was not reported, by extrapolating the number of particles released per hour, the duration of exosome release from the injectable chitosan hydrogel was approximately 16 days. In their rat model of hindlimb ischemic injury, exosome-loaded hydrogel induced less fibrotic and necrotic tissue formation, inflammatory response, and hence, faster physiological function recovery compared to rats treated with free exosomes (Zhang et al., 2018).

A more complex hydrogel system capable of delivering exosomes and antimicrobial effect was introduced by Wang et al. (2019). The hydrogel, composed of pluronic F127, hyaluronic acid, and poly-ε-L-lysine, is pH-sensitive, where the adipose-derived MSC exosome release rate was more rapid in acidic pH than in neutral pH. The skin regeneration over the wound, injected with exosome-loaded

TABLE 1 | Materials used to encapsulate exosomes derived from various cell sources.

Materials	Cell source	Duration of release	Clinical application of delivery system	Reference
Adamantane and β-cyclodextrin-modified hyaluronic acid hydrogel	Bone marrow-derived endothelial progenitor cells	21 days	Cardiac regeneration in infarcted heart	Chen et al. (2018)
Alginate hydrogel	Blood plasma	4 days	Skin regeneration in chronic diabetic wound	Guo et al. (2017)
Collagen type I Gelfoam® sponge	Cardiomyocyte-derived IPSCs	21 days	Cardiac regeneration in infarcted heart	Liu et al. (2018)
Chitosan hydrogel	miR-125-3p-overexpressing synovium MSCs	6 days	Skin regeneration in chronic diabetic wound	Tao et al. (2017)
Chitosan hydrogel	Placenta MSCs	Not reported	Angiogenesis promotion in ischemic tissue	Zhang et al. (2018)
Chitosan/silk fibroin sponge	Blood plasma	Not reported	Skin regeneration in chronic diabetic wound	Xu et al. (2018b)
HyStem®-HP hydrogel	BMSCs	Not reported	Bone regeneration	Qin et al. (2016)
pH-responsive polypeptide (Pluronic F127, oxidative hyaluronic acid and poly-ε-L-lysine) hydrogel	Adipose MSCs	21 days	Skin regeneration in chronic diabetic wound	Wang et al. (2019)
Self-assembled peptide amphiphile (C16-GTAGLIGQ-GG-GHRPS) hydrogel	Umbilical cord MSCs	21 days	Cardiac regeneration in infarcted heart	Han et al. (2019)

hydrogel, was more rapid than that injected with free exosomes over 21 days. Han et al. introduced a complex injectable peptide amphiphile (PA) that could self-assemble into a hydrogel (Han et al., 2019). The authors incorporated umbilical cord MSC-derived exosomes into a PA with a 16-carbon-alkyl tail that was functionalized with cardioprotective peptide GHRPS (His-D-2-methyl-Trp-Ala-Trp-D-Phe-Lys) and matrix metalloprotease-2 (MMP-2) degradable sequence GTAGLIGQ (Gly-Thr-Ala-Gly-Leu-Ile-Gly-Gln). The MMP-2 sequence was added to allow controlled degradation of the hydrogel over 21 days to release the encapsulated exosomes. With the hydrogel delivery system, they showed a significantly better functional cardiac recovery, reduced scarring and lower inflammatory response 28 days after the onset of myocardial infarction, when compared to the rats treated with non-encapsulated exosomes.

FUTURE DIRECTIONS AND CONCLUSIONS

The prospect of successful tissue regeneration utilizing cell-free material, such as stem cell-derived exosomes, is exciting. The cell-free nature of exosome application circumvents the primary concern of potential tumorigenesis and unwanted mutagenesis of stem cell therapies (Cairns, 2002; Erdo et al., 2003; Volarevic et al., 2018). Delivery of therapeutic dosage of exosomes to the target site, however, has been a challenge due to their short half-life in circulation. Systemic injection of exosomes has been shown to result in rapid clearance from the blood circulation, and accumulation of the exosomes in the liver, spleen, lung, and gastrointestinal tract can be found as early as 2 h after injection (Takahashi et al., 2013; Smyth et al., 2015). The half-life of topically applied exosomes, such as on the buccal mucosa or ocular surface, may even be shorter due to the rapid fluid turnover (saliva or tears), and exposure to external elements. Hence, biodegradable or highly porous hydrogels can be utilized to incorporate exosomes in their matrices, to deliver a sustained therapeutic effect. The hydrogels can prevent the loaded exosomes from being cleared prematurely, and allow the delivery of a more localized and concentrated dosage, by placing the exosome-loaded hydrogel directly at or in the proximity of the target site. This advantage can be achieved by loading only a relatively small amount of exosomes in the hydrogels. This contrasts the potential need to deliver a repeatedly large amount of exosomes to compensate for the poor bioavailability of systemic injection.

Research into techniques to encapsulate stem cell-derived exosomes in hydrogels is still in its early stage. All of the existing methods described in the literature use polymers of natural origin, such as hyaluronic acid, chitosan, and gelatin, as the main component of the hydrogels. This is due to the fact that natural origin-based hydrogels are relatively simple to fabricate, are biodegradable or highly porous, possess similarities to native ECM, and have excellent biocompatibility. However, the potential application of hydrogel-forming synthetic polymers, such as polylactic acid (PLA) and PLGA, should not be ruled out. These synthetic polymers have been used commercially in various pharmaceutical products (Zhong et al., 2018). When considering the material characteristic of synthetic polymers, polymers that are not water-soluble, such as pHEMA, may not be suitable to encapsulate exosomes. The processing of these polymers normally involves a strong organic solvent, which may degrade the structural integrity and content of the exosomes when mixed.

Other challenges include the potential toxicity of residual unreacted cross-linkers for hydrogel making, especially for injectable hydrogels, which are designed to polymerize within the tissue. Clogging of needles may occur during injection of pH- or temperature-sensitive hydrogels. Hence, it is necessary to optimize the gelling temperature, polymer concentration, and applicator system in order to prevent premature gelation in the syringe. There is also a persistent challenge in determining the kinetic release profiles *in vivo*. The release profile generated *in vitro* often does not translate *in vivo*. The development of hydrogel-based delivery systems with a delivery rate that could be modulated on-off would be beneficial for clinical applications that require varying doses of exosomes over a period of time.

The degradability and shape of hydrogels are highly tunable. The hydrogels can also be tailored to polymerize in situ. These characteristics would allow a customizable application of an exosome delivery system. For certain clinical applications at target sites that are difficult to access, such as for the treatment of myocardial infarction, one would prefer an injectable delivery system that is able to deliver the exosomes for a longer period of time. There is a future in the commercialization of exosome-loaded hydrogel products due to their potential for patient-specific applications.

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AR, HO, and GY wrote the manuscript. AR and JM conceptualized the manuscript. HO and JM obtained the grants for the study.

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Ex Vivo Human Adipose Tissue Derived Mesenchymal Stromal Cells (ASC) Are a Heterogeneous Population That Demonstrate Rapid Culture-Induced Changes

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Human adipose-derived mesenchymal stromal cells (ASC) are showing clinical promise for the treatment of a range of inflammatory and degenerative conditions. These lipoaspirate-derived cells are part of the abundant and accessible source of heterogeneous stromal vascular fraction (SVF). They are typically isolated and expanded from the SVF *via* adherent cell culture for at least 2 weeks and as such represent a relatively undefined population of cells. We isolated *ex vivo* ASC directly from lipoaspirate using a cocktail of antibodies combined with immunomagnetic bead sorting. This method allowed for the rapid enrichment of a defined and untouched *ex vivo* ASC population (referred to as MACS-derived ASC) that were then compared to culture-derived ASC. This comparison found that MACS-derived ASC contain a greater proportion of cells with activity in *in vitro* differentiation assays. There were also significant differences in the secretion levels of some key paracrine molecules. Moreover, when the MACS-derived ASC were subjected to adherent tissue culture, rapid changes in gene expression were observed. This indicates that culturing cells may alter the clinical utility of these cells. Although MACS-derived ASC are more defined compared to culture-derived ASC, further investigations using a comprehensive multicolor flow cytometry panel revealed that this cell population is more heterogeneous than previously appreciated. Additional studies are therefore required to more precisely delineate phenotypically distinct ASC subsets with the most therapeutic potential. This research highlights the disparity between *ex vivo* MACS-derived and culture-derived ASC and the need for further characterization.

Keywords: human adipose tissue-derived mesenchymal stem/stromal cells, adipose-derived stem cell, mesenchymal stem cells, paracrine effect, heterogeneity, immunomagnetic bead sorting, flow cytometry, stromal vascular fraction

INTRODUCTION

Human adipose tissue derived stromal cells (ASC) are currently being tested as cell-based therapies against a wide range of diseases and conditions in numerous clinical trials (Bateman et al., 2018) including wound healing, (Bertozzi et al., 2017) cardiovascular disease (Ma et al., 2017) and cartilage regeneration (Pak et al., 2018). ASC are isolated from adipose tissue and are therefore an abundant source of stromal cells that can be accessed with relative ease using the minimally invasive procedure of liposuction. ASC are often referred to as adipose tissue derived "stem" cells (ASC or AdMSC) or mesenchymal stem cells (MSC), inferring their suitability for a wide range of regenerative applications. However, the cellular diversity of this population, referred to hereafter as adipose tissue derived mesenchymal stromal cells (ASC), and their true therapeutic potential remains unclear. They are defined in part as being able to differentiate into fat, bone, and cartilage lineages *in vitro* (Bourin et al., 2013). They are also reported to function as bioreactors producing molecules that promote healing and inhibit over activity of the immune system (Ma et al., 2014). Although their exact therapeutic mode of action *in vivo* is unclear (Robey, 2017) increasing evidence points to mesenchymal cells exerting a paracrine effect (Zwolanek et al., 2017; Caplan, 2019) rather than cell replacement.

To isolate ASC, the by-product of liposuction, termed the lipoaspirate, is digested with collagenase and centrifuged resulting in a cell pellet known as the stromal vascular fraction (SVF). This is a heterogeneous mix of cells including ASC, preadipocytes, endothelial cells, and immune cell subsets. A widely used method to enrich for ASC involves culturing the SVF cell pellet *in vitro*. In 2006, the International Society for Cell and Gene Therapy (ISCT), formerly known as the International Society for Cellular Therapy, proposed a minimum set of guidelines to define cells isolated from tissue (Dominici et al., 2006). These included (1) that morphologically the cells are plastic adherent and fibroblastic (2) that they express the cell surface markers CD73, CD90, and CD105, and do not express haematopoietic and endothelial antigens (CD14 or CD11b, CD19 or CD79α, CD34, CD45, HLA-DR) and (3) that they display "trilineage potential" in that they able to differentiate into adipocytes, osteoblasts, and chondrocytes *in vitro*. This definition was later extended in a position statement released by the ISCT and International Federation for Adipose Therapeutics and Science (IFATS) in 2013 to include the preculture or *ex vivo* criteria to define ASC within SVF. In this position statement, the phenotypic characteristics of ASC isolated from SVF were further refined to include CD34 as a positive marker, a key difference between culture-isolated and *ex vivo* ASC (Bourin et al., 2013). Collectively, these criteria have provided a useful common ground in the mesenchymal field. Nevertheless there is now increased awareness that these definitions are no longer an up-to-date reflection of the knowledge that is rapidly accumulating. In addition, *in vitro* differentiation assays, which require cocktails of chemical cues, do not necessarily mimic the *in vivo* environment, nor demonstrate an accurate reflection of the *in vivo* activity of the cell (Locke et al., 2011; Robey, 2017). Furthermore, these defined cell surface markers are also expressed by cultured fibroblastic cells from a variety of tissue sources. It is also becoming increasingly apparent that the ASC fraction itself is heterogeneous (Merrick et al., 2019). Therefore further studies are required to identify ASC defining markers to enable the enrichment of a more defined population of cells (da Silva Meirelles et al., 2006; Crisan et al., 2008; Nielsen et al., 2016).

The plastic-adherent culturing method used to isolate a "pure" population of ASC from the SVF typically requires a minimum of 2–3 weeks in culture and even then the population can be far from homogenous (Ho et al., 2008; Baer et al., 2013). However, it should be noted that currently there is a lack of consistency or standardisation regarding the preparation of ASC for use in the clinic. Increased time in culture may increase apparent homogeneity (Mitchell et al., 2006), however culture duration could affect clinical utility and lead to increased production times, costs, and regulatory hurdles associated with getting a product to the clinic. In addition, the incidence of genetic abnormalities tends to increase with time in culture (Neri et al., 2013), therefore minimizing passage number may improve the safety profile of cells. Finally, increased passage number has been reported to result in decreased potency (Wall et al., 2007; Park et al., 2011; Lo Surdo et al., 2013). To assess what effect cell culture may have at the functional and molecular level we sought to compare culture-derived ASC with an uncultured *ex vivo* population with a defined cell-surface phenotype based on the ISCT/IFATs recommendation (Bourin et al., 2013). To this end we report here on the use of an immunomagnetic bead approach to rapidly enrich a defined and untouched population of *ex vivo* ASC from the SVF, hereafter referred to as MACS-derived ASC. To our knowledge, a side by side comparison of *ex vivo* and culture-derived ASC has not been performed previously. We hypothesised that this comparison would be important to help to elucidate the clinical utility of these two cell populations.

We found that MACS-derived ASC contain a greater proportion of cells with activity in *in vitro* differentiation assays compared to culture-derived ASC and that they exhibit an altered profile of secreted proteins. These differences may reflect the undefined nature of culture-derived ASC expanded from SVF. In addition, we show that culturing *ex vivo* MACS-derived ASC rapidly alters their gene expression profile in ways that may affect their clinical utility. This suggests that methods that enrich for a defined population of uncultured ASC may be beneficial to clinical utility in some settings. MACS-derived ASC appear homogenous based on their cell surface phenotype (according to the ISCT/IFATS definition (Bourin et al., 2013). However, using a comprehensive multicolor flow cytometry panel, here we further demonstrate that this population of cells is more heterogeneous than previously reported (Bourin et al., 2013) and is variable between donors. This highlights the need to further characterize the functionality of defined subpopulations of ASC to improve reproducibility of results in this field. Our research highlights the disparity between *ex vivo* MACS-derived and culture-derived ASC populations and the need for further characterization.

MATERIALS AND METHODS

Processing Lipoaspirate

Lipoaspirate was obtained from informed healthy, nonobese, female donors undergoing elective liposuction with protocols approved by the Northern A Health and Disability Ethics Committee (approval number NTX/07/02/003). One litre of lipoaspirate was washed twice with an equal volume of phosphate-buffered saline (PBS) and digested with 0.15% Collagenase type I (Life Technologies) in PBS for 60 min at 37° C with occasional mixing. Cells were pelleted by centrifugation at 690 g for 10 min at room temperature resulting in the SVF. The pellet was resuspended in 50 ml prewarmed ASC medium (Dulbecco's modified eagle media/Ham's F12 nutrient mixture (DMEM F-12; Life Technologies) supplemented with 10% Fetal Bovine Serum (FBS) (Life Technologies), 1% Penicillin-Streptomycin 10,000 U/ml (Invitrogen), and 1 × GlutaMAX (Invitrogen) and passed through a 100-µm Falcon™ cell strainer (BD). SVF was pelleted again and resuspended in 50% ASC media and 50% freezing media (FBS plus 20% DMSO (Sigma-Aldrich) and cryopreserved in liquid nitrogen. From eight donors the average SVF yield from 1 L of lipoaspirate was 3×10^8 cells (data not shown).

Culturing SVF to Isolate Culture-Derived ASC

Frozen SVF suspensions were thawed and for each donor vials were split to allocate half for MACS sorting and half for plastic adherent culture. Both isolation methods were conducted in parallel and cells from each donor were cultured separately. On average 5×10^6 cells were plated into a Falcon™ T75 tissue culture flask in ASC medium (see above). When cells reached 90% confluency they were detached from the flask using trypLE (ThermoFisher) and passaged at a 1 in 2 dilution, typically once a week, for 28 days (four passages). Cell purity was assessed by flow cytometry as described below for MACS sorted cells. Morphology images were taken using a Leica DMI3000 B Inverted Microscope equipped with a Leica DFC290 camera and Leica Application Suite (LAS) software.

Flow Cytometry

All FACS cell sorting and analyses were performed on a BD SORP FACS Aria II equipped with four lasers (see **Supplementary Table 1**). Voltration experiments were performed to optimize PMT Voltages across all detectors using unstained lymphocytes and Mid Intensity beads (BioLegend). This process involved stepwise increments in voltage gain to determine the minimal voltage required to ensure that dim signals were above electronic noise and within the linear detection range. These optimal settings were then saved as "application settings" and were used for all experiments to ensure consistency between experiments. All flow cytometry reagents were titrated to determine optimal dose and panels developed to minimize spectral overlap. For all experiments single-stained controls were prepared with BD CompBeads Plus, except for CD34 and CD90 where single cell controls were prepared instead. Compensation was done using the automated wizard in BD FACSDiva. All data analyses were performed using FlowJo V10.2 (BD Biosciences, San Jose, CA).

MACS Sorting Cells to Isolate MACS-Derived ASC

Frozen SVF suspensions were thawed and for each donor vials were split to allocate half for MACS sorting and half for plastic adherent culture. ASC were enriched using MACS™ anti-FITC microbeads (Miltenyi) according to the manufacturer's instructions with an antibody cocktail consisting of 2.5 µl of each of the following antihuman FITC-conjugated antibodies: CD31 (clone MW59), CD45 (clone HI30), CD146a (PIH12), and CD235a (clone H1246) (all from BioLegend). In brief, the SVF single cell suspension was pelleted and resuspended in 100 µl MACS buffer per 10^7 cells and incubated with the antibody cocktail on ice for 10 min protected from light. Cells were washed twice with MACS buffer and resuspended in 90 µl of buffer per 10^7 cells. Cells were then incubated with 10 µl of anti-FITC MACS microbeads (Miltenyi) per 10^7 cells for 15 min at 4°C and washed with MACS buffer. These were resuspended in 500 µl of buffer and applied to a precooled LS column (Miltenyi). Post sort purity was assessed by flow cytometry using CD73, CD90, CD31, CD45, CD34, and CD146 antibodies (see **Supplementary Table 2**).

Adipogenic Differentiation Assays

Adipogenic staining was performed as described previously (Eom et al., 2018). In brief cells were plated in a 96-well plate in 200 µl ASC media (DMEM/F12 media (Life Technologies) supplemented with 10% FBS, 1% GlutaMax (Life Technologies, Auckland) and 1% penicillin/streptomycin (Life Technologies) and cultured at 37°C, 5% CO_2. On day four, 100 µl of media was replaced with adipogenic differentiation media (ASC media with 1 µM dexamethasome, 10 µM insulin, 0.5 mM 3-isobutyl-1-methylxanthine (IBMX), and 200 µM indomethacin (all from Sigma Aldrich, Auckland) and standard ASC media was added to control wells. Half media changes were performed every 3 days until day 14. Cells were then subjected to immunocytochemistry using a 1:200 dilution of rabbit antihuman FABP4 polyclonal antibody (Cat #10004944, Cayman Chemicals) and then incubated with a 1:200 dilution Alexa Fluor® 488 conjugated goat antirabbit IgG secondary antibody (Cat # A11008, Molecular Probes®) and 1:2,000 diluted DAPI (Cat# D3571, Molecular Probes®). Fluorescent images were taken using the ImageXpress Micro XLS high content screening system (Molecular Devices™). Nine images were taken per well at 10 × magnification and quantitative data was generated using the MetaXpress v 5.3.0.1 (Molecular Devices™) software.

Osteogenic Differentiation Assay

ASC were seeded into a 96-well plate in standard ASC media. The following day half of the media was replaced with StemPro® osteogenic differentiation medium in experimental wells and with standard ASC medium in control wells. Half media changes were performed with osteogenic media every three days until day 21. Cells were then fixed with 4% formaldehyde for 30 min at room temperature, washed twice with water, then incubated with 2% Alizarin Red 5 min at room temperature.

Cells were washed with water, dried and imaged (12 images per sample) using a Leica DMI3000 B Inverted Microscope equipped with a Leica DFC290 camera. Alizarin Red stain was quantified using Image J software.

Microarrays

Cells were FACS sorted on a BD SORP FACS Aria II using the same antibody cocktail as described for the MACS sort, and post sort analysis was as above. Cells were washed once in ice-cold PBS and total RNA was purified using the miRVANA kit (Ambion). RNA integrity was assessed using a Bioanalyser (Agilent). 100 ng of RNA were reverse transcribed and labeled using the Genechip 3' IVT Express kit and hybridised to Primeview Arrays (Affymetrix) according to manufacturer's protocols. Fluorescent signals were recorded by an Affymetrix scanner 3000 using Gene Chip Operating Software. The Affymetrix® Expression Console™ Software was used to carry out quality control analysis. Affymetrix® Transcriptome Analysis Console (TAC) 3.0 was used to determine genes that were differentially expressed between different conditions (day 0, day 3 and day 28). Only those genes that showed an ANOVA p-value of less than 0.05 and a fold difference between > 2 and < −2 were considered as differentially expressed between the conditions. The p-value and fold differences were an average obtained from three different donors. Data was preprocessed, normalized, and summarized using the RMA (Irizarry et al., 2003) function from the R software package affy (Gautier et al., 2004). A dendrogram was used to show a pictorial representation of the relationship between the samples. The Euclidean distance between the normalized samples were calculated, then clustered together using the Ward's method. The dendrogram showing the grouping of the clustered samples was then plotted using the ape (Paradis and Schliep, 2018) package. A list of transcripts with a fold change of greater than, or equal to 10 was obtained using the Affymetrix/Thermofisher Transcriptome Analysis Software. A heatmap of these transcripts were plotted using the heatmap.2 function from the gplots (Warnes et al., 2016) package. Microarray data has been deposited in the Gene Expression Omnibus (GEO) database and can be accessed *via* accession no. GSE136633.

Quantitative Real Time (RT)-PCR

Quantitative real-time (RT)-PCR was performed as described previously (Sheppard et al., 2016). In brief total RNA was isolated from all samples using a miRVANA kit (Ambion). First-strand cDNA was synthesized for all samples using random hexamer primers and SuperScript III reverse transcriptase (Invitrogen). Quantitative RT-PCR was carried out on a 7900HT Real-Time PCR System (Applied Biosystems) using TaqMan® FAST Universal PCR Master Mix (Roche), gene specific TaqMan® probes and between 2 and 10 ng cDNA per reaction. PCR cycling parameters were 20 s at 95˚C and then 40 cycles of 1 s at 95˚C followed by 20 s at 60˚C. Results were normalized against two housekeeper genes (B2M and HPRT1).

Quantification of Paracrine Factors

MILLIPLEX® MAP kits (EMD Millipore, Merck) were used to quantify key paracrine factors present in the conditioned media of "MACS" or "cultured" isolated cells. Cells isolated by MACS were immediately plated postsort into 1 ml ASC medium in a standard 24-well plate at an equal density to ASC that had been cultured from SVF for at least 2 weeks. Four days later supernatants were removed, centrifuged for 5 min at 360 g at 4°C to pellet cell debris and stored at −20°C until analyzed. At this time, fresh media was added to the cells for further 3 days, before being and harvested again in the same manner. Three Human Cytokine/Chemokine Magnetic Bead Panels were used to quantify cytokines in the supernatents (1) Cat# HCYTOMAG-60K was used to detect IL-6, Il-8, VEGF, and IFNγ, (2) Cat# HIGFMAG-52K was used to detect IGF I and II, and (3) Cat# TGFBMAG-64K-03 was used to detect TGFβ 1 and 2. Samples were run in duplicate on a MAGPIX® analyser and results were obtained using Luminex xPONENT software.

Multicolor Flow Cytometry Analysis

For phenotypic analyses, frozen cell samples were thawed, washed, and resuspended in ASC medium and incubated at 37°C/5% CO_2 for 1 hour prior to staining. Cells were stained using an antibody cocktail containing CD26, Podoplanin, CD271, CD144, CD105, CD90, CD36, FAP, CD34, CD73, CD31, HLADR, CD45, CD146, CD141, CD73, CD31, CD45, and CD34 (see **Supplementary Table S2**). Following a 30-min incubation on ice, cells were washed twice in 1 ml of staining buffer (PBS + 1% human serum). Cells were resuspended in buffer and DAPI (1:5,000) was added to exclude dead cells immediately prior to data acquisition on a BD SORP FACS Aria II. Data in **Figure 5A** was analyzed using FlowJo V10.2 (BD Biosciences, San Jose, CA) and in **Figures 5B, C** using viSNE (Amir el et al., 2013) and FlowSOM (Van Gassen et al., 2015) in Cytobank (www.cytobank.org/). viSNE was run using equal sampling (204,977 cells) of the pregated CD90+CD73 +CD34+ population to identify heterogeneity within the populations and also to allow for donor comparisons. FlowSOM was subsequently run to identify clusters by hierarchical clustering. Four clusters (tabulated in **Figure 5C**) were identified using two dimensional gating and subsequently displayed as overlays on the viSNE plots.

Statistical Analysis

Unless otherwise stated, statistical analysis was performed using Microsoft Excel software. Significance was assessed using two-tailed type 1 t-tests with p values < 0.05 being considered significant. * denotes a p value < 0.05, ** denotes a p value < 0.01, and *** denotes a p value < 0.001.

RESULTS

MACS Enrichment of a Defined Population of ASC From SVF

Our previous analysis of the SVF from human adipose tissue using multicolor flow cytometry gave us a good understanding of the various cell types present in this tissue (Feisst et al., 2014). Based on this earlier work we used a flow cytometry panel to demonstrate that the ASC population, positive for CD90, CD73, and CD34, could be enriched by excluding all populations positive for CD45 (haemopoietic), CD235a (red blood cells),

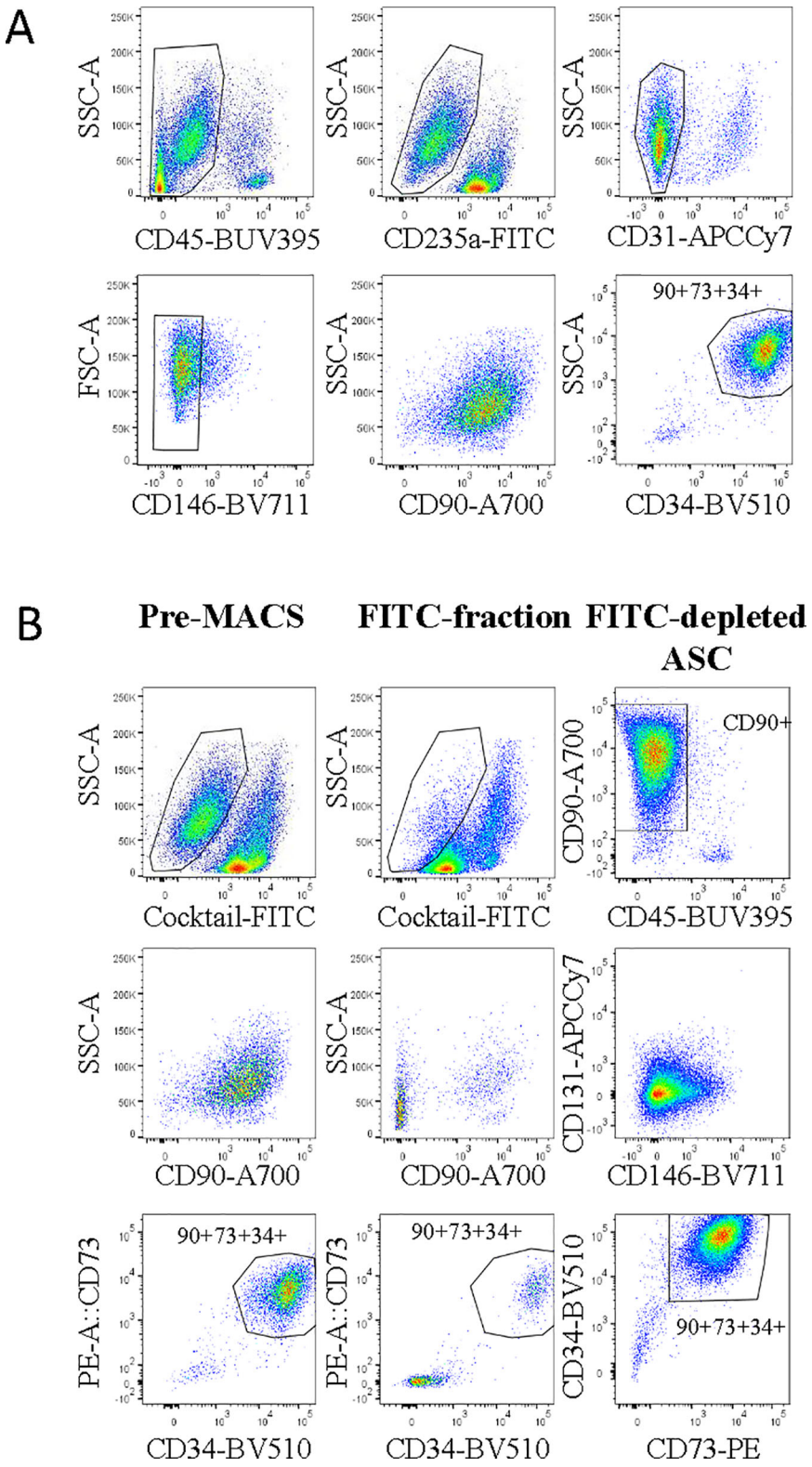


FIGURE 1 | Continued

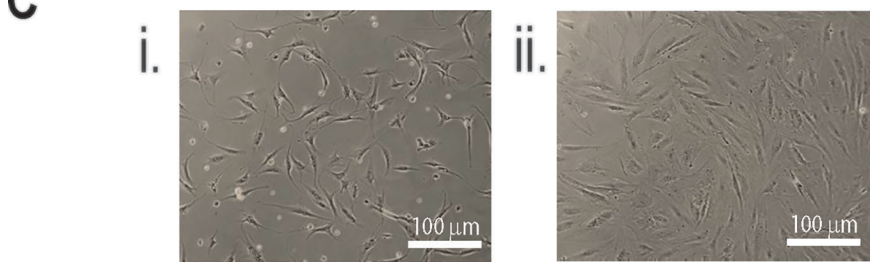


FIGURE 1 | Untouched adipose-derived stem cell (ASC) can be enriched from the stromal vascular fraction (SVF) using an antibody cocktail to deplete contaminating cell populations. A flow cytometry panel was used to demonstrate exclusion gating of CD45, CD31, CD235a, and CD146 cells enriched for CD90 +CD73+CD34+ ASC cells within the SVF **(A)**. Therefore, a FITC antibody cocktail containing anti-CD45, -CD31, -CD235a, and -CD146 and anti-FITC microbeads was used to enrich ASC from SVF single cell digests using the "MACS" protocol. **(B)** Flow cytometry was used to assess the enrichment strategy: (1) presort, (2) the FITC+ fraction, and (3) the FITC depleted fraction with a panel of markers including CD73-PE, CD90-A700, CD31-APC-Cy7, CD45-BUV395, CD34-BV510, and CD146-BV711. Data shown are representative of four biological replicates. **(C)** The morphology of MACS-derived four days post sort (i) or culture-derived ASC 48 h post passage four (28 days) (ii) was assessed. This image is representative of at least four donors.

CD31 (endothelial), and CD146 (pericytic) (**Figure 1A**). Therefore, we sought to use a cocktail of FITC-labeled antibodies (CD31, CD45, CD146, and CD235a) and anti-FITC labeled magnetic MACS™ beads to enable enrichment of an "untouched" ASC cell population from the SVF using immunomagnetic column-based sorting. Samples labeled with the anti-FITC cocktail and anti-FITC beads were passed over a magnetic column, to allow the enrichment of untouched ASC cells to flow through. The presort, column-retained (FITC-positive), and enriched/flow through fractions were analyzed by flow cytometry (**Figure 1B**). Flow cytometry analyses indicated that post sort purity of the enriched fraction was >97% for CD34+, CD73+, and CD90+ (see representative data from one donor in **Figure 1B**). Cell morphology of cells sorted using the immunomagnetic bead protocol (herein referred to as MACS-derived cells) was comparable to stromal cells isolated from the same donors in parallel using the standard method of plastic adherence and 28 days in culture (herein referred to as culture-derived cells) (**Figure 1C**).

One of the most common methods to enrich for ASC is by plastic adherence, followed by cell expansion and passaging. We have previously demonstrated that 28 days was sufficient to achieve homogeneity of ASC based on CD90, CD73, and CD34 (Feisst et al., 2014) expression and are consistent with the IFATS/ISCT definition for ASC (Bourin et al., 2013). In addition three to four passages is generally accepted as a pure population (Braun et al., 2013) and is comparable to standard methods used to enrich for ASC. We therefore compared MACS-derived cells to culture-derived cells that had been cultured for 28 days. Unsurprisingly, this results in higher cell yields compared to MACS-derived cells. The cultured-derived method generated at least six times more cells (19.12×10^6, st. dev +/− 4.0×10^6) after 28 days in culture compared to the MACS enrichment process (2.9×10^6, st. dev +/− 0.62×10^6), n = 4). This method infers that large cell numbers are more clinically relevant; however, it is also likely that this period in culture will influence the functional capacity of these cells. In addition, a loss of ASC differentiation potential over time in culture has also been reported (Wall et al., 2007; Park et al., 2011; Lo Surdo et al., 2013). However, MACS enriched cells represent a more defined population that may be more potent in some settings. Therefore, we hypothesised that the MACS-derived ASC would perform better in *in vitro* differentiation assays compared to culture-derived ASC, although we acknowledge that these assays do not necessarily reflect *in vivo* activity. When subjected to a quantitative adipogenic differentiation assay assessing FABP4 expression by immunohistochemistry, a significantly greater proportion of MACS-derived cells expressed FABP4 in comparison to culture-derived ASC isolated from the same donor (**Figures 2A, C**). When subjected to a semi-quantitative osteogenic differentiation assay, using alizarin red staining as a marker of calcium rich deposits, we also consistently observed significantly higher levels of alizarin red staining in the MACS-derived ASC cells in comparison to culture-derived ASC isolated from the same donor (**Figures 2B, D**).

Next, we sought to examine the molecular changes that might occur when enriched *ex vivo* ASC are cultured *in vitro*. In this experiment ASC were enriched by flow assisted cell sorting (FACS) using the same antibody cocktail as was used for the MACS bead enrichment described above. The sorted ASC were then grown on plastic in standard tissue culture conditions for 0, 3, or 28 days, after which total RNA was isolated. This approach meant that the same defined population of cells was being assessed at each time point with the major experimental variable being time in culture. These time-points were chosen to examine gene expression in uncultured (day 0), minimally cultured (day 3), and extensively cultured *ex vivo* FACS-derived ASC (day 28). The 28-day time-point was chosen to match the time point used to enrich for culture-derived ASC from SVF. RNA was subjected to microarray analysis using Affymetrix PrimeView arrays. Quality controlled and robust microarray average (RMA) normalized data was further analyzed using Affymetrix transcriptome analysis console software to identify any genes differentially expressed between the treatment groups.

49,372 genes were interrogated using the microarray platform. When comparing freshly sorted "day 0" cells to cells

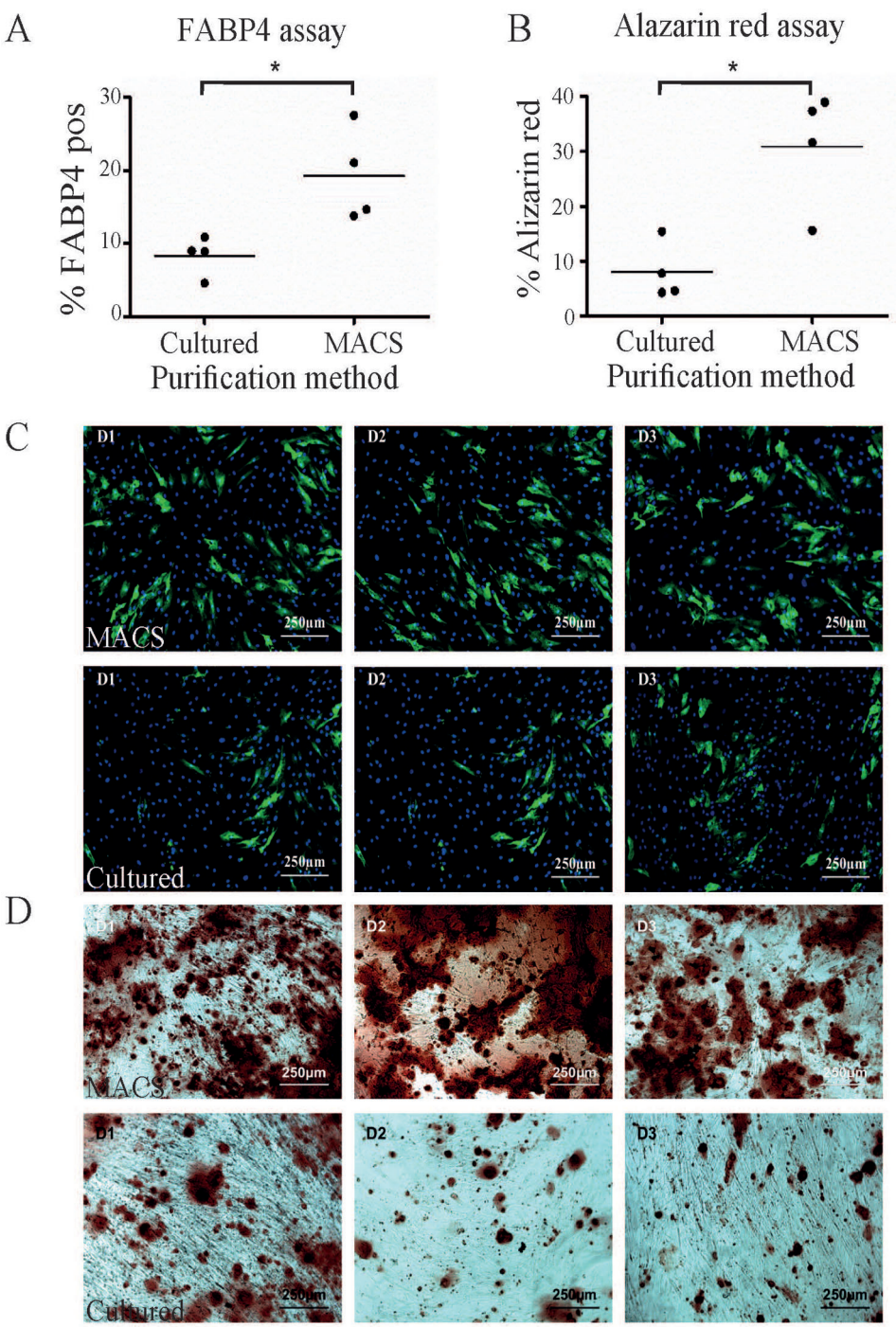


FIGURE 2 | A greater proportion of MACS-derived adipose-derived stem cell (ASC) exhibit activity in *in vitro* differentiation assays compared to culture-derived ASC. **(A)** MACS-derived and culture-derived ASC were subjected to an *in vitro* adipogenic differentiation assay. The graph represents the percentage of cells which stained positive for FABP4 expression after quantification. Panel **(C)** shows representative images from the adipogenic differentiation assay for three donors (D1–D3) where FABP4 positive cells are stained green and nuclei are stain blue. The top row contains images of MACS-derived ASC and the bottom row contains images from culture-derived ASC. The graph in **(B)** represents the percentage of cells which stained positive for alizarin red after 3 weeks of culture in commercial osteogeneic differentiation media. Panel **(D)** shows representative images from the alizarin red assay for three donors (D1–D3). The top row contains images of MACS-derived ASC and the bottom row contains images from culture-derived ASC. * denotes a p value of < 0.05.

cultured for 3 days ("day 3") from three donors 1,122 genes were downregulated > twofold (with an ANOVA p value < 0.05) and 13,659 genes were upregulated > twofold. When comparing day 0 to day 28 cells these numbers change to 3,018 genes downregulated and 6,358 genes upregulated. A comparison of differential gene expression between day 3 and day 28 cells indicated that only 197 genes are downregulated and 853 genes are upregulated (see **Supplementary Table S3** for genes upregulated and downregulated > tenfold comparing day 0 versus day 3, day 3 versus day 28, and day 0 versus day 28). This data indicates that global gene expression changes are most striking during the first three days in tissue culture conditions and changes thereafter are more modest. A heat map generated using a list of all transcripts which exhibited a fold change >10 clusters uncultured cells together and cultured cells as a separate group, regardless of time spent in culture (**Figure 3A**). This is further demonstrated by unsupervised hierarchical clustering based on all gene expression changes which again groups all cultured cells together as a separate group from the uncultured cells, regardless of time spent in culture (**Figure 3B**).

Lineage-specific genes were among the top most significantly downregulated genes following a 28-day culture period (day 0 versus day 28). This included genes typically implicated in the terminal differentiation of MSCs following commitment to the chondrogenic, osteogenic, and adipogenic fates including matrix Gla protein (MGP, > 48-fold), osteoglycin (OGN, > 330-fold down) and fatty acid binding protein 4 (FABP4, > 790-fold) respectively. KLF4, a transcription factor present in pluripotent stem cells was also downregulated > eightfold, although other pluripotent factors such as c-MYC, SOX2, OCT4, and NANOG were not found to be differentially expressed. Many immune response related genes were also downregulated between day 0 and day 28 including chemokines (CXCL14 (> 800-fold) CXCL12 (> 19-fold), CXCL3 (> 18-fold) and CXCL2 (> 29-fold)) and HLA-DRa (> 28-fold), CD14 (43-fold), and CD54 (> 17-fold).

Functional annotation analysis using the DAVID tool (Huang da et al., 2009) on genes downregulated > twofold between days 3 and 28 ranks positive regulation of cell proliferation followed by negative regulation of apoptosis as the pathways most affected by time in culture (p values of > 0.001). This suggests that over time proliferation decreases and apoptosis increases. Numerous ECM, adhesion, cytoskeletal, and matrix remodelling proteins were among the genes upregulated following a 28-day culture period (**Supplementary Table S3**). DAVID analysis on genes upregulated > twofold between days 3 and 28 ranks extracellular matrix reorganization, cell adhesion, collagen fibril organization, and regulation of cell shape as the top four pathways most affected by time in culture (p values of > 0.001).

Real-time PCR was used to validate some of the differentially expressed genes identified by the microarray analysis using ASC isolated from up to eight subsequent donors (**Figure 3C**). MACS-derived ASC (day 0) were compared to cultured expanded MACS-derived ASC grown in standard tissue culture conditions for 3 or 28 days. Significant downregulation of KLF4, FABP4, OGN, MGP, and CXCL14 and significant upregulation of SCRG1 and POSTN was confirmed. We also assessed the expression of the pluripotency associated genes c-MYC, SOX2, and OCT4 that had not been identified as differentially expressed by the microarray data. c-MYC was significantly, albeit modestly, reduced in expression whilst no differential expression was observed for SOX2 or OCT4. ASC from donors 4–8 were all sorted from the SVF *via* MACS, whereas ASC from donors 1, 2, and 3 were sorted *via* FACS. Comparison of the qPCR data derived from MACS or FACS sorted cells was performed using one-way ANOVA and indicated that there were no statistical differences between these two sorting methods (data not shown).

ASC are thought to exert beneficial clinical immune-modulatory effects in part *via* a paracrine mechanism (Kilroy et al., 2007; Ma et al., 2014). Therefore, we were interested to assess if key secreted molecules were differentially present in conditioned media taken from MACS-derived ASC compared to culture-derived ASC. The microarray data, which focused only on MACS-derived ASC, suggested that some secreted chemokines and growth factors were downregulated following time spent in culture. In particular, IGF1 and 2 were found to be significantly (> 20-fold) downregulated in the microarray dataset between day 0 and day 28. However, we also wanted to assess the expression levels of other key factors reported to be important to ASC paracrine function that may be differentially expressed in conditioned media taken from MACS-derived ASC compared to culture-derived ASC. These included IFNg and IL-8 where no changes in mRNA were observed at any time point in the microarray data, and IL-6, VEGF and TGFb 2 where a modest twofold–threefold downregulation was observed between day 0 and day 3 (data not shown). Milliplex® multiplexed assay panels were employed to compare the expression levels of VEGF, IL-6, IL-8, IFNg, TGFb 1 and 2 and IGF-1 and 2 in conditioned media harvested either at day 4 or at day 7 of culture (day 7 representing conditioned media from day 4 to day 7). At day 4 significantly higher levels of IL-8, TGFb1 and 2 were secreted from the MACS-derived ASCs compared to the culture-derived ASCs, which continued to day 7 for TGFb2 (**Figure 4**). At the day 7 time point, MACS-derived ASCs secreted significantly more (> 13-fold) IGF1 than culture-derived ASCs. No significant differences were detected for IGF2, IFNg, IL-6 or VEGF.

As the adipose stromal field develops it is becoming increasingly likely that subpopulations of cells exist within the ASC population (Merrick et al., 2019; Raajendiran et al., 2019). Therefore, the observed differences in the expression levels of some key proteins secreted from MACS-derived ASC in comparison to culture-derived ASCs may reflect the fact that these populations have a different cellular composition, even though both populations express the classical ASC markers. To investigate this further we expanded our 11-color flow cytometry panel (Feisst et al., 2014) up to 16 colors to enable a deeper interrogation of the cell surface phenotype of ASC present in uncultured SVF (i.e. the population equivalent to MACS-derived ASC). As we previously reported, ASC constitute a large proportion of the SVF and were positive for CD34, CD73, CD90, and lacked expression of CD31, CD146, and CD45. These CD90+CD73+CD34+ ASC, that are often referred to as homogenous, were analyzed for expression of CD141, FAPa,

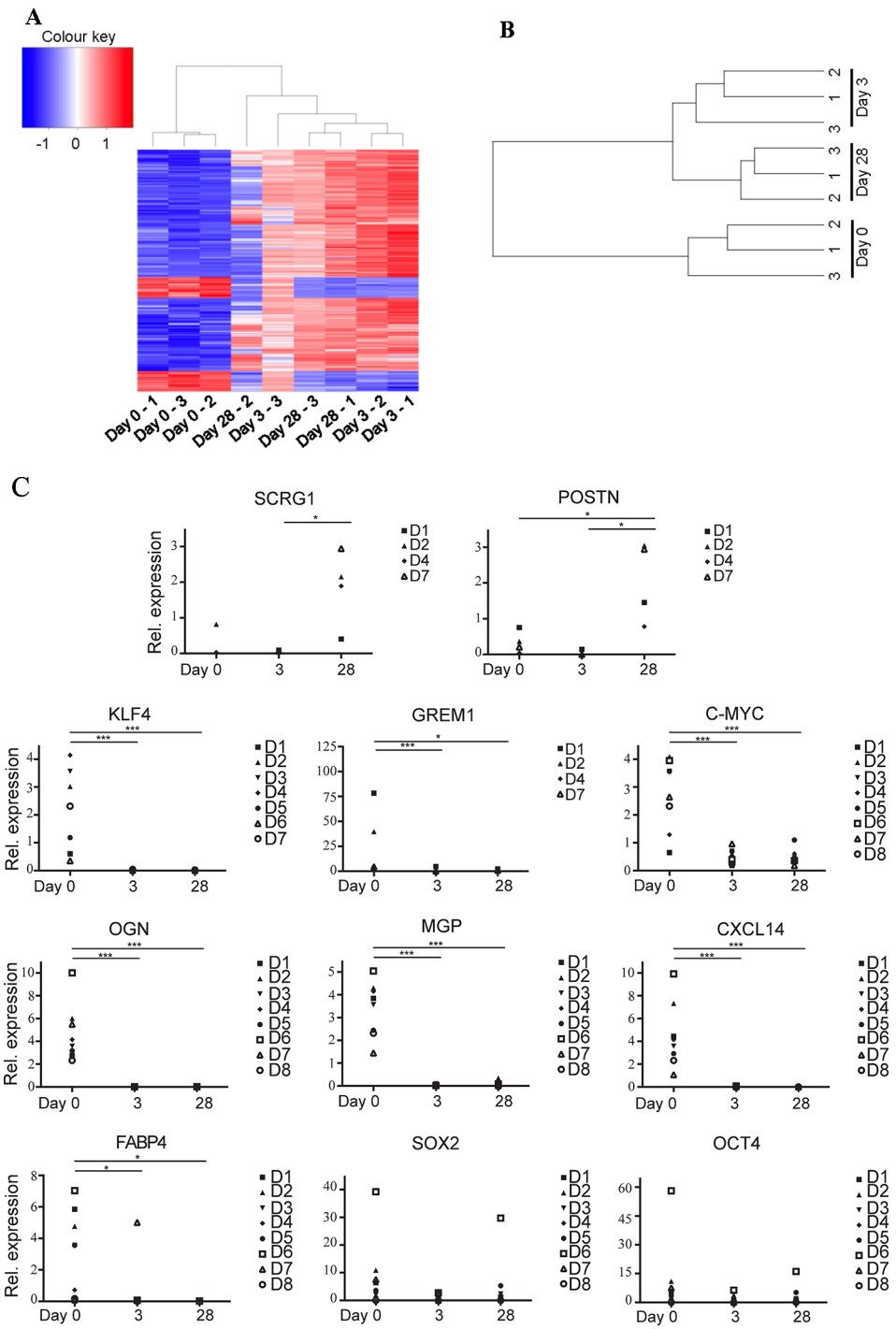


FIGURE 3 | *Ex vivo* MACS-derived adipose-derived stem cell (ASC) exhibit rapid and marked changes in gene expression when subjected to standard tissue culture conditions. MACS-derived ASC were subjected to standard tissue-culture conditions and microarray analysis was performed at day 0, day 3, or day 28 post sort. **(A)** A heatmap was generated using a list of all transcripts which exhibited a fold change >10 when microarray data was analyzed using the Affymetrix Transcript Analysis Console software. Blue represents downregulated genes and red upregulated genes from three donor samples (1–3) at day 0, day 3, or day 28 post sort. **(B)** Unsupervised clustering was performed using all transcripts detected in the microarray data when processed using robust microarray average (RMA). This was then plotted as a dendogram to pictorially represent the relationship between the three donor samples (1–3) at day 0, day 3, or day 28 post sort. **(C)** Real-time PCR was used to validate a subset of the microarray data (target genes SCRG1, POSTN, KLF4, GREM1, C-MYC, OGN, MGP, CXCL14, FABP4, SOX2, and OCT4 as indicated) in at least four, and a maximum of eight subsequent donors (D1–D8). * denotes a p value of < 0.05, *** denotes a p value of < 0.001.

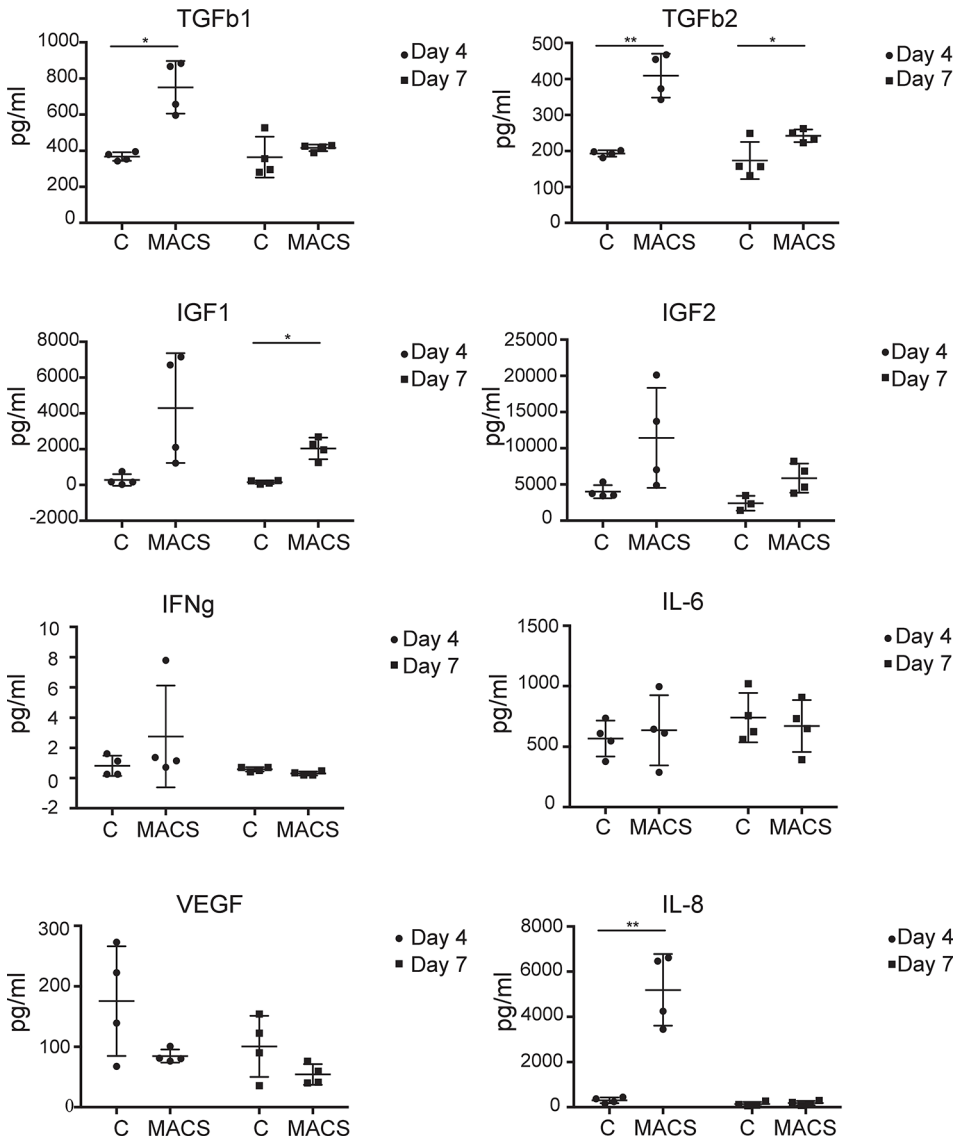
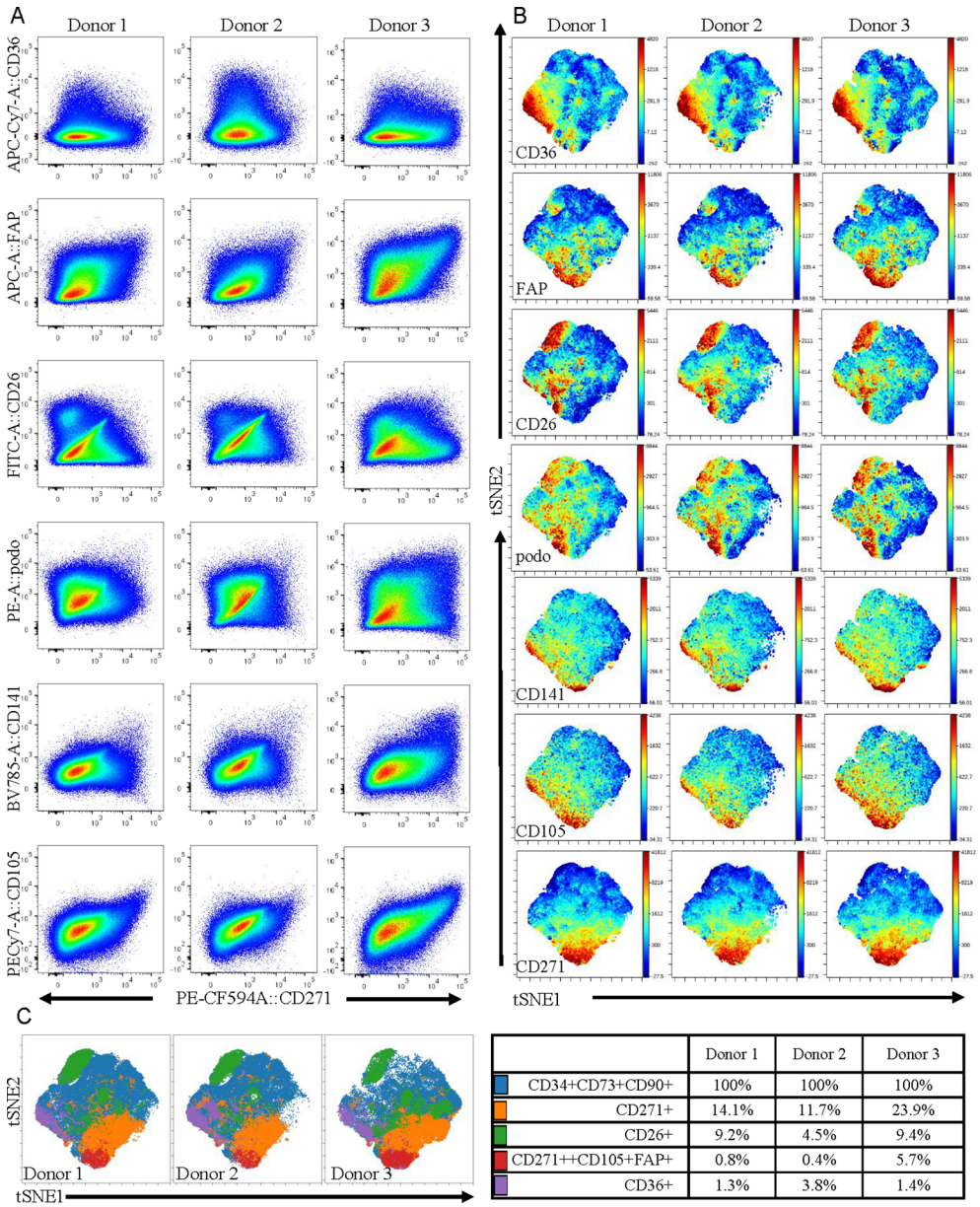


FIGURE 4 | MACS-derived adipose-derived stem cell (ASC) differentially secrete key paracrine-effect proteins compared to culture-derived ASC. Milliplex® multiplexed assay panels were used to assess the expression levels of key secreted proteins thought to be involved in the paracrine effect of ASC. Each panel represents the expression level of a specific protein (TGFb1, TGFb2, IGF1, IGF2, IFNg, IL-6, and VEGF as indicated) present in the media supernatant from culture-derived ASC (C) or MACS-derived (MACS), in both cases 4 days or 4–7 days (day 7) post plating at equal densities. Data derived from four donors. * denotes a p value of < 0.05, ** denotes a p value of < 0.01.

CD26 (also known as DPP4), CD36, CD271, and podoplanin. HLA-DR, CD144, CD31, and CD146 were also assessed, however expression of these markers was mostly confined to the endothelial and pericytic cell populations rather than the ASCs. Despite the use of a number of new markers, distinct, separable subpopulations were not overly obvious within the ASC population. However, when comparing all markers of interest against CD271, heterogeneity became more apparent (**Figure 5A**). For example, a distinct CD26 positive population was identified that was negative for CD271, suggesting these two markers to be mutually exclusive. Whereas CD271 displayed coexpression with CD105, FAPα, and CD141 across all donors, albeit at different frequencies. Interestingly, the most diverse expression occurred between CD271 and podoplanin and was also highly variable between donors. CD36, which was highly expressed on endothelial populations (data not shown), was also found to be expressed by a subpopulation of ACS. To consider all markers concurrently, we further analyzed this same data set using the advanced analysis algorithms viSNE (**Figure 5B**) and FlowSOM (**Figure 5C**). These analyses reveal the heterogeneity that exists within the CD90+CD73+CD34+ population for the markers podoplanain, CD26, FAPα, CD36, CD141, and CD271. **Figure 5B** demonstrates both the similarities and differences of each of the markers between donors while **Figure 5C** highlights



	Donor 1	Donor 2	Donor 3
CD34+CD73+CD90+	100%	100%	100%
CD271+	14.1%	11.7%	23.9%
CD26+	9.2%	4.5%	9.4%
CD271++CD105+FAP+	0.8%	0.4%	5.7%
CD36+	1.3%	3.8%	1.4%

FIGURE 5 | Multicolor flow cytometry of stromal vascular fraction (SVF) indicates that adipose-derived stem cell (ASC) defined by cell-surface expression of CD34, CD73, and CD90 represent a heterogeneous population of cells. **(A)** Multicolor flow cytometry analyses of the SVF using a 16 color flow cytometry indicates that the ASC population is heterogeneous. Following gating exclusion (CD45-CD31-CD146- see **Supplementary Figure 1**), cells with the ASC phenotype, (CD90+CD73+CD34+), identified within SVF were found to be heterogeneous when CD271 was plotted against CD26, CD141, CD36, podoplanin, CD105, and FAPα as labeled, and to differ between donors. Data is derived from three donors. Advanced data analyses were subsequently performed using viSNE **(B)** and FlowSOM **(C)**. viSNE was run using equal sampling (204,977 cells) of the pregated CD90+CD73+CD34+ population per donor to identify subset heterogeneity **(B)**. FlowSOM analyses identified clusters based on expression of podoplanin, CD26, FAPα, CD36, CD141 and CD271 and these are displayed as overlays on the viSNE plots, and percentages of cells present within clusters are tabulated **(C)**.

the key populations identified by FlowSOM clustering and the relative differences observed between the three donors. These FlowSOM plots also demonstrate that a proportion of ASC (shown in blue) do not express the markers under investigation. Therefore, our novel 16 color flow cytometry panel reveals that heterogeneity exists not only within the ASC population, but it is also variable between donors. Future studies will therefore be required to further refine the phenotype and determine whether any of these phenotypic characteristics are also functionally relevant.

DISCUSSION

We present a simple method to enrich an untouched population of defined *ex vivo* ASC from SVF with purity (based on CD34, CD73, and CD90 expression) comparable to ASC isolated using the standard 2–4 weeks in tissue culture method (Feisst et al., 2014). Other groups have reported using an immunomagnetic approach to positively sort ASC from SVF using a single antibodies targeting, for example, CD34 (Maumus et al., 2011), SSEA4 (Jiang et al., 2010; Maddox et al., 2012), CD105 (Jiang et al., 2010), CD49a, CD90, CD-105a, or CD271 (Griesche et al., 2010). Positive sorting using a single antibody will copurify other cell types that also express that marker, and to date no marker has been identified that is exclusively expressed on ASC. In addition, positively selected cells will retain bead-bound antibodies, conferring an advantage to taking a negative selection approach. As far as we are aware we report here on the most comprehensive antibody cocktail that has been employed to negatively enrich for untouched human ASC. Our data indicate that a greater proportion of MACS-derived *ex vivo* ASC exhibit activity in *in vitro* differentiation compared to culture-derived ASC. Increased passage number has previously been reported to result in decreased differentiation potential (Mitchell et al., 2006; Wall et al., 2007; Park et al., 2011; Lo Surdo et al., 2013). Our data extends on these previous results by including an *ex vivo*, uncultured, enriched population of ASC.

