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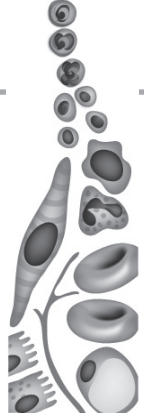

Mesenchymal stem cell exosome ameliorates reperfusion injury through proteomic complementation

The therapeutic efficacy of mesenchymal stem cell (MSC) transplantation has recently been attributed to exosomes when a single bolus of MSC exosomes prior to reperfusion of ischemic myocardium ameliorates reperfusion injury and reduces infarct size. In this article we review the therapeutic efficacy of MSC exosome in ameliorating cell intrinsic factors in reperfusion injury by focusing on the proteome complementation of exosomes and reperfused myocardium. The well-documented ATP deficit and initiation of apoptosis during ischemia and reperfusion were recently found to be underpinned by a proteomic deficit in enzymes critical for fatty acid oxidation, glycolysis and tricarboxylic acid cycle, and a proteomic surplus of proapoptotic proteins. Interestingly, this deficit in glycolytic enzymes was complemented by an abundance in MSC exosomes and the surplus of proapoptotic proteins was circumvented by CD73 that could increase survival signaling through the activation of reperfusion injury salvage kinases. Together, this provides a window of opportunity for the cells to repair and regenerate thus constituting the rationale for the therapeutic efficacy of MSC exosomes.

KEYWORDS: exosomes ■ mesenchymal stem cells ■ proteome ■ reperfusion injury

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Mesenchymal stem cells & their clinical applications

Mesenchymal stem cells (MSCs) were first described in 1968 as a population of multipotent fibroblast-like cells that reside in the bone marrow and have the potential to differentiate into osteocytes, chondrocytes, adipocytes and myoblasts [1]. They are the most extensively evaluated stem cells, with reported therapeutic efficacy against an equally extensive assortment of diseases ranging from cardiovascular diseases, graft-versus-host disease, osteogenesis imperfecta or brittle bone disease to amyotrophic lateral sclerosis.

The main allure of MSCs as the stem cell of choice for regenerative medicine lies in their ease of isolation from a wide range of ethically and socially palatable adult tissues and a large capacity for *ex vivo* expansion [2]. MSCs have been isolated from many different tissues, such as bone marrow [3,4], adipose tissue [4,5], liver [6,7], muscle [8,9], amniotic fluid [10,11], placenta [12,13], umbilical cord blood [3,14] and dental pulp [15,16]. They have also been derived from human embryonic stem cells [17,18] and induced pluripotent stem cells [19]. Although most MSCs differentiate primarily into adipocytes, osteocytes and chondrocytes [20–25], they have been reported to be capable of endothelial and cardiovascular differentiation [26], neurogenic differentiation [27–29] and neovascular differentiation [30–32]. The clinical testing of MSCs on an amazingly wide spectrum of tissue injury was predicated on this wide-ranging potential of MSCs to differentiate into appropriate cell types to replace damaged cells after homing and engrafting in damaged or diseased tissues. However, this hypothesis of engraftment and differentiation of donor MSCs is increasingly untenable, as most transplanted MSCs were found to be trapped in the liver, spleen and lung, with <1% of the cells actually reaching their target tissue [33]. Furthermore, the reported evidence for differentiation of engrafted MSCs at the target site is ambiguous and often relies on markers that could be transferred from transplanted cells to the host cells by cell fusion [34–36]. In addition, it is observed that the therapeutic efficacy of MSCs for a target tissue was not dependent on the physical proximity of the transplanted cells to the tissue [37–41].

To reconcile an increasing contradiction between the therapeutic efficacy of MSC transplantation and its hypothetical therapeutic mechanism of cell replacement through engraftment and differentiation, it was proposed that MSCs exert their therapeutic efficacy through the secretion of factors to reduce cellular injury and enhance repair [42]. Support for this paradigm shift is most evidenced by the shift in the rationale to trophic secretion for 65 of the 101 MSC clinical trials in 2010, with only 36 still rationalized on the differentiation potential of MSCs [43].

Trophic secretion of MSCs

MSCs are known to secrete a broad spectrum of growth factors and cytokines [44]. We

Regenerative Medicine

subsequently demonstrated that this secretion was far more diverse and contained cellular and membrane proteins that were not previously known to be secreted [45]. This secretion was cardioprotective in pig models [46,47]. This diversity could potentially provide a basis for the therapeutic efficacy of MSCs in a wide range of disease indications and injuries [48–55]. In recent studies, MSCs were reported to secrete small lipid vesicles such as microvesicles [56,57] and exosomes [58]. These lipid vesicles carry a cargo of proteins and nucleic acids that is sufficiently large and diverse to underpin the therapeutic efficacy of MSCs observed in a plethora of diseases. Indeed, purified MSC exosomes were found to reduce reperfusion injury in an animal model of myocardial ischemia/reperfusion (I/R) injury [58].

Exosome

Exosomes are presently the most defined class among several classes of secreted membrane vesicles reported to date, such as microvesicles, ectosomes, membrane particles, exosome-like vesicles, apoptotic bodies, prostasomes, or oncosomes. They can be distinguished according to their biogenesis pathway, size, flotation density on a sucrose gradient, lipid composition, sedimentation force, and cargo content [59,60]. However, the parameters used in this classification are generally not definitive or exclusive, and membrane vesicles are often not stringently characterized, leading to much confusion in our understanding of the biology and functions of the different classes of secreted membrane vesicles [61]. As a consequence, it is not clear if each of the previously mentioned vesicles represent unique biological entities.

Secreted membrane vesicles are known to be produced by many cell types. These vesicles have attracted much interest from the biomedical research community because of their potential as sources of biomarkers for diseases, therapeutic agents or vehicles for drug delivery. Secreted membrane vesicles carry a cargo of proteins and nucleic acids that reflect their cell sources and the pathophysiological state of the cell source, such that these proteins or nucleic acids could be used as biomarkers to diagnose, prognose or predict disease and its progress. In addition, these vesicles with their cargoes could be internalized by other cells as a form of intercellular communication, making these vesicles potential therapeutic agents or delivery vehicles of therapeutic agents [62].