The isolation of MACS-derived ASC allowed us to examine the molecular changes that occur within a defined population of cells when subjected to tissue culture. At the RNA level, as expected, we observed no significant change in the expression of stromal cell surface markers CD44, CD73, or CD90 on cultured *ex vivo* MACS-derived cells. We note a previous report showing increased expression of these stromal markers in culture-derived ASC over time until passage 3 (Mitchell et al., 2006), suggesting that at least 3 passages (or approximately 20 days in standard tissue culture conditions) are required to achieve a stromal cell phenotype similar to MACS-derived ASC based on these markers. However, upon culture we did observe a significant downregulation in the expression of stem-cell associated markers CD34 (> 37-fold decrease by day 28) and aldehyde dehydrogenase (ALDH, a 29-fold decrease in RNA levels by day 28). Down regulation of CD34 with time in culture has been reported previously (Mitchell et al., 2006; Yu et al., 2010; Feisst et al., 2014). However, the downregulation of ALDH is a novel observation. We note that a recent paper reported that FACS sorted ALDH-bright ASC were "more primitive" i.e. less differentiated, than their ALDH dim counterparts based on network connectivity parameters using single cell RNA-seq data (Hardy et al., 2017). In addition, we observed a culture-associated reduction in the expression of c-Myc and KLF4 which are genes associated with proliferation and differentiation (Paula et al., 2015; Kami et al., 2016). DAVID analysis of gene expression changes between day 3 and day 28 identified proliferation as a decreased pathway and apoptosis as an increased pathway. This is in line with a report that found a decrease in ASC proliferation rate with time in culture (Legzdina et al., 2016). Expression of some key chemokines reported to be involved in ASC differentiation, migration and wound healing, including CXCL14, CXCL12, CXCL3, and CXCL2 were also significantly downregulated with time in culture (Heneidi et al., 2013; Hayashi et al., 2015; Stuermer et al., 2015; Kusuyama et al., 2016). Collectively the rapid culture-induced changes in gene expression suggest that even a limited time in culture is likely to have a significant impact on ASC activity.

Further marked differences in the profiles of secreted key cytokines were observed when conditioned medium from *ex vivo* MACS-derived ASC and cultured-derived ASC populations were compared. Of the eight proteins we assessed, three were expressed at significantly higher levels in day four conditioned media from MACS-derived ASC compared to culture-derived ASC. These include TGFb1 and 2, which are pleiotropic cytokines with roles in differentiation (Wang et al., 2012) and wound healing (Jung et al., 2011) and IL-8 which is pro-inflammatory and promotes wound healing (MacLeod and Mansbridge, 2016). Levels of IGF1 and 2, which are thought to be antiapoptotic and to have roles in differentiation (Youssef et al., 2017), were on average secreted at greater levels from MACS-derived ASC. However, variability between donors meant that this did not achieve significance except for IFG1 at day 7. Conversely, VEGF, a protein involved in angiogenesis (Johnson and Wilgus, 2014), was secreted on average at greater levels from culture-derived ASC, although again variability between donors meant that this did not achieve significance. The different profiles of secreted proteins from these phenotypically similar populations of cells (based on standard markers of ASC cell surface phenotype) suggest that they may not be functionally similar. They are likely to have different paracrine effects and potentially different clinical utilities. Certainly in settings where high levels of IGF1 secretion are desired, such as in the treatment of myocardial infarction (Bagno et al., 2016), the use of MACS-derived ASC may be more beneficial compared to cultured-derived ASC.

The RNA data generally correlated with the protein data with a notable exception being IL-8. No change was observed at the mRNA level for IL-8 (comparing uncultured MACS-derived ASC to cultured MACS-derived cells) but significant changes were observed in the levels of secreted IL-8 protein in an experiment that compared MACS-derived ASC to cultured-derived ASC. It is possible that, in addition to culture-induced changes, differences in the cellular composition of these two populations may contribute to their different paracrine profiles and activity in differentiation assays. The latter option is feasible as heterogeneity in early passage cultured ASC has been reported previously (Baer et al., 2013; Walmsley et al., 2015; Barilani et al., 2018). Our analysis extends these previous results by showing that heterogeneity and donor-variability exists within uncultured ASC and aligns with recent reports of heterogeneity within this cell population (Merrick et al., 2019; Raajendiran et al., 2019).

Conducting flow cytometry analyses of ASC within uncultured SVF using a novel 16 color flow cytometry panel demonstrated that subpopulations of cells with different expression profiles for the markers CD26, CD36, CD271, CD141, FAPα, and podoplanin exist. Interestingly, the most prominent subpopulation identified within the ASC was positive

for CD26 but did not coexpress CD271, a marker that is often described as a key marker on mesenchymal stromal cells (Barilani et al., 2018; Kohli et al., 2019). CD26 has recently been reported to mark highly proliferative, multipotent progenitors present in adipose tissue (Merrick et al., 2019). In the study by Merrick et al. (2019) the CD26+ progenitors were shown to give rise to two distinct types of preadipocytes in the adipose niche. However, the relevance of these newly defined progenitors in terms of their multipotency beyond adipogenesis (i.e. down the osteogenic and chondrogenic mesenchymal lineages) or indeed their therapeutic potential, remains to be explored. CD271 expression has previously been associated with enhanced activity in ASC differentiation assays (Quirici et al., 2010; Barilani et al., 2018; Kohli et al., 2019) and CD36 has been associated with enhanced adipogenic potential (Gao et al., 2017). However, to our knowledge expression of podoplanin, a marker that is used to define lymphatic endothelial cells, has not been reported previously on human uncultured, *ex vivo* ASC. Podoplanin has been found to be expressed on a progenitor population in the liver (Eckert et al., 2016) and has recently been found to regulate mammary stem cell function in mice (Bresson et al., 2018). Therefore, collectively these markers, combined with ALDH as identified in our microarray experiments, may help to define functional subsets within the ASC fraction.

Cell surface marker expression has been reported to vary with time in culture and with different culture conditions (including CD34, CD105 and CD271 (Braun et al., 2013; Barilani et al., 2018)). Therefore, studies such as ours using fresh and uncultured ASC are warranted. CD26, for example, is reported to be broadly expressed in cultured ASC (Baer et al., 2013; Walmsley et al., 2015) whereas we observe a large negative fraction in uncultured cells. Cryopreservation can also affect cell-surface marker expression (Irioda et al., 2016) and based on the above it is likely that some of these markers will be relevant to identifying functional subsets of ASC. Therefore, there is a pressing need for these deeper analyses of *ex vivo* adipose tissue derived mesenchymal stromal cells. Dissecting cellular heterogeneity may lead to a better understanding and, more importantly, the identification of therapeutically relevant cell populations. Indeed, understanding this heterogeneity could enhance the clinical utility of ASC, both as an *ex vivo* or culture-derived cellular product, as well as be informative to understanding their role in the adipose niche.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the GEO using accession number GSE136633.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Northern A Health and Disability Ethics Committee. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

HS and AB conceived and obtained funding for the study. HS, VaF, and AB designed the experiments and analyzed the results. MI, EW, TD, and VJ-P executed most of the experiments. JJ provided technical expertise. RD provided guidance, expertise and obtained funding. ViF assisted with data analysis. HS and AB wrote the manuscript.

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Evaluating the Endocytosis and Lineage-Specification Properties of Mesenchymal Stem Cell Derived Extracellular Vesicles for Targeted Therapeutic Applications

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Mesenchymal stem cells (MSCs) are multipotent cells with regenerative and immunomodulatory properties. Several aspects of MSC function have been attributed to the paracrine effects of MSC derived extracellular vesicles (EVs). Although MSC EVs show great promise for regenerative medicine applications, insights into their uptake mechanisms by different target cells and the ability to control MSC EV properties for defined function *in vivo* have remained elusive knowledge gaps. The primary goal of this study is to elucidate how the basic properties of MSC derived EVs can be exploited for function-specific activity in regenerative medicine. Our first important observation is that, MSC EVs possess a common mechanism of endocytosis across multiple cell types. Second, altering the MSC state by inducing differentiation into multiple lineages did not affect the exosomal properties or endocytosis but triggered the expression of lineage-specific genes and proteins *in vitro* and *in vivo* respectively. Overall, the results presented in this study show a common mechanism of endocytosis for MSC EVs across different cell types and the feasibility to generate functionally enhanced EVs by modifications to parental MSCs.

Keywords: exosome, extracellular vesicles, mesenchymal stem cells, differentiation, endocytosis

INTRODUCTION

Human mesenchymal stem cells (HMSCs) are multipotent somatic stem cells that can be isolated from a variety of tissues such as the bone marrow, adipose tissue, and dental pulp. The regenerative, protective, and anti-inflammatory properties of HMSCs especially bone marrow derived HMSCs are well documented (Yu et al., 2014; Kim and Park, 2017) and make HMSCs attractive cells for regenerative therapies. As of 2016, about 493 clinical trials that used HMSCs were reported in the National Institutes of Health (NIH) clinical trials database (Squillaro et al., 2016). However, issues such as donor dependent variability, cellular viability, poor attachment, and aberrant differentiation

have posed significant hurdles for the use of HMSCs in clinical treatment (Zhang et al., 2015; Kim and Park, 2017).

Many existing tissue-engineering approaches focus on delivery of selected proteins (growth factors, transcription factors etc.) or nucleic acids to host or implanted stem cells to achieve lineage specific differentiation. A variety of techniques ranging from exogenous addition of growth factors and controlled release devices [reviewed in (Mao and Mooney, 2015)] to utilization of engineered biological and synthetic nano-vesicles such as liposomes and polymeric vesicles (Li et al., 2012; Soltani et al., 2015) have been investigated to deliver morphogens. Although the single morphogen system shows initial promise, when applied clinically, issues such as dosage, specificity, ectopic effects, toxicity, and immunological complications have posed significant restrictions to clinical efficiency as well as translational potential (Soltani et al., 2015). Therefore, a sophisticated system that is biomimetic in nature, provides necessary cues in physiologically relevant amounts and avoids the limitations of the single morphogen system is required. Extracellular vesicles (EVs)/exosomes can satisfy these criteria (Marcus and Leonard, 2013).

EVs are nano-vesicles (40–150 nm) secreted by cells to facilitate intracellular communication (Park et al., 2008). As these vesicles pinch off or fuse with the plasma membrane of the cell prior to release, their lipid bilayer membrane contains components of the parental cell's plasma membrane (Stoorvogel et al., 2002). Within the EV, RNA [both messenger RNA (mRNA) and microRNA (miRNA)], cytosolic proteins, as well as transmembrane proteins are present (Thery et al., 2002). These nano-packets of information are endocytosed by effector cells to trigger a cellular response designated by the parental cell to the target cell (Valadi et al., 2007; Svensson et al., 2013). Although originally believed to be mediators of cellular homeostasis by secreting cellular waste (Johnstone et al., 1991), the past decade study of EVs demonstrate their specific roles in modulating cellular function in immunology, cancer biology, and regenerative medicine (Johnstone et al., 1991; Azmi et al., 2013).

Recent evidence suggests that several of the beneficial effects of human mesenchymal stem cell (HMSC) therapy can be attributed to paracrine effects of the HMSC secretome (Dai et al., 2007; Gnecchi et al., 2008; Yao et al., 2015). More specifically, HMSC derived EVs have been implicated as the principal active agents of the HMSC secretome (Lai et al., 2010; Mokarizadeh et al., 2012; Reis et al., 2012). A recent study highlighted that HMSC derived exosomes possess better anti-inflammatory properties compared to HMSC derived microparticles (Cosenza et al., 2018). Our recent studies have shown that bone marrow and dental pulp HMSC derived EVs can be used to induce osteogenic and odontogenic differentiation of naïve HMSCs respectively (Huang et al., 2016; Narayanan et al., 2016). Additionally, a recent study by Narayanan et al. indicates that HMSC EV function supersedes the extracellular matrix (ECM) derived signals indicating the potent nature of EV signaling (Narayanan et al., 2018). These and many other studies implicate HMSC derived EVs as effective tools in clinical efforts to control inflammation and regenerative therapy and in the treatment of disease.

The paracrine aspect of HMSC function involves the directed uptake of HMSC derived EVs by target cells. Further, the multilineage differentiation potential of HMSCs suggests that lineage specific function could be reflected as lineage specific exosomal effects on naïve target cells. Harnessing the fundamental mechanistic features of EV-mediated signaling can be turned into an application-specific tool to direct lineage specific tissue repair/regeneration and disease treatment. With these goals in mind, the present study characterizes basic mechanistic aspects of HMSC EV function.

MATERIALS AND METHODS

Cell Culture

Human bone marrow derived primary HMSCs (HMSCs) were purchased from ATCC and Lonza. Over the course of this study, HMSCs from at least three individual lots spanning at least three donors were utilized. These cells were cultured in αMEM (Gibco) containing 20% fetal bovine serum (FBS, Gibco), 1% L-Glutamine (Gibco), and 1% antibiotic-antimycotic solution (Gibco). For induction of differentiation of HMSCs into osteogenic (Ravindran et al., 2012), chondrogenic (Ravindran et al., 2015) and adipogenic (Scott et al., 2011) lineages, the growth medium was supplemented with growth factors and differentiating agents as per the indicated published and standardized protocols. Briefly, osteogenic differentiation was induced by culturing the cells in αMEM growth medium containing 100 μg/ml ascorbic acid (Sigma), 10 mM β-glycerophosphate (Sigma), and 10 mM dexamethasone (Sigma) for 4 weeks. Chondrogenic differentiation was induced by culturing the cells in αMEM basal medium containing 1 μM dexamethasone, 50 μg/ml ascorbate-2-phosphate (Sigma), 1% ITS premix (BD Biosciences), 1% FBS, and 10 ng/ml TGFβ1 growth factor (Sigma) for 4 weeks. Adipogenic differentiation was induced by culturing the cells in growth medium containing 10 μg/ml insulin (Sigma), 500 μM isobutyl-l-methylxanthine (Sigma), 100 μM indomethacin (Sigma), and 1 μM dexamethasone for 4 weeks. Human gingival keratinocyte cell line TIGK was cultured as per standardized protocols using the basal medium LM-0004 (Lifeline Cell Tech) supplemented with 10% FBS and LS-1030 cell supplement kit (Lifeline Cell Tech). J774A.1 mouse monocyte-like cells were cultured in DMEM (Gibco) basal medium supplemented with 10% FBS and 1% antibiotic-antimycotic solution.

Extracellular Vesicle Isolation and Characterization

EVs were isolated from the culture medium as per our previously published and standardized protocols (Huang et al., 2016; Narayanan et al., 2016). Briefly, HMSCs were washed in serum free medium and cultured under serum free condition for 24 h. If they were subjected to supplementation for altering cell state, the

supplementation was maintained with only FBS being removed. The culture medium was harvested, removed of cell debris by centrifugation (1,500xg) and EVs were isolated using the ExoQuick TC isolation reagent (System Biosciences) as per the manufacturer's recommended protocols. To maintain consistency, the isolated EVs were resuspended in phosphate-buffered saline (PBS) such that each 100 µl of EV suspension contained EVs from approximately $1x10^6$ HMSCs. This equated to a stock concentration of 10,000 particles/µl as determined by nanoparticle tracking analysis (NTA).

The isolated EVs were characterized for number and size distribution and presence of membrane markers by NTA, immunoblotting, and transmission electron microscopy (TEM) as per established standards (Thery et al., 2018). For NTA, a 1/100 dilution of the EV suspension was analyzed in the Nanosight NS-300 instrument to obtain the size distribution plot. For quantitative experiments, the EV concentration (particles/ml) was also measured by NTA and equal number of EVs were used for each experiment.

For immunoblotting, exosomal proteins were isolated in radioimmunoprecipitation assay (RIPA) buffer and 10–20 µg of EV protein isolate was resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), transferred onto nitrocellulose membranes and probed with primary rabbit anti-CD63 (1/500, Abcam) and mouse anti-CD9 (1/500, Abcam), mouse anti-BMP2 (1/500, Abcam) antibodies and near infrared dye conjugated secondary antibodies (1/10,000 Licor) as per previously published protocols (Huang et al., 2016; Narayanan et al., 2016). The blots were then dried and imaged using a Licor Odyssey imager. For immunoblotting of the conditioned medium, the medium from which EVs were isolated was dialyzed against deionized water, lyophilized and reconstituted in 1x Laemmli buffer. SDS PAGE and immunoblotting was performed as described previously.

For transmission electron microscopy (TEM), 10 µl of 1/10 dilutions of the EV suspensions were placed on to carbon fomvar coated nickel TEM grids and incubated for 1 h followed by fixing with 4% formalin, washing with double deionized water and air drying. For immunogold labeling of CD63, the EV containing grids were blocked in PBS with 5% BSA, incubated with CD63 antibody (1/100, Abcam) followed by washing and incubation with 10 nm gold tagged secondary antibody (1/1,000, Abcam). The grids were then washed and air-dried. All the grids were imaged using a Joel JEM3010 TEM.

For all the experiments described in this study, EVs isolated from one lot of HMSCs were used for functional experiments on the same lot of HMSCs.

Quantitative and Qualitative Endocytosis of Human Mesenchymal Stem Cell Extracellular Vesicles

For endocytosis experiments, HMSC EVs were fluorescently labeled using the ExoGlow green labeling kit (System Biosciences) that labels the exosomal proteins fluorescently as per our previously published and standardized protocols (Huang et al., 2016; Narayanan et al., 2016). The EVs were resuspended in PBS at the same concentration as described previously (100 µl corresponding to EVs from 1 million HMSCs).

For quantitative experiments HMSC cells were plated on to 96 well tissue culture plates at a concentration of 10,000 cells per well and incubated for 18 h to facilitate cell attachment. The cells were then incubated with increasing amounts of fluorescently labeled HMSC EVs for 2 h at 37°C. The cells were washed with PBS and fixed in neutral buffered 4% paraformaldehyde. The fluorescence from the endocytosed EVs was measured using a BioTek Synergy2 96 well plate reader equipped with the appropriate filter sets to measure green fluorescence. The results were plotted as mean (+/− SD) normalized fluorescence intensities (normalized to background and no EV fluorescence) as a function of dosage (n = 6 per group).

For quantitative endocytosis blocking experiments, the cells were plated in 96 well plates as described previously or in 12 well culture plates (50,000 cells/well) and prior to EV treatment, were pre-treated with the blocking agents for 1 h as per our previously published protocols (Huang et al., 2016). Cell surface integrins were blocked with 2 mM arginylglycylaspartic acid (RGD) polypeptide (Sigma). Membrane cholesterol was depleted using methyl β cyclodextrin [methyl-β-cyclodextrin (MBCD), Sigma] in a dose dependent manner (0–10 mM). In addition to this, the labeled EVs were pretreated for 1 h with indicated concentrations of heparin (0–10 µg/ml, Sigma) to block the heparin sulfate proteoglycan binding sites on the exosomal membrane. For the qualitative and quantitative experiments, the fluorescently labelled exosomal volume was maintained at 2x saturation volume (determined from the saturation curve. The stock concentration of EV was 10,000 particles/µl) to ensure that saturable levels of HMSC EVs are used in the assay. Treatment with the EV suspension was carried out as described previously and the fluorescence measurement and quantitation and statistical analysis was performed as per published (Huang et al., 2016) and previously described protocols.

For qualitative endocytosis experiments, 50,000 cells (HMSCs) were plated on coverslips placed in 12 well tissue culture dishes. Fluorescently labeled EVs at 2x saturation volume were then added with/without inhibitors as described above and incubated for 2 h in the presence/absence of blocking agents as described above. The cells were then washed, fixed in 4% neutral buffered paraformaldehyde, permeablized, and counter stained using mouse monoclonal anti-tubulin antibody (1/2,000, Sigma), rabbit polyclonal anti-caveolin1 antibody (1/100, Santa Cruz Biotechnology), or rabbit polyclonal anti-clathrin antibody (1/100, Santa Cruz Biotechnology) followed by treatment with TRITC labeled anti mouse/rabbit secondary antibody. The coverslips were then mounted using mounting medium containing 4′,6-diamidino-2-phenylindole (DAPI) (Vector Labs) to label the nuclei and imaged using a Zeiss LSM 710 Meta confocal microscope.

Extracellular Vesicle Mediated Human Mesenchymal Stem Cell Differentiation

HMSCs were differentiated as described under the cell culture methods section and EVs from the differentiated HMSCs were

isolated as described under the isolation section. The isolated EVs were characterized for size and the presence of exosomal markers as described under the characterization section. For *in vitro* differentiation experiments, naïve HMSCs (250,000 cells per 1 cm x 1 cm collagen sponge) were embedded in type I collagen sponges in quadruplicates. Clinical grade collagen sponges (Zimmer collagen tape) were used for these experiments. 2x saturation volume of the different EVs (osteogenic, chondrogenic, and adipogenic) were then added to the cells and incubated for 72 h. The saturation volume was determined by the quantitative dose dependence endocytosis experiment described in the previous section. The saturation was reached at 20 µl of standardized EV suspension per 10,000 HMSCs. NTA was used to measure the amount of EVs and this amounted an average of 10,000 EV particles/µl of standardized EV suspension from HMSCs. $1x10^8$ EV particles was used per group in this experiment. Untreated cells received PBS treatment of equal volume. Post-72 h, RNA was isolated from the embedded HMSCs followed by cDNA synthesis and qPCR for selected marker genes for osteogenic, chondrogenic, and adipogenic differentiation as per our previously published protocols and primer sequences (Huang et al., 2016; Narayanan et al., 2016).

Mouse Subcutaneous Implantation Experiments

All *in vivo* experimentation was performed in either immunocompromised mice (1-month old mice, Charles River Labs) or Sprague Dawley rats (250–300 g, Charles River Labs) as per protocols and procedures approved by the University of Illinois animal care committee (ACC). All animals were housed in appropriate cages in temperature and humidity-controlled facilities. Food and water were made available *ad libitum*.

The ability of EVs from differentiated HMSCs to induce lineage specific differentiation of naïve HMSCs was evaluated *in vivo* in an immunocompromised mouse subcutaneous implantation model. Briefly, $1x10^6$ HMSCs were seeded on to a 1 cm x 1 cm square of clinical grade collagen tape (Zimmer) with 2x saturation volume (approximately $4x10^8$ EVs) of respective control (naïve HMSC EV) or experimental EV (osteogenic, chondrogenic, or adipogenic) suspension and implanted within the subcutaneous pocket bilaterally on the back of immunocompromised mice. The mice were anesthetized by intraperitoneal injection of ketamine (80–100 mg/kg)/xylazine (10 mg/kg). A 1.5 cm incision was made on the back along the midline and the control or experimental scaffolds were placed bilaterally within the subcutaneous pocket. All experiments were performed in quadruplicate. Four weeks post-implantation, the animals were sacrificed by carbon dioxide asphyxiation followed by cervical dislocation. The scaffolds were extracted, fixed in neutral buffered 4% paraformaldehyde, embedded in paraffin, and sectioned in to 5 µm sections. The sections were then immunostained fluorescently for marker proteins as per previously published protocols (Huang et al., 2016; Narayanan et al., 2016), mounted, and imaged using a Zeiss LSM 710 laser scanning confocal microscope. All primary antibodies were purchased from Abcam and were used at a dilution of 1/100 of the stock solution. The secondary anti-mouse fluorescein isothiocyanate (FITC) and anti-Rabbit TRITC were obtained from Sigma and were used at a dilution of 1/200.

Statistical Analysis

For all experiments the normal distribution of the data was evaluated using the Shapiro-Wilk test. Following confirmation to normal distribution, appropriate parametric tests were used to calculate statistical significance. For experiments involving comparison of just two groups, student's t-test with a confidence interval of 95% was utilized. For the experiments involving comparison of more than two groups, one-way ANOVA was performed with a confidence interval of 95%. Following this, pairwise comparisons were performed using Tukey's *ad-hoc* method with a confidence interval of 95%. Statistical analyses were performed using either SPSS software or Microsoft Excel.

RESULTS

Characterization of Extracellular Vesicles

EVs isolated from HMSCs were characterized for size, shape, and presence of exosomal marker proteins. The isolation procedure did not induce cell death in the source HMSCs (**Supplementary Figure 1**). NTA analysis indicated that the isolated vesicles show a particle size distribution consistent for exosomes (Shah et al., 2018; Thery et al., 2018) (**Figure 1A**). We determined that on average, after our standardized EV dilution (100 µl suspension containing EVs from $1x10^6$ cells), the EV concentration for HMSCs was approximately $1x10^8$ particles/ml of the EV suspension. Osteogenic, chondrogenic, and adipogenic differentiation of HMSCs yielded EVs with a similar average size, but an altered distribution of EV sizes (**Figure 1A**). However, the polydispersity index (PDI) was similar between the different groups (**Figure 1A**). TEM analysis revealed spherical vesicles between 100 and 150 nm in size and positive for CD63 marker (**Figures 1B, C**). Immunoblot analysis indicated the presence of exosomal marker proteins CD63 (**Figure 1D**) and CD9 (**Figure 1E**) in both naïve and differentiated HMSC EVs, but not in the EV depleted conditioned medium. Immunoblotting for tubulin revealed tubulin presence in the cell lysate, but not in the EV lysate and EV depleted conditioned medium (**Figure 1F**). The data presented here indicate that the EVs may be primarily composed of exosomes. However, as the exosomes and other EVs have overlapping properties and as we cannot conclusively determine that the purified vesicles are only exosomes, we will refer to them as EVs throughout this article.

Endocytosis of Human Mesenchymal Stem Cell Derived Extracellular Vesicles

EVs from different cell types have been shown to be endocytosed by a variety of mechanisms (Mulcahy et al., 2014). Here, we evaluated the endocytic mechanism of HMSC EVs by target

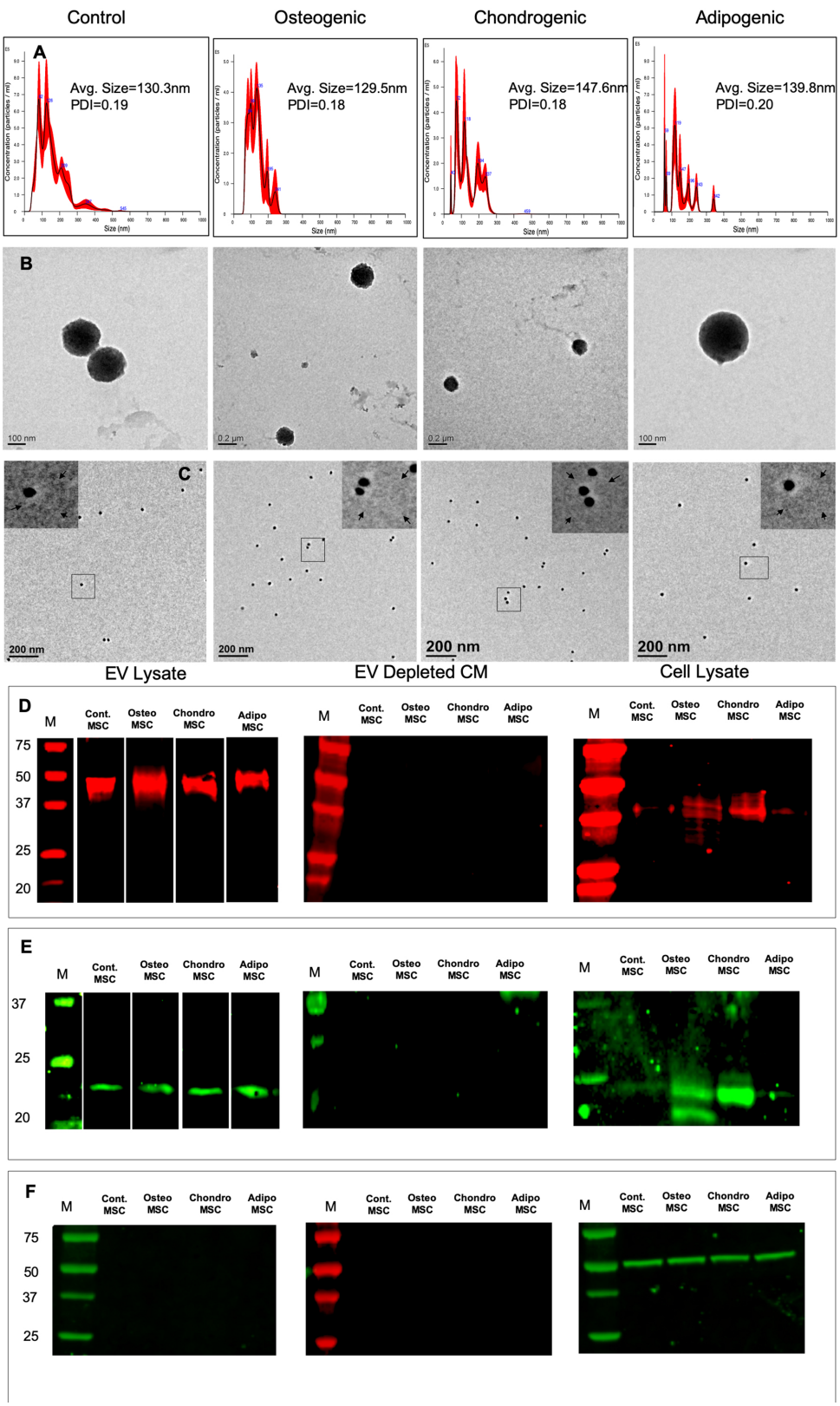

FIGURE 1 | Continued

FIGURE 1 | Isolation and characterization of extracellular vesicles (EVs): **(A)** Representative nanoparticle tracking analysis (NTA) plots of EVs isolated from naïve, osteogenic, chondrogenic, and adipogenic human mesenchymal stem cells (HMSCs). **(B)** Representative transmission electron microscopy images of the EVs isolated from naïve, osteogenic, chondrogenic, and adipogenic HMSCs. **(C)** Representative TEM images of Immunogold labeled (CD63, 20 nm gold particles) EVs from naïve, osteogenic, chondrogenic, and adipogenic HMSCs. The inserts in each of the images represent the boxed area. The arrows in the inserts point to EV membranes. **(D)** Immunoblot of EVs lysates, EV depleted conditioned medium, and cell lysates from naïve, osteogenic, chondrogenic, and adipogenic HMSCs for the presence of CD63 exosomal marker protein. **(E)** Immunoblot of EVs lysates, EV depleted conditioned medium, and cell lysates from naïve, osteogenic, chondrogenic, and adipogenic HMSCs for the presence of CD9 exosomal marker protein. **(F)** Immunoblot of EVs lysates, EV depleted conditioned medium, and cell lysates from naïve, osteogenic, chondrogenic, and adipogenic HMSCs for the presence tubulin.

HMSCs. Quantitative endocytosis experiments indicated that HMSC EV endocytosis by HMSCs was a dose dependent and saturable process (**Figure 2A**). When the quantitative and qualitative endocytosis experiments were performed at 4°C using saturable amounts of EVs, EV endocytosis was blocked indicating the temperature and thereby, the energy dependency of the process (**Figures 2D, E**). In an attempt to identify the mode of endocytosis, we evaluated the role of integrins. Published studies have shown that EV endocytosis by dendritic cells is mediated by integrins (Morelli et al., 2004; Mulcahy et al., 2014). Pre-treatment of the target cells with 2 mM RGD peptide to block the cell surface integrins did not block EV endocytosis (comparing **Figures 2D, G**) indicating that integrins are not primary receptors involved in HMSC EV endocytosis.

A recently published study shows that heparan sulfate proteoglycans (HSPGs) are involved in the endocytosis of glioblastoma cell derived exosomes (Christianson et al., 2013). HSPGs act as both receptors and co-receptors on the plasma membrane and are actively involved in endocytosis of several viruses (Shukla et al., 1999; Belting, 2003). Sulfated heparin mimics the extracellular heparan sulfate domains of the HSPGs and can competitively block endocytosis *via* HSPGs by actively binding to the EVs (Christianson et al., 2013). We therefore investigated if HSPGs are involved in the endocytosis of EVs. Pretreatment of the EVs with heparin significantly reduced the endocytosis in both quantitative (**Figure 2B**) and qualitative (**Figure 2F**) experiments suggesting the involvement of membrane heparin sulfate proteoglycan receptors (HSPGs) in the process of EV endocytosis (Christianson et al., 2013).

Depending on the target cell type, exosomes can be endocytosed by either clathrin or caveolin mediated endocytosis (Mulcahy et al., 2014). We performed immunolocalization experiments to evaluate the involvement of a clathrin or caveolin mediated pathway. In these experiments, the endocytosed EVs colocalized with caveolin 1 (**Figure 2H**). Blocking the lipid raft/caveolar endocytosis with MBCD inhibited EV endocytosis significantly (**Figure 2C**). On the other hand, no colocalization with clathrin and the endocytosed EVs was observed (**Figure 2I**).

Following these observations on HMSC EV endocytosis, we next evaluated if a change in cell state would affect the endocytosis of lineage specified, HMSC derived EVs. HMSCs were first differentiated along the osteogenic, chondrogenic, and adipogenic lineages. EVs isolated from these cells were harvested and evaluated for dose dependent and saturable endocytosis. **Figure 3A** shows representative confocal images of the endocytosis of different fluorescently labeled EVs by naïve HMSCs. Quantitative endocytosis assays with the different MSC EVs revealed no differences in the dose-dependence or saturation of endocytosis (**Figure 3B**).

To test if HMSC EVs possess a common endocytic mechanism across different cell types, we evaluated the endocytosis of naïve HMSC derived EVs in two additional cell types namely: J774A.1 monocyte-like cells and TIGK gingival keratinocytes. Results presented in **Figures 4** and **5** indicate that HMSC EVs are endocytosed using a similar pathway and display dosage and temperature dependence. The involvement of HSPGs and the role of the caveolar pathway in the endocytic process was also common across the three cell types. The layout of images in **Figures 2**, **4**, and **5** have been maintained consistent for ease of comparison. Overall, these results indicate the existence of at least one common heparin-sensitive and caveolin-mediated mechanism of HMSC EV endocytosis across diverse cell types.

Extracellular Vesicles From Differentiated Human Mesenchymal Stem Cells Induce Lineage Specific Differentiation of Naïve Human Mesenchymal Stem Cells *In Vitro* and *In Vivo*

Undifferentiated HMSCs in 3D cultures were incubated with EVs isolated from naïve and differentiated HMSCs for 72 h. Osteogenic, chondrogenic, and adipogenic EVs induced a significant increase in the expression levels of respective lineage specific marker genes with respect to untreated controls (**Table 1**). These genes included a mixture of growth factors, transcription factors, and ECM proteins representative of the individual lineages. No significant change in gene expression was observed with naïve HMSC EVs and the changes observed with lineage specific EVs were restricted to that specific lineage.

To verify these effects *in vivo*, collagen sponges loaded with undifferentiated HMSCs with or without EVs were implanted subcutaneously in the back of immunocompromised mice. After 4 weeks, the forming tissues were excised, fixed, embedded and the sections were analyzed by fluorescence immunohistochemistry for the expression of lineage-specific marker proteins. For all three different EVs, lineage-specific protein expression was observed. **Figure 6** shows representative confocal images of the sections. Similar to the qPCR results, the expression was lineage specific.

For osteogenic differentiation the expression levels of bone sialoprotein (BSP) and osteocalcin (OCN) were analyzed. Results presented in **Figure 6** show that HMSCs from the group treated with osteogenic EVs showed an increased presence of BSP and OCN compared to the control group as well as other EV treated groups adding evidence to the *in vitro* results presented in

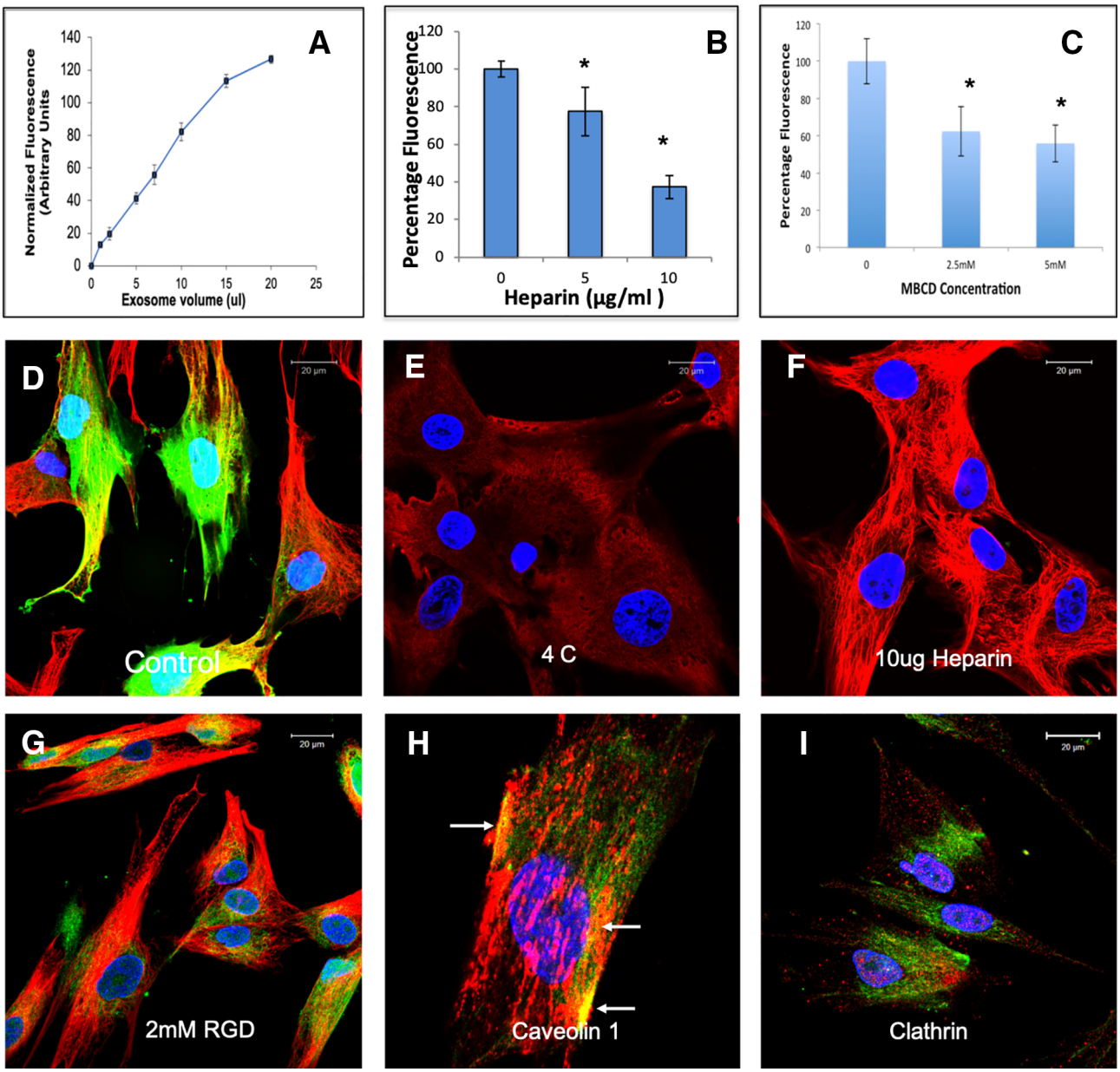

FIGURE 2 | Endocytosis of human mesenchymal stem cell (HMSC) extracellular vesicles (EVs) by HMSCs: **(A)** Graphical representation of dose-dependent and saturable endocytosis of fluorescently labeled HMSC EVs by naïve HMSCs. Data points represent mean fluorescence +/– SD (n=6). The EV volume/particle number was standardized as described under the methods section. **(B)** Graph showing the dose dependent inhibition of HMSC EV endocytosis after pre-treatment of the EVs with heparin to block interaction with the cell surface HSPGs. Data represent mean percentage fluorescence with respect to control +/– SD. **(C)** Graph showing the reduction in HMSC endocytosis after disruption of target cell membrane cholesterol with varying doses of methyl-β-cyclodextrin (MBCD). Data is presented as mean percentage fluorescence with respect to control +/– SD. Representative confocal micrograph depicting the endocytosed fluorescently labeled HMSC EVs within target HMSCs after 1 h of incubation at 37°C. **(E)** Representative confocal micrograph indicating the abrogation of HMSC EV endocytosis when the experiment is performed at 4°C. **(F)** Representative confocal micrograph showing that pre-treatment of EVs with heparin blocks HMSC EV endocytosis. **(G)** Representative confocal micrograph of HMSC EV endocytosis after pre-treatment of the cells with 2 mM arginylglycylaspartic acid (RGD) peptide to block cell surface integrins. In images **(D–G)**, green fluorescence represents endocytosed EVs. Red fluorescence represents tubulin counter stain and blue fluorescence indicates 4′,6-diamidino-2-phenylindole (DAPI) nuclear stain. **(H)** Confocal micrograph showing colocalization of endocytosed HMSC EVs (green) with caveolin1 (red). **(I)** Confocal micrograph showing the absence of co-localization between endocytosed EVs (green) and clathrin (red). * represents statistical significance ($P < 0.05$, ANOVA followed by Tukey's *ad-hoc*) with respect to control.

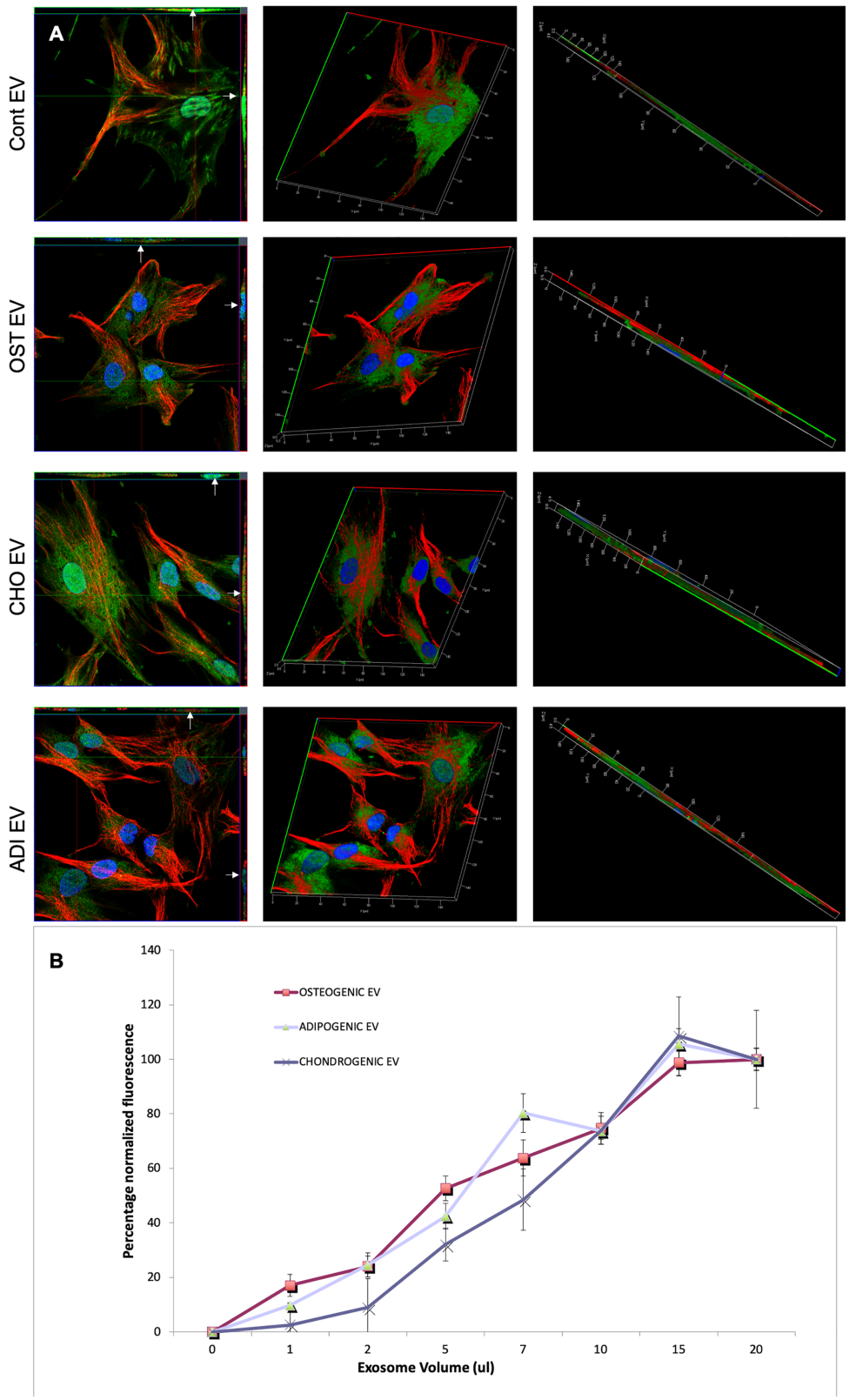

FIGURE 3 | Continued

FIGURE 3 | Endocytosis of extracellular vesicles (EVs) isolated from differentiated human mesenchymal stem cells (HMSCs): **(A)** Representative 3D confocal micrographs of fluorescently labeled EVs isolated from control (naïve), osteogenic, adipogenic, and chondrogenic HMSCs endocytosed by naïve HMSCs. The first image in each row is an orthogonal representation of the z-stacks. The arrows point to localization of the EVs within the cells in the x-z and y-z planes. The other images in the rows represent 3D reconstructions of the z-stacks showing the endocytosed EVs within the cells. In all images, green fluorescence represents labeled EVs, blue represents DAPI nuclear stain and red represents tubulin. **(B)** Graph showing dose dependent and saturable endocytosis of EVs isolated from osteogenic, chondrogenic, and adipogenic HMSCs by naïve HMSCs. Data points represent mean percentage fluorescence with respect to the highest concentration +/− SD (n = 6). Note the absence of any significant difference in endocytosis between EVs isolated from the three lineages.

Table 1. Similarly, chondrogenic differentiation was evaluated by looking at the expression levels of type II collagen, a major component of the cartilaginous matrix as well as the expression level of aggrecan (AGN), a cartilage specific proteoglycan. Results presented in **Figure 6** show that type II collagen and AGN expression was elevated in HMSCs subjected to chondrogenic EV treatment with respect to the control group as well as the other EV treated groups. Finally, adipogenic differentiation of HMSCs from the subcutaneous implants was evaluated by the expression levels of peroxisome proliferator activator receptor-gamma (PPAR-γ) and fatty acid binding protein 4 (FABP4). PPAR-γ is a nuclear receptor that controls adipogenesis and adipogenic differentiation of HMSCs (Brun and Spiegelman, 1997; Spiegelman et al., 1997; Siersbaek et al., 2010). Results presented in **Figure 6** show an increased expression of PPAR-γ and FABP4 in HMSCs treated with adipogenic EVs compared to controls indicating an induction of adipogenic differentiation. Additionally, these cells demonstrate the presence of fat-like deposits arrows in **Figure 6**.

Collectively, these results indicate that EVs isolated from differentiating HMSCs can induce lineage-specific phenotypic changes in naïve HMSCs *in vitro* and *in vivo*. The verification of the *in vitro* results *in vivo* validated the lineage specificity of the EVs and additionally, also provided evidence that the effect that was observed after a few days *in vitro* translates into a long-term effect over a period of 4 weeks *in vivo*.

DISCUSSION

Regenerative strategies require the recruiting and instructing of cells to form new tissues. HMSC EVs are of current interest because they demonstrate immunomodulatory and regenerative potential that may rival the use of HMSCs or growth factors in regenerative medicine (Cheng et al., 2017). Furthermore, studies are currently underway to engineer HMSCs to improve their ability to produce EVs by altering several secretory pathways (Phan et al., 2018). The immunomodulatory, angiogenic, and regenerative potential of HMSC EVs is well documented (Lai et al., 2010; Reis et al., 2012; Mokarizadeh et al., 2012). Others and we have shown the potential of bone marrow derived HMSC EVs for regenerative medicine applications (Narayanan et al., 2016; Martins et al., 2016; Wang et al., 2018). Albeit the presence of multiple studies documenting the potential of MSC derived EVs, several aspects of their mechanisms that are translationally relevant and important remain as key knowledge gaps.

In this study, we have provided insights into some of the basic properties of HMSC derived EVs and how they may be utilized and exploited for improving tissue engineering strategies. We began by investigating HMSC EV endocytosis. Identification of the endocytic mechanism can provide valuable information to target EVs for therapeutic delivery. With respect to EV endocytosis, the clathrin pathway, caveolar pathway, phagocytosis, and even macropinocytosis have all been implicated in endocytosis of EVs (Mulcahy et al., 2014; Li et al., 2015; Alcayaga-Miranda et al., 2016). We observed energy dependence, dose dependence as well as dependence on membrane cholesterol indicating the involvement of the lipid raft/caveolar endocytic pathway. Furthermore, we show that the HMSC EVs are endocytosed in a manner that involves the target cell surface HSPGs. Based on our observations with dental pulp HMSC derived EVs, this appears to be a common endocytic mechanism for HMSC derived EVs (Huang et al., 2016). Further studies using different HMSC sources are required to conclusively determine if this mechanism is applicable to HMSCs in general.

In this study, we also show that the HMSC EVs are endocytosed by monocytes and keratinocytes using a similar endocytic process to that of recipient HMSCs *via* the cell surface HSPGs. We chose these two cell types as representative cells for hematopoietic and ectodermal cells respectively. Further studies with other representative cell types belonging to multiple germ layers is required to conclusively establish one common pathway across all cell types. However, this important first observation identifies a pathway that can be targeted to enhance the endocytic efficiency of therapeutic EVs. For example, the HIV TAT peptide and poly arginine repeats containing peptide sequences have been implicated to be endocytosed by target cells *via* HSPGs and in addition, HSPGs have been implicated as an accessible target receptor for delivery of biological cues to enable disease treatment as well as tissue regeneration (Fuchs and Raines, 2004; Fuchs and Raines, 2006; Christianson and Belting, 2014). These results and the results presented in this study indicate that it may be possible to enhance HMSC EVs endocytosis by target cells by tagging them with HSPG binding peptides. If possible, such modifications may promote enhanced delivery by reducing dosage as well as minimizing ectopic effects.

This study also explores an important question regarding the use of EVs for therapeutic purposes: Does the state of the parental cell influence EV functionality? The results presented here show that when HMSCs were differentiated into osteogenic, chondrogenic, and adipogenic lineages, the secreted EVs from these cells maintained their morphology and expression of exosomal surface markers. We next considered if lineage-specification of parental HMSCs would inform the differentiation potential of their respective EVs. Results

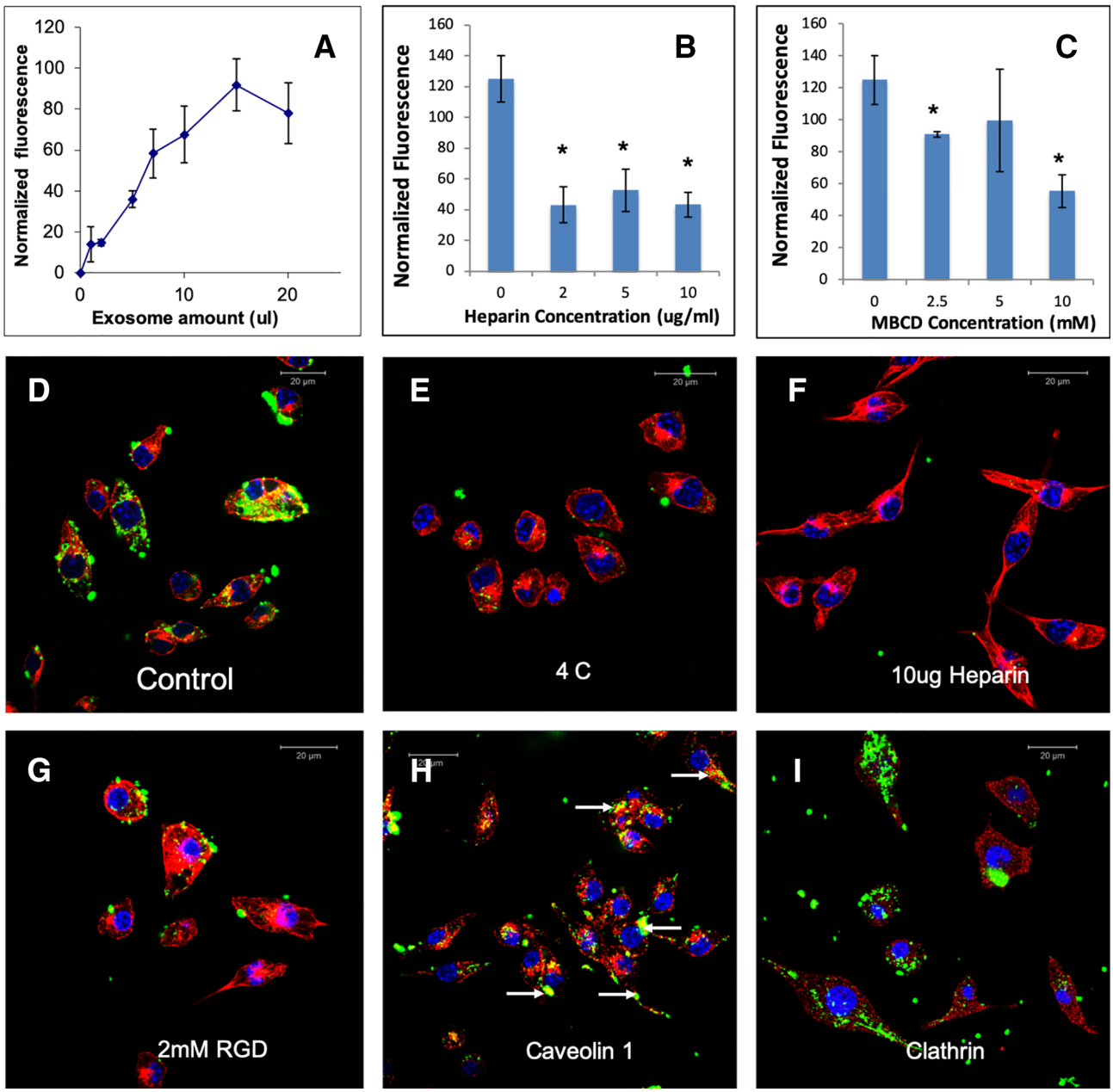

FIGURE 4 | Endocytosis of HMSC extracellular vesicles (EVs) by J774A.1 monocytes: **(A)** Graphical representation of dose-dependent and saturable endocytosis of fluorescently labeled human mesenchymal stem cell (HMSC) EV by J774A.1 cells. Data points represent mean fluorescence (n=6) +/− SD. The EV volume was standardized as described under the methods section. **(B)** Graph showing the dose dependent inhibition of HMSC EV endocytosis after pre-treatment of the EVs with heparin to block interaction with the cell surface heparan sulfate proteoglycans (HSPGs). Data represent mean percentage fluorescence with respect to control +/− SD. **(C)** Graph showing the reduction in HMSC endocytosis after disruption of target cell membrane cholesterol with varying doses of methyl-β-cyclodextrin (MBCD). Data is presented as mean percentage fluorescence with respect to control +/− SD. Representative confocal micrograph depicting the endocytosed fluorescently labeled HMSC EVs within target HMSCs after 1 h of incubation at 37°C. **(E)** Representative confocal micrograph indicating the abrogation of HMSC EV endocytosis when the experiment is performed at 4°C. **(F)** Representative confocal micrograph showing that pre-treatment of EVs with heparin blocks HMSC EV endocytosis. **(G)** Representative confocal micrograph of HMSC EV endocytosis after pre-treatment of the cells with 2 mM RGD peptide to block cell surface integrins. In images **(D–G)**, green fluorescence represents endocytosed EVs. Red fluorescence represents tubulin counter stain and blue fluorescence indicates 4′,6-diamidino-2-phenylindole (DAPI) nuclear stain. **(H)** Confocal micrograph showing colocalization of endocytosed HMSC EVs (green) with caveolin1 (red). **(I)** Confocal micrograph showing the absence of co-localization between endocytosed EVs (green) and clathrin (red). * represents statistical significance ($P < 0.05$, ANOVA followed by Tukey's *ad-hoc*) with respect to control.

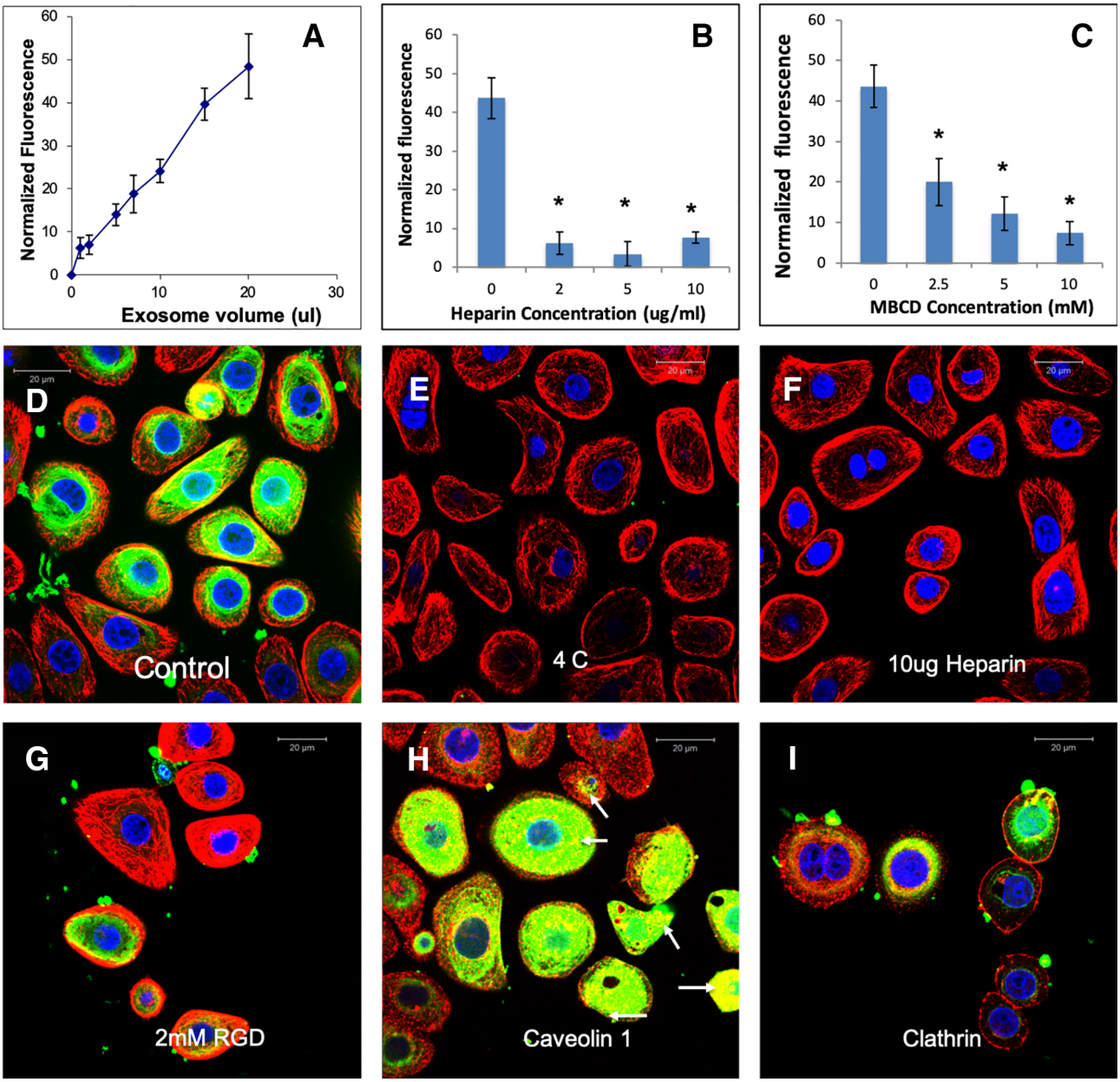

FIGURE 5 | Endocytosis of human mesenchymal stem cell (HMSC) extracellular vesicles (EVs) by TIGK gingival keratinocytes: **(A)** Graphical representation of dose-dependent and saturable endocytosis of fluorescently labeled HMSC EV by TIGK cells. Data points represent mean fluorescence (n=6) +/– SD. The EV volume was standardized as described under the methods section. **(B)** Graph showing the dose dependent inhibition of HMSC EV endocytosis after pre-treatment of the EVs with heparin to block interaction with the cell surface HSPGs. Data represent mean percentage fluorescence with respect to control +/– SD. **(C)** Graph showing the reduction in HMSC endocytosis after disruption of target cell membrane cholesterol with varying doses of methyl-β-cyclodextrin (MBCD). Data is presented as mean percentage fluorescence with respect to control +/– SD. Representative confocal micrograph depicting the endocytosed fluorescently labeled HMSC EVs within target HMSCs after 1 h of incubation at 37°C. **(E)** Representative confocal micrograph indicating the abrogation of HMSC EV endocytosis when the experiment is performed at 4°C. **(F)** Representative confocal micrograph showing that pre-treatment of EVs with heparin blocks HMSC EV endocytosis. **(G)** Representative confocal micrograph of HMSC EV endocytosis after pre-treatment of the cells with 2 mM RGD peptide to block cell surface integrins. In images **(D–G)**, green fluorescence represents endocytosed EVs. Red fluorescence represents tubulin counter stain and blue fluorescence indicates 4′,6-diamidino-2-phenylindole (DAPI) nuclear stain. **(H)** Confocal micrograph showing colocalization of endocytosed HMSC EVs (green) with caveolin1 (red). **(I)** Confocal micrograph showing the absence of co-localization between endocytosed EVs (green) and clathrin (red). * represents statistical significance ($P < 0.05$, ANOVA followed by Tukey's *ad-hoc*) with respect to control.

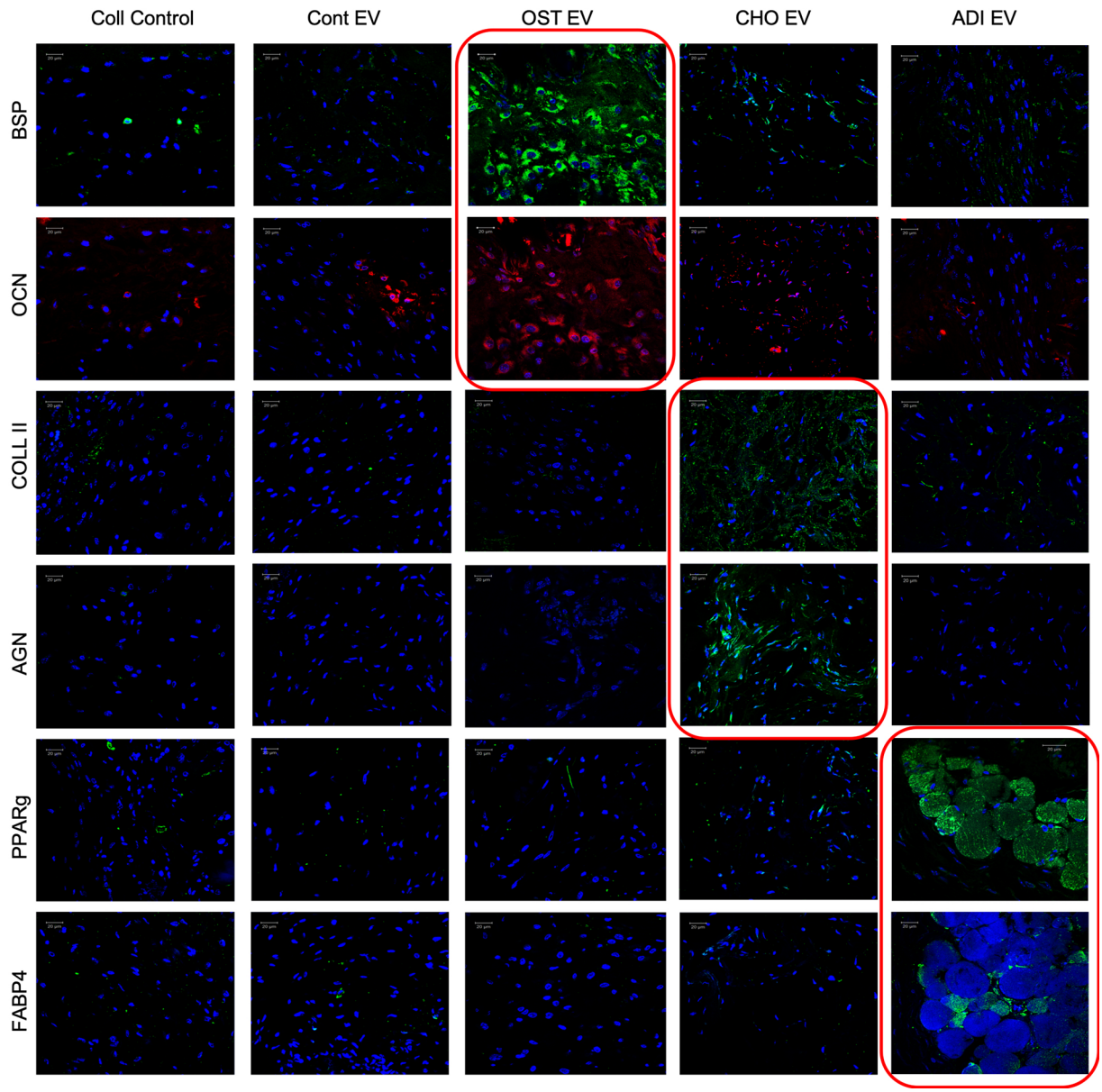

FIGURE 6 | Lineage-specific differentiation of human mesenchymal stem cells (HMSCs) *in vivo:* Confocal micrographs representing immunohistochemical staining for the presence of lineage specific proteins in the tissue explants from the subcutaneous implantation of HMSCs with and without respective control and lineage specific EVs. The red boxed images represent lineage specific protein expression. In all images, blue represents DAPI nuclear staining and red or green represents the immunolabeled protein. Scale bar represents 20 μm in all images.

indicated that the endocytic efficiency of HMSC EVs is not altered by changes to cell state. EVs isolated from osteogenic, chondrogenic, and adipogenic HMSCs did not show any significant difference in their dose-dependent ability to be endocytosed by naïve HMSCs. However, they were able to effect lineage specific changes within the target HMSCs *in vitro* and *in vivo*. We anticipate that this effect is due to the alterations to the exosomal cargo of miRNA, mRNA, and proteins. Our characterization of lineage specification by EVs from lineage differentiated HMSCs underscores the unique character of cell-type specific EVs. This novel finding that directing tissue-specific regeneration using EVs from differentiated HMSCs has wide-ranging applications in regenerative medicine.

Overall, the data presented in this study indicates that altering the HMSC cell state generates EVs with function-specific properties without altering EV characteristics, size distribution,

TABLE 1 | Extracellular vesicle (EV) mediated lineage-specific differentiation of human mesenchymal stem cells (HMSCs) *in vitro*..

GENE	Cont. EV	Ost. EV	Cho. EV	Adi. EV
Runx2	1.11 +/- 0.09	3.80 +/- 0.06	0.99 +/- 0.06	0.79 +/- 0.02
OSX	1.27 +/- 0.09	2.91 +/- 0.05	1.64 +/- 0.17	0.81 +/- 0.05
BMP2	1.09 +/- 0.07	7.97 +/- 0.26	1.20 +/- 0.11	0.89 +/- 0.07
BMP9	1.52 +/- 0.07	10.29 +/- 3.61	1.45 +/- 0.12	1.39 +/- 0.06
TGFb1	0.99 +/- 0.04	0.98 +/- 0.03	4.90 +/- 0.02	0.83 +/- 0.07
SOX9	1.09 +/- 0.08	1.12 +/- 0.09	5.71 +/- 0.41	0.63 +/- 0.03
COMP	0.97 +/- 0.05	0.98 +/- 0.13	3.15 +/- 0.33	0.72 +/- 0.02
COLL 2	0.73 +/- 0.05	0.68 +/- 0.02	10.93 +/- 2.19	0.09 +/- 0.01
PPARg	0.79 +/- 0.10	1.14 +/- 0.03	0.62 +/- 0.09	4.27 +/- 0.19
CEBPA	1.20 +/- 0.29	1.37 +/- 0.14	1.81 +/- 0.11	6.81 +/- 0.84
LPL	0.94 +/- 0.07	1.21 +/- 0.22	1.11 +/- 0.21	4.34 +/- 0.27
ADIPOQ	1.10 +/- 0.07	1.27 +/- 0.10	1.61 +/- 0.17	2.47 +/- 0.19

Table represents fold changes in gene expression levels of representative marker genes for osteogenic, chondrogenic and adipogenic differentiation of HMSCs after treatment of naïve HMSCs for 72 h with the EVs isolated from naïve and respectively differentiated HMSCs. The data are presented as mean fold change +/– SD with respect to control (n = 4). Red lettering within the table denotes lineage specific genes.

or endocytic ability. Furthermore, HMSC EVs were endocytosed by cell types from other germ layers in a similar dose-dependent and pathway-specific manner identifying a common mechanism of endocytosis. These results identify underlying mechanism and properties of HMSC derived EVs and provide an indication to how they may be manipulated for various applications in disease treatment and regenerative medicine.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding authors.

ETHICS STATEMENT

The animal study was reviewed and approved by the UIC Animal Care Committee (ACC).

AUTHOR CONTRIBUTIONS

C-CH is the first author and designed and performed most of the experiments. MK performed the experiments with the monocytes in LC's laboratory. RN was the technician that assisted C-CH in his experiments. LD's laboratory provided the keratinocytes and performed the endocytosis experiments using them. PG performed the *in vivo* experiments and edited the manuscript. SR conceptualized the original hypothesis, directed C-CH and RN, performed the imaging analyses, and wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2020.00163/full#supplementary-material

SUPPLEMENTARY FIGURE 1 | Cell viability: **(A)** Representative fluorescent images from live/dead cell assay (Life Technologies) performed on HMSCs cultured in the presence of growth medium (GM) and serum-free medium (SFM) for 24 hours. **(B)** represents results from MTS assay (Promega) performed on HMSCs cultured in the presence of GM or SFM (n=6) for 24 hours. Data in B represent mean +/- SD. No statistically significant difference was found between the two groups as measured by student's t-test.

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REVIEW
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Fat Therapeutics: The Clinical Capacity of Adipose-Derived Stem Cells and Exosomes for Human Disease and Tissue Regeneration

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Fat grafting is a well-established surgical technique used in plastic surgery to restore deficient tissue, and more recently, for its putative regenerative properties. Despite more frequent use of fat grafting, however, a scientific understanding of the mechanisms underlying either survival or remedial benefits of grafted fat remain lacking. Clinical use of fat grafts for breast reconstruction in tissues damaged by radiotherapy first provided clues regarding the clinical potential of stem cells to drive tissue regeneration. Healthy fat introduced into irradiated tissues appeared to reverse radiation injury (fibrosis, scarring, contracture and pain) clinically; a phenomenon since validated in several animal studies. In the quest to explain and enhance these therapeutic effects, adipose-derived stem cells (ADSCs) were suggested as playing a key role and techniques to enrich ADSCs in fat, in turn, followed. Stem cells - the body's rapid response 'road repair crew' - are on standby to combat tissue insults. ADSCs may exert influences either by releasing paracrine-signalling factors alone or as cell-free extracellular vesicles (EVs, exosomes). Alternatively, ADSCs may augment vital immune/inflammatory processes; or themselves differentiate into mature adipose cells to provide the 'building-blocks' for engineered tissue. Regardless, adipose tissue constitutes an ideal source for mesenchymal stem cells for therapeutic application, due to ease of harvest and processing; and a relative abundance of adipose tissue in most patients. Here, we review the clinical applications of fat grafting, ADSC-enhanced fat graft, fat stem cell therapy; and the latest evolution of EVs and nanoparticles in healing, cancer and neurodegenerative and multiorgan disease.

Keywords: adipose, stem cell, exosome, extracellular vesicles, regeneration

INTRODUCTION

Adipose dysregulation is fundamental to several important human disease states, such as obesity, chronic lymphedema and lipedema. In contrast to the unwanted effects of excess adipose tissue accumulation, however, adipose tissue also plays a critical physiological role (Fujimoto and Parton, 2011; Rajabzadeh et al., 2019). In humans, fat performs key functions, including energy storage and metabolism, thermoregulation, shock absorption and hormone metabolism (Nishimura et al., 2000; Yoshimura K, 2010). In addition, clinical use of fat tissue has revealed important potential therapeutic applications for adipose tissues in the treatment of human disease (Nishimura et al., 2000; Yoshimura K, 2010). Whilst the clinical use of fat initially began as a physical 'space filler' or 'contour correction' technique, it was through serendipitous observation of the tissues being filled with fat, that an even more important role has emerged – the role of adipose tissue as a putative therapeutic (Matsumoto et al., 2006).

An adipose derived stem cell (ADSC) is defined as a mesenchymal cell within adipose tissue with multipotent differentiation and self-renewal capacity. Adult stem cells have found an important role in tissue engineering and regenerative medicine, as they may be used to develop novel treatment approaches (Rajabzadeh et al., 2019). In particular, ADSCs are a most promising cell type for translational potential and for cell-based regenerative therapies, as they provide a new and unique source for multipotent stem cells that boasts ease and reproducibility of isolation using minimally invasive techniques with low morbidity. As multipotent ADSCs can differentiate into various cell types of the tri-germ lineages, including osteocytes, adipocytes, neural cells, vascular endothelial cells, cardiomyocytes, pancreatic β-cells, and hepatocytes; the use of fat/ADSCs and their cell products represents a paradigm of tissue regeneration and cell restoration.

Here, we review the treatment of human diseases using adipose tissue from its origins as the humble fat graft, through attempts to enrich the concentration of ADSCs within the grafts; to selective attempts to harness the potential paracrine effects of the ADSC secretome, and finally to the most recent evolution – the targeted use of ADSC exosomes (now known as EVs). We provide a review of the field to date, exploring the therapeutic application of ADSCs and small EVs as delivery vehicles of the ADSC secretome for clinical use in disease. As the focus of the review is ADSC cell products, previous theories of fat differentiating or homing in to replace tissue as 'building blocks', are not extensively addressed.

Fat Grafting, the Stromal Vascular Fraction and ADSCs

Fat Embryology, Anatomy and Physiology

Adipocytes that form adipose tissue arise from perivascular adipoblast stem cells in the third month of gestation (Matsumoto et al., 2006) *via* adipocyte precursors, which, in turn, differentiate into mature fat cells (Joseph et al., 2002). After adolescence, minimal new adipocytes are formed, and the role of fat cell replication, is thereafter undertaken by post-adipocytes. The ultimate number of fat cells formed is genetically determined, and slightly influenced by environment and nutrition (Fujimoto and Parton, 2011).

Within adipose tissue, lipid droplets may be uni- or multi-loculated (Fujimoto and Parton, 2011). Unilocular signet-ring shaped fat cells (25-200 μm diameter) are characteristic of 'white' fat. Multilocular cells, typically found in so-called 'brown' **or 'beige'** fat, consist of numerous smaller (60 μm) fat droplets (Joseph et al., 2002). Brown fat occurs in smaller quantities near the thymus and in dorsal midline region of the thorax, neck and abdomen (Nueber, 1893; Fujimoto and Parton, 2011) and plays a role in regulating body temperature *via* non-shivering thermogenesis, a mitochondrial mechanism of heat generation *via* a specific carrier called an uncoupling protein (Czerny, 1895; Joseph et al., 2002). In contrast, white fat performs three distinct functions of heat insulation, mechanical cushioning, and an energy source/storage sync; (Illouz, 1986; Joseph et al., 2002). Fat for clinical therapeutic use is sourced predominantly from areas of white fat.

Adipocytes have two different catecholamines receptors (lipolytic β -1 receptors that secrete lipase and α -2 receptors which block lipolysis) (Joseph et al., 2002). During weight gain, fat deposition occurs throughout the subcutaneous and visceral areas relatively evenly (Joseph et al., 2002), into existing adipocytes (hypertrophic growth) (Fujimoto and Parton, 2011). In contrast, when a patient is greater than thirty percent above the ideal weight (body mass index (BMI) over thirty-five), new fat cells are produced (hyperplastic obesity) (Fujimoto and Parton, 2011). Hyperplastic cells are more resistant to dieting and exercise (Tabit et al., 2012). During weight loss, visceral fat is preferential lost, due to greater sensitivity to lipolytic stimulation signals (Joseph et al., 2002). This a process associated with improved insulin resistance (Ross et al., 2014). Bariatric surgery reduces both visceral and subcutaneous fat, leading to overall improved metabolic profiles (Rajabzadeh et al., 2019), however surgery to remove subcutaneous fat (liposuction or abdominoplasty) do not lead to improved metabolic profiles (Ross et al., 2014). The largest amount of visceral fat occurs at level of umbilicus and the greatest amount of subcutaneous fat is found in the region of the buttocks; however, these distributions may vary significantly with gender (Mizuno, 2009). The abdomen and buttocks are the most commonly used areas for fat harvest for fat graft surgery (Ross et al., 2014).

The History and Evolution of Fat Grafting

An autologous graft is defined as the transfer of a tissue(s) to a distant area of the body, without its original blood supply (Nishimura et al., 2000) (**Figure 1A**). In order to survive, therefore, a fat graft needs to gain nutrients and a blood supply and from the native tissue bed into which it has been introduced. It needs early revascularization to avoid death of the grafted tissue (Nishimura et al., 2000; Yoshimura K, 2010). Unfortunately, due to poor graft re-vascularization, cell apoptosis or fat cell necrosis, up to 50%–100% of the initial injected volume may fail to engraft and become resorbed (Matsumoto et al., 2006).

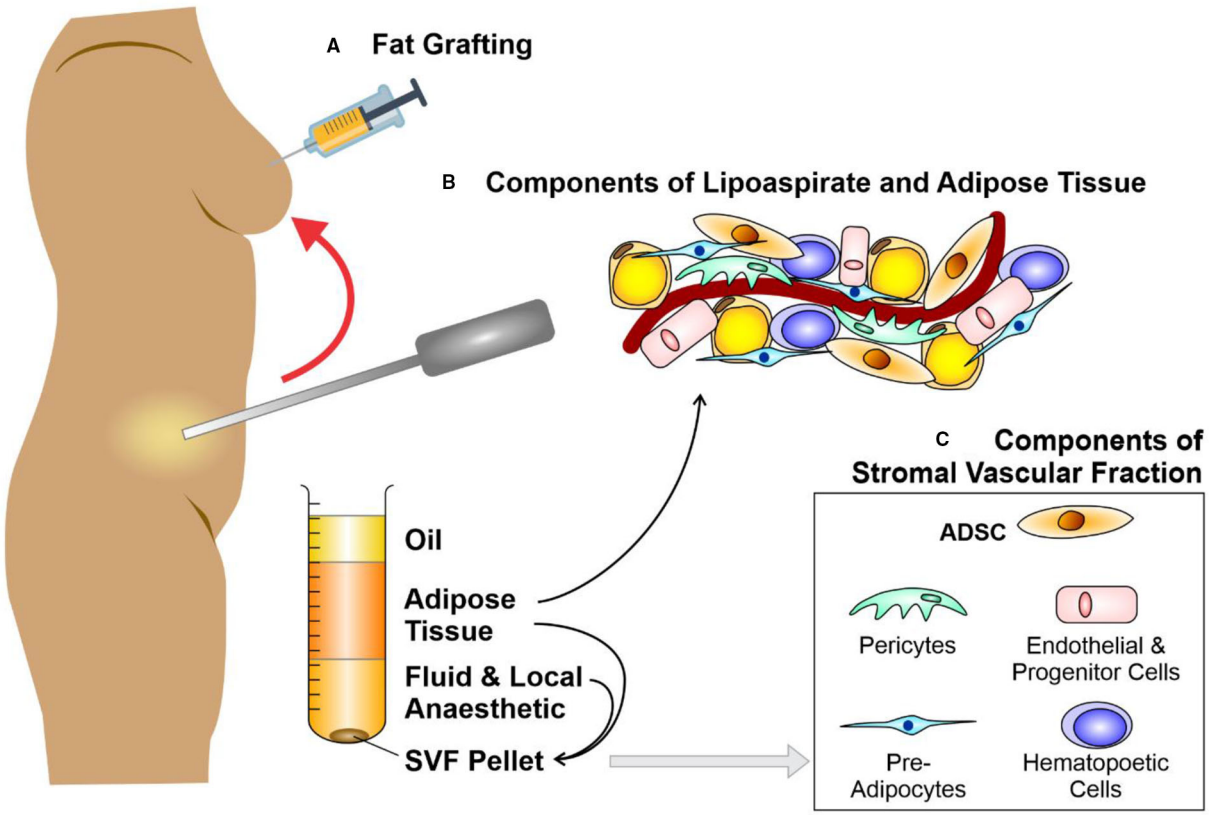

FIGURE 1 | Liposuction, fat grafting and the components of adipose tissue. Schematic diagram depicting **(A)** fat grafting after liposuction of subcutaneous fat from an abdominal donor site. The components of lipoaspirate **(B)** separate into layers of oil (discarded), aspirated adipose tissue and infranatant (composed of blood, plasma, and local anesthetic). The components of adipose tissue and the key constituents of the stromal vascular fraction (SVF) pellet **(C)** may be re-introduced to enhance the fat graft. Further processing of this adipose tissue with collagenase digestion and centrifugation allows the isolation of a SVF pellet. Figure adapted from Shukla et al. (2015) under the CC-BY license (Shukla et al., 2015).

Fat graft surgery was first performed by Neuber (1893), then expanded to breast reconstruction when a lipoma (benign fatty lesion) was transferred from the back to reconstruct a breast after cancer surgery (Czerny, 1895). By the 1980s, early rates of graft take [(approximately 50% (Illouz, 1986)] had failed to significantly improved, despite multiple technical refinements in graft harvest, centrifuge or infiltration (Carraway and Mellow, 1990). Irrespective of these loss rates, liposuction techniques using syringe harvest enhanced the popularity of fat grafting for correcting facial contour defects in the 1980s (Rohrich et al., 2004) and led to the introduction of fat grafting for the correction of soft tissue deficits in other body areas (Coleman, 2001; Yoshimura et al., 2008; Tabit et al., 2012; Ross et al., 2014) (**Figures 1A, B**). Nevertheless, significant numbers of patients who underwent fat grafting continued to suffer graft loss, and those in whom graft take was achieved endured up to 70% loss of volume (Matsumoto et al., 2006; Mizuno, 2009).

More recently, fat grafting has been used in staged breast reconstruction following oncological mastectomy, and has been adapted in some settings, to a single-stage, large volume injection procedure (Khouri R, 2009). Various authors have suggested differing methods of injection for achieving optimal graft take, ranging from individual droplet deposits (the so-called pearling technique) to a multilayered and multidirectional lattice configuration as an adaptation to the pre-existing standard 3 mm linear graft injections techniques (Coleman, 2001).

Overall, no consensus had been reached regarding the optimal technical procedure to maximize graft take. Whilst the nuances have been debated, the basic principle is that adipose tissues are removed from beneath the skin *via* minimal-access incisions using a hollow, blunt-ended but perforated steel surgical tube, attached to a source of external suction and collection reservoir.

Principles of Fat Grafting, Graft Enhancement and Treatment With ADSCs

Several technical modifications have been described to enhance fat graft reliability. It has been suggested that graft survival occurs through imbibition then angiogenesis (Kilroy et al., 2007) from surrounding tissues, promoted through hypoxic-driven protein growth factors. Therefore, various additions such as collagen, FGF, and insulin (Hong et al., 2010; Baek et al., 2011) were suggested to enhance adipocyte survival; however, did not result

in significant graft survival gains. The skin quality overlying areas of fat injection were anecdotally observed to improve, therefore, it was suggested that this may be an influence of stem cells within the introduced adipose cell population (Rigotti et al., 2007). The mesenchymal stem cells (MSCs) thought to be instrumental in these effects were hypothesized to originate from pre-adipocytes (ADSCs) within the stromal vascular fraction (SVF) of liposuction aspirate (Gimble et al., 2011); or from MSCs derived from blood vessels (Eto et al., 2011).

Regardless, of all the different variables in fat grafting, the concept of multipotent stem cells populating fat grafts became the new justification for the use of fat graft. ADSCs became the central focus of enhancing grafts and lately, a potential factor in reversing tissue injury, such as injury occurring in radiotherapy (Haubner et al., 2013). ADSCs were initially isolated nearly 2 decades ago by Zuk and colleagues (Zuk et al., 2001). Eto et al. suggested that ADSCs had lower metabolic demands and were more resistant to the mechanical trauma of fat grafting (Yoshimura et al., 2009; Eto et al., 2012; Trojahn Kolle et al., 2012), and were thus more robust compared to adipocytes (Zuk et al., 2001; von Heimburg et al., 2005; Shoshani et al., 2005; Lu et al., 2009; Tremolada et al., 2010; Suga et al., 2010; Piccinno et al., 2013). Other authors showed enhance graft survival rates due to greater levels of angiogenesis (via either imported endothelial progenitor cells or ADSCs) generating neo-vasculature (Thanik et al., 2009; Zhu et al., 2010; Kolle et al., 2013). Butala et al. on the other hand, postulated that ADSCs in a graft may themselves chemotactically recruit further stem cells, particularly from bone marrow, or differentiate into fat cells themselves (Zhu et al., 2010; Butala et al., 2010; Kolle et al., 2013).

To enhance the abundance of ADSCs within fat grafts (Caplan AI, 2006; Eto et al., 2012; Kolle et al., 2013; Wang et al., 2013) Yoshimura et al. proposed "cell-assisted lipotransfer enrichment" in which the surplus lipoaspirate was separated into components by centrifugation and the lipoaspirate supplemented with additional SVF (Matsumoto et al., 2006; Fraser et al., 2006; Yoshimura et al., 2008; Yoshimura K, 2010; Harfouche and Martin, 2010; Rigotti et al., 2010; Krumboeck et al., 2013). Briefly, SVF [comprised of 10% ADSCs (Zhu Y et al., 2008; Tabit et al., 2012; Akita et al., 2012)] is derived from a lipoaspirate component that is surplus to the volume needed to fill a particular soft-tissue deficit (Ross et al., 2014). Subsequent to digestion with collagenase, centrifugation creates an SVF pellet (**Figure 1C**). Eventually, the SVF pellet is introduced to the lipoaspirate, in readiness for injection with the ADSCs as part of a fat graft (Zuk et al., 2001; Kilroy et al., 2007; Mizuno, 2009; Yoshimura et al., 2009; Yoshimura K, 2010; Tremolada et al., 2010; Trojahn Kolle et al., 2012; Hsiao et al., 2012). A randomized control trial was designed by Kolle et al. to assess enrichment of lipoaspirate with ADSC concentrations of up to 2,000 times over physiological levels (Kim et al., 2009). Quantification using magnetic resonance scans suggested that ADSC-enriched groups yielded higher graft retention volumes (Caplan AI, 2006; Kolle et al., 2013).

Collectively, this work implied that enrichment of fat grafts could increase viability, volume retention, and neo-vascularization, whilst reducing necrosis rates. The findings also supported the theory that adding ADSCs may augment fat graft survival by bolstering adipogenesis, the supporting vasculature and/or diminishing cell apoptosis—key features of the regenerative properties of fat graft (Phinney and Prockop, 2007; Zhu et al., 2010; Collawn et al., 2012; Kolle et al., 2013).

Characteristics of ADSCs

ADSCs are defined as plastic-adherent cells (in standard culture conditions) (Dominici et al., 2006; Zimmerlin et al., 2011), cells exhibiting a $CD34^+$, $CD31^-$, and $CD45^-$ cell surface marker profile (Gronthos et al., 2001; Shayan et al., 2006; Yoshimura et al., 2006; Karnoub et al., 2007; Walter et al., 2009; Lin et al., 2010; Zimmerlin et al., 2011; AIHW, A, 2012; Authors on behalf of, I et al., 2012; Baer and Geiger, 2012; Zuk, 2013) and cells showing differentiation multi-potency into mature bone, cartilage, and fat (Zuk PA1 et al., 2002).

In adults, stem cells may uniquely differentiate into more specialized cell types to: i) replenish injured cells, ii) preserve tissue integrity, iii) maintain cell homeostasis during normal growth or healing (Caplan AI, 2006; Kim et al., 2009). Therefore, MSCs show promising utility in tissue regeneration (Ebrahimian et al., 2009; Harfouche and Martin, 2010; Bhang et al., 2011; Yan et al., 2011; Forcheron et al., 2012; Krumboeck et al., 2013; Yuan et al., 2013). As is the case in bone marrow derived MSCs, ADSCs are may undergo differentiation into a variety of distinct mature tissue types including fat, cartilage, bone, skin, muscle, endothelial, and nerve-like cells when grown with a particular set of induction factors (Zuk et al., 2001; Mizuno, 2009; Ebrahimian et al., 2009; Tremolada et al., 2010). ADSCs also boast the additional benefits that the stem cell yield from fat is 500-fold greater than that obtained from bone marrow (Fraser et al., 2006)—[$5x10^5$ ADSCs can be derived from 400-600g of fat (Zhu Y et al., 2008; Marigo and Dazzi, 2011)]; and that ADSCs easier and less invasive to harvest overall (Ross et al., 2014; Shukla et al., 2015).

In terms of the cellular secretory profile, ADSCs produce a more extensive range of chemokines, cytokines and protein growth factors (Caplan AI, 2006; Dominici et al., 2006; Kilroy et al., 2007; Locke et al., 2009; Blaber et al., 2012; Carrade et al., 2012; Cawthorn et al., 2012; Hsiao et al., 2012; Strioga et al., 2012). This secretome profile has contributed the understanding that, in contrast to previously held theories that ADSCs would differentiate to actually replace damaged cells (the "building block" or "host replacement" theories (Neuhof and Hirshfeld, 1923; Yoshimura et al., 2006; Kim et al., 2009; Zuk, 2013; Ross et al., 2014); the paracrine effects of the secretome are now considered as more likely to orchestrate the events needed tissue regeneration (Phinney and Prockop, 2007). The distinct makeup of the ADSCs secretome suggested that ADSCs may influence tissue regeneration by altering the biological and molecular cues driving (Gronthos et al., 2001; Kim et al., 2009; Baer and Geiger, 2012; Collawn et al., 2012; Forcheron et al., 2012), angiogenesis (Bhang et al., 2011; Zimmerlin et al., 2011; Matsuda et al., 2013; Yuan et al., 2013) and lymphangiogenesis (Lin et al., 2010; Yan et al., 2011); while suppressing local immune/inflammatory responses (Fraser et al., 2006; Rigotti et al., 2007; Delay et al., 2009; Tremolada et al., 2010; Marigo and Dazzi, 2011; Cawthorn

et al., 2012) and reducing fibrogenesis (Tremolada et al., 2010).

Since the time of the initial description of ADSCs, their molecular profile has been the subject of debate (Stone et al., 2003; Mazzola et al., 2011). This has been chiefly due to the description of different ADSC purification and culture protocols and differing use of sub-total vs. whole SVF (Coleman, 2001; Rigotti et al., 2007; Locke et al., 2009; Yoshimura K, 2010; Cawthorn et al., 2012; Strioga et al., 2012).

Safety Concerns and Legislative Implementation of Fat Grafting in Clinical Practice

Concerns regarding the use of ADSCs in clinical practice have been three-fold. Firstly, fears arose that introducing stem cells into a former cancer field might encourage recurrent cancer growth due to potential secretion of pro-angiongenic growth factors such as VEGF-A (Ross et al., 2014; Shukla et al., 2015). Secondly, it was hypothesized that chronic calcification occurring in the previously fat grafted areas may make screening/monitoring for the occurrence further cancer difficult (Ross et al., 2014; Shukla et al., 2015). Finally, the addition of components to enhance ADSC efficiency—such as collagenase processing—created the impression that the fat has been significantly altered and therefore ceases to be an autologous tissue transfer, but more a modified therapeutic product (Raposio and Ciliberti, 2017). The first of these reservations was addressed when it was contested that, despite *in-vitro* data that suggesting that introducing stem cells might promote cell proliferation, there was no equivalent definitive evidence *in-vivo* to that effect (Ross et al., 2014; Shukla et al., 2015; Simonacci et al., 2016). The second concern was deemed not to be an issue in the hands of an experienced radiologist, who should be expected to differentiate between benign "post-graft" and suspicious calcification (Ross et al., 2014; Shukla et al., 2015; Simonacci et al., 2016). A recommendation of the American Society of Plastic Surgeons against fat grafting for breast reconstruction was dropped in 2009, and subsequent case studies have upheld an acceptable risk profile. (Ross et al., 2014; Shukla et al., 2015; Simonacci et al., 2016). Finally, the addition of processing to fat graft to enhance take rates has rendered the fat graft unusable in some jurisdictions. In Europe, the use of collagenase digestion in fat grafting is considered to be a significant manipulation of the graft and therefore no longer to be homologous (Raposio and Ciliberti, 2017). The practical use of manipulative steps is therefore likely to remain a restricted procedure, and would likely need to pass regulatory approval steps akin to those stringent steps required of devices or genetically modified cell treatments.

Functions of ADSCs in Tissue Regeneration

Since the initial observations made in clinical fat grafting, adipogenic differentiation of ADSCs has been thought to result in restoration of tissue contour and volume. Clinical work indicates that there is new fat near the area of the fat graft introduction, which must have occurred *via* either; i) direct differentiation of introduced ADSC into adipocytes; or ii) ADSCs exerting paracrine effects to influence local stem cells to differentiate into adipocytes (Zuk et al., 2001; Rigotti et al., 2007; Delay et al., 2009; Ebrahimian et al., 2009; Kim et al., 2009; Mizuno, 2009; Uysal et al., 2009; Eto et al., 2011; Mazzola et al., 2011; Karathanasis V et al., 2013). The latter has gained favor of late.

Differentiation of Transplanted ADSC During Wound Healing

There are several studies demonstrating that transplanted ADSC can potentially promote wound healing by differentiating into specific cell types in animal models of wound healing. For example, Nie et al. showed that intradermally administered ADSCs facilitated wound closure in rats by enhancing re-epithelialization and granulation tissue deposition (Nie et al., 2011). The enhanced wound repair in these rats was attributed to differentiation of ADSC into epithelial and endothelial cells, which accelerated cutaneous regeneration and angiogenesis (Nie et al., 2011). Kim et al. assessed the efficacy of ADSCs in promoting wound healing introduced *via* three different techniques (topical application, intravenous injection and intramuscular injection) (Kim et al., 2019). This study found that mice treated with ADSC exhibited more stratified and differentiated epidermal and dermal layers, with more rapid re-epithelialization and vascularization regardless of the type of ADSC administration compared to control mice (Kim et al., 2019). Further, Wu et al. employed an ADSC-seeded silk fibroin chitosan film in a rat incisional cutaneous wound healing model, and showed accelerated wound healing and colocalization of transplanted ADSCs which displayed enhanced levels of endothelial markers CD31 and alpha-smooth muscle actin (α-SMA) (Wu et al., 2018). These findings were consistent with another study using an acute radiation ulcer model in rats, in which a portion of transplanted ADSCs were also shown to be colocalized with CD31 (Huang et al., 2013). These findings suggest that these ADSCs may have partially differentiated into endothelial cells to promote angiogenesis during wound healing. Lastly, subcutaneously injected ADSCs resulted in a significant increased angiogenesis and enhanced wound healing at 8 weeks post-implantation in rats (Kuo et al., 2016). Unfortunately, however, these studies failed to directly address the question of whether ADSCs promoted wound healing by differentiating into specific cells types, such as epithelial or endothelial cells, or whether—as the authors claimed—that the increased angiogenesis was due to the ADSC secretomes, including VEGF-A (Kuo et al., 2016). A limitation of these studies was that they were conducted using tissue immunofluorescence, which relies on optical co-localization of markers that can be more misleading in terms of positive ADSC and CD31 signals, compared to PCR that will tease out distinct cell populations that co-express numerous specific markers. Finally, no differentiation of ADSCs was detected in a rabbit model of wound healing 7 days after topical application, although the animals treated with ADSCs did increase granular tissue formation in the wound area (Hong et al., 2013). This finding may suggest that the microenvironment in wounds between rodents and rabbits is

critically different, or that ADSC differentiation may not play a significant role as the paracrine secretome of the ADSC population. Further research is required to better understand the differentiating capacity of transplanted ADSC *in vivo*.

Non-Differentiation Related Mechanisms: Enhancement of Angiogenesis and Lymphangiogenesis

Angiogenesis

Injection of the ADSCs into the recipient tissue bed is thought to increase perfusion of injured tissues and/or graft viability by: i) paracrine promotion of angiogenesis, or ii) supporting existing vascular structures. The concepts that support the existence of such regenerative mechanisms are based on several key findings regarding fat grafting in murine ischemic injury models (Eto et al., 2011). These experiments demonstrated that: i) ADSCs may differentiate into $CD31^+$ ECs *in-vivo*; ii) there was enhance density of blood vessels and co-localized fluorescent-labeled ADSCs in or near the vessels; and iii) ADSCs formed a vWF^+ vessel networks in a Matrigel matrix (Karathanasis V et al., 2013). Further, the release of angiogenic growth factors by ADSCs has been shown to promote revascularization and wound healing. These included proteins such as IGF, PDGF-bb, FGF, TGF-β, and interleukins IL-6, IL-8, stromal-related proteins MMP inhibitor 1 precursor, MCP-1, ANG, and SDF-1, and vascular-related proteins such as vascular endothelial growth factor (VEGF) -A, -C, and -D, (Rehman et al., 2004; Benvenuto et al., 2007; Kilroy et al., 2007; Kim et al., 2008; Lu et al., 2008; Ebrahimian et al., 2009; Mizuno, 2009; Pallua et al., 2009; Uysal et al., 2009; Marigo and Dazzi, 2011; Eto et al., 2011; Heo et al., 2011; Zografou et al., 2011; Baer and Geiger, 2012; Forcheron et al., 2012; Hsiao et al., 2012; Kapur and Katz, 2013; Haubner et al., 2013; Jiang et al., 2013; Karathanasis V et al., 2013; Yuan et al., 2013).

Lymphangiogenesis

ADSCs secrete lymphangiogenic factors that aid in lymphangiogenesis, improving or reversing lymphedema in damaged tissues. Lymphatic fluid stasis was found to result in increased TGF-β1, exerting a hypothesized further anti-lymphangiogenic effect. Blockade of TGF-β1 and ADSC stimulation, in contrast, lead to increased expression levels within ADSCs of lymphatic endothelial cell markers podoplanin and Prox-1 and of lymphangiogenic growth factor VEGF-C. In addition, the protein growth factors detected in ADSCs that differentiate them from other MSCs (VEGF-D, IGF-1, and IL-8) at baseline, all display pro-lymphangiogenic activity (Ji, 2007; Rigotti et al., 2007; Delay et al., 2009; Avraham et al., 2010; Mazzola et al., 2011; Yan et al., 2011).

Anti-Oxidant, Anti-Inflammatory and Anti-Fibrosis Effects

ADSCs may elicit regenerative benefits by exerting anti-oxidant effects, which in turn provide protective effects combatting cellular injury induced by radical oxygen species, hypoxia, and reperfusion effects following ischemia. Protein growth factors that have been implicated include PDGF-AA, HGF, IL-12, G-CSF, GM-CSF, IGFBPs. Pigmented epithelial derived growth factor, Superoxide dismutase may mediate these effects (Chen et al., 2008; Kim et al., 2008; Kim et al., 2009; Heo et al., 2011; Chang et al., 2013). Specific ADSC-induced cytokines have also been shown to modulate immune and inflammatory responses, as BMSCs, and ADSCs restrict the proliferation T-cells and B-cells through NFKB-mediated pathways. Further, IL-6 and IL-8 secretion act as attractants for monocytes and macrophages, which also promote wound healing processes (Ohnishi et al., 2007; Chen et al., 2008; Goh et al., 2010; Heo et al., 2011; Marigo and Dazzi, 2011; Nambu et al., 2011; Forcheron et al., 2012; Rodriguez-Menocal et al., 2012; Kapur and Katz, 2013; Haubner et al., 2013; Jiang et al., 2013).

An additional method of improving epithelialization and wound healing has been shown to be through modulation of granulation tissue formation and of fibrosis. ADSCs co-cultured with fibroblasts *in-vitro* appeared to modify extracellular matrix (ECM) remodeling through down-regulation of gene expression related to production of collagen types I and types III by fibroblasts. Functionally, treatment of keratinocyte and fibroblasts with conditioned media (CM) harvested from ADSC ($ADSC^{CM}$) lead to improved re-epithelialization (Bensidhoum et al., 2005; Francois et al., 2007; Mouiseddine et al., 2007; Ohnishi et al., 2007; Greenberger and Epperly, 2009; Gimble et al., 2010; Goh et al., 2010; Lee et al., 2010; Heo et al., 2011; Nambu et al., 2011; Lee et al., 2012; Rodriguez-Menocal et al., 2012; Zhang et al., 2012; Chang et al., 2013).

Overall, the endogenous stem cell recruitment along a chemokine gradient to the site of injury or inflammation resulted in improved wound healing, truncation of prolonged inflammatory responses and tissue regeneration (Greenberger and Epperly, 2009). Murine models have demonstrated that MSCs respond by aggregating to a site of tissue damage. Studies tracking systemically introduced human MSCs showed that they home to and became grafted into the site of ischemia or of a necrotic injury. In these studies, SDF1α, produced by ADSCs was the key chemoattractant of other stem cells to the injured area of tissue (Bensidhoum et al., 2005; Francois et al., 2007; Mouiseddine et al., 2007; Dewhirst et al., 2008; Greenberger and Epperly, 2009; Gimble et al., 2010; Suga et al., 2010; Eto et al., 2012; Zhang et al., 2012; Chang et al., 2013; Frazier et al., 2013).

Implications of Age-Related Changes to Fat Grafting in Clinical Practice

Several clinical applications for adipose-derived stem cell therapy are related to diseases that become more prevalent with age. In studies that examined the changes to the stem cell population, it was found that the differentiation and other functional profiles changes between cells from infanthood, middle age, and elderly donors (Jin et al., 2017). Other studies also demonstrated reduced proliferation and migration profile with age, however, this effect was less marked in adipose-derived cells than it was in bone marrow derived stem cell populations (Efimenko et al., 2015). When stem cells were harvested from aged patients and mice, ADSCs were more robust in terms of potential cell yield than was the case with other MSCs, however, in terms of the paracrine signaling and angiogenic potential of stem cells (e.g., in terms of VEGF-A production), there was a marked impairment seen in cells taken from older donors in both *in vivo* and *in vitro* models (Efimenko et al., 2015). Similarly, clonogenic potential in

ADSCs was reduced with age and all the effects were linked to a likely telomere shortening and accumulation of reactive oxygen species-related cellular injury (Efimenko et al., 2015). Overall, aging of donor stem cell populations may form an important limitation of the ability of ADSCs to delivery therapeutic benefits that can be derived from younger donor stem cell populations. This limitation may constitute an indication for ADSC function testing prior to clinical use, bolster the case for procedures to enhance ADSC efficacy, or herald the requirement for a delivery system that by-passes the ADSC itself to harness the paracrine secretome and cell products in a more targeted fashion—such as the use of exosomes.

Alternative Approaches to Deliver Beneficial Effects of ADSCs: Small Extracellular Vesicles

Extracellular Vesicles: Understanding Their Composition

Extracellular vesicles (EVs) are a heterogeneous population of nano- and micro-sized membrane-encapsulated cell particles that are fundamental mediators of intercellular communication. EVs constitute a diverse range of subtypes, namely microvesicles, exosomes, and several other EV populations, classified by The International Society for Extracellular Vesicles (ISEV) (Thery et al., 2018). All cell types continuously secrete EVs to the extracellular environment. EVs contain select proteins, peptides, RNA species (microRNAs, mRNAs, and long noncoding RNAs), lipids, and DNA fragments, that act locally or disseminate through circulation to act at specific distal sites to pleiotropically modulate cellular responses *via* paracrine signaling (Greening et al., 2016; Xu et al., 2018; Rai et al., 2019). The origin, nature, morphology, size and content of EVs are diverse and represent a novel signaling paradigm (Antonyak and Cerione, 2015). EV trafficking has been studied extensively in the area of oncology; however, there is now evidence of their seminal roles in intercellular communication in fetal-maternal signaling (Evans et al., 2019) and metabolism and tissue regeneration - particularly as trafficking intermediates for adipose tissue (Thomou et al., 2017). EVs may be divided into distinct classes, each with differing composition, capacity for selective packaging and potential for targeted delivery (and thus potential roles in disease). Comprehensive examination of the composition and molecular function of EVs in physiology and pathophysiology must be explored in the context of individual cell types, in order to facilitate cell-specific functions and therapeutic use [reviewed in (Greening and Simpson, 2018)].

Defining Extracellular Vesicles

Numerous terminologies have been described to define and identify EVs (Gould and Raposo, 2013). Overall, two main classes of EVs exist: large EVs (or shed microvesicles) and small EVs (or exosomes) (Colombo et al., 2014; van Niel et al., 2018). Large EVs (~150–1500 nm) are generated by outward blebbing of specific regions of the plasma membrane (Tricarico et al., 2017; van Niel et al., 2018; Mathieu et al., 2019). Small EVs (30–150 nm) originate as intraluminal vesicles (ILVs) through the endosomal maturation pathway (i.e., multivesicular bodies (MVBs)), which can release ILVs as exosomes into the extracellular space (Raposo and Stoorvogel, 2013).

During their biogenesis, EVs are selectively enriched with diverse cellular bioactive cargo molecules. RNAs (coding, non-coding), DNAs (single-/double-stranded), proteins (peptides, fusion proteins), and lipids are selectively incorporated into distinct types of EVs (van Niel et al., 2018; Mathieu et al., 2019). Further, diverse surface-bound proteins (e.g., receptors, tetraspanins) that are characteristic of the cell of origin, are selectively displayed on secreted EVs and play a crucial role in the recognition of target recipient cells and orchestrating EV localization; as well as uptake by recipient cells (Xu R. et al.,2019).

Although a growing number of studies have investigated the roles of EVs in cell–cell communication, an understanding of specific mechanisms behind their biogenesis and the heterogeneity of EVs and their subtypes remains rudimentary (Greening and Simpson, 2018). The heterogeneity of small EVs and the identification of non-vesicular extracellular content has raised concerns as to the content and function of some exosomes (Jeppesen et al., 2019). Currently, the extent to which small EVs (and exosomes) differ from other EVs in terms of their biogenesis and functions remains ill-defined; and specific markers that distinguish large from small EVs are the subject of much research (Ji et al., 2014; Greening et al., 2017; Greening and Simpson, 2018; Thery et al., 2018; van Niel et al., 2018; Xu et al., 2018; Zhang et al., 2018; Claridge et al., 2019; Jeppesen et al., 2019). This research includes the characterization of EV classes and their subtypes, imaging and tracking of EVs, mechanisms of cell and tissue targeting and internalization, post-translational and transcriptional regulation of EVs and their cargo, and administration and duration (i.e., transient vs. stable) of functional effects (Xu et al., 2016; Greening and Simpson, 2018; van Niel et al., 2018; Xu et al., 2018; Mathieu et al., 2019).

Isolating and Purifying Extracellular Vesicles for Biophysical Studies and Clinical Utility

The majority of rapid/one-step approaches for isolating EVs do not account for the fact that samples may contain a mixture of vesicle classes/subtypes and co-isolated contaminants such as high-molecular weight protein oligomers, RNA granules, and protein-RNA complexes (e.g., high-/low-density lipoproteins, argonaute-2/AGO2) complexes (Jeppesen et al., 2019). Varying methodologies for purifying (enriching) EVs and their modified versions exist, including differential (sequential) ultracentrifugation, density-based fractionation, gel permeation chromatography, affinity chromatography using bio-specific reagents (e.g., antibody targets), membrane ultrafiltration using low-centrifugal force, microfluidic devices, and synthetic polymer based precipitation reagents [for a discussion on application, yield/purity and scalability of these methods, see (Xu et al., 2016; Li et al., 2017)]. The choice of which method for EV isolation used depends on the specific research question or proposed use, as outlined below. Further detail of specific

guidelines as recommended by ISEV for studies of EVs has been reported elsewhere (Thery et al., 2018).

Stringent EV Isolation Procedures

EVs can be isolated and purified depending on the application. For stringent biochemical analysis [e.g. define their luminal cargo—RNA/DNA/lipid/protein species and surface-exposed proteins (Xu R. et al., 2019)] or specific functionality, rigorous purification strategies are critical, including immunoaffinity targeting. Antibody targets that have been successfully employed in this process include those directed against A33 (Mathivanan et al., 2010), EpCAM (Yoo et al., 2012; Tauro et al., 2012), MHC-II antigens (Clayton et al., 2001; Keryer-Bibens et al., 2006), CD45 (Coren et al., 2008; Mercier et al., 2013), CD63 (Caby et al., 2005; Oksvold et al., 2014), CD81 (Oksvold et al., 2014), CD9/CD1b/CD1a/CD14 (Wiley and Gummuluru, 2006), CD24/SWA11 (Rupp et al., 2011), and HER2 (Koga et al., 2005). Further, targeted EV capture based on bio-specific synthetic peptides (Ghosh et al., 2014) and proteoglycan enrichment (Christianson et al., 2013; Balaj et al., 2015) have been described. Other approaches to purify EVs include sequential centrifugal membrane ultrafiltration (Xu et al., 2015) and density-based fractionation using differential centrifugation (i.e., top- or bottom-loaded) (e.g., OptiPrep™/iodixanol) (Ji et al., 2013; Carrasco-Ramirez et al., 2016; Greening et al., 2016; Willms et al., 2016).

Generation of EVs for Therapeutic Studies

By virtue of their bioactive cargo EVs have inherent therapeutic potential (Dean et al., 2013; De Toro et al., 2015; Reiner et al., 2017). Small EVs from human MSCs have been used in tissue regenerative medicine to reduce infarction size in a mouse model of myocardial ischemia/re-perfusion injury (Lai et al., 2015). For these studies, large-scale production of functional homogeneous MSC-derived exosomes was accomplished using size-based fractionation. In another therapeutic application, small EVs from dendritic cells (and tumor cells) have been trialed in vaccine studies (Romagnoli et al., 2014; Kunigelis and Graner, 2015; Pitt et al., 2016; Tian and Li, 2017). Navabi et al. described a large-scale production method combining ultrafiltration and sucrose/deuterium oxide for generating good manufacturing (GMP) grade small EVs for use in clinical trials (Navabi et al., 2005).

Extracellular Vesicle Regulation of Adipose Function

Several key studies have demonstrated the role of EVs in adipose function. Recently, adipose tissue macrophages were shown to release exosomes containing a specific miRNA to facilitate glucose intolerance (from fat mice population) and insulin resistance (in lean mice population) (Wu et al., 2017). Exosome-containing miR-155 was shown to transfer into insulin target cell types, regulating cellular insulin response, insulin sensitivity, and glucose homeostasis (Wu et al., 2017). The ability of adipose tissue macrophage-derived exosomes to modulate systemic insulin and glucose tolerance *via* different miRNA compositions depended on their adipose phenotype (Wu et al., 2017). Thomou et al. further highlighted the contribution of adipose EVs to adipose function, with 653 miRNAs expressed in serum-derived exosomes from non-obese, or non-diabetic mice (Thomou et al., 2017). Importantly, adipocyte-specific Dicer KO mice were used to deplete adipocyte-derived miRNAs, revealing that exosomes from adipocytes containing miR-99b, inhibited liver FGF21 expression (Thomou et al., 2017). It was further suggested that these changes in FGF21 facilitated the overall phenotype of the Dicer KO mice. Interestingly, Ying et al. demonstrated that such changes were only marginally affected by adipose tissue macrophages-derived exosomes (Wu et al., 2017), indicating that significant differences are present between the miRNA profiles of different cell types within the source adipose tissue. Finally, it was observed that in adipocyte-specific Dicer KO, there was a substantial reduction in circulating exosomal microRNAs (Thomou et al., 2017).

A seminal study by Flaherty et al. identified that adipocytes communicate with adipose tissue macrophages through EVs (Flaherty et al., 2019). This is achieved by directly transferring lipids to differentiate bone marrow precursors into adipose tissue macrophage-like cells, with critical implications for obesity-associated pathologies (Flaherty et al., 2019). The authors highlighted the fact that adipose tissue from lean mice releases ~1% of its lipid content per day *via* exosomes *ex-vivo*, a rate that more than doubles in obese animals. Amose et al. also showed that EVs in human plasma increased significantly with BMI, supporting a role of EVs as metabolic relays in obesity (Amosse et al., 2018). This study demonstrated a key role for large EVs in the transfer of macrophage migration inhibitory factor (MIF) and the link between adipose-derived EVs and macrophage regulation.

Further investigating the role of exosomes in adipose tissue, Crewe et al. showed that adipose tissue EVs modulated crosstalk between adipocytes and stromal vascular cells for metabolic signaling and regulation (Crewe et al., 2018). Quantities of adipose tissue EVs were increased in a fasted state (compared with genetic and diet-induced obesity), partially because of glucagon-stimulated EV secretion from endothelial cells (Crewe et al., 2018). The authors showed dysregulation of important signaling proteins (antioxidant response, mitochondrial respiration) and lipid species involved in stress response. A critical finding was that extracellular molecules are internalized and packaged into EVs (Crewe et al., 2018), representing a new mechanism by which blood-borne signals are integrated into and supplied to adipose tissues.

In addition to influencing fat biology, components of the ADSC secretome have also been shown to promote wound healing and neuro-regeneration, making it an exciting focus for discovery of potential therapeutic targets (Hu et al., 2016; Yim N et al., 2016); particularly as engineering-specific EV delivery systems is now a reality (Yim N et al., 2016).

ADSCs for Therapeutic Application in Human Disease

Pre-clinical studies of ADSCs and ADSC-exosomes/EVs are listed in **Table 1** and **Table 2**, respectively. As the exosome/EV field is far less advanced than the clinical practice of fat grafting, the respective advances in the clinical application of each are considered together.

TABLE 1 | Pre-clinical studies of ADSCs.

Disease model	*In vitro* or *In vivo*	Function	Key findings with ADSC-CM	Reference
Cutaneous wound	*In vitro* and *in vivo*	Wound healing	Reduced UVB-induced wrinkles in mice. Also, ADSC-CM (conditioned media) inhibited UVB-induced apoptosis and enhanced type I collagen synthesis of human dermal fibroblasts	(Kim et al., 2009)
Cutaneous wound	*In vitro*	Wound healing	Accelerated collagen deposits in human dermis through up-regulation of fibroblasts TGF-β1	(Jung et al., 2011)
Cutaneous wound	*In vivo*	Wound healing	Promote neovascularization and wound repair by up-regulating *Tgfb-1*, *Fgfb,* & *Vegf* gene expression	(Hamada et al., 2019)
Cutaneous wound	*In vitro* and *in vivo*	Wound healing	Enhanced neovascularization and re-epithelialization of wounds by up-regulating VEGF, HGF an FGF protein expression	(Nie et al., 2011)
Cutaneous wound	*In vivo*	Wound healing	ADSC + platelet-rich plasma activated Rho GTPase signaling and lead to accelerated wound cell migration & re-epithelialization	(Zhang et al., 2019)
Secondary lymphedema	*In vivo*	Reduce tail swelling	Promote VEGF-C-mediated lymphangiogenesis and anti-inflammatory M2 macrophages recruitment	(Shimizu et al., 2012)
Radiation injury	*In vitro*	Lymph-angiogenesis	Promoted bFGF-mediated lymphangiogenesis in irradiated LECs	(Saijo et al., 2019)
Alzheimer's disease	*In vivo*	Neurogenesis	Secreted IL-10 and VEGF to reduce Aβ plaques and promote neurogenesis and cognitive functions	(Kim et al., 2012)
Alzheimer's disease	*In vivo*	Neurogenesis	Reduce oxidative stress and stimulate neuroblast proliferation to improve cognitive function	(Yan et al., 2014)
Parkinson's disease	*In vivo*	Neuroprotection	Inhibit dopaminergic neuronal cell death and reduce brain mitochondrial damage, restore mitochondrial function	(Choi et al., 2015)
Parkinson's disease	*In vivo*	Neuroprotection	Improved motor function by increasing BDNF and GFPA	(Berg et al., 2015)
Huntington's disease	*In vivo*	Neuroprotection	ADSC-extracts improve rotarod test and reduce mHtt aggregates and striatal atrophy *via* CREB-PGC1α	(Im et al., 2013)
Huntington's disease	*In vivo*	Neuroprotection	Improved rotarod performance and limb clasping, increased survival, protected striatal neurons and decreased mHtt aggregates	(Lee et al., 2009)
Acute kidney injury	*In vivo*	Renal protection	Attenuate I/R-induced renal damage by suppressing apoptosis and inflammation *via* reduction in levels of pro-apoptotic and pro-inflammatory cytokines	(Zhang et al., 2017)
Diabetic nephropathy	*In vivo*	Renal protection	Reduce oxidative stress and inflammation by inhibiting p38 MAPK signaling pathway	(Fang et al., 2012)
Breast cancer	*In vivo*	Tumor promotor or tumor suppressor	ADSC injected into tumor promote tumor growth, c.f. ADSC injected around tumor inhibits tumor growth	(Illouz, 2014)
Breast cancer	*In vivo*	Tumor promotor	Promoted pulmonary metastases by inhibiting miR-20b & activating c-Kit/MAPK-p38/E2F1 signaling	(Xu H. et al., 2019)

Wound Healing

A wound consists of an area of disrupted tissue integrity, architecture and homeostasis. It may be caused by trauma or by thermal or radiation injury (Devalia and Mansfield, 2008; Fry, 2017). The process of wound healing involves a series of organized molecular events including inflammation, neo-vascularization, scar tissue formation, and tissue remodeling (Gurtner et al., 2008); processes tightly regulated by specific growth factors, such as TGF-β, FGF, and PDGF (Grazul-Bilska et al., 2003). In most injuries, wound repair results in scar formation due to recruitment of collagen secreting fibroblasts to enhance the deposition of collagenous ECM (Gurtner et al., 2008). The beneficial effects of $ADSC^{CM}$ on wound healing have been reported in several pre-clinical studies. For example, reduced proliferative capacity and increased apoptosis seen in UVB-irradiated human dermal fibroblasts were reversed with $ADSC^{CM}$ treatment (Kim et al., 2009). Similarly, it was shown that $ADSC^{CM}$ stimulated synthesis of type I collagen by human dermal fibroblasts and reduced UVB-induced wrinkles in mice (Kim et al., 2009). Another study demonstrated that the mRNA expression of types I and III collagens were enhanced in human dermal fibroblasts following treatment with $ADSC^{CM}$ (Jung et al., 2011).

In addition, animal models have shown promising effects of ADSCs on accelerating wound repair. For example, treatment using artificial dermis as a supportive matrix impregnated with autogenic ADSCs in wounded rats resulted in increased vascularization and healing, which was mediated by increased gene expression of genes involved in tissue repair or angiogenesis [e.g., *Tgfb-1* and *-3*, *Fgfb* and *Vegf* (Hamada et al., 2019)]. Also in rats, Nie et al. employed an excisional wound healing model and demonstrated that ADSCs secreted pro-angiogenic mediators both *in vitro* and *in vivo* (e.g., VEGF-A, HGF, and FGF), in-turn promoting neo-vascularization and re-epithelial regeneration of wounds, thus accelerating the wound repair (Nie et al., 2011). Further, the wound healing effects of ADSCs in skin seems to be augmented when administered in combination with platelet-rich plasma containing several different protein growth factors and cytokines, including FGF, TGF-β and PDGF (Zhang et al., 2019). The study suggested enhanced wound closure in treated mice *via* activation of the Rho GTPase signaling pathway, which is involved in cell migration and invasion (Lawson and Ridley, 2018). Collectively, these findings suggest that ADSCs are a potential therapeutic tool for promoting wound healing.

TABLE 2 | Pre-clincial studies of ADSC-EVs.

Disease model	*In vitro* or *In vivo*	Function	Key findings	Reference
Myocardial I/R injury	*In vivo*	Cardio-protection	Reduced oxidative stress-induced necrosis and apoptosis in myocardium	(Cui et al., 2017)
Acute myocardial infarction	*In vivo*	Cardio-protection	Reduced cardiac apoptosis, fibrosis & inflammation *via* S1P/SK1/S1PR1 pathway & macrophage M2 polarization	(Deng et al., 2019)
Acute myocardial infarction	*In vivo*	Cardio-protection	miR-126-enriched ADSC-exosomes reduced cardiac inflammation & fibrosis, induce microvascular generation & migration	(Luo et al., 2017)
Stroke	*In vivo*	Neuro-protection	miR-126-enriched ADSC-exosomes induced neurogenesis, vasculogenesis & inhibit post-stroke inflammation	(Geng et al., 2019)
Stroke	*In vivo*	Neuro-protection	miR-181-b-5p-enriched ADSC-exosomes promote angiogenesis of brain microvascular ECs post O_2-glucose deprivation	(Yang et al., 2018)
Neural injury	*In vivo*	Neuro-protection	Reduced neuro-inflammation by suppressing microglia cells activation by inhibiting NF-κβ and MAPK pathways	(Feng et al., 2019)
Neural injury	*In vivo*	Neuro-regeneration	Promote axonal regeneration & myelination in atrophied gastrocnemius by stimulating secretion of neurotrophic factors from Schwann cells	(Chen et al., 2019)
Alzheimer's disease	*In vitro*	Neuro-protection	Inhibit formation of Aβ plaques and induce neuronal cells proliferation	(Lee et al., 2018)
Huntington's disease	*In vitro*	Neuro-protection	Reduce mutant Huntingtin protein aggregates, ameliorated abnormal apoptotic protein levels, & restored mitochondrial function	(Lee et al., 2016)
Parkinson's disease	*In vivo*	Neuro-protection	Reduce gene expression of GFAP, restore astrocytic injury, and increasing dopamine levels	(Meligy et al., 2019)
Acute kidney injury and chronic kidney disease	*In vivo*	Renal protection	Promoted tubular regeneration and inhibit AKI-CKD transition *via* SOX9 activation	(Zhu et al., 2017)
Acute kidney injury	*In vivo*	Renal protection	Combined ADSC + ADSC-exosomes reduce renal inflammation, oxidative stress, apoptosis, fibrosis, & glomerular & tubular damage	(Lin et al., 2016)
Diabetic nephropathy	*In vivo*	Renal protection	Inhibit podocyte apoptosis and induced podocyte autophagy through miR-486-mediated inhibition of Smad1/mTOR signaling pathway	(Jin et al., 2019)
Breast cancer	*In vitro*	Tumor promotor	Promote migration/proliferation of MCF7 human breast cancer cells *via* Wnt/β-catenin signaling pathway	(Lin et al., 2013)
Prostate cancer	*In vitro* & *in vivo*	Tumor suppressor	Inhibit tumor growth by activating caspase-3/7 pro-apoptotic miR-145 pathway	(Takahara et al., 2016)
HCC	*In vivo*	Tumor suppressor	miR-122 enriched ADSC-exosomes increase HCC chemosensitivity & inhibit tumor growth	(Lou et al., 2015)
Breast cancer	*In vivo*	Tumor suppressor	miR-379 enriched ADSC-exosomes inhibited tumor growth over 6 weeks	(O'Brien et al., 2018)

Extracellular Vesicles in Wound Healing

Geiger et al. investigated the application of human fibrocyte-derived exosomes in diabetic mice. They found that wound healing was significantly enhanced in all parameters studied (Geiger et al., 2015). Zhang et al. found human umbilical cord MSC-derived EVs to promote re-epithelialization of a wound model and improved the Wnt4 expression profile (Zhang et al., 2015). Similarly, Zhang et al. suggested that MSC-derived exosomes promote collagen formation and angiogenesis (Zhang et al., 2015). ADSC-derived exosome treatment of human dermal fibroblasts seemed to also induce enrichment of the microRNA within the fibroblasts that contribute to healing (Choi et al., 2018). In a murine wound model, Wang et al. suggested that IV administration of ADSC-exosome resulted in reduced scar size and altered metalloproteinases that may improve healing (Wang et al., 2017). Finally, Ren et al. showed that MVs from ADSCs stimulated proliferation and migration of fibroblasts, keratinocytes, and endothelial cells, particularly *via* the AKT and ERK signaling pathways both *in vitro* and *in vivo* (Ren et al., 2019).

Radiotherapy Soft Tissue Injury

Radiotherapy (RTX) is administered as part of cancer treatment, either before or after surgery or, unusually, in the absence of surgery (Ross et al., 2014; Shukla et al., 2015). The resulting injury may have devastating consequence in terms of chronic tissue fibrosis and breakdown that may expose vital underlying structures; or can cause secondary pain, contracture and functional impairment. ADSCs have been shown to enhance the quality of skin and soft tissues in clinical RTX injury and in animal models. These influences are thought to be mediated in a paracrine fashion by ADSC-secreted elements that counter the chemokine environment generated by the RTX-injury; this includes anti-inflammatory and anti-apoptotic effects (Ross et al., 2014; Shukla et al., 2015).

Haubner et al. investigated the influences of RTX in blood ECs, and showed enhanced gene expression of pro-inflammatory cytokines IL6, FGF, ICAM-1, and VCAM1. This model of co-culture with ADSCs showed restoration of expression profiles of all RTX-altered cytokines (Haubner et al., 2013). Chang et al. also utilized intra-peritoneal ADSCs after local RTX to show abrogation

of inflammation in treatment groups, with restored gastrointestinal tract (GIT) regeneration and enhanced survival (Chang et al., 2013). ADSC treatment was also linked with increased serum levels of IL10, VEGFA, bFGF, and EGF; in addition to increased SDF-1-mediated stem cells recruitment to the injured area (Chang et al., 2013). Further, Kojima et al. and Lim et al. showed protective influences of ADSC against RTX-induced salivary gland irradiation (Kojima et al., 2011; Huang et al., 2013).