Exosomes are presently the only class of membrane vesicles known to originate from the endosomes. They are formed by the invagination of endolysosomal vesicles to form multivesicular bodies. Exosomes are released to the extracellular space when multivesicular bodies fuse with the plasma membrane. They have a narrow diameter range of 40–100 nm and float at 1.1–1.18 g/ml in sucrose density gradient. While they share an evolutionarily conserved set of proteins such as the tetraspanins (e.g., CD81, CD63 and CD9), Alix and Tsg101, exosomes also carry proteins that are unique to their cellular source [63]. They are also known to contain RNA. Richard Simpson and his colleagues have set up Exocarta, a freely accessible web-based compendium of proteins and RNAs found in exosomes [64,201] and more recently, Vesiclepedia [65,202].

Exosomes were first discovered as a vehicle for discarding unwanted transferrin receptors from maturing sheep reticulocytes [66]. Subsequently, Raposo *et al.* observed that B-lymphocytes stimulate T-cell proliferation [67] and suppress tumor growth [68] by secreting exosomes that express functional MHC class I and II and T-cell costimulatory molecules. More recently, exosomes were found to contain mRNA [69] and miRNA [69–71] that could be transferred into recipient cells to modulate protein synthesis. Together, these studies suggest that the function of exosome extends beyond the disposal of unwanted proteins to include intercellular communication through protein–protein interactions and the exchange of proteins and genetic materials. For a more extensive discussion on exosome function, the reader may refer to this review [72]. Recently, this function was extended to include tissue repair when it was reported that exosomes secreted by human embryonic stem cell-derived MSCs reduced infarct size by approximately 50% in a mouse model of myocardial I/R injury [58,73].

Myocardial I/R injury

Recent advances in reperfusion therapy, for example, thrombolysis, bypass surgery or percutaneous coronary intervention to circumvent an occluded coronary artery and rapidly restore blood flow and oxygenation, have significantly reduced mortality in acute myocardial infarction (AMI) [74]. However, as many as 65% of AMI survivors progress to fatal heart failure within 5 years [75]. The progression of an AMI survivor to a heart failure patient is a complex multifactorial process that is largely dependent on the size of infarcted myocardium. It has been demonstrated that reperfusion of severely ischemic tissue itself causes lethal injury, also known as I/R injury, and contributes to the final infarct

size in AMI patients undergoing reperfusion therapy [76]. Therefore, adjunctive therapies to reperfusion therapy to alleviate I/R injury are urgently needed to reduce the final infarct size in reperfused AMI patients and improve their outcome. However, this need has been largely unmet, as I/R injury has proven to be intractable to pharmaceutical interventions [77].

Myocardial I/R injury is a highly paradoxical injury that is precipitated ironically by the restoration of blood and oxygen to ischemic heart tissue, and its severity is proportional to the duration of the ischemia. Ischemia essentially causes a collapse of ATP production through a lack of oxygen to drive oxidative phosphorylation, the main production source of cellular ATP. This is particularly detrimental to the cardiomyocytes, which maintain their relentless contractile activity by having the highest basal ATP consumption rate in a resting body. Under normal circumstances, the heart derives more than 95% of its ATP from the oxygen-dependent coupling of fatty acid (FA) β-oxidation and oxidative phosphorylation [78]. The lack of oxygen during ischemia disrupts this main production source of ATP and also aerobic glycolysis, leaving inefficient anaerobic glycolysis as the major source of ATP production, resulting in an inevitable ATP deficit. To conserve ATP, ATP-dependent cellular activities such as protein synthesis and turnover, gene transcription, ion pumps or transporters are curtailed [79]. With increasing duration of the ischemia, the derangement in cellular activities escalates to a loss of cellular homeostasis as manifested by intracellular acidification, imbalance of ions such as sodium, potassium and calcium, and increased intracellular osmotic pressure through the accumulation of metabolic products such as ADP, inorganic phosphates, reduced coenzymes (e.g., NADH and FADH) and lactic acid, and eventually cell death [78,80–83].

Rapid reperfusion of an ischemic cardiac tissue represents an intuitively logical therapeutic solution to halt the cascading and escalating ischemia-induced cellular damages. Indeed, rapid reperfusion to restore flow and oxygenation within 15 min of ischemia (or the reversible phase) restores cardiac contractibility, but delayed reperfusion after 40–60 min of ischemia (or the irreversible phase) cannot restore cardiac contractibility [80–84]. While reperfusion could salvage much of the tissue at risk, it could paradoxically exacerbate ischemia-induced injury and reduce the efficacy of reperfusion therapy [79]. For less ischemic cells, reperfusion re-energizes the mitochondria to generate ATP and restore cellular activities. By contrast, reperfusion exacerbates mitochondrial re-energization in severely ischemic cells with mitochondrial damage; not only failing to restore oxidative phosphorylation, it also causes opening of the mitochondrial permeability transition pore to generate and release reactive oxygen species (e.g., superoxide, peroxynitrite, hydrogen peroxide and hydroxyl radical) [85,86]. The restoration of flow and oxygen to severely ischemic tissues during reperfusion is also known to exacerbate the perturbed ion homeostasis, resulting in the paradoxes of oxygen, calcium and pH, sarcolemmal disruption and hypercontracture [78,87]. Together, these cellular derangements trigger the initiation of apoptosis and an acute inflammatory response. Both apoptosis and the immune system, which include innate and adaptive immune cells, play important roles in mediating reperfusion injury [87–90].

In consideration of our present understanding of the molecular pathogenesis of reperfusion injury, an expedient therapeutic strategy would target the preceding intrinsic cellular factors over the extrinsic immune factors in order to halt a cascading injury process and promote cellular repair. This rationale is further supported by the failure of anti-inflammatory agents to demonstrate efficacy against reperfusion injury in clinical trials [91]. Two immediately obvious intrinsic targets are the ATP deficit and the initiation of apoptosis, as they are paramount to cellular survival. Correction of the ATP deficit is crucial in order to reverse a shutdown of cellular activities and restore cellular homeostasis, both of which are indispensable for cellular repair and recovery. The initiation of apoptosis pre-empts any cellular attempts at repair and recovery, and its inhibition or delay provides a critical window of opportunity for cellular recovery.

Proteomic changes in ischemic & reperfused myocardium

To identify intrinsic factors in reperfusion injury, we have focused on the proteins in reperfused myocardium. In our recent study of the cardiac proteome after ischemia and then reperfusion, we observed a total of 121 proteins exhibiting significant temporal quantitative changes after 30 min of ischemia, and these changes persisted for up to 120 min postreperfusion. The proteins are largely clustered in eight functional groups: FA oxidation, glycolysis, tricarboxylic acid (TCA) cycle, electron transport chain

(ETC), redox homeostasis, apoptosis, glutathione S-transferase and heat shock proteins [92]. It is notable that 94 of the 121 altered proteins were clustered in the four functional groups that are intimately involved in the well-documented bioenergetic changes during ischemia and reperfusion [78], namely FA metabolism, glycolysis, TCA cycle and ETC.

Many of the enzymes that were clustered in FA oxidation, glycolysis and the TCA cycle play critical roles in these processes. They were significantly reduced after ischemia and remained depressed for up to 120 min after reperfusion [92], indicating that FA oxidation, glycolysis and the TCA cycle would also be depressed. By contrast, ETC proteins were elevated in the ischemic and reperfused heart tissues, but this elevation would have little impact on ATP production as the ETC relies on substrates generated by FA oxidation, glycolysis and the TCA cycle to generate ATP via oxidative phosphorylation. Therefore our proteomic analysis of the ischemic and reperfused myocardium revealed that the inhibition of cellular bioenergetic metabolism initiated by oxygen deprivation during ischemia was fortified by a proteomic depletion of critical bioenergetic proteins that persisted for 60 min after re-oxygenation. This not only provides a molecular rationale for the well-documented lethal irreversible phase of myocardial ischemia, in which reperfusion to restore oxygen supply cannot restore cardiac contractibility [80–84], but it also shows that restoration of bioenergetic metabolism in reperfused myocardium requires *de novo* protein synthesis to replenish the depleted enzymes.

Another distinctive proteomic change in reperfused myocardium was the significant increase in proapoptotic proteins. Cell death, particularly apoptosis, has long been recognized as a major cause of AMI [93–95], and its importance was demonstrated by the attenuation of I/R injury when the apoptotic signaling cascade was pharmacologically inhibited [88,96–98]. These proapoptotic proteins include NADH-ubiquinone oxidoreductase, apoptosis-inducing factor 1 and isoform pl-VDAC1 of voltage-dependent anion-selective channel protein 1.

In summary, the dominant determinants of reperfusion injury, ATP production and apoptosis were underpinned by proteomic changes that persisted for at least 60 min postreperfusion, and were therefore refractory to the restoration of oxygen for at least 60 min postreperfusion. Therapeutics that could compensate for these proteomic alterations during this interim refractory period would alleviate the ATP deficit and vulnerability to apoptosis, and provide a critical time window for cell repair and recovery. Our reported efficacy of MSC exosomes in alleviating myocardial I/R injury suggests that the exosomes may have the potential to compensate for these proteomic alterations in reperfused myocardium. Although exosomes have both protein and RNA cargo, the protein component is likely to be the active therapeutic agent, as it provides a better prospect of delivering a timely and direct intervention, which is critical for ATP-deficient and proapoptotic reperfused cells. Unlike RNA, which requires ATP-dependent RNA translation or degradation to elicit a cellular effect, proteins could elicit cellular responses without intermediaries or ATP.

Bioenergetic proteomic complementation between MSC exosomes & reperfused myocardium

To identify candidate mechanisms by which the proteome of MSC exosomes could alleviate I/R injury, we interrogated the proteome of MSC exosomes by mass spectrometry and antibody array to identify 857 unique gene products [99,201]. These proteins were distributed over a wide array of biochemical and cellular processes such as communication, structure and mechanics, inflammation, exosome biogenesis, tissue repair and regeneration and metabolism. A striking feature is a significant clustering of proteins in glycolysis, which could complement the glycolytic deficit in the reperfused myocardium.

All five enzymes in the ATP-generating stage of glycolysis, namely GAPDH, PGK, PGM, ENO and PKm2, along with PFKFB3, a key enzyme in the regulation of glycolysis, are present in MSC exosomes [201]. Of the glycolytic enzymes, GAPDH, PGK, and PKm2, which generate either ATP or NADH, were determined to be enzymatically active.

The presence of glycolytic enzymes in MSC exosomes complements their depletion in reperfused myocardium and could potentially increase glycolytic flux and ATP production in reperfused myocardium. However, an important caveat will be that the proteins in MSC exosome could be transferred from the exosome across the plasma membrane into the cytosol of reperfused cardiomyocytes. Cellular internalization of exosomes has been shown to modulate cellular activities consistent with the transfer of biologically functional proteins or RNAs. For example, exosome-mediated transfer of peptide loaded MHC class I and II complexes could

regulate immune response [100,101], mRNAs in exosomes were translated in the recipient cells [102] and siRNAs loaded in exosomes knocked down the targeted gene in the recipient cells [103].

Intracellular delivery of proteins by exosomes

The capacity of exosomes to transfer proteins and RNA across plasma membranes and elicit cellular responses has made exosomes increasingly attractive as potential drug delivery vehicles. In addition to this capacity, exosomes also have many of the highly desired attributes expected of a drug delivery vehicle [62,99]. They are physiologically well tolerated and can be found in many biological fluids such as blood, urine, bronchoalveolar lavage fluid, breast milk, amniotic fluid, synovial fluid, malignant pleural effusions and ascites [63]. The encapsulation of proteins within bilipid membranes of exosomes enhances the bioavailability of the exosome cargo and enables internalization of the protein by facilitating membrane fusion or endocytosis. Exosomes have been shown to deliver their cargo into target cells across the plasma membrane into the right cellular compartment to exert a functional response. For example, exosomes derived from dendritic cells (also known as Dex) could modulate immune cell response by transferring peptide-loaded MHC class I and II complexes to dendritic cells [100,101]. Exosomes have also been shown to mediate intercellular transfer of mRNAs and miRNAs that resulted in the translation of the transferred mRNA in the recipient cells [102]. We have demonstrated that MSC exosomes are internalized by H9C2 cardiomyocytes [104].

Exosomes could also home to specific cell types. For example, melanoma exosomes have been reported to preferentially home to sentinel lymph nodes to promote tumor metastasis [105]. Their membranes could also be modified to enhance cell type-specific targeting. For example, overexpression of a fusion gene consisting of a neuron-specific rabies virus glycoprotein encoding sequence and *LAMP2B* encoding an exosomal membrane protein in dendritic cells resulted in the secretion of exosomes with rabies virus glycoprotein peptide on their membranes. These exosomes, after loading with siRNA, could cross the blood–brain barrier to knock down more than 60% of the siRNA-targeted genes in neurons, microglia, oligodendrocytes and their precursors [103].