In terms of skin and subcutaneous RTX-induced damage, ADSC treatment resulted in improvement in mouse models of chronic RTX-related impaired wound healing and in unwounded RTX-damaged skin [marked by altered collagen-based scar index measurements, increased dermal thickening and reduced fibrosis marker Smad-3 (Sultan et al., 2011; Huang et al., 2013)]. A similar study, investigating ADSC-enriched fat grafting in larger animals exposed to RTX, showed labeled ADSC integration into skin and concomitant enhanced wound repair, epithelialization, subcutaneous fat reserves and lower apoptotic rates. In addition, recruitment and activation of lymphoid cells was seen (Forcheron et al., 2012; Chen et al., 2014).

Lymphoedema

Lymphoedema is the chronic swelling of a limb caused by an accumulation of excess interstitial fluid. In time, if unresolved, the fluid accumulation may lead to the formation of excess subcutaneous fibro-adipose tissue (Brorson, 2003). This condition most commonly occurs in a limb and may be the result a developmental malformation that leads to poor interstitial fluid drainage *via* the lymphatic system (primary lymphoedema) (Lee and Villavicencio, 2010). Alternatively, as is the case in most patients, lymphoedema may develop subsequent to a trauma to the lymphatic system. Typically, secondary lymphoedema occurs following surgery or RTX for cancer (in the developed world) or due to filarial infection (in the developing world) that damage lymphatic vessels and impair lymphatic drainage. The pathological features of secondary lymphoedema include inflammation, adipogenesis, and fibrosis.

Shimizu et al. demonstrated the therapeutic potential of ADSCs in lymphangiogenesis by implanting ADSCs into a surgical mouse model of secondary lymphoedema. They showed that ADSCs stimulated lymphangiogenesis by secreting VEGF-C, and enhanced the recruitment of anti-inflammatory M2 macrophages, which were associated with significantly reduced tail swelling in the model (Shimizu et al., 2012). A recent study by Saijo et al. suggested FGF as a novel factor in the ADSC secretome that could potentially contribute to lymphangiogenesis in irradiated human dermal lymphatic endothelial cells (LEC), implying that ADSCs may ameliorate RTX-injury in LECs (Saijo et al., 2019). Counter to this, however, early lymphangiogenesis has been highlighted as a possible risk factor associated with developing the later stages of lymphoedema in a surgical mouse model of secondary lymphoedema; and, paradoxically, pharmacological inhibition of lymphangiogenesis suppressed lymphedema development in the model (Ogata et al., 2016). Thus, whether ADSC-mediated lymphangiogenesis could be therapeutically beneficial in lymphoedema remains elusive and requires further investigation.

Mechanistic and small EV-based functional studies by Greening et al. linked key components of cancer cell-derived EVs to the modulation lymphatic vessel formation and metastasis, demonstrating that lymphatics can also be responsive to secretome components (Carrasco-Ramirez et al., 2016). This study demonstrated critical functional effects on lymphangiogenesis mediated by vesicle surface podoplanin (hitherto considered a passive marker of lymphatic endothelial tissue) on small EVs, using a specific neutralizing monoclonal surface-specific antibody. It also highlighted a key role of podoplanin in biogenesis and release of EVs, and in lymphangiogenesis function. However, the role of the ADSC secretome as a driver of lymphatic repair after RTX or other lymphatic injury, remains to be revealed.

Neurodegenerative Diseases

ADSCs in the Treatment of Neurodegenerative Diseases

The use of ADSCs has shown promising pre-clinical results in studies investigating several important neurodegenerative disorders, such as Parkinson's disease, Alzheimer's disease, and Huntington's disease. A study using a murine Alzheimer's disease model showed that treatment with human ADSCs significantly enhanced levels of the anti-inflammatory cytokine IL-10, as well as key neurotrophic (and vasculogenic) factors, including VEGF-A - which led to a marked reduction in Aβ plaques and memory impairment, and elevation of endogenous neurogenesis and dendritic stability (Kim et al., 2012). Furthermore, autologous implantation of mouse ADSCs in mice with Alzheimer's disease enhanced regeneration of neuroblasts and reduced oxidative stress in the brain, which in turn alleviated cognitive impairment (Yan et al., 2014). Exosomes from ADSCs have also been shown to transfer enzymatically active neprilysin, a Aβ-degrading enzyme, *in vitro* (Katsuda et al., 2013). Importantly, this study showed that ADSC exosome-mediated function was more significant than bone marrow derived MSCs, contributing to prevention of extracellular plaque formation, subsequent pathogenesis and a potential Alzheimer's disease therapeutic.

In terms of Parkinson's disease, a common chronic progressive neurodegenerative movement disorder characterized in patients as diminished brain dopamine levels, numerous studies have been performed assessing the therapeutic potential of human ADSCs on a 6-hydroxyldopamine (6-OHDA)-induced mouse Parkinson's disease model (Berman and Hastings, 1999). Mitochondrial dysfunction in the brain is known to contribute to pathogenesis of the disease by increasing reactive oxygen species and hence oxidative stress, which exacerbates damage to the dopaminergic neurons in Parkinson's disease (Berman and Hastings, 1999). Choi et al. demonstrated that ADSCs significantly improved behavioral performance by decreasing dopaminergic neuronal cell death and the population of damaged mitochondria in the mouse brain; as well as by recovering mitochondrial functions in the brains of ADSC-injected mice (Choi et al., 2015). It has also been shown that human ADSCs significantly enhanced expression of brain-derived neurotrophic factor (BDNF) and improved motor lost function in the 6-OHDA murine Parkinson's disease model (Berg et al., 2015), suggesting a pro-healing effect. Interestingly,

however, the levels of glial fibrillary acidic protein (GFAP), were shown to be up-regulated in the brain of ADSC-treated animals (Berg et al., 2015). GFAP is a common indicator of dysfunctional astrocytes, the most abundant central nervous system glial cells. They may contribute to the progression of Parkinson's disease and GFAP upregulation is a possible sign of neuronal regeneration, however, it should be noted that a definitive role for GFAP is not yet agreed upon (Berg et al., 2015).

Huntington's disease is a progressive, fatal hereditary neurodegenerative disorder characterized by accumulated mutant *Huntingtin* (mHtt) protein in neural cells, which affects mitochondrial energy metabolism to accelerate cell death by progressive brain atrophy. Therefore, altered mitochondrial energy metabolism due to an impaired CREB-PGC1α pathway is a key risk factor in disease progression, which is characterized by an accumulation of mHtt in the brain (Cui et al., 2006; Chaturvedi et al., 2010). Im et al. investigated the influences of cell-free extracts of human ADSC (ASC-E) on R6/2 mice, which developed Huntington's disease, and found that ASC-E induced activation of the p-CREP-PGC1α pathway and amelioration of mHtt aggregates as well as striatal atrophy in the brain of R6/2 mice (Im et al., 2013). Also, injection of ASC-E in the mouse model slowed progression of the Huntington's disease phenotype, including weight loss and declining rotarod performance; although the molecular contents of the ASC-E that exerted these therapeutic effects was not assessed in this study (Im et al., 2013). Similarly, ADSC implantation in the R6/2 murine Huntington's disease model also showed beneficial effects, such as enhanced rotarod performance, limb clasp and survival; and attenuation of striatal neurons loss; as well as diminished brain aggregation of mHtt (Lee et al., 2009). These results were found to be driven by CREB-PGC1α pathway activation (Lee et al., 2009). Altogether, these studies suggest that ADSC treatment could constitute a novel treatment tool useful in ameliorating key pathogenic steps in the development of Huntington's and other similar neurodegenerative diseases.

Exosomes in the Treatment of Neurological Diseases

There have been a few studies demonstrating critical roles of ADSC-exosomes in neuro-protection and neuro-regeneration owing to their capacity to cross the blood-brain barrier (Alvarez-Erviti et al., 2011). For instance, ADSC-exosomes have been shown to mediate functional neuro-regeneration in stroke. Geng et al. demonstrated in a rodent model that miR-126 enriched ADSC-exosomes enhanced neurogenesis and vasculogenesis after stroke (Geng et al., 2019). These results are in keeping with a rat experiment undertaken by Yang and colleagues, in which miR-181b-5p-enriched ADSC-exosomes promoted mobility and angiogenesis of brain microvascular endothelial cells in stroke (Yang et al., 2018). The manner in which exosomes transverse the blood-brain barrier by using transcytosis through endothelial cells are capable of mediating astrocytes to degrade the cell cytoskeleton (Morad et al., 2019), and have only recently been elucidated. Furthermore, neuroinflammation is a major complication of brain injury, which is triggered by the activation of microglia cells in the central nervous system (Dheen et al., 2007). miR-126-enriched ADSC-exosomes were shown to significantly inhibit post-stroke inflammation by suppressing activation of microglial cells and reducing pro-inflammatory cytokine levels in the rat brain (Geng et al., 2019). Feng et al. also demonstrated ADSC-exosomes to inhibit microglial activation by inhibiting the pro-inflammatory MAPK and NF-κβ signaling pathways, which protected rat brain neural cells from injury (Feng et al., 2019).

Potential gene candidates in ADSC-exosomes that underpin these therapeutic effects have been explored using models of neurite outgrowth and sciatic nerve regeneration. Bucan et al. showed rat ADSC-exosomes to contain a range of neurotrophic factors, such as glial-cell derived neurotrophic factor, FGF-1, BDNF, ILGF-1, as well as nerve growth factor (NGF) (Bucan et al., 2019). Schwann cells are also simulated by neurotrophic factors NGF and BDNF and elicited pro-regenerative effects in nerve regeneration after nerve damage (Jessen and Mirsky, 2019). Chen et al. also demonstrated that exosomes derived from human ADSCs enhanced secretion of BDNF and NGF by Schwann cells, which led to increased proliferation, myelination, migration of cells in a dose-dependent manner *in vitro* (Chen et al., 2019). Additionally, this study assessed the effects of ADSC-exosomes on gastrocnemius muscle atrophy (a readout of sciatic nerve injury in rats) and found that treatment with the ADSC-exosome improved muscle atrophy by promoting axonal regeneration and myelination; although exosomal components that exerted these effects remained unidentified (Chen et al., 2019). Lastly, another study showed ADSC-exosomes to inhibit apoptosis and increase proliferation of Schwann cells in rats after nerve injury (Chen et al., 2019); an additional potential mechanism by which the ADSC-exosomes may promote nerve regeneration.

Several other studies demonstrated beneficial effects of ADSC-exosomes on key neurodegenerative diseases. Lee et al. demonstrated that ADSC-EVs significantly reduced the levels of Aβ plaques in Alzheimer's disease, inhibiting apoptosis of neuronal cells and augmenting neurite outgrowth of neuronal cells *in vitro* (Lee et al., 2018). In Huntington's disease (Cho et al., 2019), Lee *el al.* showed that Huntington's disease model that ADSC-EVs profoundly decreased mHtt aggregates and inhibited apoptosis of neuronal cells *in vitro*. Mitochondrial dysfunction was attenuated by activation of the proliferator-activated receptor γ coactivator 1α (PGC1α) and cAMB response element binding protein (CREB)-peroxisome pathways (Cui et al., 2006; Chaturvedi et al., 2010; Lee et al., 2016). Finally, in Parkinson's disease (McGregor and Nelson, 2019). Meligy et al. studied a rotenone-induced rat model of Parkinson's disease to demonstrate that ADSC-EVs significantly increased levels dopamine in the treatment group compared to the control (Meligy et al., 2019). In contrast to the overexpression of GFAP seen in animals treated with ADSCs (Clairembault et al., 2014), it was shown that ADSC-EVs markedly *decreased* the gene expression of GFAP, restored astrocytic injury, and improved motor performance in their Parkinson's disease model (Meligy et al., 2019). This suggested that GFAP may play a different role in neuroprotection in the same model whether treated with

ADSCs or ADSC-EVs. Overall, these results indicated that the ADSC-EVs may have reparative potential in incurable neurodegenerative disorders. Further studies are needed to understand the neuroprotective mechanisms by EVs.

ADSCs in Renal Diseases

AKI is a complex clinical condition characterized by deteriorating renal function due to decreased renal perfusion, blood supply and glomerular filtration rates, caused by damage to nephron structures (Prowle et al., 2010; Ostermann and Joannidis, 2016). AKI may progress to long-term chronic kidney disease (CKD), for which there is currently no cure (Rafieian-Kopaei, 2013). Thus, prevention of transition of AKI to CKD is critical. Implantation of ADSCs has been shown to yield beneficial effects on rat models of acute kidney injury (AKI). For example, ADSC treatment in an ischemia/reperfusion (I/R)-induced rat model of AKI significantly decreased the number of apoptotic kidney cells and effectively restored urine protein and serum creatinine levels (Zhang et al., 2017). This finding suggested restoration of kidney function by ADSC treatment, and was consistent with the findings by Lin et al. (Lin et al., 2016). Moreover, ADSC treatment lead to markedly reduced expression levels of multiple pro-inflammatory cytokines, for example, IL-6, TNF-α, and IFN-γ; however, was associated with elevated expression of anti-inflammatory cytokine, IL-10 (Zhang et al., 2017) at the mRNA level. Furthermore, ADSC treatment effectively ameliorated diabetic nephropathy by reducing oxidative stress and inflammatory cytokines levels (e.g. IL-6 and TNF-α), by mediating the inhibition of the pro-inflammatory p38 MAPK signaling pathway (Fang et al., 2012), a factor involved in the development of human diabetic nephropathy (Adhikary et al., 2004).

Extracellular Vesicles and Renal Disease

ADSC-EVs have been demonstrated to have a pivotal role in protection from the development of AKI. Zhu et al. studied downstream effects of using ADSC-EVs to prevent transition of AKI to CKD, in a mouse model of renal I/R injury. The authors showed that mice treated with ADSC-EVs exhibited decreased renal I/R injury and increased proliferation of renal tubular epithelial cells, thus attenuating AKI (Zhu et al., 2017). Notably, treatment with ADSC-EVs resulted in upregulation of tubular SOX9 gene expression (Zhu et al., 2017), a key gene involved in renal repair and renal tubule epithelial cell regeneration (Kumar et al., 2015; Kang et al., 2016). Furthermore, reduced levels of the pro-fibrotic cytokine TGF-β1 were observed following the ADSC-EV treatment in the model, suggesting that the EVs inhibited TGF-β1-induced renal fibrosis (Zhu et al., 2017), a key feature of CKD (Humphreys, 2018). Another study by Lin et al. demonstrated that inflammation, oxidative stress, apoptosis, fibrosis, and glomerular and renal tubular damage were mitigated by a combined treatment of ADSC-EVs and ADSCs in a rat model of renal I/R injury (Lin et al., 2016).

In diabetic nephropathy, a common variety of CKD due to impaired podocyte autophagy resulting from aberrant activation of the mTOR signaling pathway, a more recent study employed a spontaneous diabetic mouse model to assess the roles of ADSC-EVs (Godel et al., 2011; Tagawa et al., 2016). It was demonstrated that serum creatinine and blood urea nitrogen and total urinary protein levels, indicators of renal dysfunction, were significantly reduced by ADSC-EVs in diabetic mice (Jin et al., 2019). This finding correlated with the study in AKI carried out by Lin et al. (Lin et al., 2016). Additionally, ADSC-EVs were shown to enhance autophagy (the body's clearance of cellular debris) and diminish podocyte apoptosis by restricting Smad1/mTOR pathway activation *via* miR-486 (Jin et al., 2019). Activation of miR-486 is important as expression of miR-486 has been found to be down-regulated in diabetic patients when compared with non-diabetic individuals (Regmi et al., 2019), implying that miR-486-enriched ADSC-EVs could be a potential therapeutic for treating diabetic nephropathy. Overall, these findings suggest a therapeutic use for ADSCs in kidney diseases such as AKI and diabetic nephropathy, given their capacity to suppress oxidative stress and inflammation; and the possible additional future efficacy of ADSC-EV in AKI.

ADSCs in Cancer

A study using a xenograft mouse model of human breast cancer showed that human ADSCs promoted tumor growth when injected *into* a tumor. In contrast, ADSCs inhibited tumor growth when injected *around* the tumor (Illouz, 2014), suggesting distinct influences of ADSCs in different tumor microenvironments. A recent study by Xu et al. showed that ADSCs could promote metastases in mice xenografted with breast carcinoma through ADSC-released stem cell factor-mediated inhibition of miR20b, which in turn, lead to activation of the c-Kit/MAPK-p38/E2F1 signaling pathway and increased expression of HIF-1α and VEGFA (Xu H. et al., 2019). Meanwhile, upregulation of miR20b reduced metastasis of 4T1 breast cancer cells to the lung, suggesting that miR20b acted as a tumor suppressor miRNA, and that ADSCs may be able to induce lung metastases *in vivo*, through miR-20b inhibition (Xu H. et al., 2019). In contrast, miR-20b was also shown to enhance breast cancer proliferation both *in vitro* and *in vivo* by inhibiting expression of the phosphatase and tensin homologue (PTEN) gene (Zhou et al., 2014), a well-known tumor suppressor gene involved in regulation of breast cancer cells (DeGraffenried et al., 2004). This discrepancy may be due to heterogenous roles of miR-20b in regulating breast cancer development in the presence of ADSCs and the ADSC secretome; or may be due to poor study design. Hence, before conclusions can be drawn, this area warrants further detailed studies. Controversies regarding the regulatory approval for use of fat grafting in a former or current tumor bed are summarized above and in (DeGraffenried et al., 2004).

ADSC-Derived Extracellular Vesicles in Cancer

Given the capacity of EVs to exert their effects by transferring proteins and RNA to target cells, the effects of EVs in promoting cancer progression has been studied extensively [reviewed in (Xu et al., 2018)]. It appears that ADSC-EVs have dual (or contradictory) functions in regulating tumorigenesis, both by promoting and inhibiting the growth of cancer cells. For instance, platelet-derived growth factors stimulate ADSCs to

TABLE 3 | Completed and ongoing clinical trials of ADSCs.

Diseases	Study phase	Intervention or treatment	Autologous/Heterologous/Allogeneic	Key findings ADSC/EVs	Reference
Continued					
Fingertip injury	Pilot study	Injections at the site of injury	Autologous	Accelerate wound healing process and recovery of sensory function	(Tarallo et al., 2018)
Idiopathic pulmonary fibrosis	Ib	Intravenous injections of ADSC-derived SVF	Autologous	Similar survival rates disease progression time in untreated populations. Fail to demonstrate any beneficial effect by ADSC therapy	(Ntolios et al., 2018)
Refractory Perianal fistula in Crohn's disease	III	Local injections of allogenic expanded ADSCs	Autologous	Remission of fistula openings and reduce perianal disease (MRI)	(Philandrianos et al., 2018)
Secondary progressive multiple sclerosis	I/II	Intravenous injections	Autologous	Safe & feasible in patients. No significant changes in safety parameters	(Fernandez et al., 2018)
osteoarthritis	I/IIa	Intra-articular injections	Autologous	Safe and improved pain, function and cartilage volume of knee joint	(Song et al., 2018)

Diseases	Study phase	Intervention or treatment	Autologous/Heterologous/Allogeneic	NCT number
Chronic kidney diseases	I/II	Intravenous injection	Autologous	NCT03939741
Diabetic foot ulcer	I/II	ADSC-enriched fibrin gel	Autologous	NCT03865394
Chronic obstructive pulmonary disease	I	Intravenous injection	Autologous	NCT02161744
Isolated Articular Cartilage Defects	Unknown	ADSC-enriched acellular dermal matrix	Autologous	NCT02090140
Moderate to Severe Chronic Kidney Disease	I/II	Allogenic injection	Allogeneic	NCT02933827
Knee Osteoarthritis	I/II	Intra-articular injection	Allogenic	NCT02784964
Scars or cutis laxa	I/II	Autologous injection combined with laser therapy	Autologous	NCT03887208
Stroke	I	Intravenous injection	Unknown	NCT03570450
Knee osteoarthritis	III	Intra-articular injection	Autologous	NCT03467919
Knee osteoarthritis	Unknown	Transplantation	Autologous	NCT03014401
Vestibulodynia	Unknown	Transplantation	Unknown	NCT03431779
Alopecia	Unknown	Transplantation	Unknown	NCT03427905
Ischemic Heart Disease and Left Ventricular Dysfunction	I	ADSC-enriched VB-C01 collagen patches	Allogeneic	NCT03746938
Facial Rejuvenation	Unknown	Intradermal injection	Autologous	NCT03928444

release EVs containing pro-angiogenic factors—such as Axl (Tanaka and Siemann, 2019), artemin (Banerjee et al., 2012) and stem cell factor (Zhang et al., 2000)—which have been shown to enhance angiogenesis in human microvascular endothelial cells (Lopatina et al., 2014). An *in vitro* study demonstrated that ADSC-EVs promoted migration and proliferation of MCF7 human breast carcinoma cells through activation of Wnt/β-catenin signaling (Lin et al., 2013), although the involvement of angiogenesis was not assessed.

In contrast, there have been a few studies demonstrating that ADSC-EVs can act as tumor suppressors. For example, Takahara et al. demonstrated notable reduction in prostate cancer growth in tumor-bearing mice following ADSC-EVs treatment, an effect mediated *via* activation of the caspase-3/7 pro-apoptotic pathway, itself signaling *via* miR-145 (Takahara et al., 2016). The therapeutic potentials of microRNA-enriched EVs have also been explored in several tumor models. For example, miR-122 is highly expressed in the liver, and loss of miR-122 correlated with development of hepatocellular carcinoma (HCC) in mice (Tsai et al., 2012). Lou et al. demonstrated that miR-122 transfected ADSC-secreted EVs were rich in miR-122, and that uptake of these EVs by cultured HCC cells lead to increased chemosensitivity to chemotherapeutic agents and significant reduction in tumor growth *in vivo* (Lou et al., 2015). Similar

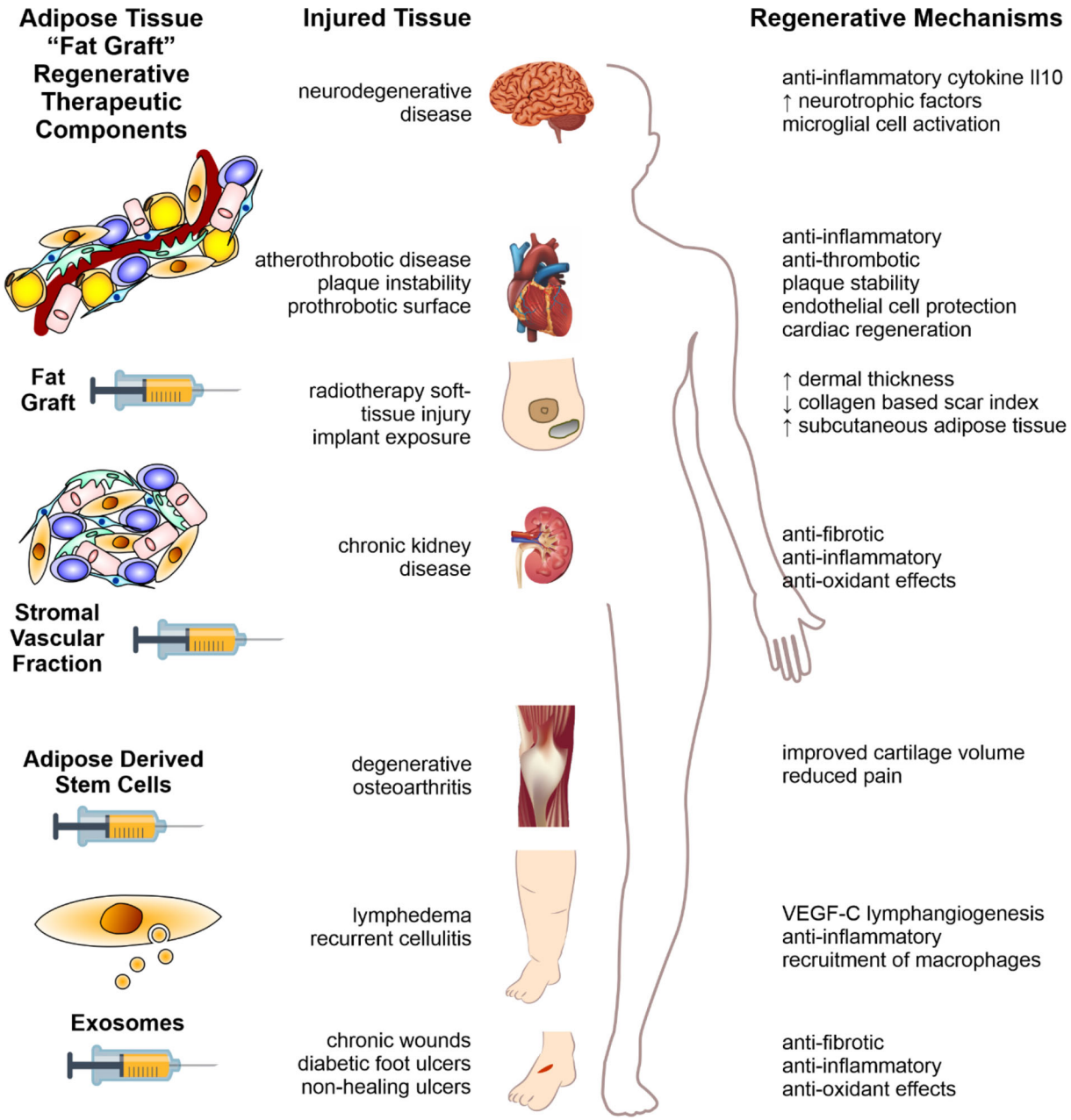

FIGURE 2 | Fat therapeutics of adipose tissue in human disease. Schematic summary of adipose tissue "fat graft" obtained *via* liposuction of subcutaneous fat. Refinement of this fat graft can has occurred at various levels from the acquisition of the rudimentary fat graft, further processed with digestion to obtain the stromal vascular fraction cell pellet, further refinement with extraction of ADSCs, and extracellular vesicle isolation (left column). Each of these components demonstrate significant therapeutic potential in reversing the pathology of human disease, across a range of body systems (middle column). The mechanisms by which these effects are mediated are illustrated in the right-hand column. Figure adapted from Shukla et al. (2015) under the CC-BY license (Shukla et al., 2015).

results were shown in a breast cancer study (O'Brien et al., 2018) employing ADSC-EVs enriched with miR-379, a tumor suppressor miRNA whose expression is down-regulated in breast cancer (Khan et al., 2013). It was found that the miR-379-enriched ADSC-EVs significantly inhibited tumor growth without adverse effects in mice over the 6 weeks of monitoring (O'Brien et al., 2018). These findings suggested a potential application of genetically engineered ADSCs to promote secretion of EVs encapsulated in tumor suppressor miRNAs may be a promising, novel strategy to treat cancer. However, whether ADSC-EVs have long-term therapeutic effects after withdrawal of administration is unknown.

Extracellular Vesicles in Cardiac Disease—Pathology and Cardio-Protection

EVs derived from human ADSCs have been shown to demonstrate cardioprotective roles through their paracrine effects rather than the direct differentiation into cardiomyocytes. Cui et al. used a rodent myocardial I/R injury model to show that ADSC-EVs protected the myocardium from ischemia- or hypoxia- induced necrosis and apoptosis (Cui et al., 2017). Implantation of ADSC-EVs in the rat model resulted in significant reduction in the levels of apoptotic proteins detected (e.g. Bax), and a significant increase in the expression of pro-survival proteins, including Bcl-2 and Cyclin D1 in rat myocardium (Cui et al., 2017). Further, ADSC-EVs exerted cardioprotective effects *via* activation of Wnt/β-catenin signaling (Cui et al., 2017). Another experiment investigating treatment of a rodent model of myocardial infarction with ADSC-EVs profoundly improved cardiac dysfunction by suppressing cardiac apoptosis and fibrosis (Deng et al., 2019). Interestingly, ADSC-EVs promoted macrophage M2 polarization by activating the sphingosine 1-phosphate/sphingosine kinase 1/sphingosine-1-phosphate receptor 1 signaling pathway, which inhibited inflammatory responses and reduced myocardial fibrosis, suggesting that ADSC-EVs may exert potential anti-inflammatory effects (Deng et al., 2019). In addition, Luo et al. employed genetically modified ADSCs to overexpress miR-126 (a microRNA shown to exhibit cardioprotective effects in myocardial infarction) in EVs (Long et al., 2012; Fei et al., 2016). The miR-126-enriched ADSC-EVs significantly decreased myocardial injury by inhibiting inflammation and fibrosis, and enhancing microvascular generation and migration in rats (Luo et al., 2017). Limitations of ADSC treatments for ischemia heart disease include low cardiac retention rates and insufficient concentrations and retained volumes (Li et al., 2019). Numerous clinical trials of ADSCs-derived products have shown promise and an account of completed and ongoing clinical trials using ADSCs are summarized in **Table 3**.

Summary of ADSC-Derived Clinical Trials

The focus of this review is pre-clinical data supporting ADSC-derived therapy; however, it is worth noting that several early clinical trials have been completed. Studies using non-adipose sourced stem cells are not discussed. Trials conducted to assess the benefit of ADSC-derived treatment of wounds, have only reached pilot study or phase I stage in simple cutaneous wounds (Kim et al., 2009; Holm et al., 2018); however, in Crohn's disease-related peri-anal fistulae, a phase III study (Panes et al., 2018) has shown good efficacy. Similarly, good efficacy has been shown in phase I and IIa studies involving treatment of osteoarthritis (Song et al., 2018) and phase III studies are ongoing at the time of writing (**Table 3**). Finally, promise has also been shown in central nervous system disease [phase I and II studies in multiple sclerosis (Fernandez et al., 2018)].

The dynamic nature of the field warrants close observation of the ongoing results of these clinical studies. It is hoped, however, that the application of genetically modified ADSC-derived small EVs may overcome issues encountered in trials of ADSCs and enhance our capacity to tailor and target future treatment approaches.

CONCLUSION

Fat has played a critical role in basic survival and function throughout the history of human evolution. Now, through evolving the role of fat, humankind may unlock critical answers that assist in novel therapeutic approaches to age-old human diseases; as well as those brought upon ourselves by the evolution of the modern lifestyle. The humble, and until recently rather unfashionable, fat cell may hold the secrets to combatting these diseases—be it through old-fashioned "en-bloc" delivery as raw fat graft, through more sophisticated ADSC-enrichment or cutting-edge discovery and harnessing of paracrine factors in exosomes and other EV types as depicted in **Figure 2**. Together, these insights and the putative treatment that result, may themselves form the cornerstone of the future treatment approaches in regenerative medicine.

AUTHOR CONTRIBUTIONS

LS, YY, RS, DG, and TK all contributed to conceptual and figure design, and writing and editing of manuscript. LS and DG contributed to figure creation and YY compiled tables.

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Electrospun Nanofiber Meshes With Endometrial MSCs Modulate Foreign Body Response by Increased Angiogenesis, Matrix Synthesis, and Anti-Inflammatory Gene Expression in Mice: Implication in Pelvic Floor

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Mukherjee S, Darzi S, Paul K, Cousins FL, Werkmeister JA and Gargett CE (2020) Electrospun Nanofiber Meshes With Endometrial MSCs Modulate Foreign Body Response by Increased Angiogenesis, Matrix Synthesis, and Anti-Inflammatory Gene Expression in Mice: Implication in Pelvic Floor. Front. Pharmacol. 11:353. doi: 10.3389/fphar.2020.00353

Purpose: Transvaginal meshes for the treatment of Pelvic Organ Prolapse (POP) have been associated with severe adverse events and have been banned for clinical use in many countries. We recently reported the design of degradable poly L-lactic acid-co-poly ε-caprolactone nanofibrous mesh (P nanomesh) bioengineered with endometrial mesenchymal stem/stromal cells (eMSC) for POP repair. We showed that such bioengineered meshes had high tissue integration as well as immunomodulatory effects *in vivo*. This study aimed to determine the key molecular players enabling eMSC-based foreign body response modulation.

Methods: SUSD2$^+$ eMSC were purified from single cell suspensions obtained from endometrial biopsies from cycling women by magnetic bead sorting. Electrospun P nanomeshes with and without eMSC were implanted in a NSG mouse skin wound repair model for 1 and 6 weeks. Quantitative PCR was used to assess the expression of extracellular matrix (ECM), cell adhesion, angiogenesis and inflammation genes as $\log_2$ fold changes compared to sham controls. Histology and immunostaining were used to visualize the ECM, blood vessels, and multinucleated foreign body giant cells around implants.

Results: Bioengineered P nanomesh/eMSC constructs explanted after 6 weeks showed significant increase in 35 genes associated with ECM, ECM regulation, cell adhesion angiogenesis, and immune response in comparison to P nanomesh alone. In the absence of eMSC, acute inflammatory genes were significantly elevated at 1 week. However, in the presence of eMSC, there was an increased expression of anti-inflammatory genes including *Mrc1* and *Arg1* by 6 weeks. There was formation of multinucleated foreign body giant cells around both implants at 6 weeks that expressed CD206, a M2 macrophage marker.

Conclusion: This study reveals that eMSC modulate the foreign body response to degradable P nanomeshes *in vivo* by altering the expression profile of mouse genes. eMSC reduce acute inflammatory and increase ECM synthesis, angiogenesis and anti-inflammatory gene expression at 6 weeks while forming newly synthesized collagen within the nanomeshes and neo-vasculature in close proximity. From a tissue engineering perspective, this is a hallmark of a highly successful implant, suggesting significant potential as alternative surgical constructs for the treatment of POP.

Keywords: mesenchymal stem cells, pelvic organ prolapse, electrospinning, nanofiber mesh, tissue engineering, foreign body response, gene expression, foreign body giant cells

INTRODUCTION

Pelvic Organ Prolapse (POP) is a debilitating urogynecological pelvic floor disorder that significantly impacts the quality of lives of 50% of parous women aged over 50 years (Nygaard et al., 2008). POP is mainly results from vaginal birth injury (Urbankova et al., 2019), which over time leads to herniation of pelvic organs, such as uterus, bladder, and bowel into the vagina. Symptoms include difficulty in passing urine and bowel motions, sexual dysfunction, feeling of a vaginal bulge, and urinary and bowel incontinence (Iglesia and Smithling, 2017). While first line conservative management using pelvic floor exercises and pessaries may delay disease progression (Li et al., 2016), it does not eliminate the need for surgical intervention for many women. Until recently, non-degradable polypropylene (PP) vaginal meshes were commonly used for reconstructive surgery to mitigate native tissue repair failures (Milani et al., 2018). However, regulatory authority warnings and recent reports indicate high adverse event rates and risks of complications such as mesh erosion and exposure (Mironska et al., 2019). A growing body of evidence shows implant failures and have established that prolonged inflammation and undesirable foreign body response (FBR) are associated with complications in patients (Deprest et al., 2009; Claerhout et al., 2010; Brown et al., 2015; Jallah et al., 2016; Nolfi et al., 2016; Tennyson et al., 2019). Such FBRs and associated adverse effects of transvaginal meshes were deemed to out-weigh PP benefits and therefore led to the ban on transvaginal meshes in Australia, UK, and USA by regulatory authorities, with no alternative treatments on the horizon. At present, there are no optimal therapies for POP. Therefore, more reliable treatment measures that promote tissue healing and repair without piquing deleterious FBR are pivotal for the treatment of POP (Siddiqui et al., 2018).

In nature, *in vivo* cell behavior and vaginal tissues are supported by the micro/nanoscale architecture of the ECM (Sridharan et al., 2012) that provides a larger surface area to adsorb proteins and more binding sites for cell membrane receptors and adhesion molecules. The standard clinical PP mesh biomaterial lacks a biomimetic character. They disrupt the vaginal microenvironment (Liang et al., 2013; Liang et al., 2015; Jallah et al., 2016) rather than mimicking its nanoarchitecture, evoking undesired complications. To overcome mesh erosion, vaginal implants must promote rather than impede cell-matrix interactions. The primary cause of complications resulting from PP mesh implants have been attributed to the prolonged chronic inflammation and poor tissue integration associated with mechanically inferior non-degradable implants (Nolfi et al., 2016; Tennyson et al., 2019). The tissue microenvironment comprises structural and functional components (e.g. collagens and elastin) that provide a scaffold to hold cells together through numerous chemical and physical stimuli at the molecular level. Nanofabrication of scaffolds recapitulates such biomimetic nanoscale architectural cues (Mukherjee et al., 2013; Liu et al., 2018a). As a result, meshes designed with nanoscale fibers using electrospinning techniques promote cell-cell and cell-biomaterial interactions. Given that current PP meshes bear little structural or biological resemblance to native vaginal tissue, we and others have shown that nanostructured meshes that impart biomimetic properties can improve mesh integration, overcome erosion and hold significant promise in POP reconstructive surgery (Sartoneva et al., 2012; Wu et al., 2016; Vashaghian et al., 2017; Gargett et al., 2019; Mangir et al., 2019; Mukherjee et al., 2019b).

Irrespective of the composition and fabrication technique, biomaterials elicit an FBR after implantation in the body (Mukherjee et al., 2019a; Hympanova et al., 2020). This response is a cascade of dynamic cellular processes involving several genes influencing the milieu of the tissue microenvironment that ultimately determines the fate of the implant and healing process. Mesenchymal stem/stromal cells (MSCs) are clonogenic, multipotent cells, widely recognised for their ability to promote tissue repair and regeneration (Dimarino et al., 2013; Ulrich et al., 2013; Le Blanc and Davies, 2015; Gargett et al., 2016). Therefore, cell based therapies for pelvic floor tissue repair, although less explored, hold significant potential for POP treatment (Darzi et al., 2016b; Emmerson and Gargett, 2016; Gargett et al., 2016; Callewaert et al., 2017; Gargett et al., 2019). Nonetheless, while undifferentiated MSCs mitigate inflammation and influence reparative processes (Kode et al., 2009; Le Blanc and Davies, 2015), several clinical trial outcomes have highlighted that mere injection of such therapeutic cells into damaged tissue leads to a rapid loss of MSC, preventing optimal repair (Dimmeler et al., 2014; Sharma et al., 2014). Bioengineering using biomimetic degradable nanofiber meshes that mimic natural ECM to allow entrapment and persistence of seeded MSCs will likely yield superior vaginal constructs with a

controlled and anti-inflammatory immune response (Gargett et al., 2019).

We discovered perivascular MSCs in the endometrial lining of the uterus (eMSC) and identified a unique marker, SUSD2, to isolate these rare perivascular cells (Gargett et al., 2016). We also discovered that a small molecule, A83-01, maintains eMSC' undifferentiated state during culture expansion, required for clinical use (Gurung et al., 2015; Gurung et al., 2018). We have established that eMSC have reparative capacity, reduce fibrosis and the FBR to nondegradable polyamide mesh by influencing macrophage polarization switching from an M1 to M2 phenotype in rodent and ovine models (Ulrich et al., 2014; Darzi et al., 2018; Emmerson et al., 2019). More recently, we have also shown that eMSC improve the tissue integration, cellular infiltration and overall FBR response to degradable nano/microstructured meshes (Mukherjee et al., 2019b; Paul et al., 2019). The beneficial effects of eMSC are characterized by upregulation of M2 markers such as CD206 and *Arg1*, *Mrc1*, and *Il10* genes in tissue macrophages, as well as reducing their secretion of inflammatory cytokines Il-1β and Tnf-α (Darzi et al., 2018). However, the key players in mediating eMSC paracrine effects on cellular migration and recruitment remain largely unknown. Furthermore, how eMSC mediate M2 immunomodulatory responses during the FBR after implantation of bioengineered constructs also remains unknown. In general, the FBR to tissue engineered constructs are often limited to measuring the *in vivo* capsule thickness and is poorly understood.

Recently, we reported the design of novel nanostructured degradable poly L-lactic acid-co-poly ε-caprolactone or PLCL meshes (P nanomesh) tissue engineered with reparative mesenchymal stem/stromal cells from endometrium (Mukherjee et al., 2019b). In this study, we assess the potential of these newly designed degradable nanofiber meshes tissue engineered with these therapeutic cells to influence macrophage mediated FBR and promote key reparative processes such as angiogenesis, cellular adhesion, extracellular matrix (ECM) synthesis as well as its regulation using gene expression profiling and histology in a subcutaneous mouse model. From a clinical perspective, it is not only important to design novel constructs for POP treatment, but also critical to understand their FBR pattern and tissue repair process that likely varies with different components and their degree of involvement (Mukherjee et al., 2019a). Such detailed understanding is also pivotal to the long term efficacy of all medical devices and the lack of this knowledge may potentially disrupt clinical practices as exemplified by the rise and fall of pelvic PP mesh usage (Heneghan et al., 2017). In this study we provide an in-depth assessment of changes in gene expression associated with eMSC-nanobiomaterial therapy. In particular, we have quantified the *in vivo* gene expression associated with ECM formation and regulation, cell adhesion, angiogenesis and the FBR to PLACL Nanomesh (P Nanomesh) with and without eMSC. We have also shown the histological effects arising from the gene expression profile of eMSC based surgical constructs, including angiogenesis and ECM formation. To our knowledge, this is the first study to show such detailed impact of eMSC based tissue engineered degradable nanostructured scaffolds *in vivo*.

METHODS

Ethics

All SUSD2$^+$ eMSC were isolated from endometrial biopsies obtained from seven women undergoing laparoscopic surgery for nonendometrial gynecological conditions and had not taken hormonal treatment for three months before surgery. Samples were collected following written informed consent as per approval from the Monash Health and Monash University Human Research Ethics committees (09270B). All methods were performed in accordance with National Health and Medical Research Council guidelines. Each patient biopsy was used to generate a single eMSC cell line and served as n=1.

Fabrication of Nanomeshes

Nanofiber meshes of PLACL were fabricated by electrospinning as described in our previous report (Mukherjee et al., 2019b). PLACL polymer (Resomer, Evonik) was dissolved in 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) on a magnetic stirrer to form 10% (w/v) clear solution. Syringe (Terumo Corporation, Japan) with this solution was attached to 23 G blunted stainless-steel needle (Terumo Corporation, Japan) for electrospinning, using a syringe pump (NE1000, New Era Pump Systems, Inc. USA) at a controlled flow rate of 1 ml/h and voltage of 18 kV (DC voltage power supply, Spellman SL150, USA) to collect nanofibers at a distance of 12.5 cm from the needle tip to the collector. The fibers were collected on grounded aluminum foil and dried for at least 1 week in a vacuum oven prior to experimental use.

Scanning Electron Microscopy

Samples of only nanofiber meshes were directly sputter coated with a thin platinum sputter coating layer (Cressington 208 HR, UK) for 120 s. All specimens were examined under the scanning electron microscope (Nova NanoSEM, FEI, USA) at an accelerating voltage of 10 kV and images were quantified by Image J software.

Atomic Force Microscopy

Atomic force microscopy (AFM) using a FastScan AFM (Bruker, Billerica, MA, USA) in PeakForce tapping mode and FastScan C probes with a nominal 5 nm tip radius and spring constant of 0.8 N/m. For imaging, 512 × 512 pixel resolution and a 2-Hz scan rate was used to measure n=3 samples and pointed AFM tip at five ROIs (region of interest) of 5 μm^2 area. Images were processed using Nanoscope Analysis software.

Isolation, Expansion, and Labelling of eMSC

Endometrial tissue was obtained from seven healthy women (no endometrial pathology) who had not used hormones for minimum three months. SUSD2$^+$ eMSC were isolated according to our established protocols (Darzi et al., 2018;

Mukherjee et al., 2019b). Briefly, endometrial tissue underwent enzymatic digestion using Collagenase I and DNAs I (Worthington-Biochemical Corporation) for 1 h at 37°C. The stromal fraction and red blood cells were separated from epithelial fraction using a 40 μM sieve (BD Bioscience-Durham) and Ficoll paque (GE Healthcare Bioscience-Bio-Sciences AB) gradient, respectively. The isolated stromal cells were incubated with PE anti human SUSD2 antibody (Biolegend) for 30 min at 4°C followed by incubation with anti-PE labelled magnetic beads (Miltenyi Biotec) for 20 min. PE labelled $SUSD2^+$ eMSC were sorted using a column and magnet (Miltenyi Biotec). $SUSD2^+$ eMSC were cultured and expanded in 10% FBS DMEM F12 (Invitrogen) supplied with growth factor bFGF (Peprotech) for 2–4 passages. Before *in vivo* implantation, eMSC were permanently labelled with mCherry lentivirus vector according to our published protocols (Darzi et al., 2018; Mukherjee et al., 2019b). Briefly, Lentivirus was generated using three plasmids; pLVX-IRES-mCherry (lentivirus plasmid which contains mCherry gene) (clontech-6312237), packaging plasmids; pSPAX2 that encodes capsid (Addgene 12260) and pMD2.G that encodes reverse transcriptase for lentivirus replication (Addgene 12259), into 293T cells. Transfection was performed using TransIT-X2 (Mirus) transfection reagent according to manufacturer's protocols.

Animal Surgery and Tissue Collection

The experimental procedure and mouse husbandry was approved by Monash Medical Centre Animal Ethics Committee A (2017/05). NSG mice were housed in the animal house at Monash Medical Centre according to the National Health and Medical Research Council of Australia guidelines for the care and use of laboratory animals and were provided sterile food and water under controlled environmental conditions. NSG mice were divided in two experimental groups P and P+eMSC and two time-points; 1 and 6 weeks (seven mice/group). The mice were anaesthetized using 3% w/v Isoflurane® and carprofen (5 mg/kg body weight) was used as analgesia. The abdomen was shaved and disinfected with 70% ethanol. A longitudinal 1.2 cm skin incision was performed in the lower abdomen and the skin was separated from the fascia by blunt dissection to make two pockets on each side of the midline. The P nanomesh was implanted into two pockets of each animal, and mCherry labelled eMSC was added on top of the nanomesh using a 50μl pipette tip. Meshes were sutured to the abdominal fascial layer using 6–0® monofilament sutures (Ethicon) on two ends and. The skin was closed with 6–0® monofilament sutures (Vicryl). Following 1 or 6 weeks the animals were euthanized in a CO_2 chamber and tissues were collected for analysis. Some animals were reused from our previous study (Mukherjee et al., 2019b) to comply with Monash Medical Centre Animal Ethics Committee's reuse and reduce usage policy. However, the tissue portions of the animals used for analysis have not been used in any other study.

Histology

Tissue sections containing constructs were fixed in 10% formalin and processed by the Monash Histology Platform at the Monash Health Translation Precinct (MHTP). Formalin-fixed tissues were processed to paraffin, sectioned (5 μm) and placed on super frost slides. Histological H&E, Picrosirius red staining and immunohistochemistry was performed by Monash Histology Platform at MHTP. For H&E staining, slides were dewaxed using xylene and stained in Haemtoxylin for 7 min. After washing in tap water, they were blued in ammoniated water for 30 seconds and stained in alcoholic Eosin for 7 min. For Picro Sirius red staining, slides were dewaxed using xylene and fixed in Bouin's fixative for 1 h. After washing in tap water, they were stained with Picro Sirius red for 1 h at room temperature followed by washing and mounting. For immunohistochemistry staining, slides were dewaxed and underwent citrate buffer antigen retrieval for 30 min followed by endogenous peroxidase blocking step using 0.3% v/v H_2O_2. Slides were then incubated with protein blocker (Dako, USA) for 40 min and washed with 1× PBS. Primary antibodies were diluted and incubated for 1 h at room temperature. HRP labelled anti mouse was used as secondary antibody for 30 min and the nucleus stained with Hematoxylin. Details of antibodies are listed in **Table1**. The sections are scanned using Aperio Digital pathology scanner (Leica) at 40X and analyzed using Imagescope software to identify the stains.

qPCR Fluidigm Biomark

Animal tissues were collected in RNA*later* (ThermoFisher) and stored in 4°C for 24 h followed by storage in −80°C. Samples were weighed and total RNA was extracted using RNeasy mini kit (Qiagen) as per manufacturer's protocol to prepare cDNA. Prior to fluidigm qPCR, preamplification was used to increase the number of copies of each gene to detectable levels as detailed in Fluidigm Gene Expression. Taqman assays were firstly pooled by combining 2 μl of each of the 94 20X Taqman assays and 12 μl Tris EDTA buffer pH 8.0 for a final volume of 200 μl. The final concentration of each assay was 0.2X (180nM). 3.75 μl of Sample Premix (Life Technologies TaqMan® PreAmp Master Mix and Pooled Taqman assays) was combined with 1.25 μl of each of the 87 cDNA samples and 8 RT negative samples for a final reaction volume of 5 μl per sample. A no template control from Single Cell Genomics Centre was also included and all 96 samples were preamplified for 14 cycles. Following preamplification, reaction products were diluted 1:5 by adding 20 μl Tris EDTA buffer pH 8.0 to the final 5 μl reaction volume for a total volume of 25 μl. Assays and Samples were combined in a 96.96 Dynamic array IFC according to Fluidigm® 96.96 Real-Time PCR Workflow. Five microliter of each assay at a final concentration of 10× was added to each assay inlet port and 5 μl of diluted sample to each sample inlet port according to the Chip Pipetting Map (GE 96×96 Standard v2). Data were analyzed using with Fluidigm Real-Time PCR analysis software (V4.1.1) to obtain Ct values.

TABLE 1 | Details of antibodies used in immunohistochemistry.

Primary Antibody	Isotype	Supplier	Dilution
CD206	Rabbit polyclonal	Abcam	1/2500
mCherry	Rat IgG1	Life technology	1/100

TABLE 2 | Details of qPCR Primers (mouse genes).

Gene name	TaqMan Code
Ang1	Mm00456503_m1
Ang2	Mm00545822_m1
Fgf1	Mm00438906_m1
Fgf2	Mm00433287_m1
Fgfr3	Mm00433294_m1
Ctgf	Mm01192933_g1
Mmp2	Mm00439498_m1
Mmp9	Mm00442991_m1
Mmp19	Mm00491296_m1
Pdgfa	Mm01205760_m1
Timp1	Mm00441818_m1
Timp2	Mm00441825_m1
Timp3	Mm00441826_m1
Timp4	Mm01184417_m1
Tgfa	Mm00446232_m1
Tgfb1	Mm00441724_m1
Tgfb2	Mm00436955_m1
Tgfb3	Mm00436960_m1
Tgfbr1	Mm00436964_m1
Vegfa	Mm00437304_m1
Serpine1	Mm00435860_m1
Itgb1	Mm01253230_m1
Itgb2	Mm00434513_m1
Ccl1	Mm01545656_m1
Ccl2	Mm00441242_m1
Ccl3	Mm00441258_m1
Ccl4	Mm00443111_m1
Ccl5	Mm01302428_m1
Ccl7	Mm00443113_m1
Ccl11	Mm00441238_m1
Ccl12	Mm01617100_m1
Ccl17	Mm01244826_g1
Ccl19	Mm00839967_g1
Cxcl1	Mm04207460_m1
Cxcl2	Mm00436450_m1
Cxcl5	Mm00436451_g1
Cxcl9	Mm00434946_m1
Cxcl10	Mm00445235_m1
Cxcl11	Mm00444662_m1
Cxcl12	Mm00445553_m1
Ccr1	Mm00438260_s1
Ccr2	Mm99999051_gH
Ccr3	Mm01216172_m1
Ccr5	Mm01963251_s1
Ccr7	Mm00432608_m1
Cxcr2	Mm99999117_s1
Cxcr3	Mm00438259_m1
Il1a	Mm00439620_m1
Il1b	Mm00434228_m1
Il4ra	Mm01275139_m1
Il6	Mm00446190_m1
Tnf	Mm00443258_m1
Il10	Mm00439616_m1
Nos1	Mm01208059_m1
Nos2	Mm00440485_m1
Cd86	Mm00444540_m1
CD80	Mm01344159_m1
Arg1	Mm00475988_m1
Mrc1	Mm00485148_m1
Tnfa	Mm99999068_m1
Cd44	Mm01277163_m1
Cdh1	Mm01247357_m1
Cdh2	Mm01162497_m1
Cd49B/Itga2	Mm00434371_m1
Icam	Mm00516023_m1
Vcam1	Mm01320970_m1
Col6a1	Mm00487160_m1
Col6a2	Mm00521578_m1
Col6a3	Mm00711678_m1
Col6a6	Mm00556810_m1
Col1a1	Mm00801666_g1
Col3a1	Mm01254476_m1
Rn18s	Mm03928990_g1
Gapdh	Mm03302249_g1

Primers are detailed in **Table 2**. Target gene expression was normalized to 18sRNA and relative gene expression and fold change was calculated using the $2^{-\Delta\Delta CT}$ method.

Data Analysis and Statistics

Fold Change in gene expression was calculated in comparison to sham controls. Statistical analysis was performed using GraphPad Prism v8. Data were analyzed using non-parametric Mann-Whitney U test (comparison between P and P+eMSC). Data are presented as median and value of $P \leq 0.0513$ was considered to be statistically significant.

RESULTS

Fabrication and Characterization of Nanomesh

Degradable nanostructured meshes were fabricated from poly (L-lactic acid)-co-poly(ε-caprolactone) (P Nanomesh), given their acceptance in medical devices, using electrospinning to mimic the precise features of native tissue dimensions as per our previous studies (Mukherjee et al., 2011; Mukherjee et al., 2019b). Electrospinning enabled the design of nanofibers of PLCL (**Figure 1**) that produced a mesh which macroscopically appeared like thin facial tissue paper. Scanning electron microscopy (SEM) micrographs confirmed that P nanomeshes had an ultrafine and beadless morphology (**Figure 1A**) with an average fiber diameter of 585 nm as previously reported (Mukherjee et al., 2019b). The nanomeshes were highly porous (**Figure 1B**) and had three-dimensional structure of randomly layered fibers to form sheets of ~406 nm in thickness (**Figures 1C, D**). The fabricated P nanomesh structures closely resembled the human vaginal microstructure at the nanoscale, comprised of collagen fibril structures (**Figure 1E** and **Figure S1**) that ranged from 55–130 nm depending on the patient age and POP severity, and are arranged in bundles 2–3 μm thick (Kim et al., 2016).

eMSC Increase Synthesis of New ECM Within Nanomeshes *In Vivo*

Histology sections prepared from mouse explants were stained with Picro Sirius red to visualize the newly synthesised ECM (**Figure 2**), mainly collagen (black arrows, **Figure 2**) inside

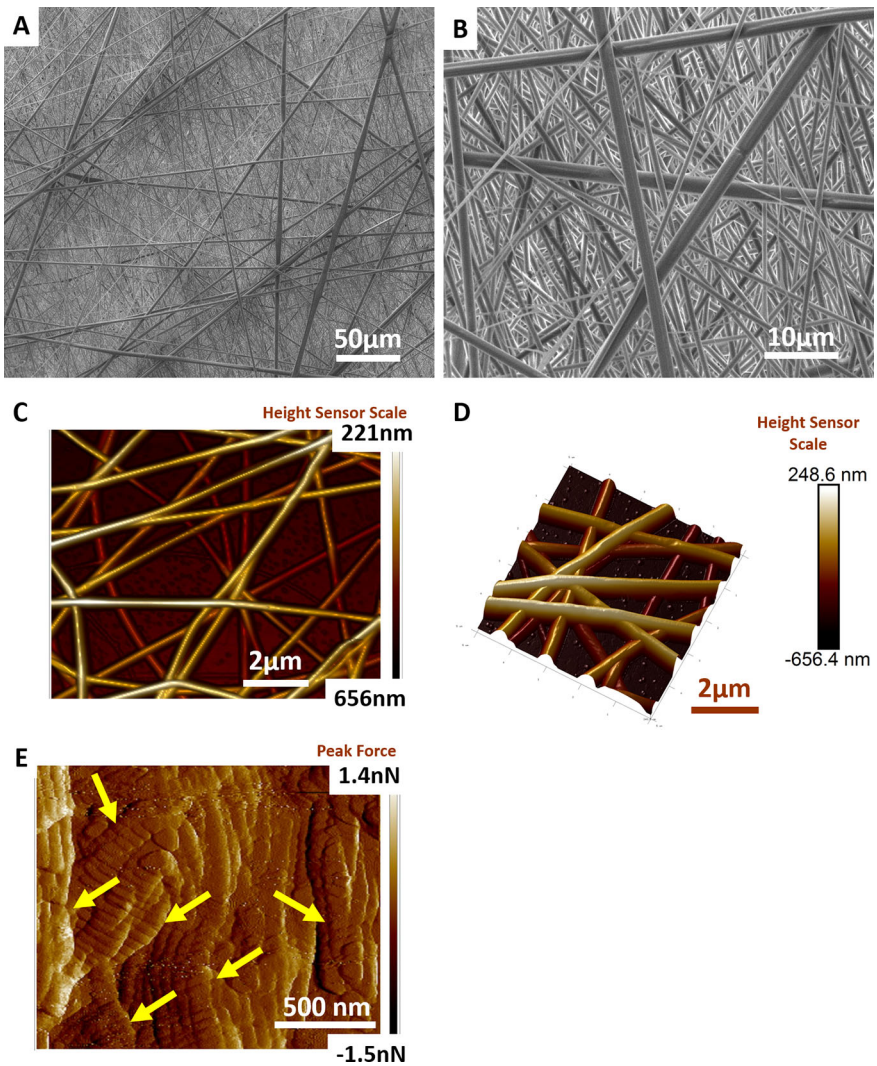

FIGURE 1 | Electrospun Nanofiber mesh. Scanning electron micrographs of PLCL nanofiber mesh structure at **(A)** 1000× magnification **(B)** 5000× magnification. Atomic force micrograph reveals structure of **(C)** randomly laid nanofiber meshes at two-dimensional (2D) view and **(D)** 3D view **(E)** vaginal extracellular matrix (ECM) structure revealing arrangement of collagen fibrils (yellow arrows).

implanted meshes (between dotted lines, **Figure 2**). At 6 weeks, there was a substantial amount of collagen inside the Nanomesh (within dotted lines, **Figure 2**) in the P+eMSC group compared to P alone. This difference was not observed at 1 week. Quantitative PCR analysis of ECM and genes (**Figure 3**) also showed a significant increase in the expression ($P < 0.05$) of several collagen genes including *Col1a1, Col3a1, Col6a1* and *Col6a2* in P+eMSC compared to P alone at 6 weeks (**Figure 3A**). Similar to histology observations (**Figure 2**), the expression of these genes was not different between the two groups at 1 week (**Figure 3B**). The expression of cell adhesion molecules and *Tgfb* genes, *Itgb1, Vcam, Icam, Cd44, Cdh1, Cdh2, Tgfb1, Tgfb3, and Tgfbr* were also significantly higher ($P < 0.05$) (**Figure 3A**), whereas expression of *Tgfb2* (**Figure 3A**) showed no difference between P+eMSC and P alone at 6 weeks. In contrast, at 1 week no collagen subunit genes were differentially expressed, neither for cell adhesion genes, except for *Cd44*, which was significantly lower in P+eMSC compared to P (**Figure 3B**). Our results show that presence of eMSC increases new collagen subunit synthesis which may be mediated by increased *Tgfb*1 and *Tgfb3* gene expression within implanted P nanomesh, may foster tissue integration *via* the expression of ECM formation and cell adhesion genes by 6 weeks but not as early as 1 week *in vivo*.

eMSC Influence Expression of Matrix Metalloproteinases and Tissue Inhibitors of Metalloproteinases

Matrix metalloproteinases (*Mmps*) are essential mediators of ECM homeostatic dynamics that degrade ECM components and

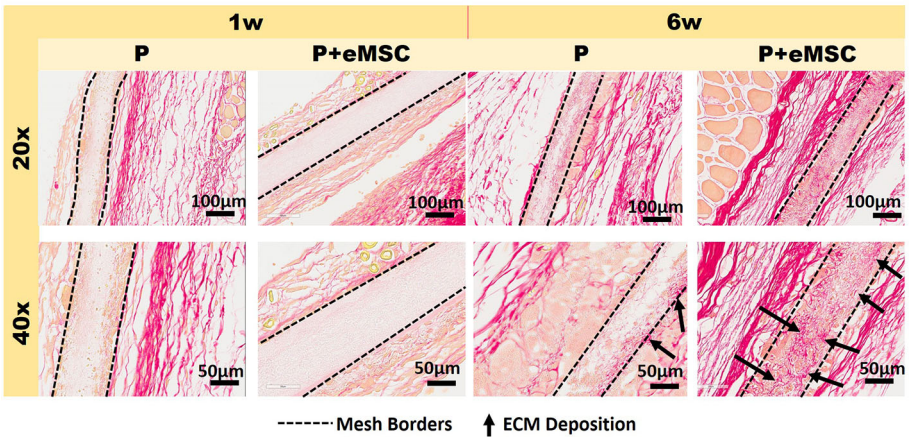

FIGURE 2 | Picro-Sirius red staining of collagen in explanted meshes at 20× and 40× magnifications. P nanomesh implants (within dotted lines), with and without endometrial mesenchymal stem/stromal cells (eMSCs), explanted from the subcutaneous skin of the flank showing red-stained collagen red in mice at 1 and 6 weeks explantation. Black arrows show new collagen deposited within the P Nanomesh.

are modulated by Tissue inhibitors of metalloproteinases (*Timps*). TIMPs reversibly bind to MMPs and regulate their proteolytic activities and their balance significantly impacts tissue homeostasis. To this end, we assessed the expression of several key *Mmp*s and *Timp*s using qPCR in *in vivo* tissues post implantation (**Figure 4**). At 6 weeks, the expression of *Mmp2, Mmp19*, *Timp2,* and *Timp3* was significantly higher ($P < 0.05$) in P+eMSC compared to P alone (**Figure 4A**). At 1 week, there was no significant differences in the expression of these MMPs and TIMPs genes in both groups (**Figure 4B**). Our results show that eMSC influence the expression of several MMPs and TIMPs when implanted as tissue engineered constructs compared with P nanomesh alone by 6 weeks.