Several mechanisms have been postulated for cellular internalization of exosomes, and these include endocytosis, phagocytosis and membrane fusion [106–108]. However, there is no unanimous consensus on whether cellular internalization of exosomes occurs exclusively through one pathway or through multiple pathways, and if so, whether there is a preferred pathway. Nevertheless, the uptake is likely to be regulated, as it was observed that the efficiency of exosome uptake correlated directly with intracellular and microenvironmental acidity [108]. This could provide a mechanism for the preferential internalization of MSC exosomes by ischemic cardiomyocytes, which reportedly have low intracellular pH [109].

As an intercellular delivery vehicle for proteins, MSC exosomes are differentiated from other exosomes or drug delivery vehicles in that they are likely to be safe and well tolerated in the body as their cell source, the MSCs, have been tested and shown to be safe in many clinical trials, and were approved for the treatment of pediatric graft-versus-host disease by Canada and New Zealand in 2012. Human MSC exosomes also do not have MHC class I or II proteins, and their infusion into non-immune-compatible animals does not induce overt immune reactions [58,73], making them suitable for use in allogenic recipients. Furthermore, we have demonstrated that MSC exosomes inhibit the proliferation of concanavalin A-activated lymphocytes [62]. The transfer of glycolytic enzymes from exosomes across the plasma membrane is likely to be sufficient for the enzymes to exert their biochemical activity, as glycolysis is a cytosolic process, unlike FA oxidation and the TCA cycle, which are mitochondrial processes. An important corollary of this hypothesis is that MSC exosomes increase ATP production in reperfused myocardium or in cells where oxidative phosphorylation is inhibited.

MSC exosome-mediated enhancement of glycolysis

The therapeutic potency of increasing ATP production in reperfused myocardium by anaerobic glycolysis is suspect if considered solely in the context of the efficiency of ATP production. Anaerobic glycolysis generates two ATPs per glucose molecule versus 38 ATPs by aerobic glycolysis. In spite of this, anaerobic glycolysis could generate three-times more ATPs in a minute than aerobic glycolysis, albeit for a very short duration of minutes [110]. This short and rapid ATP production could potentially provide the necessary ATP in the brief window of opportunity to kickstart the cellular processes in the newly reperfused ischemic cardiac tissues. Indeed,

the metabolic switch from aerobic to anaerobic glycolysis during ischemia and reperfusion has long been recognized to be critical for cardiac protection against I/R injury [111–116]. More specifically, glycolysis has been shown to be important in the functional recovery of contractibility during early reperfusion [117,118] and the restoration of calcium [119] and sodium [120] homeostasis. Loss of calcium homeostasis during cardiac ischemia is caused primarily by dysfunctional cardiac sarcoplasmic reticulum Ca^{2+}-ATPase (or Ca pump) [121]. These Ca^{2+}-ATPases are driven by ATPs from glycolysis [122] and not oxidative phosporylation. Therefore, the restoration of calcium homeostasis for the functional recovery of the reperfused heart is dependent on glycolytic ATP [117,119,123–125]. Similar to calcium homeostasis, cardiac sodium homeostasis is maintained by a complex network of several ATP-dependent Na^+ channels and transporters [126]. The Na/K pump, which plays a major role in Na^+ extrusion from cells, and the Na^+/H^+ exchanger, which is important in the regulation of cellular Na^+ and pH, are both highly reliant on glycolytic ATP for its function [127,128]. Consistent with the importance of glycolytic ATP in not only replenishing ATP supply but also energizing ion pumps to restore ion homeostasis, *TXNIP* deletion (which suppresses mitochondrial function and enhances anaerobic glycolysis) was found to protect mice against reperfusion injury [129].

In view of the importance of glycolytic ATPs not only as the only viable cellular source of bioenergy in reperfused cells, but also as the energy source for ion pump activity in maintaining ion homeostasis, the hypothesis that MSC exosomes protect against myocardial ischemia reperfusion through supplementation of glycolytic enzymes to increase glycolytic flux and enhance ATP production provides a compelling molecular rationale for the therapeutic efficacy of MSC exosomes (Figure 1). Additionally, enhanced glycolysis has an added advantage of providing glycolytic intermediates for anabolic metabolism in order to generate biomass for growth and repair as illustrated by the abnormally high rate of aerobic glycolysis in rapidly proliferating cells such as cancer cells and stem cells [130].

Verification of protein supplementation by demonstrating the physical transfer of functional glycolytic enzymes from MSC exosomes to reperfused myocardium is currently limited by a lack of suitable technology to track and monitor the metabolic fate of an exosome and its contents. Nevertheless, such a transfer could be inferred from increased ATP production in ischemic or perfused cells after exposure to MSC exosomes. Using oligomycin, which inhibits oxidative phosphorylation [131], to chemically induce a state of ischemia in H9C2 cells, we demonstrated that MSC exosomes significantly increased intracellular ATP level [99]. More importantly, ATP production was also observed in exosome-treated reperfused myocardium concomitant with a reduced infarct in a mouse model [132].

Overcoming a proapoptotic proteome in reperfused myocardium

Apoptosis plays a central role in lethal reperfusion injury and inhibition of apoptosis during reperfusion has been an important target for therapeutic intervention [88–90]. Activation of some prosurvival protein kinases, such as Akt and Erk1/2, which induce antiapoptotic effects, has been demonstrated to be protective against myocardial I/R injury when occurring at the time of myocardial reperfusion [93,133]. These kinases are now termed the reperfusion injury salvage kinases (RISKs) [134]. Therefore, activation of RISKs represents a therapeutic target in overcoming the proapototic proteome in reperfused cardiomyocytes.