Nanomesh With eMSC Promote Angiogenesis After *In Vivo* Implantation

Angiogenesis is essential to healing and growth for repair of tissues. Rapid neo-vascularisation determines the clinical success of implanted tissue constructs. Since cells must be in 100–200 µm proximity of blood vessels to receive oxygen through diffusion, spontaneous ingrowth of capillaries is highly desirable following *in vivo* implantation of meshes (Shahabipour et al., 2019). Thus, it is critical that the post implantation milieu has optimal conditions to support vascularization for tissue integration and long-term viability. Therefore, we assessed the expression of several angiogenic factors following *in vivo* implantation of P nanomeshes with and without eMSCs at 1 and 6 weeks (**Figure 5**). At 6 weeks, we observed significantly higher expression ($P < 0.05$) of the key angiogenic factor genes, *Vegfa*, *Fgf1*, *Ctgf, Ang1,* and *Pdgfa* in P+eMSCs compared to P alone (**Figure 5A**). Of these, *Serpine* and *Fgf1* were also significantly higher in the presence of eMSC acutely at 1 week (**Figure 5B**), suggesting their role in a sustained angiogenic response. Expression of *Cxcl12*, a chemokine that plays a crucial role in angiogenesis by recruitment of endothelial progenitor cells (Li et al., 2015), was also significantly higher ($P < 0.05$) in the presence of eMSCs at 6 weeks (**Figure 5A**), however not at 1 week (**Figure 5B**), indicating involvement in late angiogenic responses. Our results indicate that, in comparison to P alone (**Figure 6A**), implantation of eMSC with P nanomesh promotes early angiogenesis and neovascularization as evidenced by H&E staining (**Figure 6B**) whereby several blood vessel profiles are located inside the mesh (black arrows, **Figure 6C**) as well as within a close proximity (10–200µm) of the mesh implant (black arrows, **Figure 6D**).

eMSC Reduces the Pro-Inflammatory Response After Nanomesh Implantation

Immediately following mesh implantation, the immune system is triggered and an influx of white blood cells at the site marks the beginning of the FBR acute phase, which is characterized by several inflammatory cytokines. Several factors including components of the implants, determine the severity of this acute phase and the milieu of pro-inflammatory factors. Our analysis of pro-inflammatory factor genes showed that eMSC dampen and delay the expression of several acute pro-inflammatory genes in response to implanted P nanomesh (**Figures 7** and **8**). eMSC attenuated the inflammatory response associated with nanomesh at 1 week by significantly downregulating ($P < 0.05$) the expression of *Il1b*, *Tnfa*, *Ccl2*, *Ccl3*, *Ccl4*, *Ccl5*, *Ccl7*, *Ccl12*, *Ccl19*, *Cxcl1, Cxcl2, Cxcl10, Ccr1,* and *Ccr7* compared to P alone (**Figure 7**). In contrast to upregulating ECM and angiogenesis genes, we observed that all of these acute inflammatory genes were no longer upregulated at 6 weeks (**Figure 8**). However, the later phase inflammatory genes *Nos2*, *Ccl11*, *Ccxl9*, *Cxcl12,* and *Ccr2* which is the receptor for *Ccl11,* in P+eMSC were significantly upregulated ($P < 0.05$) compared to P alone. Our results show that eMSC seeded P nanomesh significantly and rapidly reduces the acute inflammatory response associated *in vivo* biomaterial implantation and

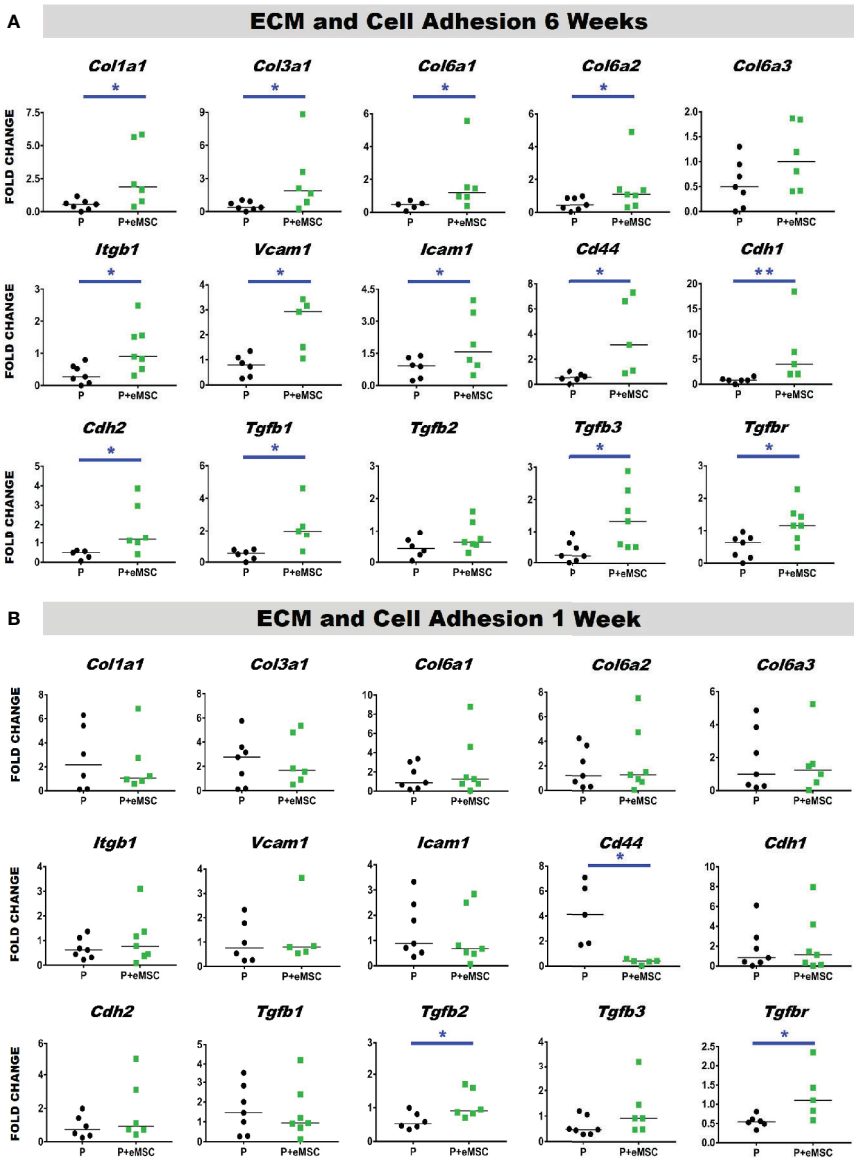

FIGURE 3 | Extracellular matrix (ECM) and adhesion molecule gene expression of P and P+eMSCs nanomesh explants. Fold change in mRNA expression of ECM and cell adhesion genes by quantitative PCR in explanted mice tissues after **(A)** 6 weeks and **(B)** 1 week consisting of P nanomesh, with and without endometrial mesenchymal stem/stromal cells (eMSCs). Data are median of n=5-7 samples/group analyzed by Mann-Whitney U test; *$P < 0.05$ and **$P < 0.01$.

therefore is likely to influence the entire subsequent FBR process. Some chemokines are upregulated in P+eMSCs at 6 weeks, associated with inflammatory cell recruitment functions such as *Cxcl12*.

eMSCs Promotes an Anti-Inflammatory Response Following *In Vivo* Implantation at 6 Weeks

Macrophages release of cytokines and growth factors induce migration and proliferation of fibroblasts, that in an anti-inflammatory environment effectively regenerate tissue (Koh and Dipietro, 2011). Accumulating evidence indicates that macrophages orchestrate the tissue response and healing process after biomaterial implantation and that macrophage polarization determines the outcome of the immune response (Ulrich et al., 2012; Roman Regueros et al., 2014; Feola et al., 2015; Darzi et al., 2016a). Herein, we observed a significantly higher ($P < 0.05$) expression of anti-inflammatory genes including *Arg1, Mrc1, Il6,* and *Il4ra* in P+eMSCs over P alone at 6 weeks (**Figure 9A**). While Il4ra is commonly associated with inflammation, recent evidence has shown it plays a role in M2 polarization by upregulating Il-6, another pro-inflammatory cytokine associated with tissue regeneration. Moreover, its

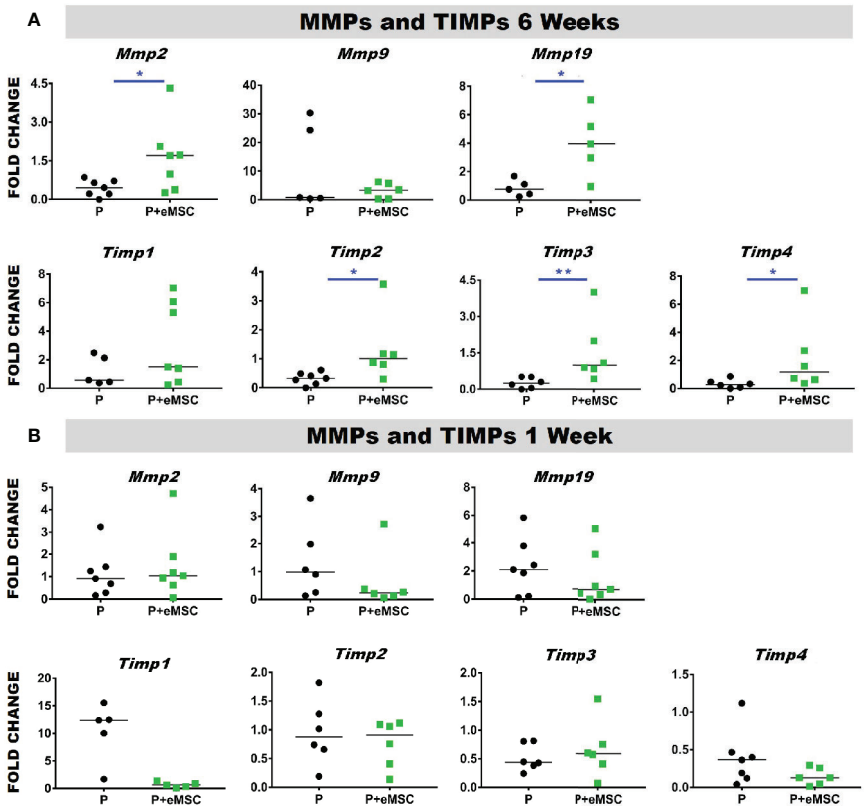

FIGURE 4 | Matrix metalloproteinase (*Mmp*) and tissue inhibitors of metalloproteinases (*Timp*) gene expression of P and P+eMSCs nanomesh explants. Fold change in mRNA expression of mouse *Mmp* and *Timp* genes by quantitative PCR in explanted mice tissues after **(A)** 6 weeks and **(B)** 1 week consisting of P nanomesh, with and without endometrial mesenchymal stem/stromal cells (eMSCs). Data are median of n=5-7 samples/group analyzed by Mann-Whitney U test; *$P < 0.05$, **$P < 0.01$.

ligand, Il-4 is commonly used to polarize M2 macrophages *in vitro* and is associated with wound repair. In contrast, *Arg1* was upregulated in the presence of P of eMSCs at 1 week (**Figure 9B**) which marks the acute phase of the FBR.

Biomaterial-Induced Multinucleated Giant Cells With M2 Phenotype

We found multinucleated foreign body giant cells (FBGC) associated with both P+eMSCs and P at 6 weeks but not at 1 week (**Figure 10**) by H&E stains, which showed fusion of macrophages mostly at the edges of the mesh (**Figure 10A**, black arrows). Our results show that the in the presence of eMSCs, the number of FBGCs were increased and smaller in size with fewer nuclei per FBGC. Immunohistological characterization revealed that these FBGCs expressed CD206, (**Figure 10B**, black arrows) a marker usually associated with M2 macrophages as shown within the mesh at 1 week in P+eMSC. These CD206 FBGCs were found both at the mesh edges and in the surrounding tissues. In P+eMSC, the intensity of CD206 in FBGC and other cells was greater, with respect to negative control (**Figure S2**) and localised to the plasma membrane compared to P alone at both time points. Although FBGC have been viewed in a negative light in FBR process, the knowledge of their role and functions remain elusive. Our study shows that these cells express CD206, a M2 marker and are present while there is a high expression of angiogenic, ECM synthesis, cell adhesion and anti-inflammatory genes in P+eMSCs at 6 weeks.

DISCUSSION

In this study, we report the difference in the profile of the FBR response, with focus on ECM, angiogenesis and inflammatory responses to degradable nanofiber meshes of PLCL (P) in the absence and presence of eMSCs. The main findings of our study are that MSCs promote better tissue integration of nanomesh through inducing increased expression of ECM, cell adhesion, angiogenesis and healing gene profiles 6 weeks following implantation, while dampening the pro-inflammatory response in the acute FBR phase at the first week. Given that the current failed vaginal meshes are associated with inadequate tissue integration and elevated chronic inflammatory FBR years after implantation (Nolfi et al., 2016), the use of eMSCs to reduce the pro-inflammatory response and promote early mesh integration and improve tissue repair is an important advance in improving outcomes for treating POP.

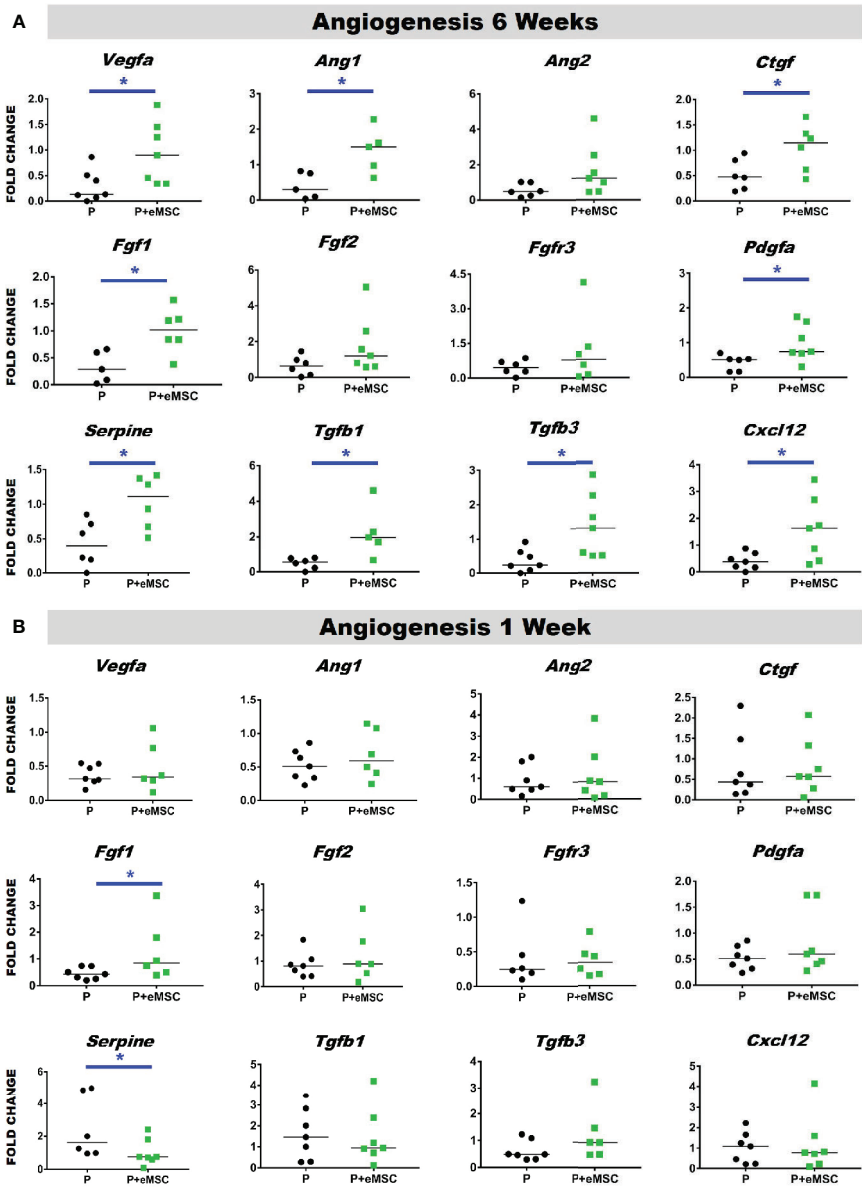

FIGURE 5 | Angiogenesis gene expression of P and P+eMSCs nanomesh explants. Fold change in mRNA expression of mouse angiogenic genes by quantitative PCR in explanted mice tissues after **(A)** 6 weeks and **(B)** 1 week consisting of P nanomesh, with and without endometrial mesenchymal stem/stromal cells (eMSCs). Data are median of n=5-7 samples/group analyzed by Mann-Whitney U test; *$P < 0.05$.

Our fabricated nanostructured porous P nanomeshes with a fiber diameter 585 nm (Mukherjee et al., 2019b) and a scaffold depth of ~406 nm have emerged as an attractive and potential alternative to nondegradable meshes owing to their biomimetic properties (Vashaghian et al., 2017; Gargett et al., 2019). Previously, we have shown that meshes with nano and micro architecture interact favorably with eMSC and promote their growth and proliferation (Mukherjee et al., 2019b; Paul et al., 2019). In the present study, our results show that the P nanomesh closely mimics the vaginal ECM architecture. Moreover, the bioengineering of nanomesh with eMSCs triggers a distinctly more favorable immune and tissue response *in vivo* compared to nanomesh alone. From a clinical perspective, in targeting POP treatment, these are highly desirable features as nanostructured meshes recapitulate structural cues for cell adhesion and prolonged retention of large numbers of MSCs after local delivery even after 6 weeks (**Figure S3**).

Following implantation of a biomaterial construct *in vivo*, a macrophage-mediated FBR is triggered whereby several molecular mechanisms are activated at each step of the process (Anderson et al., 2008; Skokos et al., 2011; Mooney et al., 2014; Mukherjee et al., 2019a). Accumulating evidence from our and

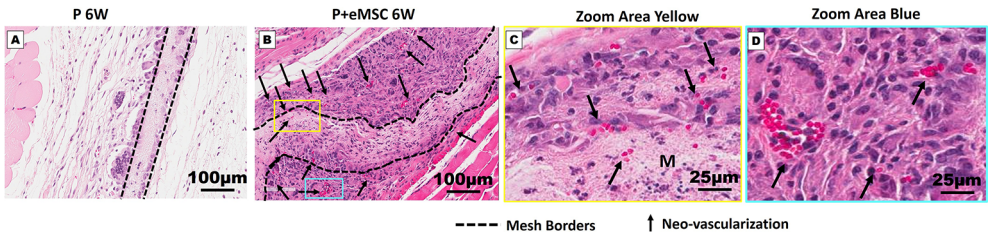

FIGURE 6 | Neo-vascularization in Nanomesh explants after 6 weeks. H&E stained section showing mesh implants of **(A)** P and **(B)** P+eMSC at 6 week (dotted line). **(C)** Neo-vascularization (black arrows) around P+eMSC is seen at optical zoom within the yellow box area showing neo-vascular structures (black arrows) inside the mesh area (M) and **(D)** blue box area showing neo-vascular structures (black arrows) around the mesh area.

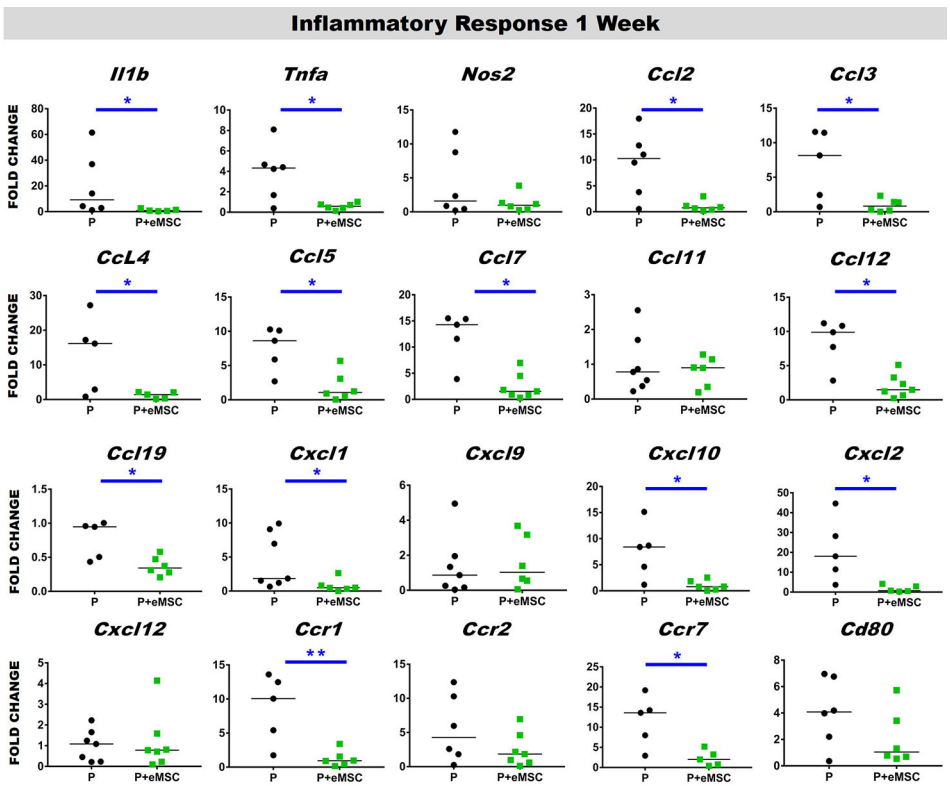

FIGURE 7 | Acute Inflammatory gene expression after 1 week implantation of P and P+eMSC. Fold change in mRNA expression of mouse inflammatory genes by quantitative PCR in explanted mice tissues consisting of P nanomesh, with and without endometrial mesenchymal stem/stromal cells (eMSCs) after 1 week. Data are median of n=5-7 samples/group analyzed by Mann-Whitney U test; *P < 0.05 and **P < 0.01.

other groups indicate that macrophages orchestrate the tissue response and healing process after biomaterial implantation and that macrophage polarization determines the outcome of the immune response (Ulrich et al., 2012; Roman Regueros et al., 2014; Feola et al., 2015; Darzi et al., 2016a; Paul et al., 2019). Our results show that eMSC-based nanomesh implants corroborate known M2 macrophage effects after 6 weeks implantation in mice by increased expression of *Il10 and Tgfb* genes which induced the M2 phenotypic genes *Arg1* and *Mrc1* on the accumulating macrophages. eMSCs also induced upregulation of angiogenic genes *Tgfbr, Vegfa, Ang1,* and *Pdgfa* promoting neovascularisation around the meshes and the chemotactic and recruitment genes *Cxcl12, Ccr2,* and *Ccl11* to promote the initial macrophage accumulation around the implanted mesh. Therefore, it is clear that eMSCs have a direct impact on the host macrophages by polarizing them to an M2 phenotype and proactively modulating their response to the implanted biomaterial *in vivo* that promotes nanomesh integration.

Once macrophages are recruited to the implant surface, they begin to accumulate and release chemo-attractive signals such as

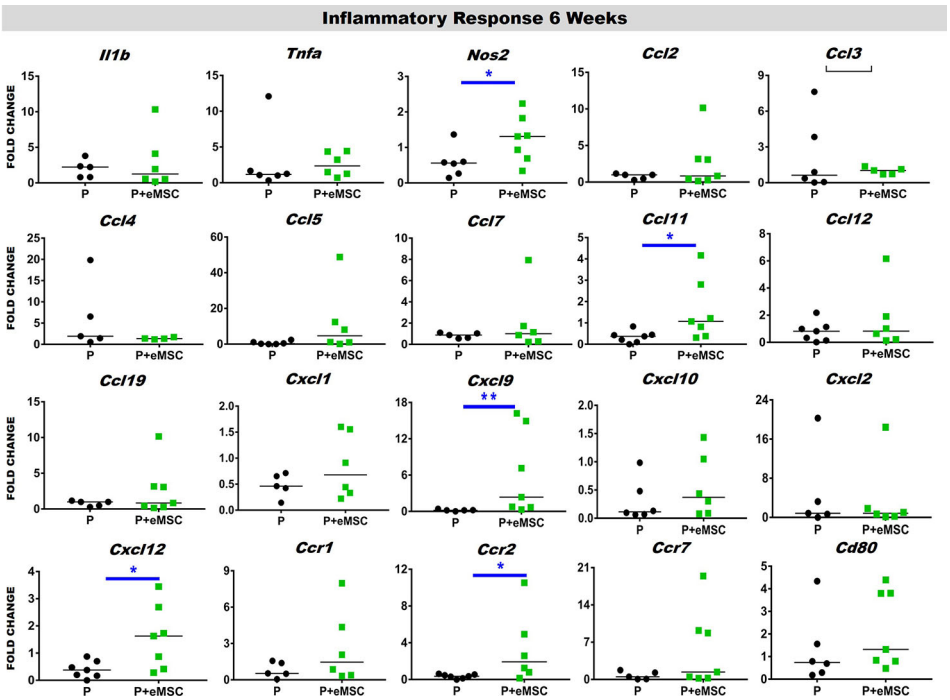

FIGURE 8 | Inflammatory gene expression after 6 week implantation. Fold change in mRNA expression of mouse inflammatory genes by quantitative PCR in explanted mice tissues consisting of P nanomesh, with and without endometrial mesenchymal stem/stromal cells (eMSCs) after 6 weeks. Data are median of n=5-7 samples/group analyzed by Mann-Whitney U test; *P < 0.05 and **P < 0.01.

TNF-α, IL-1β, IL-6, and CCL2 that further enhances macrophage assembly at the interface (Anderson et al., 2008; Van Linthout et al., 2014; Kyriakides, 2015). Our results show that a number of chemotactic genes (*Ccr2, Ccl11, Cxcl9,* and *Cxcl12*) are upregulated in the eMSC-containing constructs and their reduction over time correlates with the progression of the FBR in a modulated manner at the surface tissue interface. This is evident from significantly elevated expression of cell adhesion genes *Itgb1*, *Cdh1*, *Cdh2*, *Vcam1,* and *Cd44*. The JAK/STAT signalling pathway is activated in the FBR when IL-4 binds to its receptor on macrophages, inducing the phosphorylation of STAT6 which translocates to the nucleus and upregulates the expression of E- cadherin or N-cadherin and β-catenin (Moreno et al., 2007). Upregulation of this adhesion molecule enhances cell-cell interactions, induces the fusion of macrophages (Mcnally et al., 1996; Van Linthout et al., 2014) and modulates the M2 response, mainly *in vitro* (Van Den Bossche et al., 2015). After macrophages are bound *via* their integrin receptors, downstream signal transduction can affect cytoskeletal rearrangement and formation of more adhesion structures allowing macrophages to spread over the biomaterial surface as we observed in this study. This spreading is facilitated by specialized macrophage podosomes consist of actin filaments that are associated with both initial macrophage adhesion and subsequent macrophage fusion to form FBGCs (Kyriakides, 2015; Chung et al., 2017). Our results indicate that eMSCs modulated several cell adhesion genes to promote a coordinated interaction with the biomaterial and promote graft tissue integration, albeit at a later time point. Such a response is critical to favorable long-term outcomes after mesh implant-based POP reconstructive surgery.

Nanomeshes themselves may not have the mechanical properties to alleviate POP symptoms and therefore, we envision they augment native tissue repair surgery. However, over time, the bioengineered nanomeshes can stimulate the body to produce ECM which will not only drive tissue integration but also provide sufficient mechanical strength to the vaginal wall to prevent further herniation of pelvic organs into the vagina in POP following surgery. Herein, we observe that eMSC promoted a synergistic action between expression of matrix formation genes such as *Col1a1*, *Col3a1*, *Col6a1*, *Col6a2*, and other genes associated with fibroblast activity such as *Tgfb* as well as the ECM regulation genes, *Mmps* and *Timps* after 6 weeks of nanomesh implantation. In general, fibroblasts are the cells responsible for maintaining ECM homeostasis (Kastellorizios et al., 2015) by producing and remodelling ECM, mediated by tightly regulated and opposing activities of *Mmps* and *Timps*. Given the balanced expression of ECM forming and regulating genes, eMSCs promote recruitment of fibroblasts (**Figure S4**) to maintain this homeostatic balance in the tissue environment. Fibroblasts are chemotactically attracted to the site of injury such as *Cxcl12*, where they are induced to proliferate and secrete ECM in a process referred to as fibroplasia (Kendall and Feghali-Bostwick, 2014). Indeed, several ECM genes are upregulated in presence of

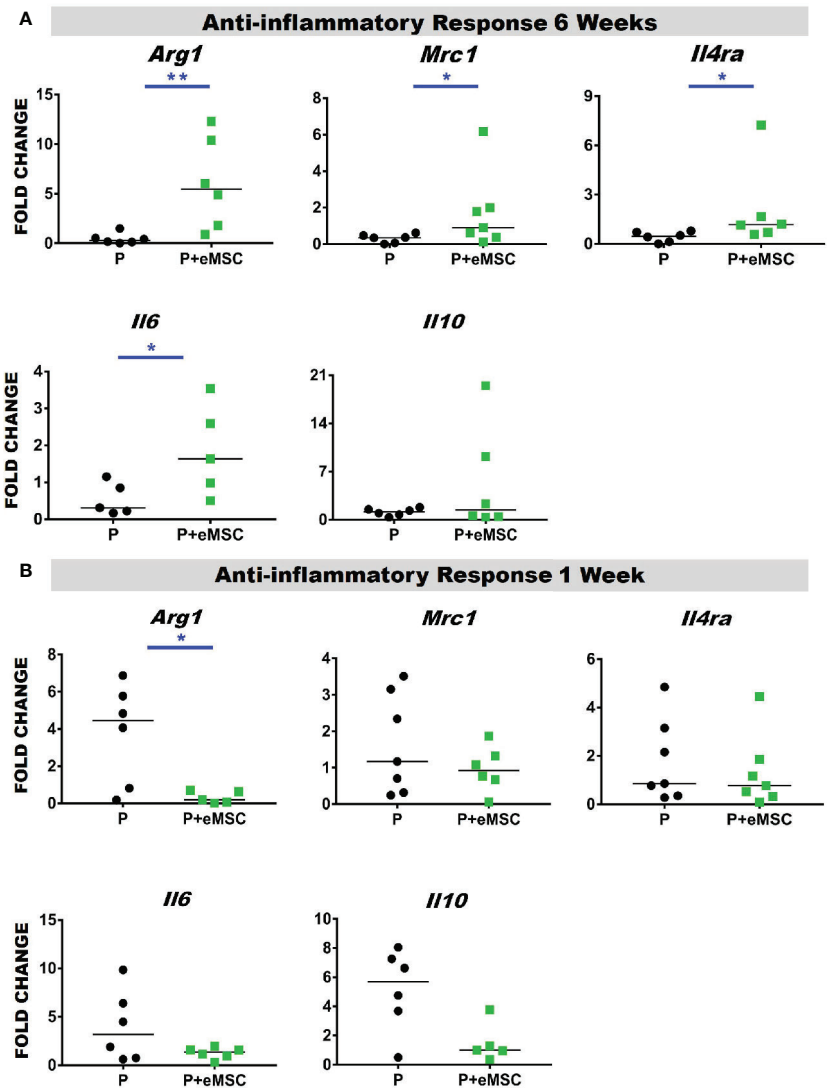

FIGURE 9 | Anti-inflammatory gene expression of P and P+eMSCs nanomesh explants. Fold change in mRNA expression of mouse inflammatory genes by quantitative PCR in explanted mice tissues consisting of P nanomesh, with and without endometrial mesenchymal stem/stromal cells (eMSCs) after **(A)** 6 weeks and **(B)** 1 week. Data are median of n=5-7 samples/group analyzed by Mann-Whitney U test; *P < 0.05 and **P < 0.01.

eMSCs. Therefore, it is likely that fibroblasts participate in the later stages of inflammation by responding to Tgfb1, Il1b and Il6, proteins which increase matrix production, in addition to lipids such as prostaglandins and leukotrienes (Kendall and Feghali-Bostwick, 2014; Jones, 2015). Fibroblasts can also produce TGFβ1, IL-1β, IL-33, CXC and CC-chemokines, and ROS, which serve to recruit and activate macrophages (Kendall and Feghali-Bostwick, 2014; Jones, 2015). Our results indicate that eMSC influence gene expression of these factors released in repairing tissues such as *Tgfbs*, *Il1b*, and *Il6*. Several CXC and CC-chemokines impacting cellular recruitment and angiogenesis such as *Ccl2*, *Ccl3*, *Ccl4*, *Ccl5*, *Ccl12*, *Cxcl2*, *Cxcl3*, and *Cxcl10* were also upregulated by the presence of eMSCs highlights their influence on host fibroblast activity. MMP and TIMP are also produced by macrophage themselves that influence remodelling in the local environment.(Nakashima et al., 1998; Laquerriere et al., 2004). Macrophages themselves are known to release MMPs such as MMP1, 2, and 9 in proportions related to biomaterial debris around the bone prosthetic materials. Nonphagocytable particles showed more MMP-9 where as phagocytable debris were associated with larger amounts of Il-1β.(Laquerriere et al., 2004) Although most of these studies are performed around bone remodelling, they show that macrophage response to biomaterials may be driven by local environment conditions. While the formation of ECM is often associated with fibrosis and its deleterious effects of encapsulating mesh, we show a higher and balanced expression of MMPs and TIMPs together with increased *Tgfb*, a fibroblast stimulator. This finding indicates that ECM formation is highly regulated and that the presence of eMSC controls and minimizes

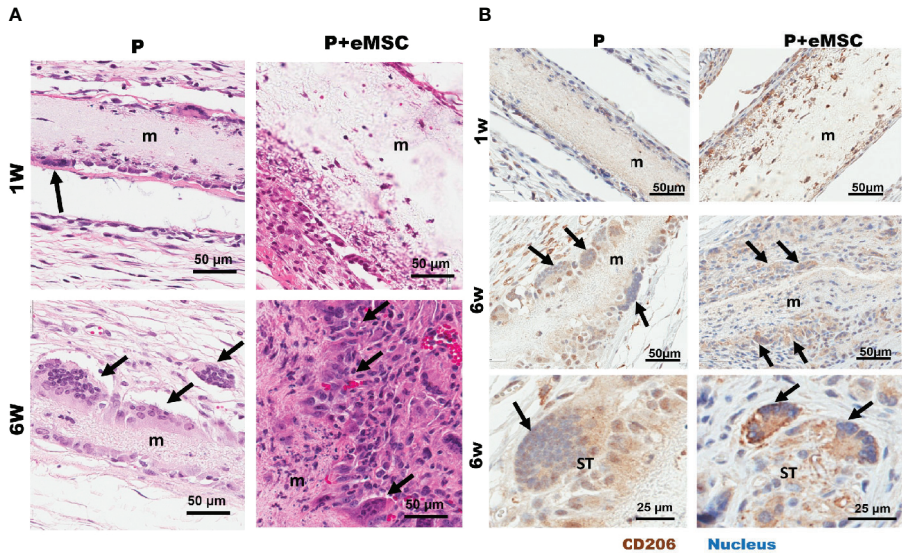

FIGURE 10 | Characterization of Biomaterial-induced Multinucleated Foreign body Giant Cells (FBGC). **(A)** H&E staining of mice tissue sections comprising P nanomesh implants (m) with and without endometrial mesenchymal stem/stromal cell (eMSCs), in the subcutaneous flank between the abdominal wall and skin showing fusion of macrophages into multinucleated FBGC after 1 week and 6 weeks. **(B)** Multinucleated (blue stain) FBGCs (black arrows) show M2 type phenotype as characterized by CD206 (brown) immunostaining and are present along the mesh edges (m) and in the surrounding tissues (ST).

fibrosis at 6 weeks and in turn stimulates *Tgfb1* expression. A balance between TGFβ and TIMP-1also aids nonfibrotic tissue repair (Jones, 2015). *Tgfb* also causes fibroblast deposition of ECM and secretion of many paracrine and autocrine growth factors, including CTGF (Leask et al., 2009) as confirmed by increased *Ctgf* gene expression in the presence of eMSCs. In humans, CTGF is involved in angiogenesis, cell migration, adhesion, proliferation, tissue wound repair, and ECM regulation and is induced by TGFβ and IL-1β (Kendall and Feghali-Bostwick, 2014; Jones, 2015). CTGF also binds to ECM proteins and growth factors including VEGF and TGFβ to maximally induce type I collagen synthesis, α-SMA and also increases *IL6* expression (Jun and Lau, 2011; Liu et al., 2012). Our gene expression results along with co-localization studies (**Figure S4**) suggest that the paracrine effects of eMSCs directly influence these molecular pathways to regulate, synthesise and maintain ECM, even within nanomeshes as seen in **Figure 2**.

Inflammatory factors and adhesion molecules such as ICAM and VCAM recruit monocytes, mast cells, and fibroblasts, all of which can produce proangiogenic factors, including VEGF and FGF through a cascade of cell and chemokine interactions (Mahdavian Delavary et al., 2011). Our results showed that eMSC increased *Vcam*, *Cxcl12*, and *Ccr2* suggesting that these in turn increased expression of the proangiogenic factors *Vegfa*, *Fgf1*, and *Ang1* to promote angiogenesis. VEGF-A directly stimulates endothelial cell proliferation by engaging with the VEGFR-2 to activate its tyrosine kinase domain and initiate the sprouting of new vessels from existing micro-vessels (Mahdavian Delavary et al., 2011). VEGF, likely produced by the recruited macrophages and fibroblasts, may also contribute to the angiogenic process by mobilizing endothelial progenitor cells and other myeloid cells to the site of angiogenesis (Antonella and Fabio, 2005). Overall, the neovascularisation of the nascent ECM is critical for ensuring viability of the new tissue surrounding the nanomesh. The upregulation of proangiogenic chemokine genes such as *Ccl11* and its receptor Ccr2, *Cxcl9*, and *Cxcl10* to some extent at 6 weeks after P implantation, suggest these are key players in initiating angiogenesis and fibroblast activity. In humans, CCL11 and well as CXCL10 are known to recruit eosinophils that subsequently induce a prolonged angiogenic effect (Van Linthout et al., 2014). This signifies that eMSC promote angiogenesis through paracrine effects even after mesh implantation. Our results also show eMSC increase *Pdgfr*, *Ang1*, and *Tgfb* expression, that are associated with pericytes to stabilize nascent endothelial cell tubes during angiogenesis (Antonella and Fabio, 2005). These results are highly encouraging given that blood vessels were found in close proximity to the P nanomesh, a highly desirable tissue engineering outcome (Shahabipour et al., 2019), and completely integrated with tissue by 6 weeks suggesting a highly influential role for eMSC in modulating the entire FBR process.

Our previous studies have shown that bioengineering of eMSCs indeed modulates the FBR process to both nondegradable (Ulrich et al., 2014; Darzi et al., 2018; Emmerson et al., 2019) and degradable nanomeshes (Mukherjee et al., 2019b; Paul et al., 2019) and can be detected *in vivo* even upto 6 weeks after implantation (**Figure S3**). Yet, we showed that eMSC facilitated M2 polarization of macrophages with immune-regulatory properties that dampen inflammation. In this cascade upregulation of cytokine and chemokine genes such as *Ccl7*, that also indirectly influence the adaptive Th2 immune system as they recruit other innate immune cells such as

basophils and mast cells (Biswas and Mantovani, 2010). They also promote angiogenesis and wound healing *via* the production of PDGFA and VEGF (Antonella and Fabio, 2005; Martinez et al., 2006; Martinez et al., 2008) genes upregulated by the presence of eMSCs. Macrophages participate in a number of ways to regenerate tissue and heal wounds through a cascade of inflammatory responses, thereby contributing to tissue ECM formation. eMSCs promote *Mmp19 and Mmp2* that degrade the ECM (Jones, 2015; Mukherjee et al., 2019a) which releases growth factors and chemokines (Detry et al., 2012) respectively. The upregulation of MMP2 and MMP19, which are promoters of angiogenesis through release of ECM growth factors (Webb et al., 2017; Liu et al., 2018b) likely contributed to the angiogenesis observed in our study. While we note the increase in *IL-6* and *IL-4ra*, which are mostly associated with pro-inflammatory responses, they also have anti-inflammatory roles (Fuster and Walsh, 2014). A recent landmark study has shown that IL-6 primes macrophages for IL-4-dependent M2 polarization by inducing IL-4RA expression *via* STAT3-mediated activation of the IL4ra (Mauer et al., 2014). Thus, macrophages have different functions during the healing, macrophage process (Galdiero and Mantovani, 2015; Rőszer, 2015). This is clearly seen in our results, where eMSCs increased the expression of genes associated with the M2 macrophage phenotype and the healing response.

The presence of FBGCs after biomaterial implantation is often viewed as a negative response and has been directly linked to FBR leading to material rejection. Recent accumulating evidence questioned the role of FBRCs in these deleterious effects. Both *in vitro* and *in vivo* studies have shown that FBGC exhibit different phenotypic profiles, in particular the expression of both pro and anti-inflammatory cytokines, depending on the physicochemical characteristics of the biomaterials (Ghanaati et al., 2010; Mcnally and Anderson, 2015). Herein, we showed FBGCs with an M2 phenotype with differences in their fusion pattern based on the cellular component (ie eMSC) of the bioengineered implant. Recent reports have indicated that FBGCs are a potent source of VEGF, promote mannose receptor mediated phagocytic processes and may be involved in the process of implant bed vascularization by stimulating angiogenesis (Mcnally and Anderson, 2011; Mcnally and Anderson, 2015; Barbeck et al., 2016). In agreement, we showed CD206 expressing FBGC in P +eMSC explants after 6 weeks, together with significant upregulation of several angiogenic genes and formation of neo-vessels. Moreover, several chemokines and cell adhesion genes which were down regulated at 1 week but upregulated at 6 weeks, may be involved in the recruitment and fusion of macrophages to form FBGC. MSC incorporated biomaterials modulate bone healing by formation of FBGC that ultimately lead to angiogenesis and long term stability of implants in humans.(Miron and Bosshardt, 2018) Given their capacity to promote both tissue inflammatory and/or tissue wound healing, the appropriate characterization of FBGCs is therefore critical. While, further studies are pivotal to establish their exact role and mechanisms of cell–cell communication, our study suggests that they may be closely associated with rapid establishment of homeostasis after implantation by aiding in key tissue repair processes.

Since the discovery of eMSCs, it has been applied to various areas of research including POP treatment (Gargett et al., 2016). Our research has shown that eMSCs can modulate FBR to various types of meshes, a phenomenon we suspected to be a paracrine effect(Gargett et al., 2019). Moreover, there is an urgent unmet health need and heavy drive in design of biomaterials that can be used for regenerative medicine, including POP treatment (Mukherjee et al., 2010; Gargett et al., 2019). These approaches include surface modifications and growth factor release from materials to modulate the FBR and repair process. This study provides an insight into the gene expression profile of host response that are modulated by eMSCs that is likely to aid researchers in the field of biomaterials and regenerative medicine with evidence and knowledge to better design constructs. Our results also help to understand FBR processes that are particularly impacted by eMSCs and will enable future studies in uncovering the exact mechanisms to hopefully overcome the current hurdles in clinical care.

CONCLUSION

In summary, our study provides the first extensive profiling of gene expression following P nanomesh implantation and the impact of tissue engineering them with eMSCs. Our results show that eMSC, most likely through their paracrine effects, significantly modulate the elicited FBR. In particular, eMSCs induce upregulation of ECM, cell adhesion and angiogenic genes, most likely through the increased expression of several chemokines and cytokines at 6 weeks but not acutely at 1week. However, in the absence of eMSCs, the acute response is pro-inflammatory, while the presence of eMSCs leads to a M2 healing response after 6 weeks following P nanomesh implantation. Thus, the initial alterations to the FBR mediated by eMSCs show longer term favorable outcomes. The expression of these genes collectively leads to the formation newly synthesized ECM within the nanomeshes and neo-vasculature in close proximity. From a tissue engineering perspective, this is a hallmark of a highly successful implant and will likely overcome the current hurdles faced in POP treatment.

STUDY LIMITATION

(1) This study used tissues that were close to the meshes implanted for the gene expression study. Although ideal, it was not feasible to extract the cells that infiltrated the mesh due to technical challenges. (2) This study was performed in a mice subcutaneous model rather than vaginal model owing to the small size of mouse vagina. Further studies in larger animal models are needed to fully understand the exact immunogenic properties of these constructs in the vaginal environment.

DATA AVAILABILITY STATEMENT

The data generated for this study can be found in GEO using accession number GSE141960.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Monash Health and Monash University Human Research Ethics committees (09270B). The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by Monash Medical Centre Animal Ethics Committee A (2017/05).

AUTHOR CONTRIBUTIONS

Study conception and design: SM, SD, JW, CG. Ethics and animal care: SM, SD, FC, KP, CG. Experiment design: SM, SD, FC. Perform experiment: SM, SD, KP. Statistical analysis: SM, SD, FC. Manuscript writing and editing: SM, SD, CG, JW.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2020.00353/full#supplementary-material

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Cancer Stem Cell-Exosomes, Unexposed Player in Tumorigenicity

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INTRODUCTION

Cancer is a well-known, yet poorly understood disease. In which, a healthy tissue is morphed into a cancerous tissue through an intricate, multistep process. This polymorphism has been the focus of cancer research for many decades. Scientists have agreed on a set of traits that are thought to be shared by all cancer tissue types, these traits include; enabling proliferation, evading growth suppressors, resisting cell death, replicative immortality, inducing angiogenesis, and initiating invasion and metastasis, along with other enabling characteristics (Hanahan and Weinberg, 2000; Hanahan and Weinberg, 2011). As researchers investigate the development and propagation of these traits, or as they are called the "hallmarks" of cancer, it became evident that cancer cell-derived extracellular vesicles (EVs), particularly exosomes, play a major role in almost all of them.

In the late 1940s, it was recognized that cells release spherical shaped particles called EVs (Chargaff and West, 1946). Then, almost 40 years later, "exosomes" were acknowledge as a distinct sub-type of EVs (Trams et al., 1981). Up tell now, it is technically challenging to obtain a pure fraction of a specific EV sub-type, due to similarities shared amongst these vesicles. However, the International Society for Extracellular Vesicles, have released a position statement on the minimal experimental requirements for definition of EVs and their functions (MISEV2014; updated in 2018; MISEV2018) (Lotvall et al., 2014; Théry et al., 2018). The MISEV distinction between the different EV sub-types realize on size, density, morphology, subcellular origin, and composition. This was done in order to make scientific reporting on EV biology more consistent and reliable. Most published literature on EVs, including the literature on the role of EVs in cancer, use the term "exosomes" to refer to the EV sub-type under study. These studies include a section that describes the method of "exosome" isolation, and at least a couple of characterization techniques, to justify their nomenclature. Characterization of exosomes in published literature is often based on size and "exosome-enriched" proteins content verification.

On the other hand, the concept of "cancer stem cell" (CSC) only emerged in the 1990s (Lapidot et al., 1994), with a lot of controversy and a number of proposed theories following it. Some say that CSC arise as a result of normal stem cell mutation, while others suggests that CSC arise as a result of a somatic cell acquiring erroneous stem cell characteristics, turning it into cancerous stem cell that can differentiate into heterogeneous population of cancer cells (Baccelli and Trumpp, 2012).

Abbreviations: EV, Extracellular Vesicle; CSC, Cancer Stem Cell; MVBs, Multivesicular Bodies; miRNA, micro Ribonucleic Acid; mRNA, messenger Ribonucleic Acid; EMT, Epithelial–Mesenchymal Transition; BMMSC, Bone-Marrow Mesenchymal Stem Cell; CD, Clusters of Differentiation; VEGF, Vascular Endothelial Growth Factor; STAT3; Signal Transducer and Activator of Transcription-3; IL, Interleukin.

Nevertheless, CSCs are now recognized as distinct population of cancer cells, and the CSC-model, is accepted as one of the two most popular models of cancer. The other model being the "clonal evolution-model", which was described earlier in 1970s. It was postulated that cancer results from the accumulation of mutations in a given group of somatic cell population, within a tissue, thus given raise to heterogeneous population of cancer cells (Nowell, 1976). As the CSC-model becomes more popular, the role of CSCs, as a sub-type of cancer cells, within the tumor microenvironment has recently come to light, especially with advances in stem cell research during the last couple of decades. However, the role of CSC-exosomes, as a sub-type of cancer exosomes, is still under the shadow. Thus, in this article we aim to provide a standpoint on the possible role of CSC-exosomes, and why it should be examined as a separate group of cancer cell-exosomes, based on published literature.

Exosomes, Devoted Messengers for Good or Bad

Exosomes originate from the inward budding of the early endosomes, which later mature into multivesicular bodies (MVBs) (Doyle and Wang, 2019). Depending on their content, MVBs are either sent to the lysosome to be degraded or released into the extracellular space, forming what's called exosomes (Doyle and Wang, 2019). Cells of different tissue types were found to release exosomes in order to facilitate intercellular communication, thus initiating different biological actions (Ma et al., 2019). Cancer cells, and cancer-associated cells, within the tumor micro-environment were also found to release exosomes. This allows them to commute their message to malignant and non-malignant cells, and initiate pathways that support tumor survival and propagation (Wortzel et al., 2019). The exosome mediated intercellular communication is enabled through "exosomal cargo". This includes functional proteins, micro-ribonucleic acid (miRNAs) and messenger RNAs (mRNAs) (Hessvik and Llorente, 2018). Exosomes will deliver its cargo from the releasing cell into the recipient cell, which contains the encrypted message. There is a growing body of published literature on the role of cancer cell-exosomes in promoting cancer progression through enabling recipient cells to acquire the mentioned "hall marks" of cancer. A number of studies, have repeatedly shown that cancer cell-exosomes, of different cancer types, significantly increase cancer cell proliferation and inhibit apoptosis by activating various proposed cellular pathways (Zhang et al., 2018; Qian et al., 2019). Studies have also shown that cancer cell-exosomes stimulate angiogenesis by stimulating endothelial cells viability, migration, and tube formation *via* the transfer of pro-angiogenic proteins and miRNAs (Yi et al., 2015; Bao et al., 2018; Lin et al., 2018; Yukawa et al., 2018). Likewise, it was reported that cancer cell-exosomes induce replicative immortality *via* the transfer of telomerase reverse transcriptase mRNA from the telomerase activate cancer cell to the telomerase silenced somatic cell (Gutkin et al., 2016). As for metastasis, it is projected that cancer cells induce metastasis by packing its exosomes with promoters of the epithelial–mesenchymal transition (EMT) cascade, to initiate EMT in the neoplastic epithelial cells, within the tumor microenvironment (Webber et al., 2015; Rahman et al., 2016; Xiao et al., 2016). It is also projected that cancer cells will establish a "pre-metastatic" niche through its exosomes. Cancer cells will release its exosomes into the circulation, where they travel to the metastasis site (Costa-Silva et al., 2015; Liu et al., 2016; Syn et al., 2016). There, cancer cell-exosomes will up-regulate the pro-inflammatory molecules, and vascular leakiness, to mobilize cells that constitute the pre-metastatic niche (Costa-Silva et al., 2015; Liu et al., 2016; Syn et al., 2016). Finally, it is projected that while traveling through the circulation, and engraftment into the new tissue, cancer cell-exosomes support cancer cells by allowing them to escape immune surveillance (Mrizak et al., 2015; Muller et al., 2016; Song et al., 2016). Moreover, in addition to the classical hall marks of cancer, it was reported by a recent study that prostate cancer cell-exosomes play a role in transforming local prostate tissue stem cells into CSCs (Ngalame et al., 2018). While another study reported that glioma cell-exosomes induced a "tumor-like" phenotype in bone-marrow mesenchymal stem cells (BMMSCs) (Ma et al., 2019). This was reported to be based on increased proliferation, migration, and invasion rates of treated BMMSCs. In addition to alteration in BMMSCs protein production, including the production of the metastasis-related proteins.

Cancer Stem Cell, the Black Sheep of the Stem Cell Family

CSCs are cancer cells (found within tumors) that possess characteristics associated with normal stem cells, specifically self-renewal and the ability to differentiate and give rise to different cell types found in a particular cancer specimen i.e. CSCs are tumor-forming cells (Sun et al., 2018). CSCs can be identified by using a set of unified surface markers (i.e. clusters of differentiation (CD); CD44, CD24, CD133), in addition to added tissue specific markers depending on cancer type (Phi et al., 2018). Within the tumor microenvironment, the CSCs are rear and reside in highly specialized niches (Sreepadmanabh and Toley, 2018). The CSCs niche is designed to maintain and protect the CSCs, allowing them to resist many current anticancer treatments (Prieto-Vila et al., 2017). The CSCs niche will also allow the cells to stay dormant for long periods of time, before initiating local recurrent and/or distant metastatic tumors (Plaks et al., 2015). Thus, it is hypothesized that targeting the whole tumor will only slow down tumor expansion while targeting the CSCs, in particular, will jeopardize tumor growth (Garcia-Mayea et al., 2019). At the same time, in regenerative medicine research, it was reported that stem cells and progenitor cells exert their tissue regeneration effects through the release of paracrine factors, mainly exosomes. Studies are consistently showing that injecting the cell-derived exosomes alone, is enough to induce the same regenerative effect as the "whole-cell" transplant approach. For example, it was reported that exosomes derived from embryonic stem cells (Khan et al., 2015), BMMSCs (Zou et al., 2019), and cardiac progenitor cells (Kervadec et al., 2016), all mimic the benefits of injecting their parent cells in a chronic heart failure and myocardial infarction animal models. Thus, it is logical to assume that CSCs function through the same

mechanism as other cancer cells and non-cancer stem cells. We can project that CSCs fulfill its "stemness duties" through the release of paracrine factors, with exosomes as a key player.

What Is Proposed?

As discussed above, cancer cell-exosomes are crucial for tumor initiation, maintenance, and propagation. However, published literature on this subject matter often don't describe the subtype of cancer cells that these exosomes were derived from. It is well established by now that cancer cell-exosomes mediate cell to cell communication within the tumor microenvironment, to support and promote tumorigenesis. It is also well established by now that any alteration to parent cell, alters exosome secretion and content, which in turn alters its message. For example, when cancer cells were subjected to hypoxia prior to exosome isolation, to reflect the tumor's hypoxic environment, these exosomes significantly increased migration and invasion of cancer cells (Li et al., 2016), and tube formation by endothelia cells (Kucharzewska et al., 2013; Hsu et al., 2017), compared with exosomes derived from normoxic cancer cells. Therefore, it could be hypothesized that the sub-population of cancer cells, CSCs, produce exosomes with unique characteristics, and thus functions. Currently, there are only few reports on "CSC-derived exosomes", and their role in cancer propagation, compared to "non-stem cancer cell-derived exosomes" (**Table 1**). One of the first studies to address this issue reported that the "macrovesicles" that had the *in vitro* and *in vivo* angiogenic effect, in renal cancer, were those driven from the $CD105^+$ cancer cell sub-population (Grange et al., 2011). Then later on, one study did a miRNA content comparison, and reported that prostate CSC-derived exosomes have in fact a different miRNA content compared with non-stem prostate cancer cell-derived exosomes (Sánchez et al., 2016). Then, a following study reported that glioma stem cell-derived exosomes promoted angiogenesis by containing a particularly high levels of miRNA-21, which upregulates the vascular endothelial growth factor (VEGF) (Sun et al., 2017). While another study identified 11 miRNAs that are characteristic of gastric CSC-derived exosomes, and suggested that a measurement of these miRNAs in patient serum could be used as a predictor of cancer metastasis (Sun et al., 2017). Other recent CSC-exosomes investigations focusing on their role in metastasis, reported that CSC-derived exosomes promote metastasis by promoting EMT in renal cell carcinoma (Wang et al., 2019) and thyroid cancer (Hardin et al., 2018) *via* the transfer of miRNA-19b-3p and non-coding-RNAs respectively. Whereas other reported on CSC-exosome role in creating a pro-tumoral microenvironment. For example, it was reported that glioblastoma stem cell-derived exosomes direct monocytes toward the immune suppressive "M2" phenotype, through the signal transducer and activator of transcription-3 (STAT3) pathway, creating an immunosuppressive microenvironment (Gabrusiewicz et al., 2018). While colorectal cancer stem cell-derived exosomes promote a pro-tumoral phenotype in neutrophils by increasing interleukin-(IL)-1β expression (Hwang et al., 2019)

Since tumor–host cross-talk is believed to be initiated by CSCs, and communication between cancer cells and other cells is conducted through exosomes, it's of great importance to take a closer look at the role of CSCs-exosomes, and its involvement in

TABLE 1 | Summary of published work on the distinct role of CSC-derived exosomes in tumorigenicity.

Exosome population	Tumorigenic action	Proposed mechanism of action	Reference
Macrovesicles derived from CD105+ cells of renal carcinoma specimens	Promoted angiogenesis and metastasis both *in vitro* and *in vivo*	miRNA screening showed 24 upregulated, and 33 downregulated miRNAs in $CD105^+$ macrovesicles compared to $CD105^-$ macrovesicles. This distinct miRNA composition favor tumor growth and invasion.	(Grange et al., 2011)
Exosomes derived from CD133+ cells of glioblastoma cell line	Increased the *in vitro* angiogenic capacity of endothelial cells	miRNA analysis revealed elevated levels of miRNA-21 in the CD133+ cells, hypothesizing that the derived exosomes promoted angiogenesis through the miRNA-21/VEGF pathway.	(Sun et al., 2017)
Exosomes derived from CD105+ cells of clear cell renal cell carcinoma specimens	Induced EMT of cancer cells *in vitro*, and promoted metastasis *in vivo*	miRNA analysis revealed elevated levels of miRNA-19b-3p in the CD105+ cell-derived exosomes. This in turn affected the protein levels of PTEN, a key mediator of cell migration.	(Wang et al., 2019)
Exosomes derived from spheroid formations of thyroid cancer cell lines	Induced *in vitro* EMT in normal and non-cancerous thyroid cells	miRNA analysis revealed elevated levels of MALTA1, EMT marker SLUG and stem cell marker SOX2, in exosome treated cells.	(Hardin et al., 2018)
Exosomes derived from glioblastoma cell lines cells cultured in stem cell-permissive medium	Polarized monocytes into M2 macrophage phenotype	Western Blot analysis revealed up regulation of PD-L1 in exosome-treated monocytes. PD-L1 correlates with increased STAT3 pathway phosphorylation, which mediate this immune suppressive switch.	(Gabrusiewicz et al., 2018)
Exosomes derived from spheroid formations of colorectal cancer cell line	Prompted a pro-tumoral phenotype in neutrophils	miRNA and ELISA analysis revealed elevated levels of IL-1β in exosome-treated neutrophils and their condition medium.	(Hwang et al., 2019)

PTEN, phosphatase and tensin homolog; MALTA1, metastasis associated lung adenocarcinoma transcript 1; PD-l1, programmed death-ligand 1; ELISA, enzyme-linked immunosorbent assay.

tumor aggressiveness. Also, to examine their miRNA content, compared to non-stem cancer cell- exosomes, in order to postulate mechanisms of actions. Then finally, develop a cancer management strategy that targets CSCs, and involves blockage of the CSC-exosome release channels.

DISCUSSION

CSCs generate tumors through the stem cell processes of self-renewal and differentiation into multiple malignant cell types. Based on advances in cell signaling biology, it's expected that these CSCs function through its exosomes. The term "exosome" was used in this article due to the fact that published literature describing EVs role in cancer often refer to the EV sub-type being examined as exosomes. These publications offer reasonable evidence that the EV sub-type being examined is in fact exosomes, *via* various methods of characterization. Other sub-types of EVs i.e. ectosomes, microvesicle particles, and apoptotic bodies, could be released by cancer cells/CSCs, and could play a role as well. However there is no adequate reporting on this in the literature. Therefore, based on findings on the role of cancer cell- exosomes, and the role of CSCs in cancer, the role of "CSC-exosomes" should be investigated as a separate entity. Such studies will encounter a significant technical and quality control issues related to harvestation of a pure CSC population, and subsequent yield of pure CSC-exosome fraction. Nevertheless, the knowledge provided by these studies will be crucial in developing a more effective approaches to control progression and metastasis of tumors and prevent recurrence.

AUTHOR CONTRIBUTIONS

BA-S conceptualized and wrote the article. Other authors were involved in manuscript review and editing.

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REVIEW
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Biological Considerations in Scaling Up Therapeutic Cell Manufacturing

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Cell therapeutics — using cells as living drugs — have made advances in many areas of medicine. One of the most clinically studied cell-based therapy products is mesenchymal stromal cells (MSCs), which have shown promising results in promoting tissue regeneration and modulating inflammation. However, MSC therapy requires large numbers of cells, the generation of which is not feasible *via* conventional planar tissue culture methods. Scale-up manufacturing methods (e.g., propagation on microcarriers in stirred-tank bioreactors), however, are not specifically tailored for MSC expansion. These processes may, in principle, alter the cell secretome, a vital component underlying the immunosuppressive properties and clinical effectiveness of MSCs. This review outlines our current understanding of MSC properties and immunomodulatory function, expansion in commercial manufacturing systems, and gaps in our knowledge that need to be addressed for effective up-scaling commercialization of MSC therapy.

Keywords: mesenchymal stromal cells, immunomodulatory, secretome, cell therapy, biomanufacturing, bioreactors, microcarriers

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INTRODUCTION

Mesenchymal stromal cells (also known as mesenchymal stem cells; MSCs) are fibroblastic precursor cells isolated in the stromal fraction of many adult tissues, including bone marrow, adipose tissue, and umbilical cord (Musiał-Wysocka et al., 2019). Originally described as bone marrow stroma-derived stem cells capable of forming ectopic hematopoietic niches (Owen and Friedenstein, 1988), MSCs were used in clinical trials for skeletal tissue repair (Owston et al., 2016). Aside from skeletal repair, it is now well recognized that MSCs have many more potential therapeutic benefits due to their immunomodulatory effects on innate and adaptive immune cells. These effects have been largely attributed to their secretory products, including immunoregulatory cytokines and molecules, growth factors, and exosomes (Castro et al., 2019). Animal and other preclinical studies have shown MSCs to be highly efficacious in wide range of ischemic, degenerative, metabolic, inflammatory, or autoimmune disease conditions (Galipeau and Sensebe, 2018; Martin et al., 2019), fuelling enthusiasm for their clinical translation. However, the large quantities of MSCs required for clinical application necessitate generation by larger scale manufacturing processes, including microcarrier-based systems in bioreactors. It is not well understood how these manufacturing processes may alter the MSC immunophenotype and secretome, compared to smaller scale, more conventional planar culture, potentially impeding

therapeutic application (Vizoso et al., 2017). In this review, we detail cell manufacturing technologies used currently for MSC expansion and examine the knowledge gap in how such processes may impact on the biological properties and function of MSCs.

MESENCHYMAL STROMAL CELLS

As defined by the International Society for Cellular Therapy (Dominici et al., 2006), MSCs are plastic-adherent when cultured in tissue flasks under standard conditions, express CD73, CD90, and CD105, but lack CD45, CD34, CD14/CD11b, CD79α/CD19, and HLA-DR, and can differentiate into osteoblasts, adipocytes, and chondroblasts under standard differentiating conditions (Ullah et al., 2015). As this set of minimal criteria does not require clonal analyses or stringent *in vivo* studies, the MSCs used in different studies display significant batch-to-batch variations in phenotype and function (Wilson et al., 2019).

Tissue Sources of MSCs

Early MSC research focused on bone marrow-derived MSCs (BM-MSCs). However, bone marrow aspiration is highly invasive, painful, and increases the likelihood of donor-site morbidity (Strioga et al., 2012). MSCs have since been isolated from almost all postnatal tissues (da Silva Meirelles et al., 2006), including umbilical cord (Bieback and Kluter, 2007), placenta (Wu et al., 2018), dental pulp (Gronthos et al., 2000), and adipose tissue (Zuk et al., 2001). Of these tissue sources, adipose-derived MSCs (A-MSCs) are the most commonly investigated alternative to BM-MSCs. The approach of obtaining MSCs from subcutaneous adipose tissue obtained *via* lipectomy or liposuction has several advantages. The procedures involved are well established, conducted under local anesthesia, relatively non-invasive, and carry minimal risk and discomfort (Zuk, 2013). Excess adipose tissue, frequently discarded as medical waste, provides a valuable source of MSCs which are at approximately 500 times the concentration of BM-MSCs in bone marrow (Fraser et al., 2006; Hass et al., 2011). While BM-MSCs display increased osteoblast and chondroblast differentiation potential, A-MSCs have greater proliferative and secretory capacity (Li et al., 2015). Several studies have reported that A-MSCs exhibit greater immunomodulatory potential (Melief et al., 2013b; Menard et al., 2013), mainly due to increased production of a key molecule involved in T cell suppression, indoleamine-2,3-dioxygenase (IDO) (Menard et al., 2013). Whether these differences translate to increased therapeutic efficacy in clinical settings remains to be determined. However, in a mouse models of multiple sclerosis, A-MSCs were found to be more potent in inhibiting disease due to their broader expression of homing molecules (Payne et al., 2013). Thus, aside from proprietary concerns motivating the commercial use of A-MSCs, comparative analysis of A-MSCs and BM-MSCs from the same donors has indicated that A-MSCs may have increased immunomodulatory capacity (Menard et al., 2013).

MSC isolation from the bone marrow or adipose tissue is, however, associated with contamination from cell types inhabiting the anatomical region of the source tissue (Xu et al., 2010; Schneider et al., 2017). Of the cells that compose the adipose stromal-vascular cell fraction, stromal fibroblasts, and dermal fibroblasts are plastic adherent and may persist alongside cultured A-MSCs (Phinney et al., 1999; Blasi et al., 2011). Furthermore, the growth kinetics, differentiation potential, and immunogenicity of isolated BM-MSCs and A-MSCs can vary depending on donor age and health (Siegel et al., 2013; Choudhery et al., 2014). A-MSCs isolated from aged rats failed to elicit T cell suppression while BM-MSC mediated immunosuppression was noted to be more effective in young rats (Wu et al., 2014). A-MSCs derived from obese and type 2 diabetes patients were also less effective in suppressing lymphocyte proliferation and activating M2 macrophage phenotype (Serena et al., 2016). Therefore, although the ease of accessibility, greater yield, and immunosuppressive qualities of A-MSCs make them more suited to clinical application, caveats relating to MSC purity and donor health must be considered.

IMMUNOMODULATORY PROPERTIES OF MSCs

Part of the initial excitement with using MSCs as a therapeutic product resulted from their supposedly immune privilege status as MSCs do not express major histocompatibility complex (MHC) molecules involved in immune recognition (Le Blanc et al., 2003). This meant that MSCs could be expanded as an off-the-shelf, allogeneic product, and be administered to patients across MHC barriers (i.e., transplantable between HLA-mismatched patients), which is commercially attractive and clinically practical. However, it became apparent that MSCs do express MHC class I constitutively and upregulate MHC class II in the presence of inflammatory cues (Tse et al., 2003). Moreover, repeated injections of MSCs can elicit antibodies and lead to sensitization and rejection (Eliopoulos et al., 2005; Badillo et al., 2007; Campeau et al., 2009; Zangi et al., 2009). MSCs are susceptible to lysis by allogeneic $CD8^+$ T cells and NK cells (Crop et al., 2011). Recent findings have also indicated that injected MSCs are killed by cytotoxic T and NK cells in a tissue environment rich in these cells (Galleu et al., 2017). Nevertheless, despite the lack of cell differentiation or sustained engraftment in injured tissues, it was clear that MSC treatment led to resolution of inflammation.

Effects on Adaptive Immunity

In the early 2000s, studies demonstrated that BM-MSCs dampen T cell proliferation *in vitro* and *in vivo*, in response to polyclonal stimuli (Bartholomew et al., 2002; Di Nicola et al., 2002). This was soon followed by the demonstration that MSCs can inhibit T cell proliferation, interferon-gamma (IFN-γ) production, and cytotoxic activity in response to antigen-specific stimuli, but do not require MHC molecules or antigen presentation by antigen presenting cells (Krampera et al., 2003). When co-cultured with

alloreactive T cells, MSCs can directly induce the proliferation of $Foxp3^+$ regulatory T (Treg) cells, specialized T cells with immunosuppressive activity that help maintain tolerance to tissue antigens (Selmani et al., 2008). MSCs have also been shown to generate Treg cells by inducing the expression of Foxp3 in T cells and inhibiting their differentiation to Th17 cells, another T cell subset with inflammatory activity (Ghannam et al., 2010).

As B cell responses are mainly dependent on T cell help, inhibition of T cell function by MSCs can impair B cell function and humoral immunity. In murine co-culture experiments of MSCs with purified B cells, MSCs were shown to also directly inhibit B cell proliferation and differentiation into antibody-producing effector B cells (Augello et al., 2005; Asari et al., 2009). Co-cultures of MSCs with human B cells, on the other hand, have yielded conflicting results, with some studies showing inhibitory effects on antibody production and chemotactic properties (Corcione et al., 2006), while others showed that MSCs can promote B cell function by supporting B cell survival, expansion and differentiation (Traggiai et al., 2008), and antibody secretion (Rasmusson et al., 2007).

The initiation of adaptive immune responses depends crucially on dendritic cells (DCs), which survey the skin and mucosal tissues, capturing and processing antigens for display to T cells in an MHC-restricted manner. MSCs have been shown to interfere in the differentiation of monocytes to DCs (Nauta et al., 2006; Spaggiari et al., 2009), and inhibit the upregulation of MHC class II and co-stimulatory molecules associated with DC maturation and antigen presentation (Zhang et al., 2004) to skew their phenotype to an immature state (Zhang et al., 2009). MSCs have also been shown to reduce the capacity of DCs to activate alloreactive T cells (Zhang et al., 2004), modulate their cytokine secretion profile towards production of anti-inflammatory molecules, such as interleukin (IL)-10, and block the release of pro-inflammatory cytokines, such as tumor necrosis factor-alpha (TNF-α), IFN-γ, and IL-12 (Aggarwal and Pittenger, 2005).

Effects on Innate Immunity

MSCs also interact with the innate immune system by conferring immunomodulatory effects on other immune cell types, including monocytes, macrophages, neutrophils, and natural killer (NK) cells (Le Blanc and Mougiakakos, 2012).

Monocytes and macrophages form the mononuclear phagocyte system and are essential components of inflammation and tissue repair (Jung, 2018). Blood monocytes that enter inflamed sites in the body respond to local inflammatory stimuli and differentiate into monocyte-derived cells that resemble macrophages or DCs (Teh et al., 2019). At early stages of inflammation, tissue-infiltrating monocytes secrete pro-inflammatory TNF-α and IL-1, while monocytes found at later stages of inflammation exhibit anti-inflammatory properties (Teh et al., 2019). Macrophages exhibit similar plasticity in their phenotype and function in response to signals in the local microenvironment, differentiating either into M1 macrophages that release pro-inflammatory factors (e.g., IFN-γ and TNF-α) or M2 macrophages that promote tissue repair by secreting anti-inflammatory factors (e.g., IL-10 and transforming growth factor (TGF)-β) (Biswas and Mantovani, 2010; Murray and Wynn, 2011). While recognized as an overly simplified classification scheme, polarization of monocytes and macrophages is evident in studies reporting MSC-mediated resolution of tissue injury. In particular, MSCs produce IDO and prostaglandin E_2 (PGE_2), which polarise macrophages toward an M2 phenotype that is characterized by secretion of IL-10 (Németh et al., 2009; François et al., 2012; Melief et al., 2013a). MSC-driven polarization of macrophages has been reported to underlie the immunomodulatory effects of MSC therapy in various disease models, including sepsis (Németh et al., 2009), wound healing (Zhang et al., 2010) and renal ischemia-reperfusion injury (Li et al., 2013).

The interactions between MSCs and monocytes/macrophages are bidirectional, as several studies have shown that MSCs are activated by inflammatory cytokines produced by macrophages at early stages of inflammation. For example, in a murine model of sepsis, MSC treatment attenuated disease by inducing IL-10 production by macrophages (Németh et al., 2009). This increase in IL-10 production was dependent on PGE_2 secretion by MSCs, which was in turn dependent on TNF-α and iNOS signalling from the macrophages. Similarly, in a mouse model of zymosan-induced peritonitis, inflammatory cytokines secreted by peritoneal macrophages activated human MSCs to produce TNF-α–stimulated gene 6 protein (TSG-6), which in turn inhibited NF-κB signaling in macrophages and attenuated the release of inflammatory cytokines in a negative feedback loop (Choi et al., 2011). The central role of macrophages in MSC therapy has been demonstrated in several disease models, including sepsis (Németh et al., 2009), allergic asthma (Mathias et al., 2013) and GvHD (Galleu et al., 2017), whereby the beneficial effects of MSCs were abrogated in the absence of macrophages.

Recent studies have linked the immunosuppressive effects of MSC treatment to the phagocytic properties of monocytes and macrophages. Lung entrapment of intravenously administered MSCs is a well-documented phenomenon (Fischer et al., 2008; Kidd et al., 2009; Lee et al., 2009; Eggenhofer et al., 2012; Mathias et al., 2013). Entrapped MSCs are phagocytosed by circulating monocytes, neutrophils, and lung macrophages, which adopt an immunoregulatory phenotype and may elicit non-specific immunosuppressive effects (Galleu et al., 2017; de Witte et al., 2018).

Neutrophils, being the most abundant innate immune cells, are the first responders to microbial challenge and accumulate at the wound site within minutes of injury (Joel et al., 2019). MSCs have been shown to enhance neutrophil phagocytic activity, aiding pathogen clearance (Hall et al., 2013). Since neutrophils are non-proliferative cells with a short lifespan, their survival is pivotal to their role in pathogen elimination (Luo and Loison, 2008). Through constitutive release of IL-6, MSCs act to inhibit apoptosis of neutrophils (Le Blanc and Mougiakakos, 2012), extending their lifespan and providing an enhanced opportunity for pathogen elimination and tissue repair to take place. MSCs express functional Toll-like receptors (TLRs), which recognize

"danger" signals and activate immune responses to fight infection or resolve inflammation (Hwa Cho et al., 2006; Pevsner-Fischer et al., 2007; Tomchuck et al., 2008). Activation of TLR3 on MSCs enhanced neutrophil viability and function (Cassatella et al., 2011). Similarly, TLR-activated BMMSCs promoted the survival of resting and activated neutrophils through the production of IL-6, IFN-β, and GM-CSF (Hirano et al., 2000; Raffaghello et al., 2008). Although neutrophils have the capacity to phagocytose apoptotic MSCs, how this relates to the immunomodulatory effects of MSC therapy remains to be clarified, particularly in view of the short lifespan of neutrophils.

NK cells mediate innate immunity by recognizing and lysing cells that are unable to display or have downregulated MHC class I molecules, such as tumor cells (Malmberg et al., 2017). When co-cultured with MSCs, IL-2-activated NK cells downregulated their expression of activating receptors, NKp30 and NKp44, and NKG2D, produced less IFN-γ, and exhibited decreased cytotoxicity to tumor cells (Spaggiari et al., 2008).

The plethora of studies demonstrating that MSCs exert potent immunomodulatory capacity prompted a shift in the focus of the field, away from utilizing their differentiation potential to harnessing their capacity to modulate immune function. This immunomodulation of various effector functions seems necessary for allogeneic MSCs to establish a tolerogenic environment that can grant MSC-specific anti-inflammatory and reparative processes to take place. The precise mechanistic pathways that lead to this tolerogenic environment are yet to be delineated; however, it is apparent that MSCs modulate the immune system *via* direct cell contact and an indirect mechanism through the production and secretion of soluble factors (Uccelli et al., 2008) (**Figure 1**).

Cell Contact-Dependent Immunomodulation

The involvement of cell-to-cell contact in MSC immunomodulation was made evident in transwell experiments in which MSCs and T cells were physically separated by a membrane. MSCs inhibited allogeneic T cell proliferation in transwells, which was further reduced when MSCs and T cells were co-cultured together (Di Nicola et al., 2002). This indicated that the immunosuppressive effects of MSCs in a mixed lymphocyte reaction are due predominantly to soluble factors and are greatly enhanced by contact with their target cells. Cell contact is facilitated by various chemokines and adhesion molecules expressed by MSCs upon activation by inflammatory cytokines (Castro et al., 2019). For example, MSCs express high levels of CXCL9, CXCL10, and CXCL11 in response to inflammatory cytokines (Ren et al., 2008). These potent T cell attractants bind to CXCR3 on activated T cells, and antibody blockade of CXCR3 binding inhibited T cell chemotaxis toward MSCs and abrogated the inhibitory effects of MSCs (Ren et al., 2008). In another study, activated T cells induced the expression of adhesion molecules, ICAM-1 and VCAM-1, on MSCs, which was positively correlated with the immunosuppression of various T lymphocyte subsets (Ren et al., 2010). Accordingly, genetic deletion of both adhesion molecules in MSCs led to a significant decrease in their immunosuppressive capacity (Ren et al., 2010).

The inhibitory effects of mouse MSCs on antigen-specific T cell activation were also greatly reduced in transwell experiments (Krampera et al., 2003). The requirement for cell contact suggests that MSCs act to directly inhibit T cell activation. Indeed, contact-dependent inhibition of T cell activation was

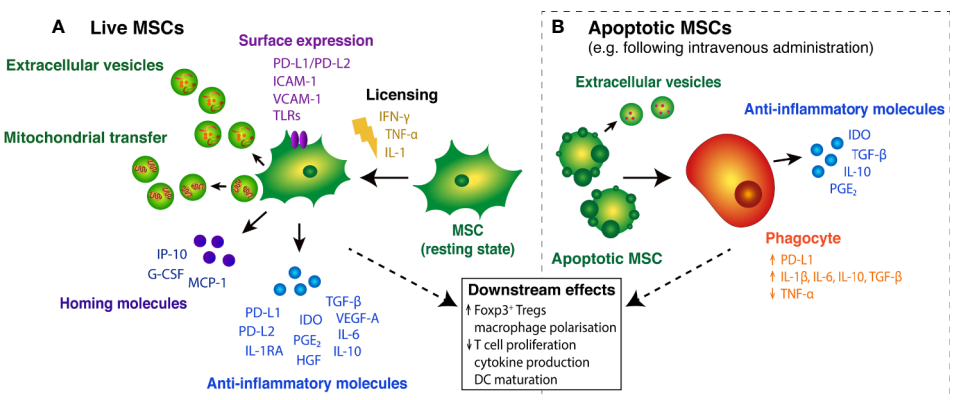

FIGURE 1 | Immunosuppressive effects of live and apoptotic MSCs. **(A)** In the presence of pro-inflammatory cytokines, resting MSCs become "licensed" to secrete key anti-inflammatory molecules, including PD-L1/PD-L2 (Davies et al., 2017), TGF-β (Niu et al., 2017) and IDO (Kim et al., 2018). Licensed MSCs also secrete homing molecules that promote MSC migration and Treg cell recruitment to tissue injury sites (Yu et al., 2011; Lunardi et al., 2014). Surface expression of various molecules on MSCs mediates interactions with T cells and guides MSC migration into inflammatory tissues (Ren et al., 2008; Chinnadurai et al., 2014). MSC cargo, in the form of extracellular vesicles (EVs) and subcellular components, such as mitochondria, may also play a role in MSC-mediated immunosuppression. **(B)** Following intravenous administration, MSCs can become apoptotic and are engulfed by circulating phagocytes, triggering the expression and release of immunomodulatory molecules (Galleu et al., 2017; de Witte et al., 2018; Cheung et al., 2019). Apoptotic cells can secrete immunosuppressive cargo packaged in extracellular vesicles (Caruso and Poon, 2018). Together, the host response elicited by live and engulfed MSCs leads to broader downstream effects on immune cell function.

demonstrated to occur *via* ligands expressed by human and mouse MSCs that bind to programmed cell death protein-1 (PD-1) on activated T cells to provide an inhibitory signal (Augello et al., 2005; Chinnadurai et al., 2014). However, it should be noted that PD-1/PD-L1 inhibition of T cell activation by MSCs can occur independent of cell contact, as human MSCs also secrete PD-1 ligands (PD-L1 and PD-L2) constitutively and in response to inflammatory cytokines (Davies et al., 2017).

Immunomodulation by Soluble Factor Secretion

MSCs separated from effector cells in transwell experiments exhibited reduced, rather than total loss of, immunosuppressive effects on T lymphocyte proliferation, indicating that MSCs exert effects through the secretion of soluble factors, such as cytokines, growth factors, and chemokines, in addition to direct cell contact (Di Nicola et al., 2002). In the past 15 years, a plethora of studies have investigated the effects of MSCs on cell-mediated and humoral responses in the innate and adaptive immune system. These studies have identified a broad range of soluble factors that are critical for MSC-mediated immunosuppression. The array of mechanisms employed by MSCs may reflect the heterogeneous composition of cells in current MSC preparations. The current view is that, while MSCs employ both cell-cell contact and soluble factors for robust pleiotropic immunomodulation, primary immunosuppressive effects are exerted *via* cytokines *in vivo*. Importantly, in inflammatory conditions, MSCs have been shown to utilize signals from the immediate cytokine milieu to fine-tune their immunosuppressive effects for tissue repair and wound healing, according to the required intensity, duration, and site of inflammation resolution (Kusuma et al., 2017).

Pro-Inflammatory Cytokines—For Priming MSC Immunosuppression

It is well accepted that immunosuppression is not an inherent feature of MSCs but rather a result of activation, or "priming," by an inflammatory environment (Krampera et al., 2006; English et al., 2007; Hemeda et al., 2010; Ren et al., 2010). Upon T cell activation, IFN-γ is released and continues to promote T cell activation and expansion. However, in the presence of MSCs, IFN-γ binds to its receptor on MSCs and results in in the suppression of T cell proliferation (Krampera et al., 2006). This effect has been confirmed by IFN-γ receptor-negative MSCs that fail to inhibit T cell proliferation (Ren et al., 2008). In addition, IFN-γ levels serve to regulate MSC proliferation and differentiation *via* IDO secretion (Croitoru-Lamoury et al., 2011). Similarly, TNF-α "primes" MSCs, which in turn upregulates a host of immunosuppressive factors that may, for example, contribute to tissue repair mechanisms (Ren et al., 2010).

Anti-Inflammatory Cytokines—For Driving MSC Immunosuppression

MSCs secrete an array of cytokines that have immunoregulatory effects. A key regulatory factor secreted by IFN-γ-primed MSCs is IDO (Kim et al., 2018). IDO is a rate-limiting enzyme of tryptophan catabolism, resulting in decreased levels of this enzyme (Grohmann et al., 2003). Since tryptophan is required for T cell proliferation, its depletion leads to T cell suppression (Yang et al., 2009) *via* direct (Meisel et al., 2004) and indirect pathways (François et al., 2012). In addition, IDO induces Treg cells *in vitro* and is responsible for B cell growth arrest and apoptosis (Maby-El Hajjami et al., 2009). With increasing Treg cell levels during MSC-mediated immunosuppression (Erkers et al., 2013; Hsu et al., 2013), there is a stimulation of IL-10 production (Engela et al., 2013), a cytokine that has been associated with inflammation resolution.

In order to confer their anti-inflammatory effects, MSCs may need to home to the site of injury (Kean et al., 2013). This homing is made possible by a range of soluble factors operating to ensure MSCs reach the appropriate site of tissue injury (Musiał-Wysocka et al., 2019). Vascular endothelial growth factor (VEGF)-A is known to stimulate angiogenesis *via* promotion of endothelial cell survival, proliferation, migration, and differentiation (Shibuya, 2011; Ge et al., 2018). IL-8-induced VEGF production by MSCs leads to increased angiogenesis and allows MSCs to utilize these blood vessels to reach the injury site (Hou et al., 2014). Interferon gamma induced protein (IP)-10 secretion by MSCs recruits Treg cells to sites of inflammation, resulting in an immunosuppressive microenvironment (Lunardi et al., 2014). IP-10 production also induces MSC migration to inflammatory sites (Rice and Scolding, 2010). Additionally, paracrine release of monocyte chemoattractant protein (MCP)-1 by MSCs enables MSC migration towards tissue injury sites (Boomsma and Geenen, 2012) and induces Fas ligand-dependent apoptosis of lymphocytes (Akiyama et al., 2012). Granulocyte-colony stimulating factor (G-CSF) release by MSCs increases both their mobility into peripheral blood systems and homing to the site of injury (Yu et al., 2011). Intracellular adhesion molecule-1 (ICAM-1) and vascular adhesion molecule-1 (VCAM-1) are both vital for the activation, rolling, and transmigration of leukocytes in immune responses (Musiał-Wysocka et al., 2019). Upregulation of ICAM-1 and VCAM-1 on the surface of MSCs has been shown to mediate MSC homing to the secondary lymphoid organs, allowing MSC-T cell interactions to take place (Ren et al., 2010). These interactions, in turn, lead to suppression of T cell proliferation (Ren et al., 2010).

Another notable regulatory factor secreted by MSCs is PD-L1. Secretion of PD-L1 by MSCs suppresses CD4+ T cell activation, downregulates pro-inflammatory IL-2 secretion, and suppresses T cell proliferation and cytokine production (Davies et al., 2017). PD-L1 also regulates Treg cell function, thus inhibiting pro-inflammatory T cell responses (Francisco et al., 2009). MSCs also produce PGE_2, a lipid mediator that acts *via* paracrine mechanisms to alter several arms of the immune system (Castro et al., 2019). PGE_2 release suppresses T cell activation and proliferation, both *in vitro* and *in vivo* (Aggarwal and Pittenger, 2005; Najar et al., 2010). It has also been shown to bind to $CD4^+$ T cells in order to inhibit Th17 differentiation (Duffy et al., 2011). In addition, MSC-secreted PGE_2 inhibits DC

maturation (Spaggiari et al., 2009) and induces a shift in M1 macrophages to adopt a M2 phenotype (Vasandan et al., 2016). IL-6 release by MSCs inhibits MSC differentiation and protects it from apoptosis in a paracrine manner (Pricola et al., 2009). IL-6 also enhances plasma interleukin-1 receptor antagonist (IL-1RA) and IL-10 release by MSCs *in vivo* (Steensberg et al., 2003). Another important soluble factor secreted by MSCs is TGF-β which acts to inhibit T cell proliferation, differentiation, and effector functions in a soluble manner and *via* direct cell contact (Kong et al., 2009; Niu et al., 2017). Furthermore, it promotes the conversion of naïve $CD4^+$ T cells to Treg cells (English et al., 2009). Other MSC-secreted cytokines like hepatocyte growth factor (HGF) mediate anti-inflammatory, anti-apoptotic, and antifibrotic mechanisms to resolve inflammation (Kennelly et al., 2016). It is apparent from accumulative studies that there are several cytokines operating in redundancy to ensure that MSC-mediated immunosuppression is established in times of tissue injury, infection, and trauma.

MSC Licensing

Importantly, to become immunosuppressive, MSCs need to be activated, or primed, by inflammatory cytokines in a multistep process called licensing (Krampera, 2011). MSC activation is mediated primarily by IFN-γ, which is one of the first cytokines produced upon T cell activation (Polchert et al., 2008; Ren et al., 2008). Blocking IFN-γ receptor with neutralizing antibodies was shown to abolish the immunomodulatory capabilities of human MSCs (Krampera et al., 2006). Similarly, MSCs isolated from knockout mice that were unable to respond to IFN-γ were incapable of inhibiting lymphocyte proliferation (Ren et al., 2008). Although the presence of IFN-γ is enough to prime MSCs, the combination of IFN-γ and either TNF-α, IL-1α, or IL-1β greatly enhances the inhibitory effects of MSCs (Ren et al., 2008).

The requirement for MSCs to be activated by inflammatory signals may explain why MSCs were only effective in treating graft-versus-host disease (GvHD) after inflammation had been established but did not show immunomodulatory properties when infused before inflammation was present (Sudres et al., 2006). In this context, differential triggering of TLRs on MSCs induces modulation of their immunosuppressive potency, with TLR-3 activation promoting an anti-inflammatory phenotype, whereas activation by TLR-4 promotes a pro-inflammatory phenotype (Waterman et al., 2010). Thus, MSCs can act either as a suppressive or pro-inflammatory cell, and this immune plasticity or functional polarization can be driven by the ligand, kinetics, and strength of the TLR stimulation (Krampera, 2011).

LIVE VERSUS APOPTOTIC MSCs

The efficacy of MSCs in various preclinical models of inflammatory diseases is well documented. In these settings, MSCs are exposed to pro-inflammatory cytokines, which are reported to "license" MSCs (e.g., IFN-γ, TNF-α, and TLR activation), but can also induce cell death (Salaun et al., 2007; Li et al., 2019). MSCs are also susceptible to activated NK cell-mediated killing *via* tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and Fas ligand (FasL) pathways (Spaggiari et al., 2006; Götherström et al., 2011).

A series of recent studies has indicated that MSC survival in the inflamed tissue may not be pertinent for the manifestation of MSC-mediated immunosuppression. In fact, apoptotic MSCs can confer immunosuppressive effects upon their administration into inflammatory sites *in vivo* (Galleu et al., 2017), suggesting that cell viability does not necessarily correlate with therapeutic efficacy. Recent studies have linked MSC apoptosis with their therapeutic effects in animal models of GvHD, sepsis, acute lung injury, and allergic airway inflammation (Luk et al., 2016; Galleu et al., 2017; Laing et al., 2018). The clinical response to MSC therapy in GvHD patients directly correlates with the ability of their immune cells to induce MSC apoptosis (Galleu et al., 2017). Whether the immunomodulatory effects in MSC-based therapies are directly mediated by factors produced by apoptotic MSCs or *via* the host response to apoptotic MSCs remains to be established. Furthermore, most MSCs are cleared shortly after infusion, with limited evidence of engraftment. The rapid clearance of these cells has been attributed to apoptosis (Eggenhofer et al., 2012) and this may be orchestrating local immune responses that lead to the anti-inflammatory effects seen as part of MSC administration (de Witte et al., 2018; Cheung et al., 2019). Although these findings challenge the longstanding view that viable MSCs are critical for therapeutic efficacy, studies have also shown limited efficacy with fixed or necrotic cells (Gupta et al., 2007; Németh et al., 2009; Kavanagh and Mahon, 2011; Mathias et al., 2013), suggesting that MSCs are most efficacious when viable at the time of administration.

MSC-Derived Extracellular Vesicles

Recent efforts in dissecting the mechanisms of MSC therapy have focused on the role of extracellular vesicles (EVs) as biological modulators. Cells produce three main types of EVs — exosomes (50–100 nm in diameter) and microvesicles (0.1–1 μm in diameter) produced by healthy cells, and apoptotic bodies produced by apoptotic cells (Caruso and Poon, 2018). Exosomes have the capacity to influence several aspects of immunity by activating or suppressing cytokine secretion, immune cell differentiation and polarization and T cell activation (Phinney et al., 2015; Chen et al., 2016). Exosomes derived from healthy MSCs in culture have been found to have anti-inflammatory effects in human disease models (Del Fattore et al., 2015; Anderson et al., 2016; Chen et al., 2016). Apoptotic cells also produce exosomes which have important immunomodulatory function such that they form a means through which dying cells communicate with their surroundings to bring about the anti-inflammatory effects (Caruso and Poon, 2018). To establish a therapeutic platform based on the delivery of MSC-derived exosomes would require a greater understanding of the quantity and quality of exosomes derived from both viable and apoptotic cells. Additionally, a greater understanding of exosomes in various disease settings is required since each disease varies in its profile, key players, and the nature of manifestation. Despite these gaps, it is evident that

exosome-based MSC therapy would be an alternative drug delivery system that would circumvent the costs and complexities associated with propagation of whole cells.

Mitochondria in Secreted EVs

Since mitochondria regulate the energy metabolism of a cell, the health and state of mitochondria will have a direct impact on oxidative stress and cell death (Guo et al., 2013). Therefore, it becomes evident that mitochondria can impact MSC immunosuppression. Mitochondrial transfer has been shown to pivotal in the therapeutic efficacy of MSCs in various pre-clinical models, such as brain injury, cardiac myopathies, acute ARDS, and chronic respiratory disorders (Li et al., 2014; Jackson et al., 2016; Torralba et al., 2016; Morrison et al., 2017). Mitochondria can be released as part of EVs in a functionally active state that enhances oxidative phosphorylation and dampens oxidative stress in recipient cells (Torralba et al., 2016). Overall, this leads to repair and healing of injured and inflamed sites. As part of MSC therapy, it is vital to reduce mitochondrial dysfunction that causes pathophysiology and strive to utilize healthy mitochondria to drive anti-inflammatory functions. Despite preliminary evidence and understanding of the significant role that mitochondria plays at the cellular level, the precise mechanisms by which mitochondria eject as part of EVs remains to be uncovered. In addition, an understanding of how EV-packaged mitochondria is taken up by recipient cells will be key in tailoring MSC therapy around the bioenergetics of this organelle.

THERAPEUTIC APPLICATIONS OF MSCs

There is much clinical interest in utilizing the immunomodulatory properties of MSCs in cellular therapy. Several MSC products have already been approved for various clinical applications with many others undergoing investigation in clinical trials. *Cartistem* is licensed for treatment of degenerative arthritis in South Korea, *Cupistem* and *Alofisel* for treatment of Crohn's anal fistula in South Korea and Europe, respectively, TEMCELL as an acute GvHD treatment in Japan, and *Prochymal* for the same indication in Canada and New Zealand (Gao et al., 2016; Galipeau and Sensebe, 2018).

Clinical use of MSCs necessitates large-scale expansion that cannot be sustained through tissue culture dishes or flasks in a laboratory setting. A constant supply of high cell numbers requires robust and economically viable culture processes. Meanwhile, risks that may compromise clinical use — such as cell transformation, secretion aberrations, and xenogeneic contact (e.g., animal serum) — must be reduced. To improve the feasibility of clinical use, there must be compromise between obtaining high cell numbers while ensuring the MSC immunophenotype is unaltered.

Another significant aspect of MSC therapy revolves around utilization of "frozen" or cryopreserved versus fresh MSCs. It is common practice for fresh MSCs to be used in preclinical models versus the predominant use of cryopreserved cells in the clinical setting (Moll et al., 2016). This contrasting practice has led to discrepancies in the protective effects of MSCs as outlined in the literature, compared to clinical outcomes observed in patients with MSC therapy. To date, it has been well documented that MSC potency can be affected by tissue origin, culture conditions, and modes of cell delivery, including the use of fresh versus thawed cells (Galipeau, 2013; Marquez-Curtis et al., 2015). Furthermore, upon recovery from cryostorage, thawed cells show various changes in molecular and physical integrity compared to fresh cells that may also impact immunomodulatory properties of MSCs when used from cryopreservation rather than fresh (Moll et al., 2014; Chinnadurai et al., 2016). The choice between the two will impact how MSC therapy products should be developed and whether an "off-the-shelf" approach would allow for therapeutic effects to be delivered without compromising the potency and immunomodulatory profile of the cell product.

MSC CULTURING SYSTEMS

Cell Culture Supplements

A regulatory requirement for the therapeutic use of cells is that they are manufactured under a quality system or using Good Manufacturing Practice (GMP) (Abbasalizadeh et al., 2017). In this system, all inputs to the process (media, supplements, growth factors) also need to be manufactured under GMP conditions, which include reagent validation, batch testing, and release under appropriate release criteria before use. This means that when considering all of the parts of a manufacturing process, an ability for the bioreactor to be utilized under a quality system is imperative if the cells produced are to be used clinically. A number of cell culture systems meet these criteria and are used currently (see **Figure 2**). Inputs to these culture systems include cells, media, supplements (often animal-derived serum) and growth factors. Synthetic media (serum-free or xeno-free) media typically have the molecules to support cell growth already included in the media but may require pre-coating of the growth surface with recombinant proteins or fragments which support cell attachment.

Serum (usually bovine or human) is included in MSC expansion media to provide nutrients for growth, attachment-promoting proteins (e.g., fibronectin, and vitronectin) for cell adhesion, and hormones and lipids to stimulate cell proliferation *in vitro* (Oikonomopoulos et al., 2015). However, the use of fetal bovine serum (FBS) has recently raised concerns that animal proteins and peptides may contaminate human MSCs during culture (Gregory et al., 2006). This could lead to viral or prion transmission and cause aberrant immune reactions in a clinical setting. In some cases, antibodies to FBS proteins have been detected in clinical settings where transplanted cells have been exposed to FBS (Horwitz et al., 2002; Sundin et al., 2007). In addition, there are ethical concerns associated with the use of FBS (Tekkatte et al., 2011). Further issues involve batch to batch variability and the requirement for extensive qualification of FBS for cell manufacturing purposes (Witzeneder et al., 2013). The limited supply and high cost of FBS is also a limitation in the

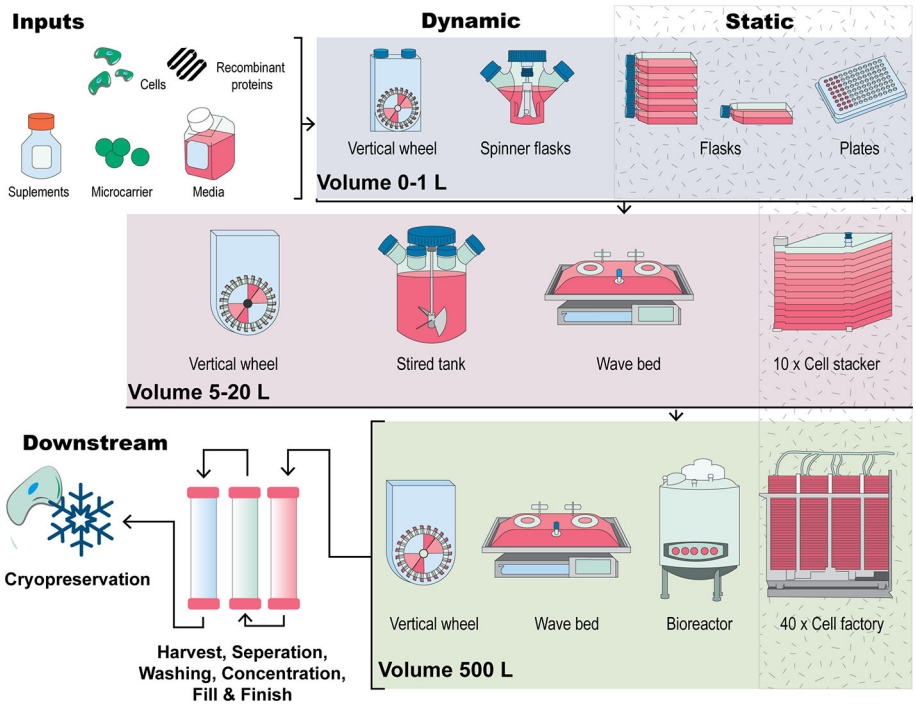

FIGURE 2 | A visual comparison of expansion strategies for human MSCs. Inputs to the process include cells, media, supplements, a culture surface (flask, cell stacker, microcarriers) and other additives including growth factors. Figure adapted from (Kropp et al., 2017). Traditional culturing methods encompass 2D, planar technologies such as expanding MSCs in a culture dish or flask by continual passaging. Scale out of this approach uses cell stackers or multilayered flasks which work in this manner through multiplication of the culturing flask. In comparison, scale-up manufacturing methods involve MSCs forming aggregates or being seeded onto microcarrier in suspension in bioreactor systems such as stirred tank, vertical wheel or wave bag bioreactors. Downstream processes such as cell harvesting cell washing, cell concentration, finish and fill and storage through cryopreservation are also critical parts of the manufacture of MSCs for clinical applications.

application of cell therapies (Fang et al., 2017). For example, estimates of FBS availability indicate that in the future it is unlikely that supply will keep up with demand, particularly given that FBS is a by-product of the meat industry (Karnieli et al., 2017). Cell culture supplementation with human serum (both allogeneic and autologous) has been studied (Gottipamula et al., 2013) and the use of pooled human AB serum (hABS) is becoming increasingly widespread, at least in *in vitro* studies. In one such study, use of hABS was found to significantly enhance MSC expansion in 2D cultures compared to FBS and had similar immunosuppressive effects (Thaweesapphithak et al., 2019). In this study, hABS was also used in the isolation of MSCs from tissue and cryopreservation. Savelli et al. (2018) cultured MSCs in a hollow fiber, perfused bioreactor and found that a particular population of cells, the mesodermal progenitor cells (MPCs), were enriched compared to cultures in media supplemented with FBS, where only a MSC phenotype was observed. Supplementation with human AB serum was tested in a comparative study of MSC expansion in planar and microcarrier culture at reasonable scale (2 L stirred tank systems utilizing microcarriers) (Tozetti et al., 2017). The microcarrier-based systems were found to give significantly greater cells/cm^2 than planar systems, however efficient harvesting was identified as a hurdle to obtaining maximum cell yields. Of course, there are some limitations such as the amount that can be supplied and the risk of spreading previously unknown or new human pathogens (Karnieli et al., 2017).

A common alternative for large-scale MSC manufacture is human platelet lysate (HPL) prepared under a quality system or good manufacturing practice (GMP) guidelines. HPL provides strong growth-promoting activity to support the expansion of a variety of cells (Choi et al., 1980; Eastment and Sirbasku, 1980; Hara et al., 1980). In fact, there are now ample studies demonstrating that proliferation of MSCs from various tissue sources is higher when HPL is used (Schallmoser et al., 2007; Bieback et al., 2009; Gottipamula et al., 2012; Gottipamula et al., 2013; Gottipamula et al., 2016; Czapla et al., 2019; Kakudo et al., 2019) and generally studies utilizing HPL for *in vitro* expansion of MSCs have found it to be an acceptable alternative to FBS in terms of maintaining cellular features for clinical applications (Fekete et al., 2012; Becherucci et al., 2018). However, studies on the effects of HPL on the immunosuppressive capacity of MSCs have been contradictory. In one study, HPL-expanded MSCs displayed altered expression of surface molecules, impaired lymphocyte, and natural killer cell suppression when compared to FBS (Abdelrazik et al., 2011). In another study, a higher immunosuppressive effect was observed for BM-MSCs expanded in HPL-supplemented media (Gottipamula et al., 2012). Other

studies comparing cell expansion in HPL- or FBS-supplemented media have reported no difference in the immunosuppressive effects of BM-MSCs (Bieback et al., 2009), or in the secretion profiles of A-MSCs (Czapla et al., 2019). Chromosomal stability appeared to be the same if not better for cells grown in HPL (Shih and Burnouf, 2015; Astori et al., 2016). Although considered a safe tool for clinical expansion purposes, there are limitations to the use of HPL as an FBS alternative for MSC expansion. Given the current literature is unclear on the consensus effects of HPL on MSC immunosuppression, further research is required to clarify the effects (if any) of HPL on the immunosuppressive capacity of MSCs *in vivo*. There have been a number of clinical studies involving MSCs that have been expanded using HPL as the supplement for MSC production, the result of which have indicated that HPL can safely replace FBS for clinical-scale MSC manufacture (von Bonin et al., 2009; Centeno et al., 2011; Introna et al., 2014; Bieback et al., 2019). In addition, a recent survey of European centers manufacturing cells for GvHD survey of showed that 77% of the centers were using HPL in preference to FBS (which was mostly supplemented at 5% in media) (Trento et al., 2018).

There is an increasing number of synthetic cell culture media available commercially, optimized for MSCs to avoid the issue of batch-to-batch variability of biologically derived media supplements. These media typically do not contain animal- or human-derived supplements and can be described as serum- or xeno-free (SF or XF). For example, Gottipamula et al. compared the growth kinetics, cell surface markers, morphology, differentiation potential, and immunosuppressive properties of BM-MSCs expanded in small volume cultures in a range of SF and XF media and one media was also used in a 10-layer cellSTACK® (Gottipamula et al., 2016). Cell yields were lower in the cellSTACK®, compared to FBS media highlighting that scaling up production even from small to moderate scale can present some challenges. Optimization may need to be carried out at each scale tested. These media are still rather expensive (approx. the same as FBS and HPL per unit volume) meaning that they are not currently being used to expand cells for clinical application to our knowledge. Costs are expected to reduce as with the economies of scale associated with more widespread use. A summary of the relative advantages and disadvantages of each media supplement type discussed above is presented in **Table 1**, particularly for human-derived and synthetic media supplements over animal-derived supplements such as FBS.

2D Versus 3D Culture of MSCs for Therapeutic Applications

Traditionally, undifferentiated MSCs are maintained and expanded at low density in two-dimensional (2D) monolayer conditions in culture vessels with planar surfaces, with cells adhering to the plastic surface of culture plates or flasks (Fang and Eglen, 2017). Cells adhere to and grow on a flat surface,

TABLE 1 | Summary of cell culture media growth supplements commonly used; fetal bovine serum (FBS), pooled human AB serum (hABS), human platelet lysate (HPL), and synthetic media and their relative advantages and disadvantages in a cell therapy context.

Supplement	Advantages	Disadvantages
Fetal Bovine Serum (FBS)	Long history of use	Limited supply (Fang et al., 2017)
	Extensive clinical experience	Animal disease transmission to humans (Gregory et al., 2006)
		Possible immune response (Horwitz et al., 2002)
		Less preferred from regulatory viewpoint (Karnieli et al., 2017)
		Batch-to-batch variability, requiring qualification (Mendicino et al., 2014)
		High cost
		Ethical concerns (Tekkatte et al., 2011)
Pooled hAB Serum (hABS)	Human origin	Limited supply
	Universal donor - meets most HLA requirements	Relies on donation
	Appears to have a higher proliferative capacity (Thaweesapphithak et al., 2019)	Ethical issues associated with use of human-derived products (Jacobs et al., 2019)
	GMP grade available	Potential spread of human diseases (Karnieli et al., 2017)
Human platelet lysate (HPL)	Human origin	Limited supply
	Higher proliferative capacity established (Bieback et al., 2009; Kakudo et al., 2019)	Relies on donation
	GMP grade available	Ethical issues associated with use of human-derived products (Jacobs et al., 2019)
	Widely used clinically (77% centers in Europe) (Trento et al., 2018)	Potential spread of human diseases
	Chromosomal stability (Juhl et al., 2016)	
	Enhanced MSC immunosuppressive effects observed (Gottipamula et al., 2012)	Still some debate on effect on MSC immunomodulatory effects (Abdelrazik et al., 2011)
Synthetic media	Potentially unlimited supply	Use with cells for clinical studies not established (Lensch et al., 2018)
	Chemically defined	Expensive
	Higher proliferative capacity (Patrikoski et al., 2013)	Proliferative capacity dependent on cell type/origin, optimized media composition (Cimino et al., 2017)
	Minimal batch-to-batch variability (Cimino et al., 2017)	May rely on animal-derived or recombinant cell adhesion molecules

flattening morphologically and receiving nutrients and growth factors on one side during expansion (Neuhuber et al., 2008). This process is labor-intensive and susceptible to contamination due the open nature of the culture and to the number of cell passages required to generate sufficient cells for research purposes. Typically, 2D culture conditions are static and also lack monitoring *via* sensors and the ability to control culture conditions, which is undesirable for cell manufacturing (Martin et al., 2004). Primary MSC monolayer cultures can also become senescent and lose their phenotype following extensive passaging (Goepfert et al., 2010), which may impact on clinical efficacy (von Bahr et al., 2012). Thus, from a manufacturing perspective, given the relative rareness of MSCs in tissues and the quantity of cells required for clinical use, multiple master cell banks from multiple donors may have to be produced every year. This driver towards higher passage number and maximal expansion to derive the maximum number of patient doses from a single master cell bank needs to be balanced against potential reduced clinical efficacy. Further drawbacks of planar culture systems include the large surface areas required for cell growth at clinical scales, sizeable volumes of liquids to be manipulated during media changes, passages, and cell harvesting, and large incubators are required which occupy considerable space in clean rooms (Campbell et al., 2015; Merten, 2015).

To increase cell number under 2D conditions, the surface area of the culture dishes used is increased using multi-layered flasks, or cell stackers (Rowley et al., 2012). Small-, medium-, and large-scale cell manufacture in planar, 2D static culture are represented in **Figure 2** as tissue culture flasks through to 10-layer and 40-layer stacked systems. Several cell stackers are commercially available, including the Corning® CellSTACK and Nunc™ Cell Factory™. This manufacturing method is referred to as "scale-out" expansion, wherein the expansion unit size remains constant and parallel units are multiplied (**Figure 2**). However, this technique results in restricted surface-to-volume ratio, creating a bottleneck in the manufacturing process. The environment within cell stackers is also non-homogenous: each flask constitutes a different microenvironment that is susceptible to contamination, batch-to-batch variability and non-uniform surface treatment between suppliers (Jossen et al., 2018). Furthermore, manual handling and downstream cell processing constraints limit the potential of scale-out techniques. The high MSC doses required for therapeutic infusion [around 10^6 cells per kg of patient (Jung et al., 2012)] necessitate "scale-up" methods.

Scale-up expansion refers to the increase in overall manufacturing scale that occurs in technologies such as bioreactors. A number of bioreactor types are depicted in **Figure 2**, including stirred tank, wave bag, and vertical wheel. In the microcarrier culturing system devised by van Wezel in 1967 (van Wezel, 1967), cells are propagated on the surface of microcarriers and expanded in suspension of growth medium *via* slow agitation. From this, stirred or mixed bioreactor systems incorporating microcarriers have been developed to provide densities of 10^6 to 10^7 cells/mL, becoming preferable to cell stackers for the generation of therapeutic cells (Fan et al., 2015). Furthermore, the shorter culture time bioreactor systems required to generate comparable cell numbers to tissue culture flasks can minimise the risk of MSC senescence and phenotypic changes due to culturing in serum (Mizukami et al., 2016). Other approaches used to increase the cell growth surface area, without increasing the footprint of the bioreactor include the use of hollow fibre bioreactors (Tozetti et al., 2017; Savelli et al., 2018) as well as fixed bed perfusion systems (Sart et al., 2014). An important feature of many scale-out systems is the ability to be able to operate them in a functionally closed manner. This means that the bioreactor can be opened to make a connection and then returned to the closed state. In this way, the contents of the bioreactor are not exposed to the room environment. This presents a distinct advantage since a number of units can operate in the same room without physical separation from each other.

Stirred-Tank and Other Dynamic Bioreactors

Typical stirred-tank bioreactors are usually cylindrical vessels with an impeller providing constant movement and are the most widely used scaled up bioreactor system used for MSC-based cell therapies, particularly allogeneic cell therapies where large cell numbers are required to be manufactured. The stirred tank configuration results in effective mixing, however, with non-homogeneous flows which can be turbulent in some conditions or regions within the bioreactor (Berry et al., 2016; Tsai and Ma, 2016). Bioreactor scale-up techniques facilitate dynamic suspension cultures which are very different to static 2D cultures. Cells within bioreactors can be expanded as suspended cell aggregates or seeded onto small solid spheres called microcarriers. For MSCs, expansion using this approach has generally been found to retain a stable phenotype (Caron et al., 2012) at least when only the minimum definition of an MSC is considered. As self-assembling cell aggregates or spheroids mimic *in-situ* conditions, cell morphology is more representative of that in bodily tissue(Edmondson et al., 2014). The medium in which the cells aggregate to form spheroids includes the need for adhesive molecules to facilitate cell-cell attachment, including laminins, integrins, E-cadherin, and vitronectin (Badenes et al., 2016). However, for GMP production, these recombinant human proteins are expensive, making viable large-scale manufacture difficult (Villa-Diaz et al., 2013).

Microcarrier-based culture systems are, in principle, particularly well-suited for MSC expansion. Microcarrier beads have a large surface area compared to 2D systems, maximizing MSC attachment. Bioreactors using microcarriers can also operate at higher densities, reducing supply costs, or cost of goods (COGs). For example, a study investigating the use of microcarrier-based MSC expansion of 2.5 L cultures in a stirred tank bioreactor system found that the larger volume cultures outperformed small 100-mL volume "spinner flask" cultures, producing cells with the phenotype, key morphology, and differentiation capacity that conformed to the ISCT definition of MSCs (Rafiq et al., 2013). Microcarriers are made from various

materials and may be coated with biologically active proteins and peptides (e.g., vitronectin and fibronectin) (Melkoumian et al., 2010). Furthermore, microcarrier-based technology can be operated as a closed culture system and is compatible with sterilization procedures, which is essential when considering therapeutic applications (Schop et al., 2008).

Despite their advantages, three-dimensional (3D) scale-up manufacturing systems utilizing microcarriers and stirred tank systems raise potential issues. Further improvements tailored to the expansion of MSCs in dynamic culture systems are required to achieve unchanging and reproducible MSC production for biological research and eventual clinical application. In addition, research is still required to fully understand the link more broadly between manufacturing methodology and clinical efficacy and how to optimise manufacturing to achieve the best clinical outcomes. This is particularly relevant for MSCs as they are applied to a wide range of disease indications, which may require different properties which can be tailored on a disease basis using optimized manufacturing.

MSC Scale-Up in Stirred-Tank Bioreactor Systems

Bioreactor systems commonly used pose a number of possible issues for MSC scale-up production. This is largely because such systems were initially designed to carry out chemical reactions at scale and later adapted to cell culture in the form of bioprocessing or therapeutic protein production from non-adherent cells (e.g., CHO cells) (Nienow, 2006). For the manufacture of cell-based therapies, retention of cell function and quality is of principal importance, yet this aspect is often overlooked when adapting scale-up manufacture systems to large-scale production of MSCs.

A range of different commercial bioreactors are available for scale-up MSC manufacture (Badenes et al., 2016) (**Figure 2**). Bioreactor performance in supporting MSC growth and phenotypic maintenance cannot be the only variable considered when selecting a bioreactor. Criteria such as the ability to operate in a functionally closed way, simplicity of operation, disposability, sterility, single use, ability to incorporate online monitoring and control, automation, ease of harvest and time- and cost-effectiveness must also be taken into account (Caruso et al., 2014; Badenes et al., 2016). This must be balanced with practical considerations, such as low costs and the ability to achieve high cell densities.

Stirred-tank bioreactor systems can be readily operated and cell culture volumes can be scaled up with computer-controlled online monitoring equipment which control process variables such as pH, temperature, and dissolved oxygen and carbon dioxide concentrations (Tsai and Ma, 2016). However, stirred bioreactors also introduce an important complication: fluid mechanics (Odeleye et al., 2014; Berry et al., 2016). Cells in a bioreactor are constantly exposed to shear stress induced by mechanical agitation of impellers or wheels. MSCs are particularly sensitive to this stress, which can lead to cell damage, premature detachment from microcarriers, priming to a specific differentiation lineage or affect immunomodulatory properties (Stathopoulos and Hellums, 1985; Dos Santos et al., 2014; Das et al., 2019). These effects must be recognized and controlled for when expanding MSCs on microcarriers in a stirred bioreactor system. Ultimately, a dynamic culture system utilizing microcarriers is complex and presents different challenges to 2D systems. Aggregation of microcarriers is of particular relevance as their presence may reduce cell harvest efficiency. An approach taken to minimise aggregation is to periodically add more microcarriers, increasing the culture surface and allowing cells to migrate from confluent microcarriers to sparely populated or empty microcarriers (Ferrari et al., 2012; Rafiq et al., 2018). From a feasibility point of view expansion of bone marrow derived MSCs has been carried out in single use stirred tank bioreactors at 3 and 50 L (Lawson et al., 2017). Expansion in HPL supplemented media was enhanced compared to FBS and a 43-fold expansion was obtained in 11 days at a 50 L culture volume scale. Maintenance of MSC phenotype according to the ISCT definition was maintained as well as immunosuppressive properties.

As MSCs are anchorage-dependent, they must be easily separated from the substrate on which they are cultured without changing their immunophenotype, secretome or differentiation capacity, all of which are strongly related to clinical efficacy. Cell harvesting in dynamic systems is often conducted with a proteolytic enzyme such as trypsin (alone or in combination with chelating agents such as EDTA) to separate cells from microcarriers and cell-microcarrier aggregates, followed by filtering through an appropriate mesh to remove the microcarriers and large aggregates (Lindskog et al., 1987). Unlike monolayer cell culturing strategies, microcarrier–MSC complexes require especially complex disassociation methods and detachment efficiencies tend to vary. Several studies have treated cell-microcarrier complexes with trypsin at high concentrations or for long periods of time (Frauenschuh et al., 2007; Schop et al., 2008; Dos Santos et al., 2014). This treatment is known to cause MSC damage or induce phenotypic changes. For example, MSCs treated with 0.25% trypsin-EDTA solution for 5, 30, and 90 min at room temperature demonstrated decreased CD105 expression with time (Potapova et al., 2008). Other studies have investigated alternative proteolytic enzymes, such as collagenase and dispase, to harvest MSCs by digesting macroporous microcarriers. This approach limits cell damage and increases detachment numbers (Rubin et al., 2007; Sart et al., 2009). However, certain cell surface molecules have also been shown to be downregulated or cleaved upon cell treatment with these enzymes (Autengruber et al., 2012; Taghizadeh et al., 2018).

Alternatively, the use of thermosensitive microcarriers, which detached MSC-microcarrier complexes by decreasing the culture temperature, showed that cell detachment *via* temperature change reduced MSC apoptosis and cell death during harvesting, suggesting that thermosensitive microcarriers are effective in MSC culturing (Yang et al., 2010). There are a number of potential issues for thermosensitive microcarriers, including cell aggregates which may also need enzymatic digestion. In any case, it is crucial to consider the cell type and microcarrier type and identify an optimal enzymatic protocol to maximise the quantity and quality of cells harvested.

Stirred-tank bioreactors offer a promising approach for generating sufficient cell numbers under controlled scale-up conditions. However, they are not tailored to or optimized for MSC expansion. Considerations must be made towards maintaining batch-to-batch standardization, cell yields, and cytokine and growth factor secretions for industrial and clinical translation. The effects of microcarrier culture systems on the MSC secretome must be taken into consideration, as the secretome is considered an integral indication of therapeutic functionality. An outstanding question is whether the MSC secretome is changed in dynamic by scale-up manufacturing systems from that obtained in 2D culture systems. A newer technology, that of a vertical wheel bioreactor (see **Figure 2**) which is scalable to 500 L culture volumes, has been evaluated in HPL-supplemented media for umbilical cord-derived MSCs (UC-MSCs) and A-MSCs and an economic evaluation against static 2D culture carried out (de Sousa Pinto et al., 2019). It was found that significant cost reductions could be obtained (up to 50% in some cases) using this type of bioreactors system and microcarriers. Another advantage of using a vertical wheel instead of an impeller for mixing is that of reduced shear stress (Sousa et al., 2015), as the impact of shear stress on cell phenotype, differentiation capacity and secretome is largely unknown.

MICROCARRIERS

Microcarriers are small, spherical beads which allow production of cells at a high culture density due to the much larger culture surface area to media volume ratio. Stirring in the bioreactor maintains the microcarriers in suspension in a bioreactor (Caruso et al., 2014). They were traditionally employed to culture primary cells and anchorage-dependent cell lines for vaccine production, pharmaceutical production, and cell population expansion (Nilsson, 1988). Commercially available microcarriers are engineered for specific applications and vary in chemical composition, charge, surface coatings, and porosity (Malda and Frondoza, 2006) and allow cells to be cultured at a higher surface area per media volume than in planar culture.

Microcarriers are composed of various materials including polystyrene, dextran, and glass. Their surface can be functionalized with in different ways (e.g., *via* a coating) to maximize cell attachment and cell culture performance. This is largely accomplished by chemically derivatizing the microcarrier surface with functional groups, such as positively or negatively charged groups, biological materials (e.g., gelatine, collagen, fibronectin) or other small molecules such as peptides (Badenes et al., 2016). Unless chemically modified with a positively charged group, synthetic microcarriers (e.g., glass, dextran, and polystyrene) are generally negatively charged.

MSC Attachment to Microcarriers

As MSC growth is anchorage-dependent, interactions between the microcarrier surface, cells, and surrounding medium are critical for the manufacture of healthy cells. The microcarrier surface is quickly "conditioned" by non-specific protein adsorption from media supplements, which facilitates cell attachment (Wang et al., 2012). Protein adsorption onto the microcarrier surface is driven largely by electrostatic, ionic or van der Waals forces, hydrophobic interactions, and hydrogen bonding interactions (Petry et al., 2016). Alternatively, microcarrier surfaces can be functionalized with biologically derived molecules (such as proteins or protein fragment), to which MSCs attach *via* adhesion motifs (Melkoumian et al., 2010). Alternatively, a synthetic coating containing chemically synthesized cell adhesion motifs, such as RGD peptides, can be chemically attached to the surface of the microcarriers (e.g., Synthmax microcarriers). These types of microcarriers, which are generally known as chemically defined, would generally be preferred from a regulatory point of view (**Figure 3**).

As MSCs attach to microcarriers (known as the induction period of the culture), their phenotype changes from rounded to spread and fibroblastic (Battista et al., 2005) (**Figure 4**). Following the induction period, MSC expansion occurs. During cell expansion, the microcarrier growth surface interacts with cell surface integrins, the principal receptors mediating cell-matrix or cell-surface adhesion (Berrier and Yamada, 2007). Cell surface integrins are activated, adopt a heterodimer formation, and initiate signaling cascades which activate downstream gene expression and ultimately regulate cell morphology and behavior including attachment, spreading, proliferation, migration, and differentiation (Berrier and Yamada, 2007).

It is important to note that growth and harvesting of MSCs on microcarriers is different than in 2D microenvironments, as indicated in **Figure 4**. The surfaces are curved on the length scale of MSCs, which can wrap around the microcarrier surface and even bridge across microcarriers. There is a limited surface area per bead which means that cells on individual microcarriers will become confluent at different times, depending on how uniform the attachment density carries from bean to bean. Cells can transfer between beads or onto the surface of pristine beads added at different time points through the culture period (Derakhti et al., 2019). Aggregation of microcarriers through cell bridging is common and can impact ease of harvesting for cells trapped between beads.

The surface properties of commercial microcarriers can be tuned to facilitate this attachment process. For example, microcarrier surfaces are often coated with growth- and attachment-promoting proteins to encourage cell adhesion. Many media proteins can be used, including native or denatured collagen, fibronectin, laminin, and vitronectin (Melkoumian et al., 2010). Each protein is recognized by specific integrin heterodimers on the MSC surface (Plow et al., 2000; Docheva et al., 2007; Niehage et al., 2011) (**Table 2**). Integrin expression in MSCs differs by harvest tissue source: A-MSCs express the integrin subunits α_1, α_2, α_3, α_5, α_7, α_8, α_{11}, α_v, β_1, β_3, and β_5 which bind *via* integrin receptors to their respective attachment proteins (De Ugarte et al., 2003; Goessler et al., 2008) (**Table 2**). In one study, actin organization was linked to more efficient expansion of MSCs on a range of microcarriers (Sart et al., 2013).

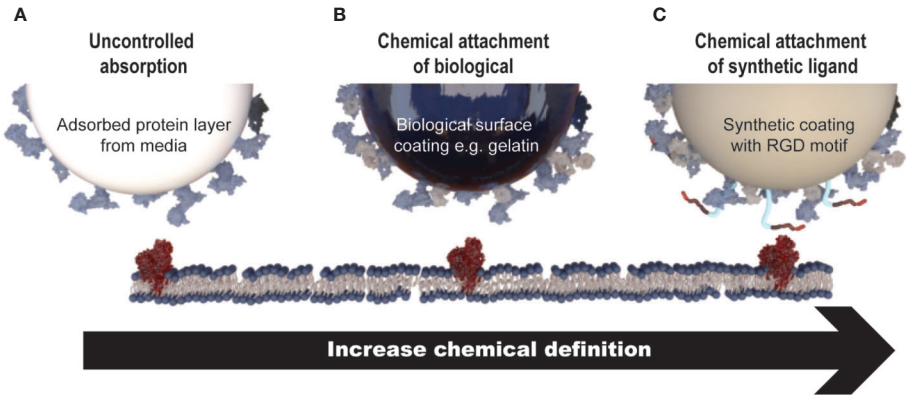

FIGURE 3 | Mechanisms of MSC attachment to microcarriers. **(A)** Cell attachment is facilitated through non-specific protein adsorption on the surface of microcarriers that do not contain a coating of any description (e.g., Solohill Plastic). **(B)** Microcarriers that contain a coating of a biologically derived molecule (e.g., gelatin) which facilitates cell attachment through native cell attachment motifs. **(C)** Microcarriers which contain a synthetic coating with a chemically synthesized cell attachment motif, for example a short peptide sequence (e.g., Synthemax®).

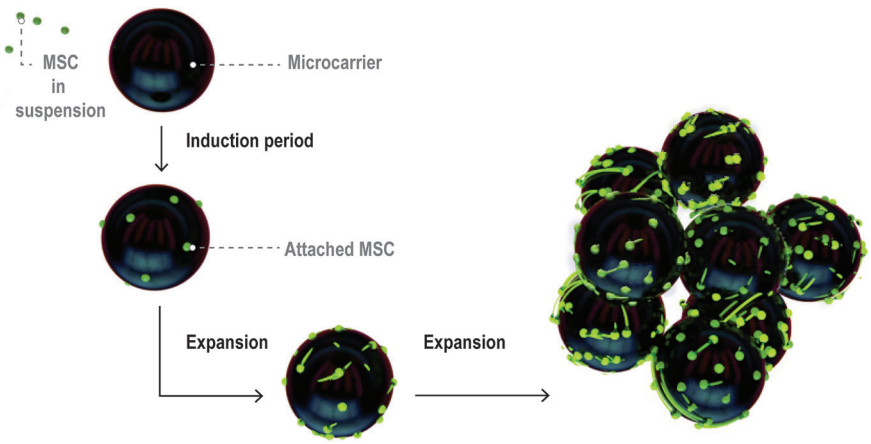

FIGURE 4 | Visual representation of MSC expansion on microcarriers over time within a bioreactor. Figure adapted from (Caruso et al., 2014). MSCs initially attach at low coverage in a rounded morphology then flatten and spread over the induction period. The cells then enter a growth phase and expand to cover a large proportion of the microcarrier surface area.

TABLE 2 | MSCs express integrin heterodimers that attach to specific media proteins (Plow et al., 2000, Docheva et al., 2007, Niehage et al., 2011).

Attachment protein	MSC-expressed integrin subunits
Native collagen	$\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_{11}\beta_1$, and $\alpha_{Ib}\beta_3$
Denatured collagen	$\alpha_5\beta_1$, $\alpha_v\beta_3$, and $\alpha_{IIb}\beta_3$
Fibronectin	$\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_4\beta_1$, $\alpha_4\beta_7$, $\alpha_5\beta_1$, $\alpha_8\beta_1$, $\alpha_v\beta_1$, $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_v\beta_6$, $\alpha_v\beta_8$, and $\alpha_{IIb}\beta_3$
Laminin	$\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_6\beta_1$, $\alpha_7\beta_1$, $\alpha_6\beta_4$, and $\alpha_v\beta_3$
Vitronectin	$\alpha_v\beta_1$, $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_{IIB}\beta_3$

Microcarrier matrix materials can also be selected from three categories: natural polymer, synthetic, and glass. Natural polymers, such as collagen, gelatine, dextran, and pectin, are commonly used as they are easily obtained, biocompatible, and inexpensive (Zhang et al., 2009; Zhou et al., 2011). Collagen- or gelatine-based microcarriers or coatings express attachment molecules to facilitate attachment (Geiger et al., 2001; Bertolo et al., 2015). Thus, they have the advantage of easy cell detachment, limiting cell damage. Furthermore, gelatine microcarriers are capable of enzymatic digestion, leaving only cells in suspension (Lönnqvist et al., 2015). However, biological materials can be problematic in the context of cell manufacture as regulatory agencies recommend the avoidance of mammal-derived materials to reduce the risk of contamination when MSC products are used in the clinic (Halme and Kessler, 2006; CHMP, 2013).