Akt and Erk1/2 belong to two of the most important survival signaling pathways, namely Ras/Raf/MEK/ERK (MAPK) and PTEN/PI3K/AKT/mTOR [135], and are the prototypic RISKs. Activation of these pathways has been shown to be important in tissue repair and amelioration of tissue injury, including myocardial ischemia reperfusion injury [93], sepsis [136] and epithelial wounds [137]. Pharmacological activation of PI3K and ERK1/2 through insulin, IGF-1, TGF-β1, CT-1, urocortin, atorvastatin and bradykinin during post-ischemic reperfusion protects the heart from I/R injury by exerting an antiapoptotic effect [93,134]. Incidentally, ischemic postconditioning, which consists of repeated interruptions of coronary blood flow following ischemia, is effective in limiting reperfusion injury in animals [138] and humans [139–142], and is also associated with the activation of PI3K–Akt and ERK1/2 pathways. Inhibition of either pathway by small molecules abrogated the protective effect of ischemic postconditioning [143].

From the proteomics of MSC exosomes, we postulate that CD73, which is present on the surface of MSC exosome, is the most likely protein candidate to overcome the proapoptotic proteome of reperfused myocardium through the activation of RISKs (Figure 2) [99]. CD73 is an ecto-5′-nucleotidase and is the only enzyme known to

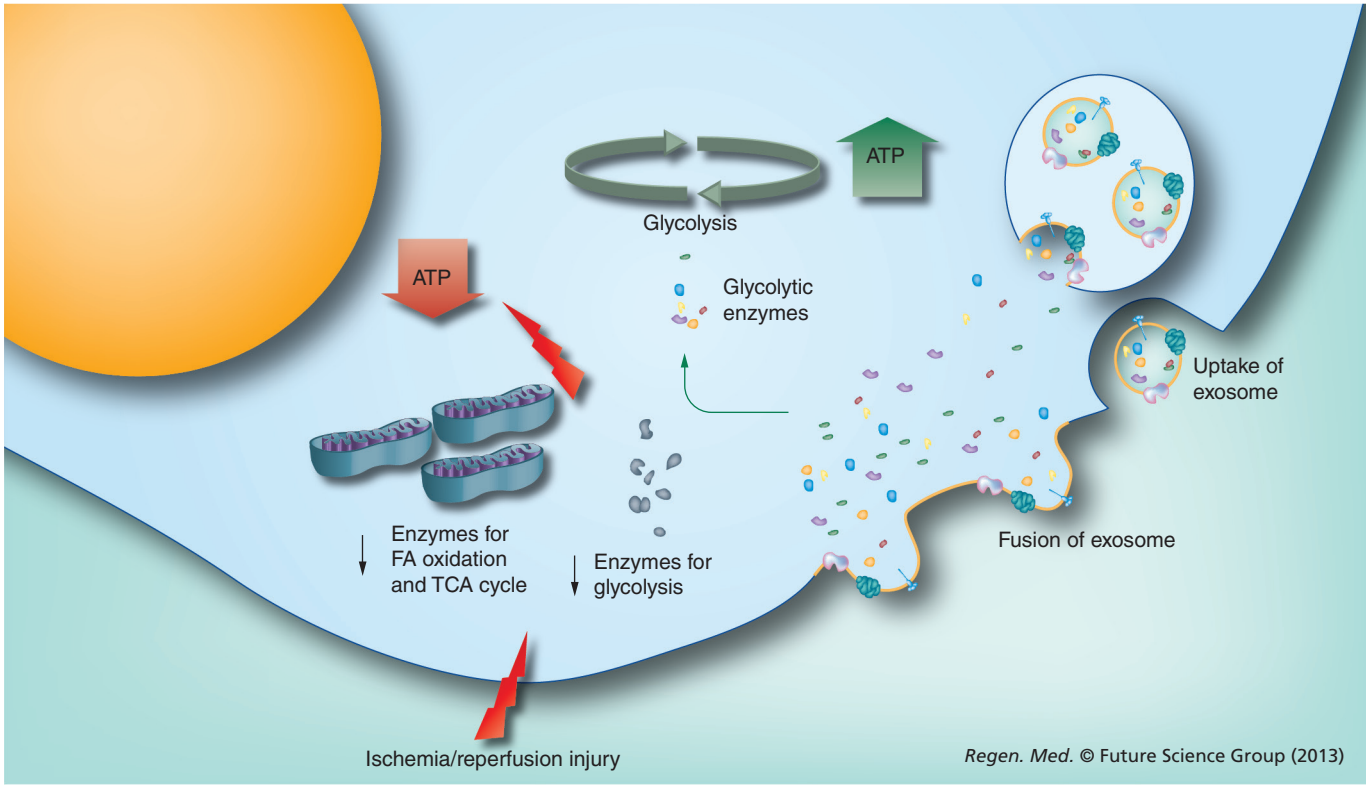

Figure 1. Proteomic complementation of mesenchymal stem cell exosomes and reperfused myocardium in glycolysis. The proteome of reperfused myocardium is deficient in critical enzymes for FA oxidation, the TCA cycle and glycolysis. Proteins for FA oxidation and the TCA cycle are localized primarily in the mitochondria, while glycolytic proteins are cytosolic. Mesenchymal stem cell exosomes are enriched in glycolytic enzymes. Cellular internalization of mesenchymal stem cell exosomes through either fusion or endocytosis results in the transfer of glycolytic enzymes from exosomes to the cytosol. The replenishment of cellular glycolytic enzymes enhances glycolytic flux and increases ATP production for cellular activity.
FA: Fatty acid; TCA: Tricarboxylic acid.

hydrolyze extracellular AMP to adenosine [144]. Adenosine is an activator of ERK and AKT phosphorylation that has been demonstrated to reduce myocardial I/R injury in animals [143,145] and clinical trials [146,147]. During tissue trauma such as shear stress-induced hemolysis of red blood cells, working skeletal muscle, perfused heart or isolated heart muscle cells under hypoxic conditions, ATPs and ADPs are released into the extracellular space [148]. The half-life of extracellular ATP and ADP in the blood is estimated to be less than 1 s [149] and 3.2 min [150], respectively. They are rapidly degraded by ecto-enzymes into AMP, which is the substrate for CD73. Consistent with the presence of enzymatically active CD73, exposure of H9C2 cardiomyocytes to MSC exosomes and AMP resulted in phosphorylation of ERK1/2 and AKT. This phosphorylation was abolished in the presence of theophylline, a nonselective adenosine receptor antagonist that antagonized A1, A2A, A2B and A3 receptors [151]. We also observed that MSC exosome treatment increased pAKT level in the reperfused myocardium of mice [132].