Therefore, cell manufacturers are increasingly focusing on synthetic polymer-based microcarriers which are largely

composed of polystyrene (Rafiq et al., 2016). When the microcarrier surface does not contain biological attachment molecules, media attachment factors (particularly fibronectin or vitronectin) adsorb to the microcarrier surface and promote cell attachment and integrin binding (van Wachem et al., 1985; Evans et al., 2004). Alternatively, a chemically defined synthetic attachment substrate can be developed by coating the microcarrier with chemically synthesized materials that mimic the ligands of cell surface adhesive molecules. Thus, treatment allows synthetic microcarriers to facilitate cell adhesion and proliferation. Studies have indicated that various microcarrier matrix materials can support MSC growth, including polystyrene (Tseng et al., 2012), glass (Elseberg et al., 2012), decellularized adipose tissue (Turner and Flynn, 2012), gelatine (Eibes et al., 2010; Chen et al., 2011) and dextran (Hewitt et al., 2011).

Commercially Available Microcarriers

A wide range of microcarriers are commercially available, enabling researchers to select one that suits their cell line, type, and purpose of cell expansion. Commercially available microcarriers can be categorized into six groups (**Table 3**). This section will discuss in further detail three popular microcarriers which have been in most cases widely used for MSC expansion: GE Healthcare's Cytodex®, Corning® Synthemax®, and SoloHill® Plastic. Selection of microcarriers is generally carried out be screening a range of microcarriers for attachment, growth, differentiation potential (Sart et al., 2013). Other parameters such as actin organization of attached MSCs may influence these outcomes as noted above. While this review is focussed on the use of commercially available microcarriers for scaled manufacturing of cells (Badenes et al., 2016; Chen et al., 2020), there are a number of other microcarrier systems in development which are used in other applications including tissue engineering (Shekaran et al., 2016; Lam et al., 2017; Zhou et al., 2020).

Dextran Beads: Cytodex® 1 and Cytodex® 3

Produced by GE Healthcare, Cytodex® 1 and 3 are dextran beads. Cytodex® 1 is positively charged while Cytodex® 3 features a denatured collagen coating. These biologic properties lead to positive results when culturing MSCs (Chen et al., 2013).

As MSCs express multiple integrin subunits that facilitate attachment to denatured collagen, microcarrier-MSC attachment on Cytodex® 3 is expected to be high. This is consistent with results from Goh et al. (2013), who achieved a 12- to 16-fold expansion efficiency (6×10^5–8×10^5 cells/mL) of human fetal MSCs on Cytodex® 3 microcarriers, compared to 4- to 6-fold expansion using traditional monolayer culture (1.2×10^5–1.8×10^5 cells/mL). The human fetal MSCs maintained colony-forming capacity, doubling times, and immunophenotype post-Cytodex® 3 expansion. Similarly, Frauenschuh and colleagues found that MSCs had greater than 80% attachment on Cytodex® 1 microcarriers following three hours of incubation (Frauenschuh et al., 2007). However, recent research by Lin et al. established that similar levels of cell adhesion, growth, and differentiation outcomes were achieved on Cytodex® 1 and 3 microcarriers (Lin et al., 2017). Thus, it may be concluded that microcarrier size, matrices, and surface nature are unlikely to be as crucial in determining MSC yield and differentiation outcomes as might be expected. Despite previous successes using Cytodex® microcarriers, their use is limited in a therapeutic context as these microcarriers are not xeno-free, leading to a risk of contamination through the introduction of adventitious xenogeneic agents (Felka et al., 2010).

Synthetic Peptide Surface Microcarrier: Synthemax®

The xeno-free Corning® Synthemax® Surface features a short peptide sequence derived from the vitronectin protein to mimic the biological ligand for cell adhesion (Melkoumian et al., 2010). The peptide is based on the Arg-Gly-Asp (RGD) motif and immobilized on an acrylate coating. Synthemax® microcarriers may be obtained already coated in two different peptide surface densities or the Synthemax® surface can be added to synthetic or biological microcarriers through an adsorption process to support MSC attachment and growth.

Previous research has found that the Synthemax® Surface can replace ECM proteins to facilitate efficient MSC attachment, support the long-term culture of BM-MSCs and maintain cell

TABLE 3 | Commercially available microcarriers (Chen et al,. 2013).

Microcarrier type	Non-porous/smooth	Collagen coated	ECM coated	Non-modified	Macroporous	Weighted
Example	Polystyrene microcarrier, e.g., plastic microporous microcarrier	Cytodex® 3	Pronectin-F	Glass beads, tissue culture polystyrene microcarriers	Cytopore, Cultispher®	Cytoline®
Properties	May incorporate a surface charge	Chemically coupled collagen	Coated with recombinant protein with a repeat RGD sequence	A high negative surface charge	Pore ranges in the range of 10–70 μm on microcarrier surface	Macroporous and the microcarrier matrix is made denser using silica
Suitable conditions	Enable culturing of adherent cells that form a continuous monolayer of cells on the surface of microcarriers in suspension	Enable culturing of sensitive cells with low plating efficiency, coating increases efficacy of cell harvest	Enable culturing of sensitive cells in serum-free conditions	Enable culturing of any anchorage dependent cell line in suspension	Provide higher surface areas for growth and offer better mechanical protection to cells from shear stress	Enable culturing in fluidized bed perfusion cultures

surface antigen expression profile following expansion (Dolley-Sonneville et al., 2013). The study calculated cell yield to be significantly higher compared to traditional BM-MSC culture in serum-containing medium. A similar study demonstrated that the Synthemax® Surface peptide recapitulates integrin-ECM engagement of human embryonic stem cell (hESC) comparable to those grown on Matrigel-coated substrates (Jin et al., 2012). The synthetic ligand interacted with human induced pluripotent stem cells (hIPSCs) *via* the integrin $\alpha_v\beta_5$ units, demonstrating its comparability to vitronectin. Lambshead *et al.* observed human pluripotent stem cells (hPSCs) cultured on Synthemax® coated plates and flasks were morphologically indistinguishable from those cultured in control flasks coated with Geltrex (Lambshead et al., 2018). Accordingly, the genetic stability and pluripotency of hPSCs was maintained on Synthemax® surface as assessed by the PluriTest™ assay (Muller et al., 2011).

Findings regarding cell yield are consistent with other reports in the literature regarding the performance of the Synthemax® Surface (Meng et al., 2010). Importantly, the Synthemax® Surface is xeno-free and therefore compatible with serum-free media. This eliminates the risk of xeno-contamination inherent in the use of animal-derived products, a strong advantage as compared to Plastic, Plastic Plus and Star-Plus microcarriers in a therapeutic context. However, its use may be limited by financial considerations: the cost of goods may be higher for microcarriers with synthetic coatings than for uncoated styrene microcarriers.

Cross-Linked Polystyrene Microcarriers: Plastic, Plastic Plus, Star-Plus

The SoloHill® range of styrene copolymer microcarriers have no specialized coating and may incorporate a surface charge to enhance protein adsorption from media supplements which facilitates MSC and attachment at an acceptable level. In the case of the Plastic microcarriers, the surface of the particles is modified to make them more hydrophilic than the base polystyrene material and is most likely negatively charged. Attachment of MSCs to Plastic is facilitated by the adsorption of extracellular matrix (ECM) proteins present in the media (Dolley-Sonneville et al., 2013). Relatively little is known about the proteins adsorbed from culture media onto microcarrier growth surfaces. The adsorbed layer on SoloHill® microcarriers are likely a complex mixture of partially denatured proteins which is highly difficult to characterise (Wang et al., 2012).

Cells derived from vertebrates (such as MSCs) carry a heterogeneous negative surface charge (Varki and Gagneux, 2012). During the cell-growth surface adhesion process, electrostatic forces and van der Waals forces play an important role in the interaction of the cell and growth surface (the microcarrier plus adsorbed protein layer from the media) (Petry et al., 2016). Initially positive surfaces (e.g., Plastic Plus, Star-Plus) become less positively charged over time as more proteins are attracted to and adsorb to its surface, changing the overall net charge to negative. Plastic, which is not chemically modified to incorporate a positive charge, is negatively charged. Relatively hydrophobic surfaces such as the SoloHill® microcarriers may attract the types of proteins that facilitate MSC attachment (Grinnell and Feld, 1981). The initial surface sign, magnitude of charge, and degree of hydrophobicity are determinants for the types, quantity, and nature of adsorbed proteins on the surface of microcarriers. Microcarrier properties which are conducive to MSC attachment and growth are generally discovered by screening a range of microcarriers, often in small volume, static cultures (Rafiq et al., 2016).

It is proposed that uncoated microcarriers with positive (e.g., Plastic Plus, Star-Plus) or negative (e.g., Plastic) charge will demonstrate better cell-surface attachment due to their ability to encourage protein adsorption from the media onto their surfaces which facilitates MSC attachment and growth. In a previous study, a greater yield of UC-MSCs was obtained on Plastic and Plastic Plus microcarriers compared to Pronectin-F (an RGD polymer-coated microcarrier) and glass microcarriers (Petry et al., 2016). A slightly higher cell yield was obtained on Plastic Plus microcarriers compared to Plastic. This establishes the preference of UC-MSCs for polymer substrates over glass. Furthermore, Rafiq and colleagues selected Plastic microcarriers as optimal for BM-MSC expansion following a systematic evaluation of 13 microcarriers (Rafiq et al., 2016). BM-MSC immunophenotype and differentiation capacity was unchanged following harvesting on polystyrene microcarriers.

In comparison to the well-characterized abilities of Plastic and Plastic Plus, Star-Plus is a relatively new microcarrier and extensive research on its relative usefulness in MSC scale-up expansions has not yet been conducted. All plastic microcarriers discussed here are xeno-free and, therefore, pose no risk for contamination of cells for therapeutic purposes. However, a significant disadvantage of these types of microcarriers is that they cannot be readily used in serum-free or chemically defined synthetic media as these do not contain serum proteins typically. Thus, a pre-conditioning step with recombinantly produced, GMP-grade human ECM proteins may be required, increasing costs and process complexity.

Microcarriers and MSC Fate

The effects of substrate stiffness on MSC properties must be considered, MSCs specify cell lineage with respect to tissue-level elasticity (Engler et al., 2006). The spectrum of stiff to soft substrates can alter MSC surface markers, with MSCs lineage markers primed to neurogenic following growth on low-stiffness substrates, myogenic on medium-stiffness substrates and osteogenic on stiff substrates. Although the effect of MSC substrate stiffness on cell differentiation pathways are well known, there is a gap in the literature regarding substrate effect on MSC secretome, and thus immunomodulation. Furthermore, studies focussing on MSC expansion on microcarriers have not elucidated the effects, if any, of microcarrier stiffness on the MSC secretome.

The attachment of microcarriers to MSCs *via* ligand-receptor complexes has been shown to transmit physiochemical signals within the cell *via* mechanotransduction mechanisms, thereby altering cell fate (Nomizu et al., 1995). ECM proteins from cell culture supplements (or derivative motifs found on the surface of microcarriers) bind to specific MSC cell surface integrin receptors, which activate intracellular signaling pathways and

controls gene expression, cytoskeletal organization, and cell morphology (Nomizu et al., 1995). Each integrin receptor can bind to a multitude of ECM proteins and stimulate at least six different classes of intracellular signaling molecules: protein tyrosine kinases, serine/threonine kinases, lipid kinases, lipid phosphates, protein phosphatases, and intracellular ion fluxes (Schwartz and Ginsberg, 2002). Through differential attachment, different microcarriers may alter MSC immunophenotype, differentiation capacity, and possibly secretome (**Figure 4**).

A study by Salasznyk and colleagues determined that culturing hMSCs on vitronectin and collagen I substrates can promote their osteogenic differentiation *via* ECM contact, inducing differentiation (Salasznyk et al., 2004). These findings have been expanded by the demonstration that MSCs propagated and harvested from microcarriers demonstrate higher osteogenic potency than those cultured in traditional monolayer cultures (Goh et al., 2013). Their results suggest that MSC culture on microcarriers resulted in a change in cell phenotype, perhaps caused by the activation of different intracellular signaling molecules following attachment. There is a body of evidence that suggests mechanical properties may prime MSCs for particular differentiation pathways, and potentially alter gene expression (Frith et al., 2010; Frith et al., 2012a; Frith et al., 2012b; Kusuma et al., 2017; Lin et al., 2017; Frith et al., 2018). This raises the question of whether the mode of MSC growth in the expansion phase affects other aspects of MSC immunophenotype, such as their secretome.

Teixeira and colleagues considered modulating MSC secretome by changing the culture environment and concluded that dynamic culture conditions may be a strong asset in regenerative strategies revolving around the use of the MSC secretome (Teixeira et al., 2016). Although the study focussed on computer-controlled bioreactors, the findings can be expanded to MSCs cultured on a range of microcarriers. A recent novel study investigated the role of microenvironment surface structure on cytokine secretion profile (Leuning et al., 2018). The group cultured BM-MSCs and kidney perivascular stromal cells (kPSCs) on unique topographies and measured any changes in cytokine and growth factor secretion compared to the same cells grown in planar culture. Although functionally different, both BM-MSCs and kPSCs displayed different cell morphologies and cytokine secretion profiles when grown on varying topographies. Their findings support the hypothesis that MSC secretome is influenced by microenvironment structure such as focal adhesion density, size, and protein recruitment. Thus, MSC immunomodulatory function may be capable of manipulation in an engineered setting (such as microcarrier expansion). The implication that microcarrier surface topography in bioreactor expansion should be taken into account to preserve therapeutic properties of MSCs should be examined in further detail.

Apart from the study by Leuning *et al.*, research where screening of microcarriers is carried out for the purposes of selecting the best microcarrier for growth of MSCs has not considered changes in cell secretome, other than testing the cells produced in simple, immunosuppression tests. This may not be predictive for how the cells will behave *in vivo*. Furthermore, a relationship between the expansion surface (such as microcarriers) and MSC cell contact-dependent immunosuppression has not been investigated thoroughly in the prior literature. Thus, a microcarrier best suited for the desired MSC secretome for clinical application has not been identified in previous research, which is remiss in the field as the therapeutic benefits of MSCs are often attributed to their secretome. Any changes in cell contact-induced immunosuppression or secretome may affect MSC immunomodulatory potential, which must be studied in detail prior to the licencing of therapeutics.

CONCLUSIONS

MSCs exert immunomodulatory effects on innate and adaptive immune cells. They induce their effects through cell-to-cell contact and the release of cytokines and other bioactive molecules (Di Nicola et al., 2002). Research involving MSCs is intensifying due to their therapeutic potential for a variety of diseases, largely mediated by their immunosuppressive properties.

The large number of cells required for therapeutic infusions requires 3D scale-up technologies such as stirred-tank bioreactors. These technologies have advantages and disadvantages which are thoroughly researched in the literature. Microcarriers, on which MSCs are propagated in bioreactors, have a high surface area allowing high rates of attachment (Caruso et al., 2014). They can be chemically modified to further increase MSC attachment. Bioreactors themselves can be monitored by online sensors, allowing cell microenvironment variables to be maintained in tight parameters (Badenes et al., 2016). However, culturing in bioreactors presents issues such as shear stress on cells, inconsistent temperature and pH, and removal from microcarriers which may change MSC phenotype (Stathopoulos and Hellums, 1985; Lindskog et al., 1987; Dos Santos et al., 2014).

MSCs are known to actively respond to their culture microenvironment, including substrates they are propagated on, by secreting various cytokines and growth factors. These soluble factors are important constituents of the MSC secretome that underlie many of their immunomodulatory properties. However, scale-up manufacturing methods are not currently tailored for MSC expansion, and there is a lack of knowledge about whether MSC expansion on microcarriers alters the secretome and cell function. The establishment of a 3D MSC culture method that does not compromise the immunomodulatory properties of MSCs would drastically improve clinical feasibility. Advances in this area will need to take into account recent findings that challenge the tenet that MSCs need to remain viable for therapeutic efficacy.

In addition to the manufacturing considerations, the extensive efforts toward understanding MSC biology, their secretome, fate upon administration and interactions with a range of immune cells, and soluble factors need to intensify in order to delineate pathways through which MSC-mediated immunosuppression takes place. This will provide substantial foundation and direction to the engineering and pharmaceutical

groups whose efforts in developing a commercial MSC product currently are blindsided by the lack of knowledge and immense speculation regarding MSC application. Shedding light in these aspects will almost certainly ensure a more translatable MSC product for tissue regeneration.

AUTHOR CONTRIBUTIONS

DC, TB, LM, and TH contributed conception and design of the manuscript. DC wrote the first draft of the manuscript. TB, LM, and TH wrote sections of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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Semipermeable Cellulose Beads Allow Selective and Continuous Release of Small Extracellular Vesicles (sEV) From Encapsulated Cells

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The clinical benefit of therapies using Mesenchymal Stem Cells (MSCs) is attributable to their pleiotropic effect over cells and tissues, mainly through their secretome. This paracrine effect is mediated by secreted growth factors and extracellular vesicles (EV) including small EV (sEV). sEV are extra-cellular, membrane encompassed vesicles of 40 to 200 nm diameter that can trigger and signal many cellular responses depending on their cargo protein and nucleic acid repertoire. sEV are purified from cell culture conditioned media using several kits and protocols available that can be tedious and time-consuming, involving sequences of ultracentrifugations and density gradient separations, making their production a major challenge under Good Manufacturing Practices (GMP) conditions. We have developed a method to efficiently enrich cell culture media with high concentrations of sEV by encapsulating cells in semipermeable cellulose beads that allows selectively the release of small particles while offering a 3D culture condition. This method is based on the pore size of the capsules, allowing the release of particles of ≤ 200 nm including sEV. As a proof-of-principle, MSCs were encapsulated and their sEV release rate (sEV-Cap) was monitored throughout the culture and compared to sEV isolated from 2D seeded cells (sEV-2D) by repetitive ultracentrifugation cycles or a commercial kit. The isolated sEV expressed CD63, CD9, and CD81 as confirmed by flow cytometry analysis. Under transmission electron microscopy (TEM), they displayed the similar rounded morphology as sEV-2D. Their corresponding diameter size was validated by nanoparticle tracking analysis (NTA). Interestingly, sEV-Cap retained the expected biological activities of MSCs, including a pro-angiogenic effect over endothelial cells, neuritic outgrowth stimulation in hippocampal neurons and immunosuppression of T cells

in vitro. Here, we successfully present a novel, cost, and time-saving method to generate sEV from encapsulated MSCs. Future applications include using encapsulated cells as a retrievable delivery device that can interact with the host niche by releasing active agents *in vivo*, including sEV, growth factors, hormones, and small molecules, while avoiding cell clearance, and the negative side-effect of releasing undesired components including apoptotic bodies. Finally, particles produced following the encapsulation protocol display beneficial features for their use as drug-loaded delivery vehicles.

Keywords: small extracellular vesicles, stem cells, cellulose sulphate microbeads, secretome, Cell-in-a-Box® encapsulation, cell therapy, drug delivery system

INTRODUCTION

Cell therapy is a constantly growing field as medical needs move toward more targeted and specific solutions. In this context, mesenchymal stem cells (MSCs) represent one of the main actors in basic and translational research. MSCs can be isolated from adult and post-natal tissues, including bone-marrow, adipose tissue, dental tissue, umbilical cord and menstrual fluid (Samsonraj et al., 2017). The therapeutic properties of MSCs mainly reside in their secretions and the paracrine signaling to target cells. The signals themselves are composed of soluble biomolecules (proteins or nucleic acids) or extracellular vesicles (EV) containing them.

EV represents a wide classification of secreted vesicles and comprises microvesicles (MV), apoptotic bodies, microsomes, and sEV among others (Margolis and Sadovsky, 2019). sEV correspond to the smaller in diameter with sizes of > 200 nm, are characterized by the expression of the tetraspanins CD9, CD63, and CD86, and their cellular origin is diverse (Théry et al., 2018). sEV are emerging as key mediators in intercellular communication through horizontal transfer of information *via* their molecular cargo, which includes proteins, DNAs, mRNAs, and miRNAs, that could trigger specific intracellular cascades in the recipient cells (Pegtel and Gould, 2019). For these reasons, the interest nowadays is to obtain large fractions of pure sEV to be used as therapeutic agents without the need for using exogenous cells in patients.

Most commonly, sEV are isolated from cell culture supernatant through methods comprising magnetic particles, immunoaffinity capture-based techniques, ultrafiltration, dialysis, precipitation, size exclusion chromatography (SEC), microfluidics-based isolation techniques, tangential flow filtration (TFF) and ultracentrifugation (Li et al., 2017). Ultracentrifugation is the most commonly used technique, in fact, it is estimated that is used in more than half of isolation protocols for sEV researchers. Differential ultracentrifugation consists in several steps with different centrifugal forces and times that allows the isolation of sEV based on their size and shape and involves the sedimentation of large particles first (such as cells, cell debris, and membrane fragments, apoptotic bodies, and others) that represent a contamination in these cases. After every centrifugation cycle, the supernatant is preserved and the pellet containing the larger vesicles fraction is eliminated. Finally, after the last cycle, sEV are found in the pellet and PBS is usually used for their final resuspension (Gardiner et al., 2016; Li et al., 2017).

Despite the number of different techniques available for sEV isolation, most have significant challenges for upscaling to therapeutic level and for generation of GMP-grade sEV. Therefore, the need for more efficient protocols is justified and could accelerate the translation of sEV into the clinical field. Additionally, the potential use of sEV in patients implies that the challenges should be resolved. For example, how to guide such vesicles to the desired area or how to avoid the rapid clearance that happens in tissues (Liu et al., 2017). For example, some groups have taken a different approach by using hydrogels to directly encapsulate sEV for controlled release in chronic diabetic wounds, which requires long treatments (Shi et al., 2017; Wang et al., 2019) and for cardiac repair that also depends on a continuous supply of the biotherapeutic agent (Han et al., 2019). Some of the limitations of these approaches are the limited number of sEV that can be encapsulated leading to an interrupted supply over longer periods.

Cell encapsulation is a classic technique that has been applied for the delivery of active therapeutic agents from entrapped cells (Acarregui et al., 2013; Gonzalez-Pujana et al., 2017). Their application ranges from insulin release therapy for type 1 diabetes (Orlando et al., 2014) to other life-threatening pathologies, such as cancer (Löhr et al., 2014; Michałowska et al., 2014); also, capsules in general represent the possibility of their localization in a desired area (Dangerfield et al., 2013). The capsule structure must be permeable in order to enable nutrients and waste flux but also the release of the therapeutic agent(s). This makes the development of the encapsulation material as highly challenging. Cellulose sulphate has been developed since more than 20 years (Dautzenberg et al., 1999) and is one of the most used materials due to its inert presentation to the immune system and other relevant properties, such as representing a safe microenvironment for the survival of the

Abbreviations: MenSCs, menstrual blood-derived stem cells; sEV, small extracellular vesicles; NTA, nanoparticle tracking analysis; EV, extracellular vesicles; MSCs, mesenchymal stem cells; MV, microvesicles; TEM, transmission electron microscopy; PBMC, peripheral blood mononuclear cells; PHA, phytohemagglutinin.

cells. Additionally, their handling in the lab does not represent any complications and can be treated as cells and be frozen without damage. Importantly, the use of cellulose sulphate encapsulated human cells is safe in patients as has been demonstrated in two human clinical trials (Löhr et al., 2014) and in a veterinary application (Michałowska et al., 2014).

Encapsulated cells remain viable inside the capsules due to nutrient and waste products exchange with their environment. Moreover, the system presents longer cell viability with the advantageous consequence of a longer secretion time of the molecules of interest (Emerich et al., 2014; Gonzalez-Pujana et al., 2017). This occurs because the cells become contact inhibited once reaching the capsules' capacity but maintaining its metabolic activity, therefore extending the secretion of the therapeutic molecule. The outflux of many biomolecules, such as insulin, cytokines, antibodies, and enzymes, has been described (Löhr et al., 2014; Salmons and Gunzburg, 2018) but the flux of sEV has not previously been demonstrated.

In this work, we first encapsulate MSCs using semipermeable cellulose beads: Cell-in-a-Box® by Austrianova is a straightforward encapsulation process. The sEV released from encapsulated MSCs derived from the menstrual fluid (MenSCs) (Meng et al., 2007) were characterized. Some of the paracrine properties described for MenSCs include the induction of angiogenic responses *in vitro* and *in vivo*, support the proliferation of $CD34^+$ $CD133^+$ hematopoietic stem cells *in vitro* (Alcayaga-Miranda et al., 2015a), anti-microbial effect over clinically relevant bacterial strains and protection in an animal model for sepsis (Alcayaga-Miranda et al., 2015b). Safety of MenSCs in patients has been demonstrated in clinical trials (Chen L. et al., 2019).

sEV derived from encapsulated MenSCs (sEV-Cap) were compared with sEV derived from the same cells in a 2D setup and isolated by ultracentrifugation (sEV-2D) in terms of shape, size, and paracrine properties. Here we show that capsule-derived sEV (sEV-Cap) retain these trophic properties *in vitro* meaning that encapsulated cells represent a new and promising technique for the generation and isolation of sEV and their use in the clinical field.

MATERIALS AND METHODS

Ethics Approval

All the procedures were approved by the Ethics Committee of Universidad de los Andes. Samples were obtained with the informed consent of donors.

MenSCs Isolation

MenSCs were isolated as previously described (Alcayaga-Miranda et al., 2015a). Briefly, menstrual fluid was collected in a menstrual silicone cup (Mialuna®, Santiago, Chile) from healthy donors and transferred to a 50-mL conical tube containing 2 mM ethylenediaminetetraacetic acid (EDTA). Mononuclear cells were isolated by a Ficoll-Paque Plus gradient (GE Healthcare, Amersham, UK) and were abundantly washed with PBS 1×. Isolated cells were seeded in T25 flasks and were nourished with high glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, 1% amphotericin B, and 1% L-glutamine. Non-adherent cells were discarded the next day. MenSCs were subcultured when reached 80% confluence using 0.05% trypsin-EDTA. Cell cultures were maintained in a humidified incubator at 37°C with 5% CO_2. All the mentioned cell cultures regents were provided by Thermo Fisher.

MenSCs Encapsulation (MenSCs-Cap)

Cell-in-a-Box® capsules containing the MenSCs cells were provided by Austrianova Singapore Pte Ltd essentially according to the protocol as previously described (Ortner et al., 2012). Briefly, frozen MenSCs cells from a single donor were sent to Austrianova where they were cultured and trypsinized to give a single-cell suspension. After pelleting, 3.5×10^6 cells were resuspended in Gel8 (proprietary cellulose sulphate solution) and jet-sprayed using an encapsulation machine into a bath of poly-diallyl dimethyl ammonium chloride (pDADMAC). The encapsulated cells were cultured for 1 day in DMEM supplemented with 10% FBS, penicillin/streptomycin, and 2 mM L-glutamine. Each capsule contained approximately 800 to 1,000 cells.

Capsules Handling

MenSCs-Cap were stored frozen in liquid nitrogen. To defrost, vials were tempered at 37°C in a water bath, and when MenSCs-Cap were settled at the bottom of the vial, the supernatant was eliminated. Next, MenSCs-Cap were transferred to a T25 flask containing culture medium supplemented with additional 50% FBS and incubated for 1 h at 37°C. Next, the medium was eliminated, and MenSCs-Cap were washed with culture medium to finally be maintained with the same culture medium used for MenSCs monolayers. Medium was changed two to three times a week.

Viability of MenSCs-Cap

Viability was determined after the encapsulation process as a control of the technique and during 16 days to show their behavior over time under cell culture conditions. Post-encapsulation, capsules were frozen and thawed, then, the capsules were incubated in Cell-in-a-Box® Decapsulation Solution as outlined by the supplier (Merck, Cat Nr. CIB002). After the cells had been released from the capsules, cell viability was determined by trypan blue exclusion. The process of encapsulation-freezing, storage, and thawing was used because all the experiments were performed using thawed capsules.

To determine encapsulated cells viability for 16 days, a determined number of MenSCs-Cap was added to a 96-well plate and 10% v/v WST-1 reagent (Quick Cell Proliferation Assay Kit, BioVision, CA, USA) was added to the culture medium. After 2 h incubation at 37°C the supernatant was transferred to a new 96-well plate for absorbance measure at 450 nm/570 nm (Tecan Reader), according to the manufacturer's guidelines.

sEV Isolation

For the characterization and comparative sEV studies with MenSCs-Cap, two distinct protocols were used: (1) a commercial Total Exosome Isolation reagent and (2) ultracentrifugation. The Total Exosome Isolation kit (Thermo Fisher) was used according to manufacturer's instructions. The selection of the isolation protocol was made according to the intended use of sEV: for small volumes, the commercial kit was used and for larger volumes, ultracentrifugation. We denominated sEV-Cap to the EV isolated from encapsulated cells and sEV-2D those EV isolated from MenSCs seeded in monolayers.

MenSCs were cultured in DMEM supplemented with 10% FBS, 1% penicillin/streptomycin, and 1% L-glutamine. When MenSCs monolayers reached 80% of confluence, cells were washed 3 times with PBS 1× and DMEM (phenol red-free) supplemented with 1% penicillin/streptomycin and 1% L-glutamine was added. After 48 h of incubation, the supernatant was recovered and subjected to sequential centrifugation steps for 600*g* for 10 min and 2,000*g* for 10 min, the supernatant recovered correspond to the EV fraction. Next, the EV fraction was centrifuged to 10,000*g* for 30 min to eliminate MV, and finally, the sEV fraction was recovered at 100,000*g* using a TH-641 rotor after 70 min of ultracentrifugation (Thermo Fisher). The supernatant was eliminated, and the resulting pellet was resuspended in PBS 1×, stored at −20°C, and used for experimental procedures.

To obtain supernatant from encapsulated cells, 50 MenSCs-Cap were maintained in 500 µl of DMEM (phenol red-free) supplemented with 1% penicillin/streptomycin and 1% L-glutamine at 37°C and 5% CO_2, for 48 h. Since this volume is small, we used the commercial kit for sEV isolation. The medium was collected and centrifuged at 600*g* and 2,000*g* for 10 min each to eliminate any cell debris. The supernatant was transferred to a new tube and mixed with Total Exosome Isolation reagent to further incubation at 4°C overnight. Next day, the samples were centrifuged at 10,000*g* for 1 h at 4°C, and the supernatant was eliminated. The pellet, containing the sEV fraction (sEV-Cap), was resuspended in PBS 1× and stored at −20°C for further analysis.

Quantification of Protein Content of sEV

Protein content from sEV samples were quantified through Bradford assay, measuring absorbance at 590/450 nm using a standard curve of bovine serum albumin (BSA).

Comparison of sEV Production (sEV-Cap vs sEV-2D)

MenSCs-Cap were added into a 24-well plate in 500 µl (50 capsules per well are equivalent to 50,000 cells approximately). In parallel, 50,000 MenSCs were seeded in another 24-well plate in the same media volume. The serum enriched medium was eliminated for the capsules and cells in monolayer and were washed with PBS 1×, then cells were induced with the same medium described previously. The supernatants were collected after 24, 48, and 72 h and were submitted to sEV isolation using the Total Exosome Isolation kit. The yield was estimated quantifying the protein content through Bradford assay.

Particle Size and Concentration Characterization With NTA

Isolated EV suspensions were analyzed using the NanoSight NS3000 instrument (Malvern Instruments). The settings were optimized and kept constant between samples for capture settings (laser type, green; camera level, 8; slider shutter, 317; slider gain, 15; temperature, 25°C) and for analysis settings (detection threshold, 3; blur size, auto). Five videos of 60 s each were recorded per sample.

Flow Cytometry Analyses of sEV

7×10^8 total sEV (quantified by NTA analysis) obtained from MenSCs-Cap or MenSCs monolayers were resuspended in a final volume of 100 µl PBS 1× and 0.5 µl Aldehyde/Sulfate beads (Thermo Fisher, cat. #A37304) were added to the solution and mixed using a benchtop rotator for 10 min. Then, 100 µl of PBS 1× was added to the mixture, and mixing was continued overnight at 4°C. Next day, 100 µl of 1 M glycine in PBS 1× was added, and mixing was continued for 1 h at room temperature. The mixture was spun down at 8,000*g* for 1 min, and the precipitate was resuspended in 100 µl of 10% bovine serum albumin (BSA) in PBS 1× and mixed for 45 min at room temperature. The mixture was spun down at 8,000*g* for 1 min, and the supernatant aspirated. The beads with sEV attached (pellet) were then resuspended to a final volume of 20 µl of 2% BSA in PBS 1× and immunolabeled for CD63, CD81 and CD9 or an isotype control. The sEV bound to beads were incubated with 1 µl of one of the following antibodies: anti-CD63 antibody (BD Pharmingen, cat. 556019), anti-CD81 (BD Pharmingen, cat. 555675), anti-CD9 (BD Pharmingen, cat. 555370) or 10 µl IgG1 isotype control (BD Biosciences, cat. 349040) and mixed for 30 min at room temperature. The mixture was centrifugated at 8,000*g* for 1 min, the supernatant was aspirated, and the pellet was resuspended in 20 µl of 2% BSA in PBS 1×. Then, 1 µl of secondary antibody conjugated with Alexa Fluor 488 (BioLegend, cat. 406626) was added to the samples and isotype control. All samples were mixed at room temperature for 30 min in darkness. The samples were then centrifugated at 8,000*g* for 1 min, the supernatant was aspirated, the pellet was resuspended in 100 µl PBS 1× and washed 2 times with PBS 1×. The expression of sEV markers (CD63, CD81 and CD9) was analyzed using the FACS Canto II flow cytometer (BD Biosciences). Data were analyzed using FlowJo software V10 (Tree Star, Ashland, OR, USA). The flow cytometry data were acquired side by side for both isotype control and samples for each experiment. The gating strategy was similar to the analysis of cells: the beads population was selected from the SSC-A vs FSC-A dot plot and doublets data was eliminated. The data for isotype and the antibodies are shown separately to show the heterogeneity of expression of CD63, CD81, and CD9 in each sample. The MFI (mean fluorescence intensity) values are representative of the entire positive beads.

Transmission Electron Microscopy (TEM) Analysis

EV visualization of the different fractions by TEM was performed as previously described (Rosenberger et al., 2019). Briefly, EV were stained with uranyl acetate and loaded on a formvar/carbon grid with copper mesh for electron microscopy (Ted Pella, No. 01753-F, US). Images of EV were taken at 60,000× magnification using the Philips Tecnai 12 Biotwin transmission electron microscope with Olympus iTEM software (Laboratorio de Microscopía Electrónica de Barrido SEM, Pontificia Universidad Católica de Chile). Circularity of EV was determined by analyzing these images in ImageJ using the parameters "area", "perimeter" and "shape descriptors" and the "circularity" measure from the "Analyze Particles" tool. The highest value for circularity is 1.

Uptake of sEV

sEV-Cap were stained with PKH26 dye (Sigma) to track them in an uptake assay. First, sEV-Cap were mixed with PKH26, previously prepared in Diluent C. PBS 1× was used as control. The samples were incubated for 1 h at room temperature and 1% w/v BSA was added. After incubation, PKH26-stained sEV-Cap were mixed with culture medium and added to previously seeded MenSCs monolayers, as control we used PBS instead of sEV. MenSCs and PKH26-stained sEV were incubated at 37°C for 4 days. Cells were analyzed using an Olympus CX41 microscope and photos were taken for analysis. PKH-26–positive cells were quantified using ImageJ, to show any unspecific stain, we also quantified positive cells in the PBS condition.

Neurite Growth Assay

The protocol used for neuronal cultures have been described previously and was developed with some modifications (Kaech and Banker, 2006). Briefly, E18 Sprague-Dawley rat fetuses were extracted, and brains were dissected to obtain the hippocampi. Hippocampi were disintegrated with 2.5% trypsin/EDTA and mechanically disaggregated with a glass pipette. 15,000 cells were seeded on poly-L-lysine-coated plates in Minimum Essential Media (MEM) and were incubated in a 5% CO_2 oven at 37°C for 24 h. The next day, all the media were eliminated and replaced with neurobasal medium, supplemented with 2% B27, 0.03% L-glutamine, and 1% penicillin/streptomycin antibiotic. Same day, 3 μg of total protein of sEV were added to the medium and left for 5 days.

Immunostaining and Neurite Growth Analysis

Neurons maintained for 5 days *in vitro* (DIV) were fixed and dehydrated in a 4% paraformaldehyde (PFA)–4% sucrose solution for 15 min at room temperature. Cells were permeabilized for 5 min at room temperature in 0.25% Triton X-100 in PBS, washed twice with PBS, and incubated for 30 min with PBS containing 10% BSA for blocking. Cells were incubated overnight at room temperature with the primary antibody anti-MAP2 (Abcam). After washing 3 times with PBS, cells were incubated with the secondary Alexa Fluor 555-conjugated anti-mouse antibody (Life Technologies A21429) diluted 1/5,000 in PBS containing 3% BSA for 45 min at room temperature in darkness. Cells were washed twice with PBS and mounted with ProLong Gold Antifade Reagents containing 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI). Photographs were taken in a Nikon epifluorescence microscope and analyzed in the ImageJ program (NIH). For neurite length and number analysis, the Sholl analysis was used (https://imagej.net/Sholl_Analysis).

In Vitro Tube Formation Assay

Angiogenic potential of sEV-Cap was evaluated through an *in vitro* tube formation assay as described (González et al., 2015). Human umbilical vein endothelial cells (HUVEC) were seeded in 24-well plates (6×10^4 cells per well) previously coated with 250 μl Matrigel® growth factor reduced (GFR) (BD Biosciences). EGM-2 medium was used as positive control and DMEM (without FBS) as negative control. 1 μg of sEV were suspended in DMEM and added to HUVEC. Cells were incubated at 37°C for 5 h, and tube formation was examined with a phase-contrast microscope. Five representative images were captured per well using an Olympus U-RFL-T camera. Quantification of tube formation was analyzed using WimTube software (Wimasis GmbH, Munich, Germany) and the parameters evaluated were total tube length, total loops, and covered area.

Immunosuppression Assay

The capacity of sEV-Cap to suppress T cells proliferation was evaluated as previously described (González et al., 2015). First, human peripheral blood mononuclear cells (PBMC) were isolated from healthy donors by Ficoll density-gradient centrifugation at 400*g* for 30 min. PBMC were stained with 1 μM carboxyfluorescein succinimidyl ester (CFSE, Thermo Fisher) and treated with 1 μg sEV-Cap. PBMC were maintained in Roswell Park Memorial Institute (RPMI) medium supplemented with 10% FBS, 1% L-glutamine, 1% nonessential amino acids (NEEA), 100 mM sodium pyruvate, 25 mM β-mercaptoethanol, and 15 mg/ml phytohemagglutinin (PHA) for lymphocytes activation, when indicated. After 72 h, PBMC were recovered and stained with anti-CD45 and anti-CD3 antibodies (BD Pharmingen, cat. 55548 and 555333, respectively) for analysis in FACS Canto II Flow cytometer (BD Biosciences) using FlowJo software V10 (Tree Star, Ashland, OR, USA). The percentage of immunosuppression was determined as described previously (Killer et al., 2017).

Statistics

All assays were performed at least in duplicate or triplicate as indicated. Values are shown as mean ± SD, and statistical significance was estimated using Student's unpaired *t* test or ANOVA test. $P < 0.05$ was considered as statistically significant. The software GraphPad Prism 5.0b was used for statistical analysis.

RESULTS

Efficient Encapsulation of Viable MenSCs

Austrianova's Cell-in-a-Box® encapsulation process is a straightforward process. After MenSCs expansion in a 2D condition, cells were sub-cultured and mixed with Gel8 (proprietary cellulose sulphate solution) and posteriorly, the cell suspension was added dropwise into a bath of pDADMAC. Machine generated capsule sizes are in the range of 750 µm ± 25 µm and can be easily manipulated, frozen, cultured, and maintained in standard cell culture conditions (**Figure 1A**). Capsules were observed under a traditional optic microscope and cells were visualized as denser areas inside the capsule (**Figure 1B**). Encapsulation is a safe process, but some levels of cell apoptosis or necrosis may occur with some cell types as Live Dead staining showed (**Figure 1C**), in fact, viability was close to 65% after the encapsulation protocol but cells remained viable over time as measured by a WST-1 assay (**Figure 1D**), which measures the reduction of tetrazolium salt into formazan by mitochondrial enzymes. Additionally, we compared the efficiency of sEV production of MenSCs and MenSCs-Cap and determined a higher production from encapsulated cells during a period of 24 to 72 h (**Figure 1E**).

Isolation of sEV From Encapsulated MenSCs (sEV-Cap)

One of the strategies for the production of sEV is the continuous release of vesicles from a carrier, in this case, correspond to encapsulated MenSCs. This could lead to a decrease in the processing time of large volumes of supernatants, but before proposing this alternative, we must describe the characteristics of the sEV generated by encapsulated cells. For that, the supernatant was submitted to the mentioned isolation protocols in order to concentrate the EV and proceed with the characterization.

As a first step, we evaluated the expression of described sEV surface markers: CD63, CD9 and CD81 (Théry et al., 2018). The analysis showed that sEV isolated from 2D or encapsulated cells were positive for CD63, CD81 and CD9, but differences were detected for CD81 with higher MFI in sEV-2D with respect to sEV-Cap (**Figures 2A, B**, **Supplementary Figure 4**). Considering that the same number of particles were used for the analysis (according to the NTA determination) we can infer that sEV isolated from encapsulated cells expressed lower levels of CD81 than sEVs isolated from cells in 2D. This could be due to a different vesicle population secreted by these cells or these differences can rely on the number of vesicles present in each fraction. Even though the same number of particles were used in the experiment, we cannot discard the presence of contamination in the sEV-Cap due to the isolation protocol (commercial kit), that could underestimate the expression of the different proteins evaluated.

In order to compare the purity and quality of the samples obtained by different methods, vesicles from several fractions were analyzed through TEM and Nanoparticle Tracking Analysis (NTA). TEM analysis showed the characteristic cup-shape of EV, sEV-Cap, and sEV-2D (**Figures 3A–C**). Additionally, we determined the circularity of the vesicles in order to analyze whether the isolation technique altered the shape of the sEV. As expected, there was a variety of shapes in EV due to their heterogeneous composition and origins. sEV-Cap and sEV-2D presented similar circularity confirming the validity of the

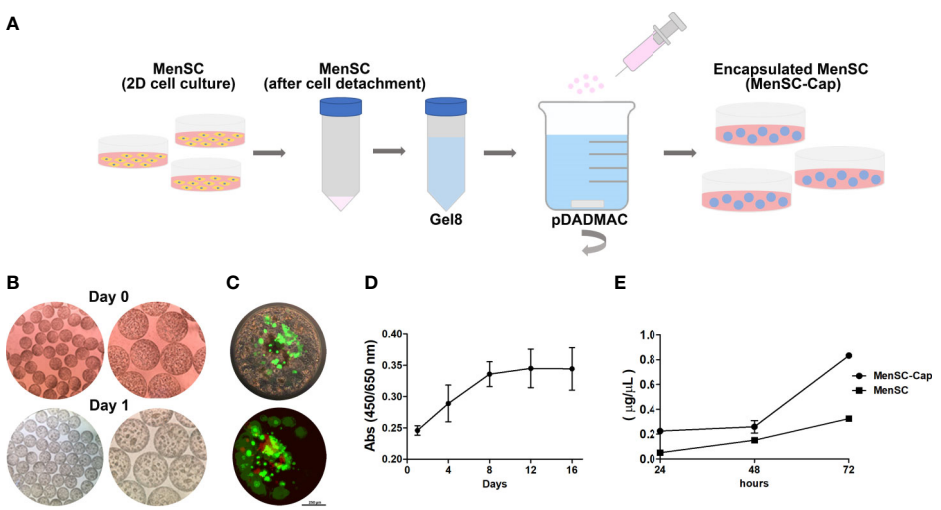
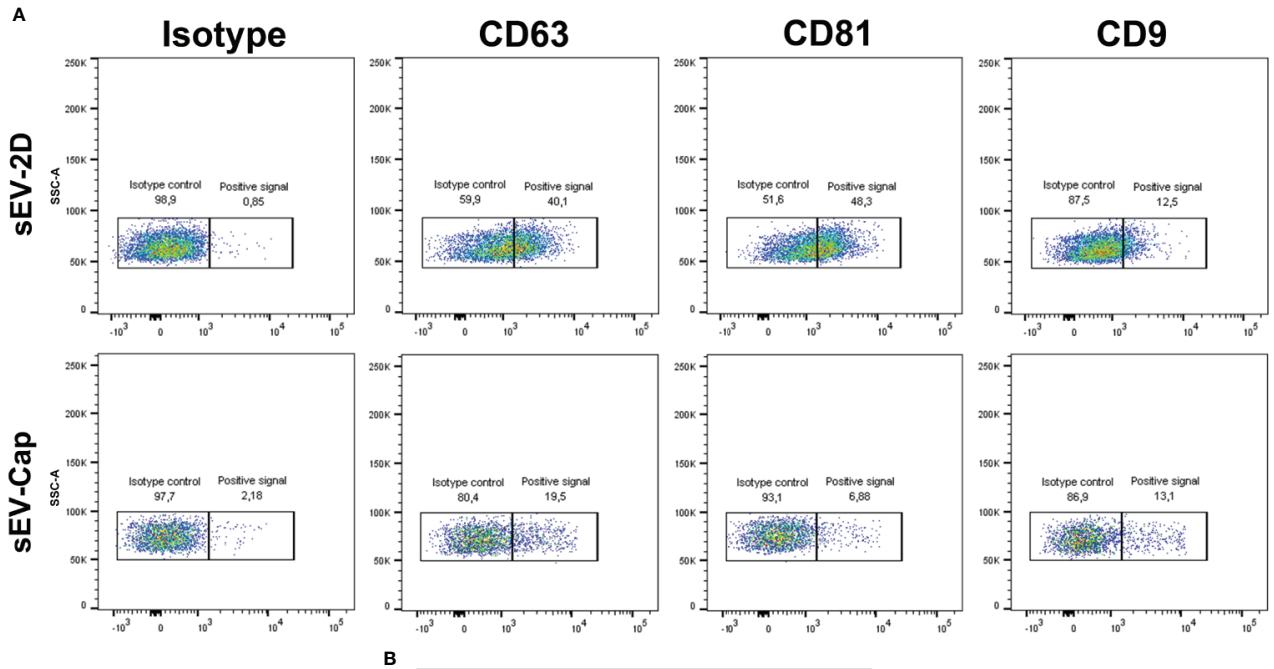


FIGURE 1 | Characterization of MenSCs-Cap. **(A)** Graphic representation of the encapsulation process. MenSCs are cultured in standard conditions, after detaching, cells are mixed with the Gel8 solution. Next, the cellular suspension is jet-sprayed into a bath of poly-diallyl dimethyl ammonium chloride (pDADMAC). The encapsulated cells are maintained in cell culture medium or can be frozen. **(B)** Cellulose capsules containing MenSCs at the same day of encapsulation and after 24 h. **(C)** Live Dead staining shows cell distribution inside the capsule after the defrosting protocol. Live (green) and death (red) cells are shown. **(D)** Cell proliferation of MenSCs-Cap while maintained in standard cell culture conditions measured through absorbance at 450 nm of WST-1 reduction (n = 2). **(E)** sEV production from MenSCs seeded in a standard 2D well plate compared with MenSCs-Cap (n = 2).



B

	Isotype	CD81	CD9	CD63
sEV-2D	274	1544	797	1349
sEV-Cap	204	544	857	1065

FIGURE 2 | Expression of classic sEV markers in sEV-cap and sEV-2D. **(A)** Flow cytometry characterization of sEV-Cap and sEV isolated from 2D-cultured MenSCs shows that are positive for CD63, CD9, and CD81, characteristic markers for sEV. **(B)** Mean fluorescence index from the isotype controls and the markers analyzed.

isolation techniques (**Figure 3D**). Next, the different fractions were analyzed by NTA (**Supplementary Figures 1–3**), EV diameters were close to 218.7 nm ± 75.4 nm, presenting a varied distribution in the size of the vesicles. sEV-2D enriched fraction had sizes around 162.1 ± 54.2 nm, but with the presence of larger vesicles which could represent EVs contamination (**Figures 3E–G**). On the other hand, sEV-Cap size was 123.9 ± 21.8 nm, with a narrower distribution compared to sEV-2D, showing the purity of the sEV released from the porous capsules. Finally, with the NTA data we determined the percentages of sEV according to their size, observing interesting differences in the distribution of vesicles between 40 and 200 nm. For the 40 to 80 nm, 80 to 120 nm, and 120 to 160 nm fractions, there was a higher percentage in sEV-Cap, but in the 160- to 200-nm range sEV-2D contains the higher fraction. Interestingly the fraction of 40 to 160 nm was significantly higher in sEV-Cap, with 78.5% ± 16.5% versus the 11.11% ± 5.51% for sEV-2D. Moreover, in both sEV-Cap and sEV-2D, there was a contamination of vesicles with sizes ≥ 200 nm but in sEV-Cap was significantly lower confirming that encapsulation favors the liberation of sEV with low or absent MV contamination (**Figure 3H**).

Functional Properties of sEV-Cap *In Vitro*

The optimization and development of more efficient techniques for sEV isolation aims to facilitate the access of these cellular products for their applications in research and translation to the clinic. A novel protocol not only needs to show the capacity for optimizing the process but also the quality of the sEV obtained. Hence, it is crucial to demonstrate the functionality of the isolated vesicles by the new protocol and to compare it with the ones obtained by standard methods. Therefore, we evaluated whether sEV-Cap are internalized by cells in order to produce their *in vivo* effect. MenSCs monolayers were incubated with PKH26-stained sEV, and it was determined that cells were capable of internalizing the sEV-Cap in a 59.7% ± 6% directly from the supernatant of capsules. These results confirmed that endocytosis signals in the vesicles surface were functional (**Supplementary Figure 5**).

sEV-Cap Induce Pro-Angiogenic Responses in a Tubule Formation Assay

As MenSCs and other MSCs are recognized as trophic mediators *in vitro* and in their native niche, we sought to evaluate whether the purified sEV contained these properties as well by performing different functional assays. First, we evaluated the potential of sEV-Cap and sEV-2D to induce a pro-angiogenic response (**Figures 4A–D**), performing a tubule formation assay evaluated by the quantification of total tube length, total loops, and covered area. The effect of both sEV-Cap and sEV-2D were comparable among them in all 3 parameters analyzed (**Figures**

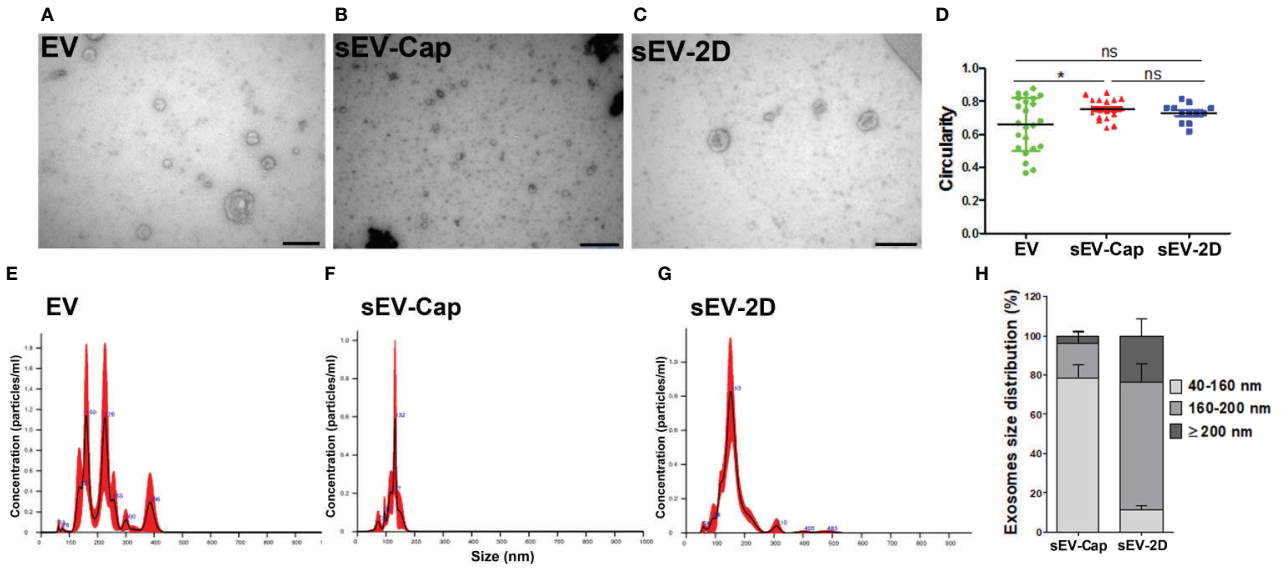


FIGURE 3 | Characterization of sEV-Cap. Shape and size characterization of EV. Representative images from TEM analysis of **(A)** EV, **(B)** sEV-Cap, and **(C)** sEV-2D. Scale bar = 200 nm. **(D)** Circularity of EV respect to their origin. *$P < 0.05$ Student's *t* test, comparison was made between two respective conditions. NTA analysis shows the size distribution of **(E)** EV (8.83×10^7 particles/mL), **(F)** sEV-Cap (1.36×10^7 particles/mL), and **(G)** sEV-2D (4.76×10^7 particles/mL). **(H)** Size distribution of sEV isolated from capsules by ultracentrifugation and isolation from 2D cell culture by the same technique (percentage of particles), data are shown as mean ± SD. There are statically significant differences between the percentage's distribution: 40–160 nm and 160–200 fractions, for the ≥ 200 nm, there were no differences (*$P < 0.05$ unpaired Student's *t* test). ns, not significant.

4E–G) and respect to EGM-2, the positive control (**Figure 4B**). These results indicated that sEV retain the trophic abilities of parental MenSCs.

sEV-Cap Promotes Neuritic Outgrowth in Hippocampal Neurons

Another tested scenario was the potential of MenSCs-derived sEV to induce neuritic growth. To confirm this property, primary cultures of rat hippocampal neurons were treated with either sEV-Cap or 2D-sEV during neurites elongation phase (**Figure 5**). We determined that the presence of the sEV induced a significant increase in the number of neurites (**Figure 5C**) with no differences between sEV-Cap and 2D-sEV. The same trend was observed for the longest neurite and total branching (**Figures 5E–G**), showing that sEV from MenSCs contained growth factors that transduced a cellular signal into the cytoskeleton, promoting the elongation of neurites. Remarkably, the critical value was lower for sEV-Cap respect to 2D-sEV, meaning that the ramifications were closer to the soma in sEV-Cap treated-neurons (**Figure 5F**). These results indicated that sEV-Cap and 2D-sEV possessed similar contents and functions but with some differences in the mechanism by which the cytoskeleton was modulated.

These results confirmed that the functionality of sEV-Cap and sEV-2D were equivalent even though the size of the sEV slightly differed between both groups. More important, sEV derived from MenSCs recapitulated the paracrine functions described when the cells themselves are used in the assays.

sEV-Cap Retain the Immunosuppressive Properties of MenSCs

Finally, we evaluated another classical property of MSCs, which is immunosuppression of T cells in an *in vitro* assay. This role of MSCs represents one of the properties of greatest interest for clinical use in autoimmune diseases. In this assay, PBMC were activated with PHA to induce their proliferation and stained with CFSE. After 72 h, we evaluated the effect of sEV-Cap in the proliferation of T cells measured as a decrease in the number of division cycles (**Figure 6A**). Our data suggest that the presence of sEV inhibited partially T cells PHA-induced proliferation by approximately 30%, supporting the fact that the paracrine properties of MenSCs were maintained in their derived sEV from encapsulated cells (**Figures 6B, C**).

Altogether, these results confirm the rational of using encapsulated cells for the generation and isolation of sEV without time-consuming protocols and with higher purity.

DISCUSSION

The application of cells or their derivates have been a field of constant growth in modern medicine. Lately, sEV have aroused the interest of researchers due to the innumerable reports showing their biological properties *in vitro* and *in vivo* [reviewed in (Zhang et al., 2019)]. Today there are more than 100 trials registered in www.clinicaltrials.gov in different developmental stages, in which sEV are being tested as

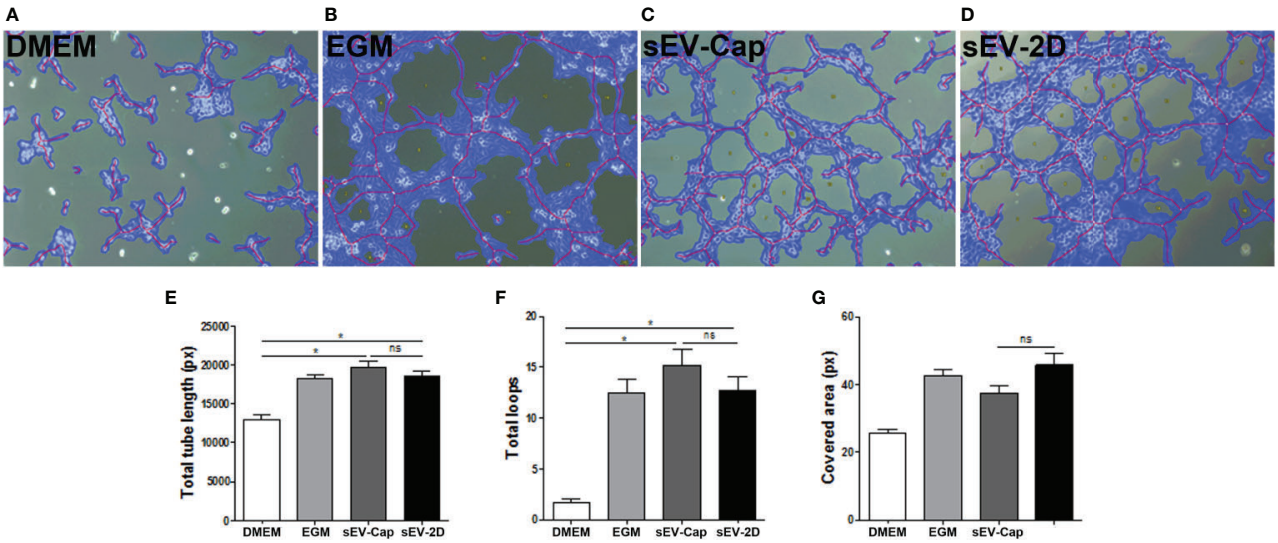


FIGURE 4 | sEV-Cap induces an angiogenic response. sEV elicit a pro-angiogenic response in HUVEC in a tubule formation assay. **(A)** DMEM, serum deprived, as negative control. **(B)** EGM, positive control. **(C)** sEV-Cap. **(D)** sEV-2D. Quantification of **(E)** total tube length, **(F)** total loops, and **(G)** covered area shows that sEV-Cap induce a response similar to sEV-2D (n = 2), and three microscope fields per condition were analyzed for each assay. *$P < 0.05$ one-way ANOVA. ns, not significant.

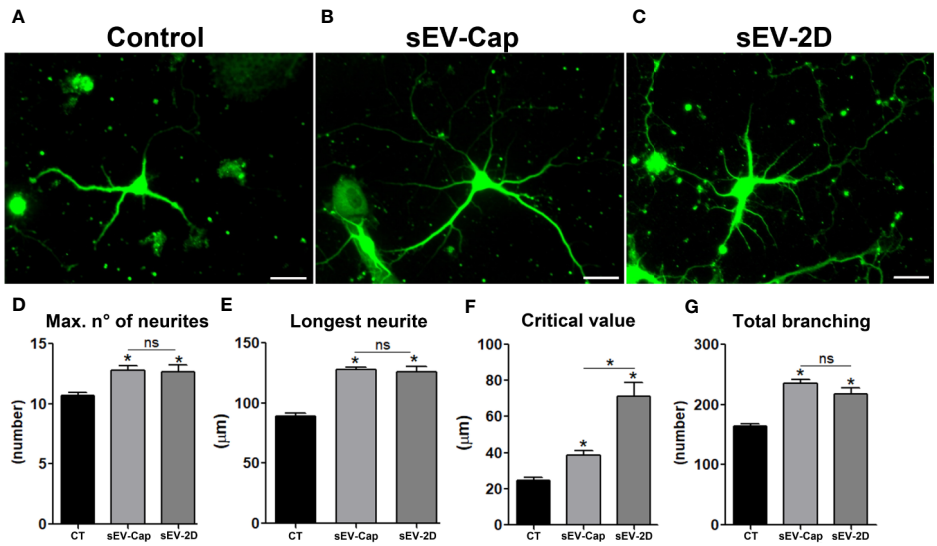


FIGURE 5 | sEV-Cap promotes neurite outgrowth. Rat hippocampal cortical neurons were treated with sEV and neurite elongation was evaluated. **(A)** Control, **(B)** sEV-Cap **(C)** sEV-2D, scale bar = 10 µm. The effect of sEV was evaluated through Sholl analysis; **(D)** Maximum number of neurites, **(E)** longest neurite, **(F)** critical value, and **(G)** total branching (n = 3), 20 neurons were analyzed per condition. *$P < 0.05$ one-way ANOVA. ns, not significant

treatment in varied pathologies, such as lymphoma, sepsis, wound healing, type I diabetes mellitus, among others, and in another perspective, as diagnostic targets mainly in cancer (such as in lung and pancreatic cancer and squamous cell carcinoma) by analyzing body fluids from patients.

Several techniques have been developed for the isolation of sEV from fluids, tissues, and cell cultures, with different challenges according to the source (Chen B-Y. et al., 2019). However, in this work, we are focused in sEV isolation from cells supernatant.

Multiples reports have shown that the paracrine properties of MSCs can be recapitulated by their secreted vesicles, therefore, one of the current strategies has been to develop new therapies based in MSCs-derived products (Mendt et al., 2019; Yin et al.,

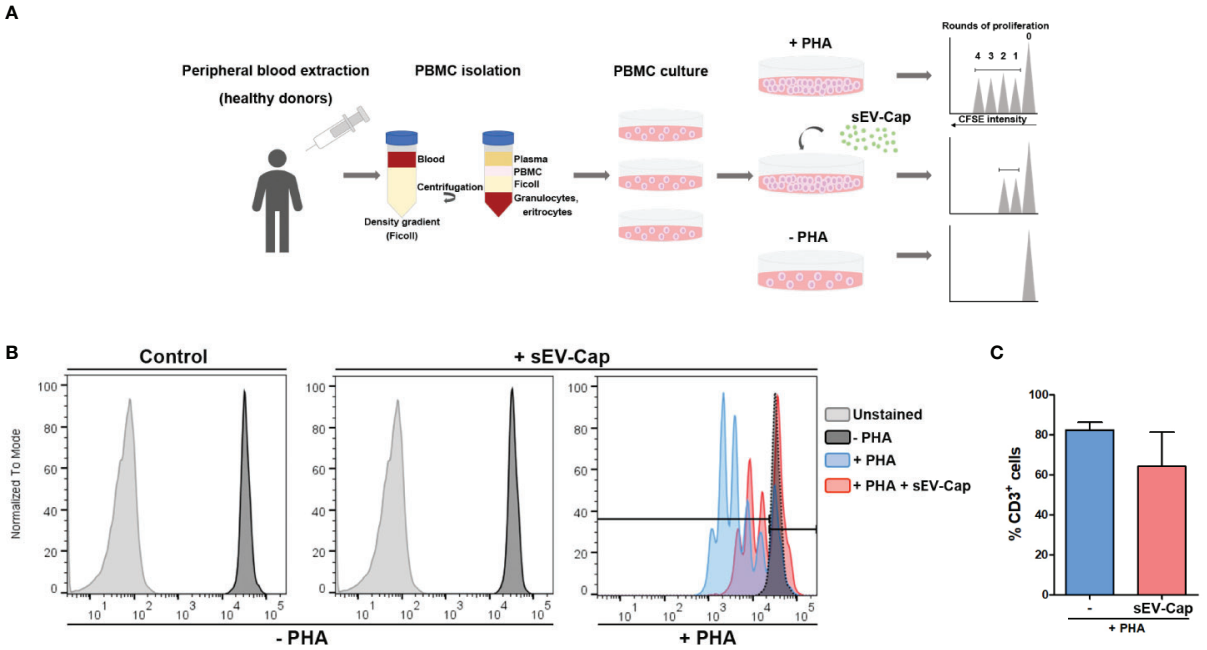


FIGURE 6 | sEV-Cap immunosuppress T lymphocytes proliferation *in vitro*. **(A)** Experimental setup of immunosuppression assay. Blood is obtained from a healthy donor and PBMC (peripheral blood mononuclear cells) are isolated using a density gradient which separated the blood into their components (plasma, mononuclear cells, granulocytes, and red blood cells). The mononuclear fraction contains the population of T lymphocytes (along with B lymphocytes and NK cells). PBMC are dyed with CFSE and maintained in culture under standard conditions. PHA (phycoerythrin) and sEV are added when indicated. After 72 h, PBMC is recovered and stained with CD3 and CD45 antibodies (for the recognition of lymphocytes). **(B)** The dilution of the dye CFSE in mitotic cells is evaluated by flow cytometry. **(C)** 76.4% of PHA-activated T cells proliferate after 72 h but these percentage decreases when sEV-Cap are present up to 52.1% (n = 2).

2019). The benefits of using encapsulated cells are diverse, from a productive point of view, the use of capsules with a determine pore size determines that the system itself will be cleaner that regular cell media since particles greater than ~200 nm will not be released from the capsules, additionally, capsules can be maintained in bioreactors in order to generate large volumes of supernatant and offers the option of a 3D-culture what can optimize the cells-volume ratio. Additionally, in the case that cell-free agents are needed, encapsulated cells can be used for the production of sEV in order to diminish some steps needed when using ultracentrifugation. However, more research is needed to evaluate differences in the yield of sEV when using encapsulated cells in comparison with cells seeded in a 2D standard fashion.

Additional challenges will be presented for the traceable production of sEV beyond the manufacture process itself. It is well known that there is a high biological variability between different sources of MSCs and also from different donors from the same source (Mendicino et al., 2014; Russell et al., 2015; O'Connor, 2019). This also has been detected in MenSCs and is explained by multiple parameters, such as cell culture conditions, and mostly for the epidemiologic and hormonal background of the donor (Chen L. et al., 2019). Alcayaga et al. reported differences in the CFU-potential and progenitors numbers in MenSCs from 10 different donors (Alcayaga-Miranda et al., 2015a) but differences in this and other parameters are proper of all MSCs and has been shown in multiple publications that even do the variability is a fact, the therapeutic properties of MSCs are also a fact (Galipeau et al., 2016). In the same line, the concept of "potency test" or "potency assay" has become very important in the field of cellular products (Bianco et al., 2013; Deskins et al., 2013; Galipeau et al., 2016; de Wolf et al., 2017), for MenSCs and their derivates. It remains to be determined which test will be the most appropriate considering which properties are of interest for a specific pathology or condition.

Another growing area resides in the intersection between pharmaceutical drugs and cell therapy. This advanced drug delivery resides in loading sEV post-isolation with specifics clinically approved chemical compounds. Recently, evidence has shown that sEV-mediated chemotherapeutic delivery has much improved anti-tumor effects when compared to free drugs in animal tumor models (Wang et al., 2016). As an example, when Paclitaxel was loaded into sEV by sonication, the loaded sEV showed 50 times more anti-tumor effect than free paclitaxel in drug-resistant cancer cells (Kim et al., 2016). The final product will need to meet both cell manufacturing and pharmaceutical industry standards and therefore, requires a homogenous population of particles. Our results show a 7-fold higher presence of sEV (40–200 nm fraction) with a more uniform size distribution, making sEV-Cap a more appropriate protocol for drug-loaded sEV.

From a therapeutic point of view, by using the encapsulated cells system, capsules can be located in the specific tissue where

the therapeutic effect is needed, and some complications related with the systemic injection of MSCs can be avoided. Specifically, MSCs which get trapped in a high percentage in the lung microvasculature causing vascular obstructions and the death of the injected cells (Wang et al., 2015). It has been reported that after 48 h of injection, less than 0.1% of total cells may be detected using a high-resolution quantitative 3D imaging system (Schmuck et al., 2016). The high clearance of injected cells and low percentage of MSCs found at the injury site or in non-target organs can lead to off-target toxicity and overdosing problems. With the localized positioning of capsules, this situation can be prevented, and cloistered cells could maintain a continuous secretion of growth factors and sEV. This point is relevant because sEV are known to have a shorter half-life (Morishita et al., 2017; Göran Ronquist, 2019) compared with their parental cells (Parekkadan and Milwid, 2010; Leibacher and Henschler, 2016) requiring highly repetitive injections to obtain the desired outcome. On the other hand, encapsulated cells secrete sEV continuously but also respond to environmental changes, avoiding undesired effects from multiple injections and from cell byproducts including MVs and apoptotic bodies. Implantation of encapsulated cells producing sEV also allows physical targeting, thereby increasing efficacy as well as acting as a safety device by holding the stem cells at the site needed and physically separating them from the body (Gunzburg and Salmons, 2009). The long-term maintenance of cell viability and the quality of their secretions over time are fundamental questions that remain unanswered. With respect of time, we have previously demonstrated a steady release of 90 µm retrovirus vector particles from encapsulated cells for at least 6 weeks during cell culture and for the same time, during an *in vivo* assay (Saller et al., 2002). Also, a number of different cell lines that have been encapsulated in Cell-in-a-Box have been shown to survive for many weeks to months *in vivo*, and so it might be expected that encapsulated MSCs will present similar survival timeline (Dangerfield et al., 2013).

sEV-Cap were characterized by size, expression of *bona fide* sEV markers, and by their function. With respect to the size, the media size is lower compared to sEV-2D, and this can be explained by the sieve effect of the capsule itself, but we cannot rule out that encapsulated cells secrete smaller vesicles. Lee et al. (2019) have described a subpopulation of sEV inside this fraction, called P100, isolated by an additional ultracentrifugation step after the standard procedure, and with differential functional effects respect to "conventional" sEV or P200 fraction. Interestingly, this P100 fraction express lower levels of CD81, similar to sEV-Cap. This raises the question of whether sEV-Cap are smaller due to the sieve effect of the capsule or are in fact a sub-fraction of the entire sEV production and just the smaller ones are able to be secreted. Further investigation is needed to better define this population and address its biological relevance.

Another theory to explain the smaller size is that the density inside the capsule resembles a confluent cell culture as cells are in close contact in all dimensions. This scenario may affect cellular metabolism and influence the process of formation and secretion of sEV. According to the literature, a high degree confluence induces a major production of sEV (Gurunathan et al., 2019; Thippabhotla et al., 2019) and a decrease in EV secretion (Gudbergsson et al., 2016; Palviainen et al., 2019), and stimulates the secretion of sEV in 3D HeLa cell cultures (Thippabhotla et al., 2019). With respect to the size of sEV, we observed an increase in the abundance of smaller sEV, and some reports indicate that the 3D growth conditions can be the cause (Thippabhotla et al., 2019), and most importantly, sEV shared comparable trophic properties in the assays evaluated here, regardless of their the origin.

Along the same lines, the fate of larger vesicles inside the capsules and their impact on the encapsulated cells needs to be determined. We already mentioned that a high degree of confluency induces a decrease in EV secretion, possibly due to entrapment in the bead (Gudbergsson et al., 2016; Palviainen et al., 2019). In accordance with the mentioned data, we suspect that encapsulated cells sense an increase in the concentration of EV inside the capsule which can activate some auto-regulatory pathways that inhibits the generation of EV. As sEV freely diffuse from the capsules, this process might not be inhibited, but more research is needed to understand this phenomenon.

Nevertheless, in summary, we have successfully showed a novel, less expensive, and faster method to generate sEV from MenSCs. Due to its simplicity, it is possible to assemble the protocol under GMP conditions, since (i) we already confirmed the feasibility of isolating MenSCs for clinical use and (ii) GMP production for Cell-in-a-Box has already been established. Moreover, encapsulated cells may be used as a device for releasing sEV *in vivo* constantly, until the capsules are removed. Finally, particles produced under the encapsulation protocol display advantageous properties positioning them as prominent vehicles for drug-loaded exosome strategies.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/ **Supplementary Material**.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by The Ethics Committee of Universidad de los Andes. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

M-PR and MK were responsible for conception, design and data analysis. M-PR, GZ, PC, MH, and AF-V were responsible for data acquisition and analysis. GZ and MK were responsible for interpretation of the results and manuscript writing. PT performed the cell encapsulations and BS, JD, and WG were responsible for cell encapsulation experiment planning and data

analysis. PC and UW performed the neurite growth assays. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2020.00679/full#supplementary-material

SUPPLEMENTARY FIGURE 1 | NTA report for EV.

SUPPLEMENTARY FIGURE 2 | NTA report for sEV-Cap.

SUPPLEMENTARY FIGURE 3 | NTA report for sEV-2D.

SUPPLEMENTARY FIGURE 4 | Flow cytometry histograms derived from the data showed as dot blots.

SUPPLEMENTARY FIGURE 5 | Uptake analysis. **(A)** PKH26 stained sEV-Cap (red) are effectively taken up by MenSCs monolayers. **(B)** Quantification of PKH26+ cells compared to PBS control, *$P < 0.05$ unpaired Student's t test.

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frontiers in Pharmacology

REVIEW
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Arming Mesenchymal Stromal/Stem Cells Against Cancer: Has the Time Come?

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Since mesenchymal stromal/stem cells (MSCs) were discovered, researchers have been drawn to study their peculiar biological features, including their immune privileged status and their capacity to selectively migrate into inflammatory areas, including tumors. These properties make MSCs promising cellular vehicles for the delivery of therapeutic molecules in the clinical setting. In recent decades, the engineering of MSCs into biological vehicles carrying anticancer compounds has been achieved in different ways, including the loading of MSCs with chemotherapeutics or drug functionalized nanoparticles (NPs), genetic modifications to force the production of anticancer proteins, and the use of oncolytic viruses. Recently, it has been demonstrated that wild-type and engineered MSCs can release extracellular vesicles (EVs) that contain therapeutic agents. Despite the enthusiasm for MSCs as cyto-pharmaceutical agents, many challenges, including controlling the fate of MSCs after administration, must still be considered. Preclinical results demonstrated that MSCs accumulate in lung, liver, and spleen, which could prevent their engraftment into tumor sites. For this reason, physical, physiological, and biological methods have been implemented to increase MSC concentration in the target tumors. Currently, there are more than 900 registered clinical trials using MSCs. Only a small fraction of these are investigating MSC-based therapies for cancer, but the number of these clinical trials is expected to increase as technology and our understanding of MSCs improve. This review will summarize MSC-based antitumor therapies to generate an increasing awareness of their potential and limits to accelerate their clinical translation.

Keywords: mesenchymal stromal/stem cell, cancer, tumor necrosis factor-related apoptosis-inducing ligand, gene therapy, cell therapy

MSCs AND CANCER

Mesenchymal stromal/stem cells (MSCs) play an important role in restoring tissue homeostasis when injury or damage affects the structural integrity of the tissue (Vizoso et al., 2019). MSCs can be attracted to injury sites by following the gradient of chemoattractant molecules released by inflammatory cells. At the site of damage, local factors such as hypoxia, cytokines, and Toll-like receptor ligands induce the recruited MSCs to proliferate and express growth factors that accelerate tissue regeneration (Rustad and Gurtner, 2012). Tumors can also mobilize MSCs from distant organs, including bone marrow and adipose tissue, driving their engraftment into the tumor microenvironment by inflammatory signals (Kidd et al., 2012; Chen and Song, 2019). It has been shown that MSCs are strongly recruited by hepatic carcinoma (Xie et al., 2017), breast cancer (Ma et al., 2015), and glioma (Smith et al., 2015). These tumor environments consist of many immune cells, which, alongside cancer cells, secrete soluble factors that can directly regulate MSC chemotaxis and recruitment to damaged tissues. For instance, interleukin (IL)-6 facilitates MSC attraction into tumor sites (Rattigan et al., 2010). An IL-8-dependent recruitment of MSCs was detected in glioma (Ringe et al., 2007), and it has also been shown that platelet-derived growth factor subunit B (PDGFB), vascular endothelial growth factor (VEGF), and transforming growth factor beta-1 (TGF-β1) can induce MSC migration (Schar et al., 2015). Recently, it was revealed that C-X-C motif chemokine receptor 4 (CXCR4) is one of the primary chemokine receptors involved in the enrollment and tumor tropism of MSCs (Kalimuthu et al., 2017). Other chemokines and their receptors with a central role in MSC tumor homing are C-C motif chemokine receptor 1 (CCR1), CCR7, CCR9, C-X3-C motif chemokine ligand 1 (CX3CL1), CXCR5, and CXCR6 (Honczarenko et al., 2006; Feng and Chen, 2009; Bao et al., 2012). In osteosarcoma, it has been shown that stromal cell-derived factor 1 alpha (SDF-1α) is implicated in MSC recruitment to neoplastic tissue. MSCs, in turn, stimulate the migration of osteosarcoma cells by C-C motif chemokine ligand 5 (CCL5)/RANTES secretion (Xu et al., 2009), thereby favoring the spread of cancer by providing metastatic osteosarcoma cells with a favorable microenvironment (Tsukamoto et al., 2012). Due to their well-documented tumor homing, MSCs become part of the tumor stroma, generating fibrovascular cellular elements, including endothelial cells or pericytes, and possibly differentiating into tumor-associated fibroblasts, which are involved in extracellular matrix remodeling (Kidd et al., 2012). The natural and specific ability of MSCs to home and engraft into malignant tissues, along with their immune privileged status, availability, genotypic and phenotypic stability, expandability, and proven safety record in clinical trials, make MSCs the ideal cellular vehicle for the delivery of anticancer agents improving their bioavailability versus more conventional approaches (Housman et al., 2014; Christodoulou et al., 2018). Thus, the engineering of MSCs to induce or enhance the production of biomolecules can counteract cancer growth while (ideally) sparing normal tissues. To achieve this, MSCs can be functionalized to release molecules capable of inducing tumor cell death (**Figure 1**) (Grisendi et al., 2011). The strategies used to convert MSCs into cellular vehicles for anticancer molecules can be classified into two different types. The first category includes nongenetic modifications of MSCs, such as loading with nanoparticle carriers or drugs. The second consists of approaches based on genetic modification of MSCs to induce the expression of anticancer proteins or suicide genes.

USING DRUG-LOADED MSCs TO TARGET CANCER

Uptake and Release of Chemotherapeutic Agents by MSCs

Because MSCs are relatively resistant to cytostatic and cytotoxic chemotherapeutic agents, they can be loaded with drugs and used for targeted anticancer therapy (**Figure 1A**). One method to do so is to dissolve active compounds in the MSC culture media. The MSCs can incorporate the anticancer drugs into the cytoplasm and release it into the culture medium in a time-dependent manner. Pessina et al. demonstrated that MSCs can efficiently take up the chemotherapeutic agents doxorubicin (DOXO), paclitaxel (PTX), and gemcitabine (GEM) and release them in an active form, resulting in an inhibition of tumor cell growth *in vitro* (Pessina et al., 1999; Pessina et al., 2013; Pascucci et al., 2014; Cocce et al., 2017a; Cocce et al., 2017b). In a leukemia xenograft mouse model, authors demonstrated that PTX-primed MSCs exerted a strong anticancer effect, inhibiting the proliferation of tumor cells and vascularization of the neoplasia (Pessina et al., 2013). The antitumor impact of primed MSCs is currently being investigated in different types of cancer cells. Among others, Bonomi et al. demonstrated in an *in vitro* 3D dynamic culture system that PTX-MSCs suppress the growth of human myeloma cells (Bonomi et al., 2017). Recently, the authors investigated the mechanisms driving PTX release by loaded MSCs, discovering that MSCs can also liberate PTX associated with extracellular vesicles (EVs) acting as "natural anticancer liposomes" (Perteghella et al., 2019). The use of EVs for drug delivery is detailed later in this review.

MSCs and Nanoparticles

MSCs can also deliver drug-loaded nanoparticles (NPs) to specific target sites (**Figure 1A**). Initial studies introduced MSCs loaded with magnetic and fluorescently labeled NPs in the field of diagnostic. Roger et al. showed that coumarin-6 dye-loaded poly-lactic acid NPs (PLA-NPs) and lipid nanocapsules (LNCs) were efficiently absorbed by MSCs in a concentration- and time-dependent way without influencing the viability and differentiation of MSCs (Roger et al., 2010). These findings prompted the use of NPs loaded with anticancer compounds in MSC-based drug delivery strategies. Originally, NPs were developed to facilitate targeted drug delivery by increasing drug stability; protecting nucleotides from degradation, thus facilitating their entry into the nucleus; and prolonging the effect of the delivered drug, allowing a dose reduction and a

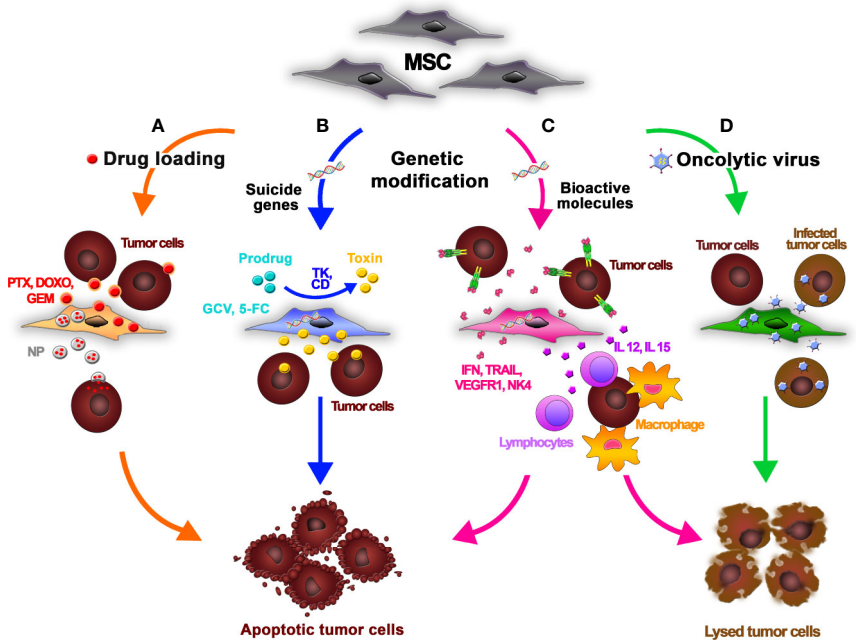

FIGURE 1 | Mesenchymal stromal/stem cells (MSCs) can be functionalized using different strategies to release antitumor agents for cancer treatment. **(A)** An anticancer drug is dissolved in the MSC culture media. MSCs incorporate the chemotherapeutic into the cytoplasm and then release it into the tumor microenvironment. MSCs efficiently absorb doxorubicin (DOXO), paclitaxel (PTX), and gemcitabine (GEM) and release them in their active forms, inhibiting tumor cell growth. MSCs can also take up drug-loaded nanoparticles (NPs), improving their biodistribution. **(B)** Using genetic modification, MSCs can be forced to express suicide genes encoding specific enzymes (e.g., TK, CD) that convert nontoxic prodrugs (GCV, 5-FC) into active derivatives. The prodrugs are systemically administered and then engineered MSCs are intravenously infused. Once injected, MSCs home into the tumor and convert the inactive prodrug into cytotoxic metabolites inside the neoplastic tissue, thus minimizing the off-target toxicity. **(C)** Genetic modification of MSCs can be also performed to induce the production of bioactive molecules and immunomodulatory cytokines such as interferons (e.g., IFN-α, IFN-β, IFN-γ), interleukins (e.g., IL-2, IL-12, IL-15,IL-18), chemokines (e.g., CXC3L1), proapoptotic molecules (e.g., TRAIL), antiangiogenic molecules (e.g., Alpha-1 antitrypsin, NK4, VEGFR1), or molecules with other antitumor properties (e.g., TNF-a, HNF4-a). These proteins can both act directly on tumor cells, inducing apoptosis, and potentiate the host inflammatory response through crosstalk with tumor-infiltrating leukocytes. **(D)** MSCs act as carriers and amplifiers of oncolytic viruses, protecting the viruses from host immune responses and delivering them into tumor sites.

possible decrease in side effects. However, their immunogenicity and uneven intratumoral distribution (due to the dense network of collagen and the high interstitial fluid pressure in the tumor environment) often limits their therapeutic potential and clinical application (Li et al., 2016). Nonetheless, the use of MSCs as cellular vehicles for drug-loaded NPs may be an effective option to overcome the limitations in NP biodistribution. MSCs could circumvent the activation of the immune system against NPs, and because MSCs have the ability to migrate within tumor tissue, they could enable entry of NPs into the tumor core (Aggarwal and Pittenger, 2005). Cellular uptake of NPs can be mediated by different mechanisms, including passive transport and active endocytosis (Banerji and Hayes, 2007). NP internalization by MSCs can be facilitated by receptor-mediated uptake and is also affected by the cell proliferation rate, time of exposure, and MSC culture conditions (Sadhukha et al., 2014). To overcome inefficient drug loading by MSCs, NPs can be linked to the cellular membrane of MSCs by covalent conjugation or by physical association obtained by electrostatic and hydrophobic interactions (Li et al., 2011). In addition, smart NPs that control drug cargo release under tumor-specific or external conditions, such as heat, low pH, the presence of enzymes, and light, have also been designed (Lei et al., 2008; Wang et al., 2011; Huang et al., 2013; Qian et al., 2015; Tian et al., 2015). Sadhukha et al. demonstrated an effective tumor-targeting strategy that consisted in engineering MSCs to carry poly(d,l-lactide-co-glycolide) (PLGA) NPs loaded with PTX. In this study, MSCs showed both concentration- and time-dependent absorption of NPs, with scarce impact on key MSC features and a dose-dependent cytotoxicity in lung and ovarian cancer cells both *in vitro* and *in vivo* (Sadhukha et al., 2014). In other studies, PLGA-PTX- or PLGA-DOX-loaded MSCs were found in different cancer types, like prostate, lung and glioma (Pacioni et al., 2015; Levy et al., 2016; Wang et al., 2016). In an orthotopic lung tumor model, Layek et al. demonstrated that MSCs carrying PTX-loaded NPs homed to cancer tissues and created cellular drug storage that released the drug over the time. Although containing significantly lower doses of PTX, treatment with MSCs carrying PTX-NPs resulted in relevant reduction of tumor growth, increased animal survival, and lower toxicity compared to treatment with PTX solution or free PTX-NPs (Layek et al., 2018).

Most of the nanoengineering strategies previously described depend on simple endocytosis of drug-encapsulated NPs into

MSCs. The rapid exocytosis of internalized NPs may lead to an adequate drug loading and retention. To increase drug loading in MSCs, Moku et al. developed PLGA NPs conjugated to the cell-penetrating peptide transactivator of transcription (TAT). It was found that TAT functionalization enhanced the intracellular uptake and retainment of NPs in MSCs. Further, treatment with MSCs carrying TAT-functionalized NPs loaded with PTX resulted in a significant inhibition of tumor growth and higher survival in a mouse orthotopic model of lung cancer compared to free drug or NP-encapsulated drug (Moku et al., 2019). In addition to these chemical NP delivery strategies, biological NPs have recently emerged as new MSC-based delivery tools.

GENETIC MODIFICATION OF MSCs TO TARGET CANCER

Methods to genetically modify MSCs generally use viral vectors, including retroviral, lentiviral, or adeno-associated viral vectors, and DNA plasmids (Marofi et al., 2017). The choice of genetic modification is driven by the aim and the target of the therapy.

Suicide Genes and MSCs

One approach to cancer treatment involves the delivery of suicide genes by MSCs (**Figure 1B**). After gene manipulation with an appropriate viral vector, MSCs can produce specific enzymes that convert nontoxic prodrugs into active derivatives (Zhang et al., 2015). The prodrugs are administered systemically following intravenous infusion of engineered MSCs. The MSCs home to tumors and convert these prodrugs into cytotoxic metabolites inside the neoplastic tissue, thus minimizing the off-target toxicity. The main advantage of this anticancer approach is the amplification of the toxicity of the drug *via* the bystander effect, which leads to the death of neighboring target cells due to indirect effects caused by engineered MSCs. The cytotoxic effect exerted by the activated prodrug additionally promotes the release of toxic substances that activate immune cells, including cytotoxic T cells and macrophages, leading to more effective cancer death (Zhang et al., 2015). The production of drug metabolites is also highly toxic for the MSC carriers themselves; thus, they die in the process, reducing a remote risk of adverse effects (e.g., transformation events or protumorigenic effects) related to the long-term persistence of homed and nonhomed MSCs in patients at the end of treatment. Drugs with a short half-life or high systemic toxicity, such as ganciclovir (GCV) or 5-fluorouracil (5-FU), may be ideal candidates for gene-directed enzyme prodrug therapy. For these agents, the systemic concentrations required for a therapeutic effect are significantly higher than the tolerated dose. Delivery of the agent directly into the tumor would permit durable effects without the toxicities seen with systemic delivery (Tsao et al., 2004). The most common enzyme-prodrug complexes used in combination with MSCs to target various tumors are herpes simplex virus thymidine kinase complexed with GCV (HSV-TK/GCV system) and yeast cytosine deaminase (CD) with 5-fluorocytosine (5-FC) (Kucerova et al., 2007; Alieva et al., 2012). Adipose tissue-derived MSCs modified to express yeast CD given in combination with 5-FC significantly inhibit the growth of colon cancer in immunocompromised mice (Kucerova et al., 2007). In this approach, MSCs home to the tumor tissue and CD produced by the MSCs converts 5–FC to 5–FU, a tumoricidal chemotherapeutic agent that can then diffuse into the tumor tissue. Co-administration of CD–expressing MSCs and 5–FC was also effective in treating melanoma and human prostate cancer in mouse xenograft models (Kucerova et al., 2008; Cavarretta et al., 2010). Similarly, it has been shown that TRAIL and HSV-TK-modified MSCs in the presence of GCV significantly reduced tumor growth and increased survival in mice bearing highly malignant glioblastoma multiforme (GBM) (Martinez-Quintanilla et al., 2013).

MSCs Delivering Bioactive Molecules

Genetic modifications of MSCs can be also used to induce the expression of anticancer bioactive molecules (**Figure 1C**). In 2002, MSCs were used for the first time for the targeted delivery of interferon-beta (IFN-β) in an *in vivo* preclinical model of human melanoma (Studeny et al., 2002). MSCs carrying IFN-β were administered into tumor-bearing mice, provoking a significant reduction in tumor growth and an increase in survival compared to the control group. In addition, the authors demonstrated that, after intravenous injection, the engineered MSCs efficiently migrated and engrafted into lung metastases, delivering IFN-β into the tumors. In addition to IFN-β, other therapeutic genes encoding regulatory proteins and immunomodulatory cytokines such as interferons (e.g., IFN-α, IFN-β, IFN-γ), interleukins (e.g., IL-2, IL-12, IL-15, IL-18), and chemokines (e.g., CX3CL1), as well as molecules with proapoptotic functions (e.g., tumor necrosis factor-related apoptosis-inducing ligand [TRAIL]), antiangiogenic activities (e.g., Alpha-1 antitrypsin, NK4, VEGF receptor 1 [VEGFR1]), or other properties (e.g., tumor necrosis factor alpha [TNF-α], hepatocyte nuclear factor 4-alpha [HNF-4α]) have been implemented in preclinical studies (Shah, 2012). There are two advantages of using genes coding for these molecules: first, these proteins may act directly on tumor cells, blocking their proliferation or inducing apoptosis; and second, because of their physiological roles in the immune response, they can potentiate the host inflammatory response *via* crosstalk with leukocytes infiltrating the tumor microenvironment. IL-12 released by engineered MSCs not only exerts a direct antitumor effect in mice with melanoma, lung cancer, and hepatoma, but also activates cytotoxic lymphocytes and natural killer (NK) cells, thereby significantly reducing metastasis (Chen et al., 2008). Similar results were obtained in mouse models of human glioma, renal carcinoma, and Ewing sarcoma (Duan et al., 2009; Gao et al., 2010; Ryu et al., 2011). Umbilical cord MSCs with enhanced IL-15 gene expression significantly suppressed pancreatic tumor growth in mice and stimulated accumulation of NK cells and CD8+ T lymphocytes in the tumor microenvironment, thus supporting the antitumor immune response (Jing et al., 2014). Co-expression of IL-18 and IFN-β by bone marrow MSCs inhibited glioma growth *in vivo* and prolonged the survival of glioma-bearing rats (Xu et al., 2015). One of the most promising antitumor cytokines is TRAIL, which selectively induces apoptosis in cancer cells, but not in most normal cells. TRAIL is the ligand for death receptors that are

commonly overexpressed on the membrane of tumor cells. In tumor cells, TRAIL can induce caspase-mediated apoptosis by binding with its receptors death receptor 4 (DR4) and DR5 (Wong et al., 2019). MSCs display resistance to TRAIL due to their low expression of both DR4 and DR5 (Grisendi et al., 2010). In addition, is possible to consistently isolate and modify MSCs from human adipose tissue by minimally invasive surgical procedures (Foppiani et al., 2019; Starnoni et al., 2019). The wild-type gene coding for membrane-bound TRAIL, as well as modified cassettes expressing soluble ligand forms, have been used in MSC-based therapeutic strategies, demonstrating antitumor effects *in vitro* and *in vivo* in a wide variety of human solid neoplasms, including lung cancer, pancreatic cancer, glioblastoma, sarcoma, and hepatocarcinoma (Loebinger et al., 2009; Sasportas LS et al., 2009; Grisendi et al., 2010; Grisendi et al., 2011; Yan et al., 2014; D'Souza et al., 2015; Grisendi et al., 2015; Guiho et al., 2016; Golinelli et al., 2018; Candini et al., 2019; Rossignoli et al., 2019; Spano et al., 2019).

MSCs and Oncolytic Viruses

In addition to producing therapeutic molecules, MSCs have also been used as carriers and amplifiers for the delivery of oncolytic viruses into tumor sites (**Figure 1D**). An oncolytic virus is an attenuated virus that can infect and kill cancer cells. After infection, cancer cells are destroyed by oncolysis, releasing new infectious virus particles that can stimulate a proinflammatory environment to counteract immune evasion by malignant cells. In this sense, oncolytic viruses not only cause direct destruction of the tumor cells, but also stimulate host antitumor immune responses to help destroy the remaining tumor. Most available oncolytic viruses are engineered to increase tumor tropism and to reduce virulence for nonneoplastic host cells. A number of viruses, including adenovirus, reovirus, measles virus, herpes simplex virus, Newcastle disease virus, and vaccinia virus, have been clinically tested as oncolytic agents (Raja et al., 2018). When oncolytic viruses are systemically administered, the host immune cells recognize viruses as "non-self" and eliminates them before they can reach the tumor site. Autologous MSCs, however, are not recognized as foreign by the host immune system; thus, those incorporating oncolytic viruses can reach the tumor without major limitations (Nakashima et al., 2010). For this reason, introduction of MSCs infected by an oncolytic adenovirus demonstrated better antitumor effects and increased survival compared to direct delivery of the oncolytic adenovirus in xenograft models of ovarian cancer, glioma, and metastatic lung cancer (Yong et al., 2009; Shah, 2012). This effect was due to MSC-mediated defense of the oncolytic virus from host immune system and transport of the viral particles to the tumor location as it has been demonstrated in human glioma, melanoma, breast cancer, lung metastasis, and liver cancer models (Stoff-Khalili et al., 2007; Yong et al., 2009; Xia et al., 2011; Ong et al., 2013). Interestingly, an engineered oncolytic adenovirus carrying a TRAIL gene has been used to treat a mouse model of pancreatic ductal adenocarcinoma (PDAC), a malignant and deadly cancer characterized by an unfavorable prognosis and limited therapeutic options. In this gene therapy strategy, the oncolytic progeny released by engineered MSCs efficiently infects and lyses the tumor cells while simultaneously provoking the apoptosis of noninfected tumor cells *via* the expression of TRAIL molecules. The results collected in this study indicated that in a PDAC mouse model, adipose tissue-derived MSCs delivering TRAIL selectively homed to the tumor site and strongly hampered tumor growth with no evident toxicity or side effects (Kaczorowski et al., 2016).

MSC-EXTRACELLULAR VESICLES FOR ANTICANCER DRUG DELIVERY

How cancer cells recruit surrounding noncancer cells into the tumor microenvironment remains a relevant and complex topic (Kikuchi et al., 2019). In the last decade, investigators have begun to focus on structures similar to dust particles that are released by cells. These nanoparticles, known as extracellular vesicles (EVs), are now studied worldwide and are recognized to be key carriers of information in cell-to-cell communication. EVs are membrane-bound nanostructures released by cells under physiological and pathological conditions. They are classified based on their size: exosomes (50–100 nm), microvesicles (100–1,000 nm) and apoptotic bodies (over 1000 nm) (Colombo et al., 2014; Lotvall et al., 2014). Present data suggest that tumor cell-derived EVs are biologically important in cancer development, suppressing tumor-directed immune responses and accelerating tumor growth and invasiveness (Kikuchi et al., 2019).

The previously mentioned synthetic NPs used as drug delivery systems to target cancer (Li et al., 2016) have raised concerns due to their instability after administration, which may be caused by immune reactions, the impact of uncontrolled *in vivo* NP degradation on biocompatibility, and a lack of target specificity (Feliu et al., 2016). In contrast, EVs may be a promising therapeutic tool since they act as intercellular messengers, carrying nucleic acids, lipids, proteins, and miRNA, while maintaining their stability and integrity in circulation, as demonstrated by their presence in most biological fluids (Bruno et al., 2019). EVs are considered nonimmunogenic and are able to protect their cargoes from serum proteases and the immune system, avoiding phagocytosis or degradation (Baek et al., 2019). The specific content of EVs reflects the specific role of the producer cells and determines the biological effect of the vesicles (Isola and Chen, 2017). The current challenge among researchers is to convert this biological message into a therapeutic one. Due to their immunomodulatory capacity, their ability to home to tumor sites, and their robust paracrine factors, MSCs may be a reliable source of EVs for this purpose (**Figure 2**) (Witwer et al., 2019). Growing evidence suggests that MSC-derived exosomes can mediate the transfer of proteins and RNA to tumor cells. However, whether these molecules suppress or promote tumor growth is controversial (Parolini et al., 2009). Interestingly, Roccaro et al. demonstrated that the content and the role of exosomes differ depending on their source. Normal bone marrow MSC (BM-MSC)-derived exosomes are associated with tumor promotion, whereas those derived from multiple

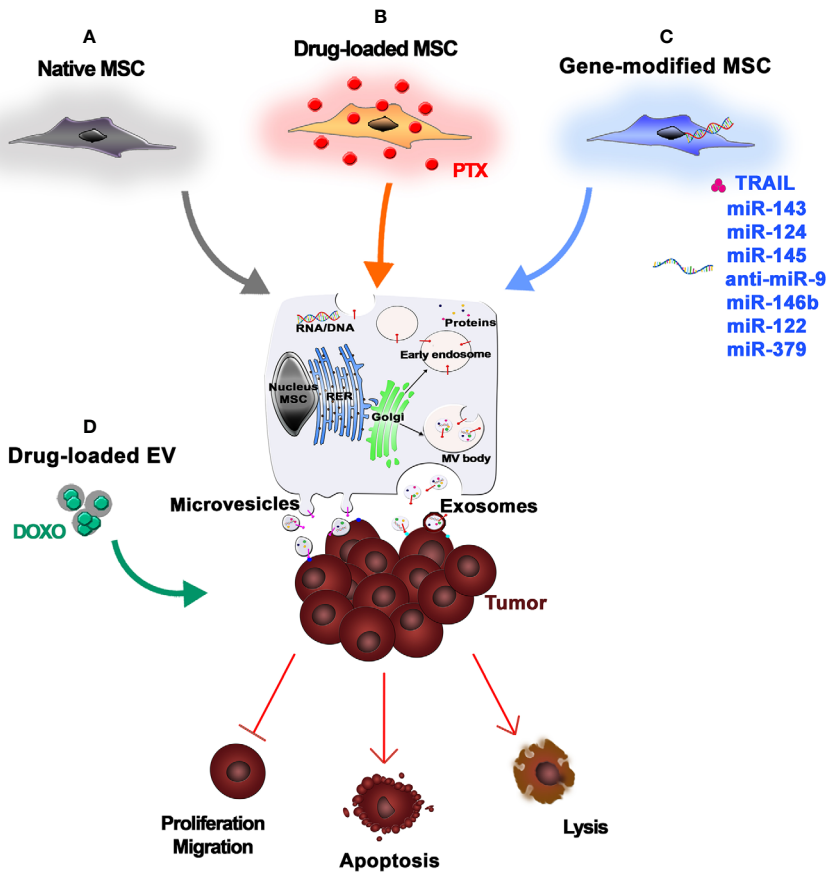

FIGURE 2 | Mesenchymal stromal/stem cell (MSC)-Extracellular Vesicles (EVs) as anticancer drugs. **(A)** Native MSCs are a reliable source of EVs, which are able to influence tumor cell proliferation, migration, and invasion. **(B)** Upon *in vitro* exposure to chemotherapeutic agents [e.g., paclitaxel (PTX)], MSCs internalize and pack the drugs into therapeutic EVs that can efficiently deliver the active drugs to the neoplastic tissue, thus inducing tumor cell apoptosis or lysis. **(C)** MSCs can also be genetically modified to express anticancer molecules (e.g., TRAIL) or miRNAs that can be secreted by MSC-derived EVs. **(D)** Alternatively, EVs are isolated from MSCs and then loaded with drugs or biological cargo by simple diffusion or electroporation.

myeloma-associated BM-MSCs are linked to tumor suppression (Roccaro et al., 2013). Several studies focused on the intrinsic ability of MSC-derived EVs to counter tumor progression (**Figure 2A**). S. Wu et al. demonstrated the capacity of EVs produced by human Wharton's Jelly-derived MSCs to abolish tumor cell proliferation *via* G0/G1 phase arrest in a dose-dependent manner (Wu et al., 2013). More recently, an *in vitro* study demonstrated that BM-MSC-derived exosomes can inhibit proliferation, migration, and invasion of pancreatic cancer cells by transporting miR-126-3p, a known tumor suppressor (Wu et al., 2019). Similarly, miRNA-100 seems to be involved in tumor suppression mediated by MSC-derived exosomes. Pakravan et al. demonstrated the ability of MSC-derived exosomes to significantly decrease the expression and secretion of VEGF in a dose-dependent manner in breast cancer-derived cells (Pakravan et al., 2017). However, because MSCs are heterogeneous, MSC-derived EVs may consequently exhibit heterogeneity, which could be an important barrier to their clinical use and should be taken into account (Del Fattore et al., 2015). To circumvent the potential issues caused by the unpredictable effects of native MSC-derived EVs on tumor growth, engineered EVs could be used instead. Current strategies to obtain anticancer EVs are based on the ability of MSCs to take up and release drugs, such as chemotherapeutic agents (**Figure 2B**), or on genetic manipulations of donor cells (**Figure 2C**) (Pessina et al., 2011). Interestingly, Pascucci et al. demonstrated that BM-MSCs exposed to high concentrations of PTX were able to survive and pack PTX into exosomes that could efficiently deliver this active drug to human pancreatic adenocarcinoma cells (Pascucci et al., 2014). The use of exosomes to deliver miRNAs to treat malignant tumors with poor prognosis, such as osteosarcoma or glioblastoma, has also been investigated. *In vitro* studies demonstrated that the introduction of synthetic miR-143 into MSCs increased the secretion of exosome-encapsulated miR-143, which was able to suppress the migration of the osteosarcoma cell line 143B (Shimbo et al., 2014). Further *in vitro* studies investigated the impact of exogenous miRNA mimics delivered by MSCs on glioma cells and glioma stem cells (GSCs) (Bao et al., 2006). MSCs derived from multiple sources can transfer miR-124 and

miR-145 mimics to both glioma cells and their GSCs, decreasing migration and self-renewal, respectively (Lee et al., 2013). This evidence demonstrates that exosomes can deliver miRNAs. This ability, combined with their capacity to penetrate the blood–brain barrier, makes exosomes a promising therapeutic tool (Ha et al., 2016). Munoz et al. investigated the role of anti-miR-9-loaded BM-MSC-derived exosomes in reversing the chemoresistance of GBM cells (Munoz et al., 2013). Moreover, *in vivo* studies in a rat brain tumor model demonstrated the efficacy of intratumorally injected miR-146b-expressing MSC-derived exosomes, once again supporting the use of exosomes delivered by MSCs to treat malignant glioma (Katakowski et al., 2013). Likewise, *in vitro* and *in vivo* studies showed that miR-122-transfected adipose tissue-derived MSCs generate exosomes containing miR-122, which is able to increase the sensitivity of hepatocellular tumor cells to chemotherapeutic agents, thereby providing a new therapeutic strategy (Lou et al., 2015). Similarly, the decrease of miR-379 expression in breast cancer is connected to its role as a tumor suppressor. Genetic manipulation of parental MSCs resulted in the release of exosomes containing miR-379 that, upon delivery to the tumor site, showed therapeutic effects (O'Brien et al., 2018). As previously mentioned, TRAIL is a promising anticancer agent (Wong et al., 2019), and TRAIL secretion *via* EVs has been described as a natural approach to deliver messages to near or distant sites that is used by several cell types, including normal T cells upon activation (Monleon et al., 2001) or human placental syncytiotrophoblasts (Stenqvist et al., 2013). Yuan et al. reported an innovative potential anticancer therapy based on EVs expressing surface TRAIL molecules produced by TRAIL-transduced MSCs. These "armed" EVs selectively induced apoptosis in cancer cells, supporting the use of this alternative system for TRAIL delivery (Yuan et al., 2017). The use of MSC-derived EVs in cancer therapy is promising because they, like their producer MSCs, are able to home to cancer sites (Wiklander et al., 2015). However, the exact functions of MSC-derived EVs in tumor biology remain largely elusive, and there are data suggesting that the acidic tumor microenvironment is a key factor that drives the paracrine traffic of EVs within the tumor mass (Parolini et al., 2009). To generate therapeutic EVs, the most common method is to manipulate parental/producer cells to generate EVs containing important cargo, such as regulatory miRNAs or tumor suppressors. However, a passive approach for drug or biological cargo incorporation into EVs is also possible, as EVs can be loaded with drugs by diffusion, or by electroporation when needed (**Figure 2D**) (Raimondo et al., 2019; Vakhshiteh et al., 2019). Although EVs, particularly those derived from MSCs, show promising properties, including high stability, slow clearance, small size, lack of toxicity, and target specificity, many challenges remain to be solved. In particular, exosome isolation would need to be scaled up for clinical applications (Vakhshiteh et al., 2019). This would require a robust standardization of EV manipulation methods and, critically, strict regulations for their clinical use in order to reduce variability in their intracellular content and, consequently, in their biological activities. Large-scale production requires controlled conditions for EV isolation and purification, with attention to donor variability and differences between cell sources. Moreover, the delivery route is critical for EV biodistribution (Raimondo et al., 2019). Several studies support the idea that MSC-derived EVs are able to accumulate in tumors due to their capacity to identify the site of tumors or metastases (Wiklander et al., 2015; Abello et al., 2019). Drug delivery can be further improved by implementing new *ex vivo* modifications, such as surface functionalization. Adding a synthetic multifunctional peptide to EV surfaces substantially increases the ability of the EVs to cross the blood–brain barrier and accumulate in gliomas, enhancing the therapeutic effect of loaded methotrexate (Ye et al., 2018). Despite the advantages of using EVs instead of cells, several challenges remain. For example, potency assays must be developed and appropriate dose findings studies must be conducted (Phinney and Pittenger, 2017). Though the enthusiasm for EVs may be warranted, we are currently far from the safe and controlled clinical use of these biological shuttles.

IMPROVING MSC TUMOR TARGETING

MSCs are currently evaluated in clinical trials to treat a variety of diseases, with variable degrees of efficacy. For both locally and systemically injected MSCs, there are issues with MSC fate post-implantation, cell localization, and cell engraftment and survival in the target tissue (Mastrolia et al., 2019). Once locally injected, cells can be lost due to washout, cell death, and rejection by the immune response (Kean et al., 2013). For systemic delivery, the homing ability of MSCs has been showed for several tumors, including gliomas (Nakamizo et al., 2005), breast (Yang et al., 2019), colon (Knoop et al., 2015), ovarian (Komarova et al., 2010), and lung carcinomas (Loebinger et al., 2009). However, only a small amount of systemically administered MSCs effectively reaches the target site (De Becker and Riet, 2016). Current studies indicate that most MSCs accumulate in the lung, liver, and spleen and are subsequently eliminated from the body, which negatively impacts engraftment into the target site (Kean et al., 2013). This suggests that a higher absolute number of cells is needed to guarantee that a sufficient number of MSCs reaches the damage site. However, producing a high number of MSCs is technically challenging in the clinic, in particular for autologous products generated within a cGMP environment. Hence, novel targeting methods are needed to ameliorate MSC engraftment and increase the therapeutic efficacy while reducing the number of cells required and minimizing off-target effects (De Becker and Riet, 2016). MSCs are amenable to various targeting strategies, including physical, physiological, and biological methods aimed at increasing their concentration in the target site (Roth et al., 2008). Physical targeting (**Figure 3A**) involves using either surgical procedures or guiding strategies, such as catheters or external magnets, to place cells directly into the site where the therapy is needed (Arbab et al., 2004; Fiarresga et al., 2015; Silva et al., 2017). Alternatively, therapeutic cells can be restrained in matrices or devices that retain cells at the transplant site (Roth et al., 2008). Notably, Shah et al. reported that MSC encapsulation in a biodegradable, synthetic extracellular matrix significantly increased

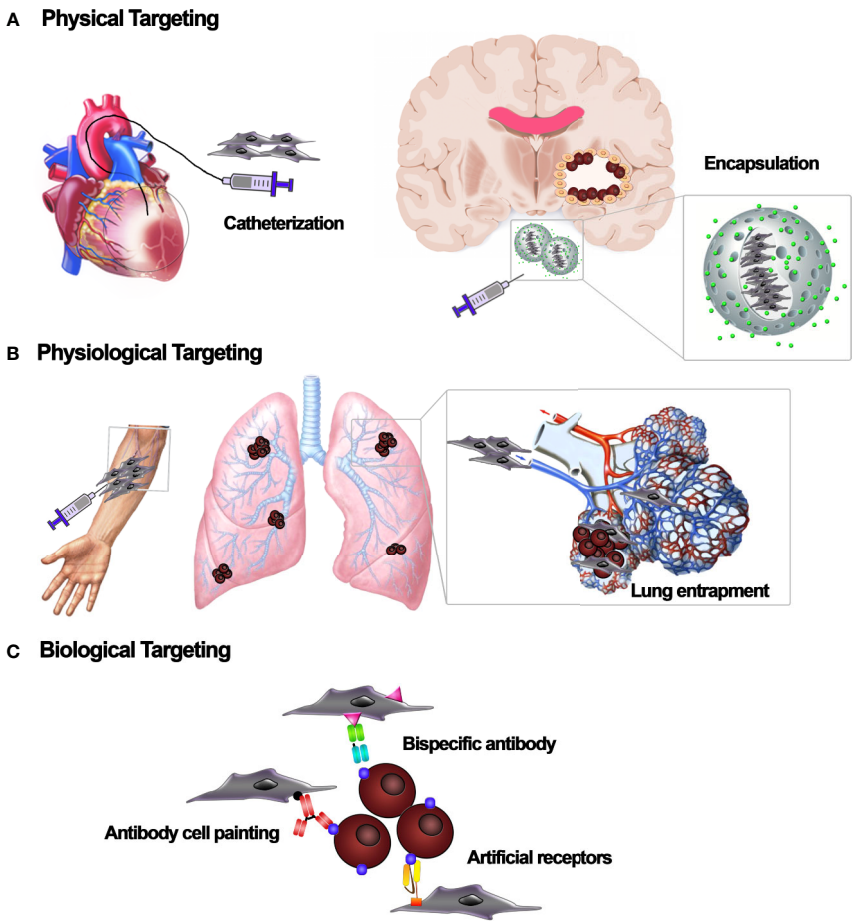

FIGURE 3 | Cell-based targeting strategies. Different targeting strategies to localize mesenchymal stromal/stem cell (MSC) carriers to the tissue of interest. **(A)** Physical targeting relies on the use of devices, such as catheters or scaffolds, to position the cells where they are needed. **(B)** Physiological targeting takes advantage of natural forces that route transplanted cells to specific sites or organs. Based on the infusion site, cells can be physiologically entrapped by the vascular bed of specific tissues. **(C)** Biological targeting strategies embody a range of molecular techniques to target cell vehicles. Adapters such as bispecific antibodies, "cell painting" with antibodies or peptides, and expression of artificial receptors enable the affinity-based retention of cell vehicles at the target site.

their retention in the GBM resection cavity while allowing secretion of antitumor proteins (Kauer et al., 2011; Shah, 2013; Duebgen et al., 2014). An additional strategy relies on physiological processes (**Figure 3B**), as the systemic circulation, to move the cells, instead of using active cell-mediated migration (Roth et al., 2008). For example, cells have a tendency to be trapped in the capillary of the lungs. This is a first-pass mechanical barrier to systemic delivery. However, this effect can be exploited to deliver MSC-mediated therapies to the lungs (Hakkarainen et al., 2007; Stoff-Khalili et al., 2007). Recently, biological targeting strategies (**Figure 3C**) have been designed to meet the need for higher target stringency upon systemic infusion of MSCs, especially when the pathology to be treated is widespread, as it is for metastases (Rosenblum et al., 2018). It involves knowledge-driven approaches aimed at improving MSCs homing, binding specificity to target tissue, and retention inside the target environment (Roth et al., 2008). Different strategies have been developed to manipulate MSC homing potential, including modifying the MSC culture conditions to boost the expression of homing-related molecules, engineering the cell membrane to increase homing, and manipulating the target tissue to better recruit MSCs (De Becker and Riet, 2016). For example, the inherent homing potential of MSCs has been exploited by exposing MSCs to glioma-conditioned media (Smith et al., 2015) or to proinflammatory cytokines, such as TNF-α (Egea et al., 2011). The ectopic expression of trafficking machinery components, such as CXCR1, significantly improved MSC tropism toward gliomas secreting high levels of IL-8 (Kim et al., 2011). In addition, radiation augments inflammatory signaling in the cancer site and may be used to improve site-specific MSC migration (Klopp et al., 2007). In parallel to efforts to improve MSC homing, researchers are developing methods to improve MSC affinity for the target site. Affinity-based targeting is dependent on binding interactions and therefore exploits molecules that are exclusively or highly expressed by the cells or tissue that we aim to target and that have affinity for specific receptors on MSCs (Roth et al., 2008). Methods to improve MSC affinity that do not involve genetic modification include

antibody- and peptide-based "cell painting" and the use of bispecific antibodies, with applications currently restricted to regenerative medicine (Gundlach et al., 2011; Kean et al., 2013). Most of the work on tumor targeting strategies based on affinity has been done in adoptive immunotherapy, the field in which the highest binding capacity has been achieved, due to immune molecules such as T-cell receptors (TCRs) and their derivatives and chimeric antigen receptors (CARs) (Liu et al., 2019). Affinity-based cell targeting has also recently been applied to MSCs to further optimize their tumor-localizing potential (Golinelli et al., 2018). Balyasnikova et al. genetically modified MSCs to express an artificial receptor (AR) that recognizes EGFRvIII. This allowed the MSCs to specifically target GBM cells expressing EGFRvIII, a mutated form of epidermal growth factor receptor (EGFR) that is not present in healthy tissues but has a high prevalence in GBM. The retention of modified MSCs in EGFRvIII-expressing GBM was significantly increased compared to unmodified MSCs (Balyasnikova et al., 2010). Similarly, Komarova et al. showed that MSC surface modification with an AR that binds to erbB2 increased MSC engraftment and persistence in erbB2-positive ovarian tumors (Komarova et al., 2010). However, evidence supporting targeted anticancer molecule delivery by MSCs expressing an AR remains sparse. The concept of targeted drug delivery as a "magic bullet" was presented in 1908 by Paul Ehrlich and has inspired recent efforts aimed at increasing the concentration of a drug in the tumor site by modulating its affinity for a specific biological target (Strebhardt and Ullrich, 2008). Taking inspiration from strategies used to redirect lymphocyte specificity using CARs or bispecific adaptors, our group coupled affinity and cytotoxicity by genetically modifying therapeutic MSC-TRAIL to express an AR against the disialoganglioside GD2 (Golinelli et al., 2018). The GD2-based targeting allowed MSCs delivering TRAIL to be specifically directed to GD2-expressing cancers, strengthening their adherence to tumor cells. In developing this CAR-based anticancer strategy, we aimed to reach site-specific and lasting retention of MSCs within the tumor bed, thereby effectively delivering proapoptotic TRAIL molecules to GD2-expressing tumors (Golinelli et al., 2018). Combinatorial targeting has recently been applied by Segaliny and colleagues, who produced MSCs that express P-selectin glycoprotein ligand-1 (PSGL-1)/Sialyl-Lewis X (SLEX) together with modified versions of CD and osteoprotegerin (OPG) to treat bone metastases of breast cancer (Segaliny et al., 2019). MSC delivery to bones has been improved through interactions between PSGL-1/SLEX and selectins on activated endothelial cells, megakaryocytes, and platelets in the tumor microenvironment. Once in the tumor niche, engineered MSCs induced local cancer killing through a CD/5-FC suicide gene therapy system and reduced osteolysis by expressing modified OPG (Segaliny et al., 2019). Also noteworthy is the technology developed by Zhu et al. aimed at simultaneously targeting cell proliferation and death pathways in tumor cells using MSCs armed with a bi-functional molecule comprised of a nanobody targeting the EGFR (Enb) and TRAIL (Zhu et al., 2017). EGFR is an excellent target, as it is commonly overexpressed and/or altered in tumor, leading to abnormal cell proliferation and activation of prosurvival pathways. The authors demonstrated that the Enb-TRAIL bi-functional molecule simultaneously engages both EGFR and DR5 on the surface of tumor cells, leading to amplification of the apoptotic signal and proving to be more effective than a combination treatment with Enb and TRAIL. Using an orthotopic resection model of primary glioblastoma, they showed that *in vivo* treatment with encapsulated Enb-TRAIL MSCs reduced tumor growth and considerably increased survival of tumor-bearing mice (Zhu et al., 2017). Although each of the aforementioned tumor targeting approaches individually improves MSC delivery, a combination of different targeting approaches will be likely required to ameliorate both the efficiency and the specificity of cell-based therapies in cancer (Roth et al., 2008).

MSCs AND CANCER TOWARD THE CLINIC: ARE WE THERE YET?

Several trials have been designed to study MSCs and their possible implications in cancer treatment. A proportion of these are based on genetically modified MSCs. However, only four are using MSCs as anticancer vehicles (**Table 1**). Among these trials, the Phase I/II clinical trial TREAT-ME1, with the aim of evaluating the safety and efficacy of MSCs delivering HSV-TK under the control of a CCL5 promoter (Einem et al., 2017). Preclinical studies had demonstrated tumor growth reduction in models of hepatocellular and pancreatic cancer, as well as a reduction in metastases (Niess et al., 2015). Patients enrolled in the study were affected by advanced, recurrent, or metastatic gastrointestinal or hepatopancreatobiliary adenocarcinoma. The clinical trial protocol includes intravenous injection of HSV-TK-engineered MSCs, followed by repeated GCV injections. Intriguingly, this technology is based on CCL5, a chemokine produced by MSCs upon contact with tumor cells, which allows the activation of the CCL5 promoter driving HSV-TK genes only in tumor-infiltrating MSCs, restricting expression of the prodrug-converting enzyme to the tumor microenvironment. This selective activation was introduced to reduce systemic adverse effects. As primary endpoint, they demonstrated acceptable safety and tolerability of the combined cell and gene therapy applied (Einem et al., 2017). An ongoing Phase I clinical trial is studying the best calibrated dose and the side effects of BM-MSCs loaded with the oncolytic adenovirus DNX-2401 in patients affected by recurrent GBM, gliosarcoma, or isocitrate dehydrogenase 1 (IDH1) wild-type anaplastic astrocytoma. DNX-2401 (Delta-24-RGD; tasadenoturev) is a tumor-selective oncolytic adenovirus

TABLE 1 | Mesenchymal stromal/stem cell (MSC) clinical trials targeting solid tumors.

Therapeutic Options	Targets	References
1. MSC-HSV-TK	Gastrointestinal cancer	(Niess et al., 2015; Einem et al., 2017)
2. MSC-TRAIL	Nonsmall cell lung cancer (NSCLC)	(clinicaltrials.gov, 2017)
3. MSC-IFN-β	Ovarian cancer	(clinicaltrials.gov, 2015)
4. MSC- MV-NIS	Ovarian cancer	(clinicaltrials.gov, 2014)

(clinicaltrials.gov, 2019). The virus has been genetically modified to make it safe for patients and capable of specifically targeting brain cancer cells. This clinical trial has enrolled 36 patients who will be monitored to determine the maximal tolerated dose and local/systemic toxicity (clinicaltrials.gov, 2019). In 2017, a Phase I/II clinical trial (TACTICAL) designed to evaluate the safety and antitumor activity of allogenic MSC-TRAIL in combination with chemotherapy in patients with metastatic nonsmall cell lung cancer (NSCLC) was announced (clinicaltrials.gov, 2017). In Phase I, patients received traditional chemotherapy on the first day, followed by MSC-TRAIL cells on the second day. Each patient received three cycles of treatment at 21-day intervals (clinicaltrials.gov, 2017). Phase I was designed to assess safety and to determine the recommended Phase II dose (RP2D) of MSC-TRAIL when combined with chemotherapy. In Phase II of this trial, which is double-blind, patients will be randomized to the intervention group or the control one. All patients enrolled will be treated by chemotherapy on the first day (clinicaltrials.gov, 2017). However, patients randomized to the intervention group will receive the RP2D of MSC-TRAIL on the second day, while the control group will receive a placebo. The aim of Phase II will be to determine tolerability and efficacy of treatment with MSC-TRAIL in combination with traditional chemotherapy. In summary, TACTICAL will be a key trial to verify the potential of MSC-TRAIL to become a cell-based therapy for patients with advanced lung cancer (clinicaltrials.gov, 2017). A similar therapeutic approach using MSCs to treat PDAC has been announced. In this study, a soluble trimeric and multimeric variant of TRAIL (sTRAIL) is continuously released by adipose (AD)-MSCs and induces apoptosis (Spano et al., 2019). The sTRAIL produced by AD-MSCs that infiltrated the tumor stroma was able to significantly inhibit tumor growth *in vivo*: substantial reductions in tumor mass and in cytokeratin-7-positive cells, as well as an antiangiogenic effect, were observed (Spano et al., 2019). The multiple roles of MSCs in the tumor and their future applications in the clinic, were recently reviewed by Lin and colleagues (Lin et al., 2019), who emphasized the need to focus attention on the molecular mechanism(s) of antitumorigenic activity. Additional studies using MSC-based therapeutic approaches against cancer have been reported. For example, nanodrug carriers can accumulate in tumors due to the leaky tumor vasculature. In 2018, Layek et al. investigated the use of MSCs carrying chemotherapy-loaded NPs as cellular drug carriers. The goal was to generate cellular drug storage capable of migrating to tumors and releasing the drug over a long period of time (Layek et al., 2018). The ability of MSCs to release drugs is commonly employed in cancer therapies. Two registered clinical trials are investigating MSCs for the treatment ovarian cancer. The first one, is a Phase I clinical trial to test the safety and to find the maximum tolerated dose of modified BM-MSCs producing IFN-β that can be given to patients with ovarian cancer (clinicaltrials.gov, 2015). The second, is a Phase I/II clinical trial using AD-MSCs infected with an Edmonston's strain measles virus genetically engineered to produce sodium iodine symporter (MV-NIS) to treat patients with recurrent ovarian cancer. In Phase I of this trial, the maximum tolerated dose will be defined, and Phase II will consist of intraperitoneal infusion of MV-NIS alone or MV-NIS-modified MSCs. A successful five-year follow-up could lead to an approval for the clinical use of MSCs carrying tumor-killing substances directly to ovarian cancer cells (clinicaltrials.gov, 2014).

In conclusion, the use of MSCs for the treatment of cancer is a promising option. The MSC-mediated delivery of genes, proteins, oncolytic viruses or small molecules in the clinic will take advantage of the abilities of MSCs to be modified and deliver cargoes. While research have to address the MSC tumoral migration/persistence to possibly overcome the limits of nonspecific homing, the potential of combining cells with chemotherapy agents will initiate and write new therapeutic chapters in oncology.

AUTHOR CONTRIBUTIONS

GGo, IM, BA, VM, MP, LP, GC, MD'O, MS, PD, and DS participated in the literature search, wrote the manuscript parts, and prepared the figures and tables. MD and GGr conceived the manuscript concept, wrote and final edited the manuscript. All authors contributed to the article and approved the submitted version.

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