Conclusion

The proteomic potential of MSC exosomes in overcoming fundamental biochemical derangements in ischemic and reperfused myocardium is likely to be the underpinning mechanism for their efficacy in reducing myocardial I/R injury. The two derangements discussed in this article, namely ATP deficit and the proapoptotic proteome, represent the most critical but not the only derangements in myocardial I/R injury, and are therefore likely to dominate cell or tissue repair and recovery. We postulated here that the effectiveness of MSC exosomes in correcting these derangements could be attributed to protein complementation of biochemically active enzymes. This use of enzymes is a significant feature of exosome-based therapeutics, as enzymes have the potential of amplifying their rate of activity over a sustained period of time depending on substrate concentration and microenvironment. Therefore, a small dose of exosomes could potentially elicit a large response. In our reported studies, we routinely used 0.1–0.4 µg exosomes per mouse and 0.1 µg/ml exosomes for cell culture. Such small

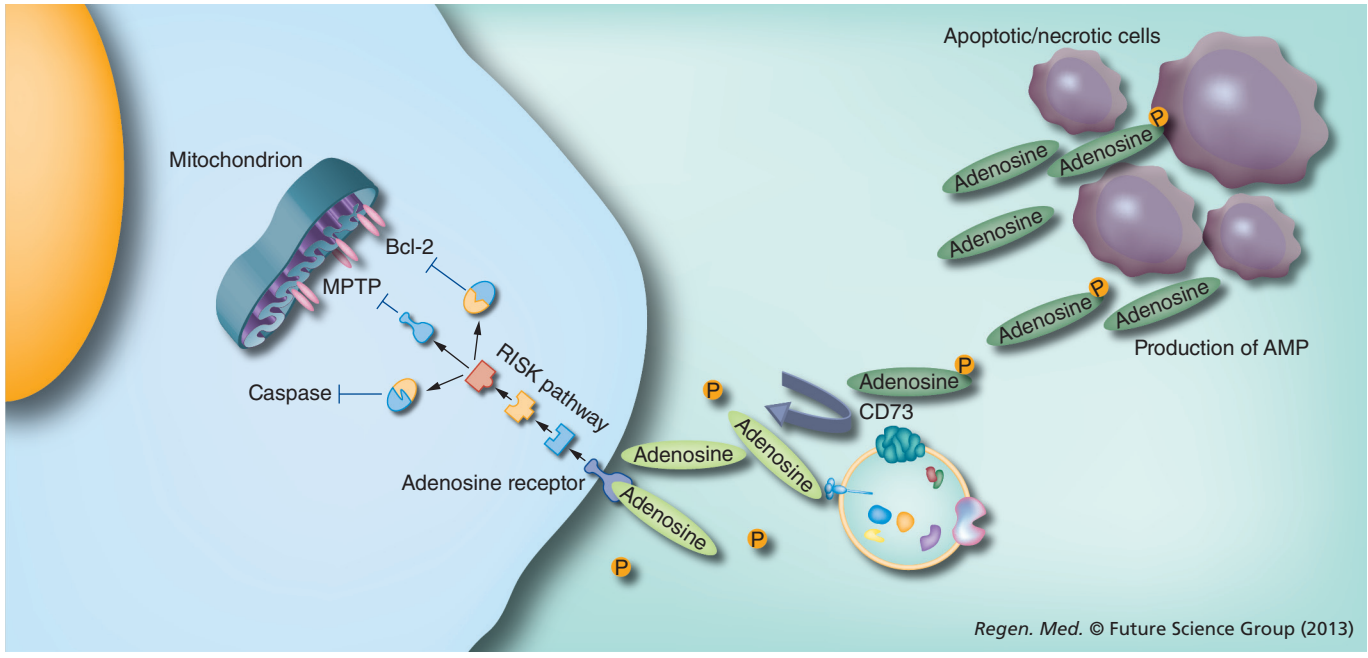

Figure 2. Circumventing a proapoptotic proteome. Reperfused myocardium has a proapoptotic proteome. Injured or dying cardiomyocytes release ATPs and ADPs that are rapidly degraded to AMP in the extracellular matrix. Membrane-bound CD73, which is present on mesenchymal stem cell exosomes, is the only enzyme known to hydrolyze AMP to adenosine in the extracellular matrix. Binding of the adenosine receptors phosphorylates RISKs such as AKT and ERK1/2 to induce survival signaling and circumvent the proapoptotic proteome of reperfused myocardium.
RISK: Reperfusion injury salvage kinase.

doses make the use of exosomes, which are produced in relatively small quantities by cells, feasible. MSC-conditioned medium routinely yields approximately 1 mg of exosomes per liter [152].

Reperfusion is a highly complex injury that extends far beyond the critical ATP deficit and the proapoptotic proteome. Other critical complications include the opening of mitochondrial permeability transition pore, release of reactive oxygen species (e.g., superoxide, peroxynitrite, hydrogen peroxide, and hydroxyl radical) [85,86], perturbed ion (calcium, potassium, sodium and hydrogen) homeostasis, hyper contracture, resulting in the paradoxes of oxygen, calcium and pH, sarcolemmal disruption and hypercontracture [78,87]. The common features of these latter derangements are their initiation by an ATP deficit and subsequent aggravation by reperfusion before converging towards activation of apoptosis or necrosis. These intrinsic cellular damages and the resulting cellular debris in turn activate the immune system including the monocytes, T and B cells, platelets and complement to destroy damaged cells and frequently healthy bystanders as well [87–90].

Although MSC exosomes could ameliorate the ATP deficit and circumvent the proapoptotic proteome in the reperfused myocardium, these activities only provide a transient window of opportunity for the injured tissue to correct the other intrinsic and extrinsic cellular derangements as discussed above, and repair itself. Hence, the tissue remains highly vulnerable until these other derangements can be rapidly resolved. The efficacy of MSC exosomes in reducing reperfusion injury in pig and mouse models, and the extensive exosome proteome in which the glycolytic enzymes and CD73 constitute a small fraction, suggests that MSC exosomes may possess additional therapeutic activities against the other complications of reperfusion injury. Some of these therapeutic activities include functionally active 20S proteasomes capable of removing aberrant peptide deposits in reperfused hearts of mouse models [153] and the inhibition of complement-mediated lysis by exosome-associated CD59 [99]. We hypothesize that the efficacy of MSC exosomes is due, in large part, to their potential to rapidly correct a critical ATP deficit and circumvent a proapoptotic proteome to generate a small window of opportunity for the other biochemical activities in the exosomes to rectify the other molecular derangements in the reperfused cells.

Future perspective

The diverse MSC proteome suggests that MSC exosomes could exert their therapeutic efficacy on an equally diverse spectrum of biochemical

derangements found in injured cells or tissues, including factors that are extrinsic to the injured cells. A more exhaustive and systematic investigation of the MSC proteome is underway to dissect and evaluate the potency of MSC exosomes. One preliminary finding includes the presence of fully assembled 20S proteasomes with lactacystin-sensitive proteolytic activity, suggesting the capacity of MSC exosomes to remove aberrant peptide deposits and reduce or delay protein aggregation [153]. Consistent with this, exosome-treated heart tissues had significantly reduced denatured proteins [153]. Although we have focused on MSC exosome activity against factors intrinsic to the injured cells in this review, the proteome of MSC exosomes also has the potential to target factors extrinsic to the injured cells. For example, MSC exosomes possess CD59 and could inhibit complement-mediated lysis of sheep red blood cells in a CD59-dependent manner [99], demonstrating that MSC exosomes could attenuate complement-mediated destruction in injured tissues. The diverse and biochemically potent proteome of MSC exosomes further suggest that MSC exosomes could act simultaneously against multiple biochemical aberrations commonly found in most injured cells and their microenvironment to induce a synergistic therapeutic effect. The generic cell-intrinsic and -extrinsic targets of MSC exosome proteome suggest that MSC exosomes could promote restoration of homeostasis in most injured cell types and tissues. MSC exosomes therefore provide a compelling rationale for the therapeutic efficacy of MSCs against a myriad of diseases, as discussed earlier in our review and could be efficacious in a similarly wide array of diseases.

Financial & competing interests disclosure

*This work was funded by the Agency for Science, Technology and Research – Biomedical Research Council (A*STAR BMRC). The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.*

No writing assistance was utilized in the production of this manuscript.

Executive summary

Mesenchymal stem cells

- Mesenchymal stem cells are currently a often-used stem cell for the treatment of myriad diseases in clinical trials.
- The rationale for mesenchymal stem cell (MSC) clinical trials has shifted from one based on engraftment and differentiation of MSCs into reparative cell types to one based on secretion of trophic factors to reduce injury and enhance repair.

MSC secretion

- MSC-conditioned medium reduced infarct size in a pig and a mouse model of myocardial ischemia/reperfusion (I/R) injury.
- Purified exosomes were sufficient to mediate the therapeutic effects of MSC-conditioned medium against reperfusion injury.

Exosomes

- Secreted bilipid membrane vesicles of endosomal origin.
- Function as intercellular conveyors of proteins and nucleic acids.
- Potential sources of biomarkers for diseases, therapeutic agents or vehicles for drug delivery.

Myocardial I/R injury

- A paradoxical injury precipitated by restoring oxygen to oxygen-starved myocardium.
- Major biochemical derangements are disruption of ATP production and initiation of apoptosis.
- The proteome of reperfused ischemic myocardium is:
  - Deficient in bioenergetic processes: fatty acid oxidation, glycolysis and tricarboxylic acid cycle;
  - Proapoptotic, with elevation of apoptosis-related proteins.

Mechanism for efficacy of MSC exosome against I/R injury

- Proteome complementary to that of reperfused myocardium. Potential to restore cellular homeostasis in bioenergetics and apoptosis through:
  - Intracellular transfer of glycolytic enzymes by increasing glycolytic flux to generate:
    - ATP for cellular activities;
    - Glycolytic ATP that is critical in driving ion pumps for ion homeostasis;
    - Glycolytic metabolites for anabolic metabolism to repair and replace cellular damage.
  - Enzymatic activity of exosome CD73:
    - AMPs produced by the breakdown of cellular ATPs and ADPs that are released from dying cells;
    - CD73 hydrolyzed AMPs to adenosine;
    - Adenosine activates reperfusion injury salvage kinases to inhibit apoptosis.
- Restoration of cellular homeostasis enables tissue repair and regeneration.

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Review

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Future Medicinal Chemistry

Exosome manufacturing status

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Exosomes are secreted by mammalian cells and are widely distributed in cellular systems. They are a medium of information and material transmission. The complexity of exosome nature and function is not thoroughly understood. Nevertheless, they are being confirmed as mediators of intercellular communication and play significant roles in many physiological and pathological processes. Significant obstacles to the efficient and robust isolation of large quantities of pure and specific exosomes still exist. These include a lack of understanding of the relationship between exosome characteristics and function, and a shortage of scalable solutions to separate specific exosomes from other large entities remain. Hence, generic production platforms are desired. While solutions suitable for exosome manufacturing under GMP are available, most have been developed for other purposes.

First draft submitted: 24 August 2018; Accepted for publication: 30 January 2019; Published online: 8 July 2019

Keywords: exosome • manufacturing • production

Exosomes are a type of extracellular vesicles (EVs) having a unique generation pathway and characteristic [1]. Microvesicles are formed by outward budding from the plasma membrane, and cells undergoing apoptosis release apoptotic bodies whereas exosomes are generated through a fusion of the plasma membrane with particular endosomal compartments known as multivesicular bodies (Figure 1). The contents of EVs characteristics and cargo vary by the type and state of the cells involved in their generation. They carry such diverse cargo as microRNA, mRNA, lncRNA, DNA, lipids, peptides and vast array of proteins (including oncoproteins, tumor suppressors, transcriptional regulators and splicing factors) [2,3]. An EV classification solely based on their origin (exosomes, microvesicles and apoptotic bodies) is not sufficient to distinguish different EVs. Additional categorization based on morphological characteristics has been suggested [4].

A single definition of exosome subtypes, characteristics and purity has, however, not been universally established. This is because researchers from various disciplines have been approaching them through an evolving understanding of their essential nature, and from different arenas of biotechnology, monitored characteristics and applications. Exosomes are produced by many types of cells and have been discovered in nearly every bodily fluid. Their presence has been reported in the growth medium of many cultured cells, including B lymphocytes, dendritic cells, cytotoxic T cells, intestinal epithelial cells, neurons, oligodendrocytes, platelets, mast cells and Schwann cells. They have been demonstrated to be active in immune response; neural communication; reproduction and development; as well as in cell proliferation, homeostasis and maturation. Interest in exosomes is growing due to the discovery of their potential in so many research, diagnostic, analytic and therapeutic procedures.

Lately, quite a number of analytical techniques have been applied to their detection, characterization and isolation – with each emphasizing a distinct set of exosome characteristics. Relevant properties for both detection and isolation include morphological characteristics, size, density, ζ potential and composition – as well as particular chemical and biological characteristics [4,5].

Reasons for interest in the large-scale production of purified lots of exosomes include their observed paracrine-like activity (replacing other communication exhibited by their cells of origin) and their use as a vector of proteins or nucleic acid in therapeutic applications [6–8]. Exosomes have been, for example, suggested as a delivery system for therapeutic compounds – especially as they can pass the blood–brain barrier [9].

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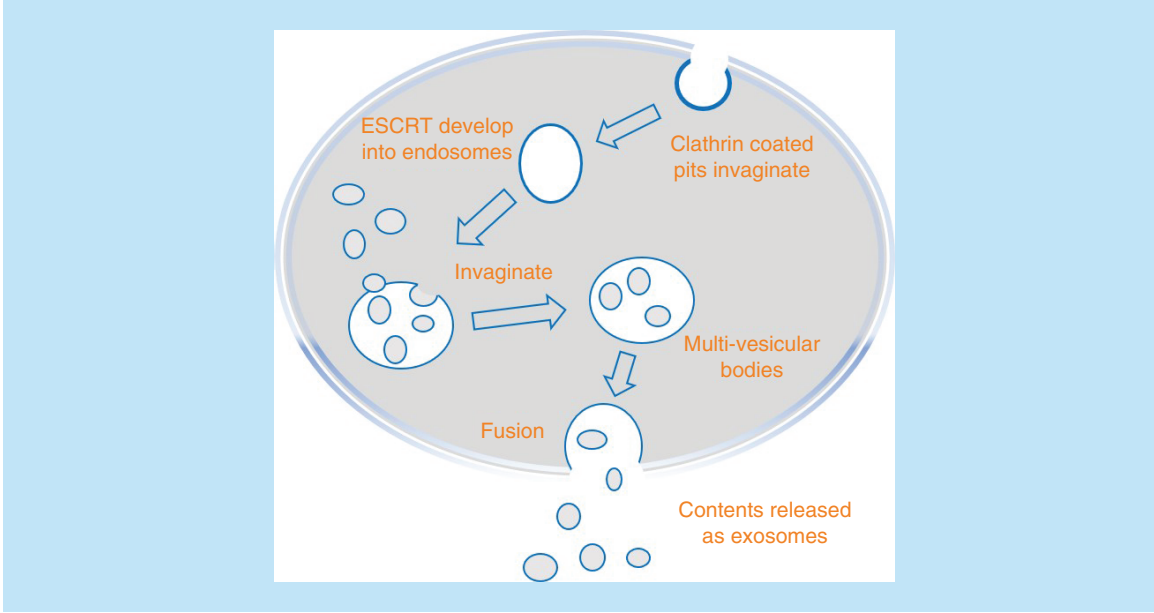

Figure 1. A simplified schematic of exosome biogenesis.

Large-scale production of exosomes is influenced by the specific therapeutic application. For such applications as tissue regeneration and immune response modulation, it is important to produce a therapeutic exosome composition that reflects the expression pattern from the parent cells. The interplay of different exosome subpopulations may have a significant impact upon the therapeutic effect [5]. When using exosomes as therapeutic vectors, such tailored composition of exosome subpopulations may be less crucial.

Isolation and purification of exosomes has, in general, proven to be difficult, and certainly it is even more difficult to isolate a particular subpopulation of exosomes. For example, the use of size-exclusion chromatography, ultrafiltration, precipitation, affinity capture or various centrifugation techniques will greatly increase the concentration and purity of exosome samples, however, no approach alone will produce a lot of highly purified vesicles or separate exosomes from other vesicle types present, and certainly not distinguish between different types of exosomes. A relatively pure population of exosomes can only be achieved through combination of techniques based upon such orthogonal properties as physical size or density and such biochemical parameters as the presence or absence of certain contents. Microfluidic techniques present another very successful category of exosome analysis, segregation and isolation. However, these are not of satisfactory capacity for large-scale production purposes. The therapeutic applications of exosomes, in combination with the delicate challenge to effectively manufacture these new therapeutic entities, motivate a current status update regarding exosome manufacturing alternatives.

Production of exosomes

Cellular platforms

Many cultured cells will secrete exosomes using standard T-flasks and cell culture media. As products of cells, the manufacture of exosomes is dependent on the ability to produce large quantities of cells in ways that do not alter certain cell behavior and characteristics. The potential for changes in cellular phenotype during technical transfer (e.g., scale-up and equipment change) must be considered. As the dynamics of exosome biogenesis is only beginning to be understood, such alterations in any number of characteristics of the cell culture platform might alter the production, composition, attributes or function of the exosomes. The types of cell lines desirable in the manufacturing of exosomes does not aid in this concern. Unlike such platforms as CHO, HeLa or Jurkat lines, it is often necessary to produce exosomes from less established, often primary or stem cell lines [10].

Evidence that alterations in such culture environmental parameters as pH gradients, mass transfer and hydrodynamic (or shear) force during scale-up can result in changes to such characteristics of the culture platform as induction of apoptotic blebs, downregulation of interleukin receptors, induction of mechanically stimulated protein-kinase pathways and modulation of extracellular signal-related kinases. The consequences of such cellular

changes upon the exosome product are not well understood [11]. In scaling up, some primary cell lines exhibit a much reduced proliferative capacity, limiting the ultimate culture size, duration, number of production batches or reproducibility. Solutions to this include immortalization by various means, via overexpression of the *MYC* gene [12,13].

Because many of the cells cultured for exosome research are anchorage-dependent, serum-free media are often not advised, and some degree and schedule of serum supplementation is often required. However, there are reports of protocols for the efficient production by some particular cell types in serum-free, even chemically defined, medium. For example, human-induced pluripotent stem cell-derived cardiovascular progenitors produced exosomes when plated on fibronectin-coated plates and cultured in a serum-free medium supplemented with only an added energy source and bFGF I [14]. Some have reported that shifting from fetal bovine serum (FBS)-containing to serum-free medium resulted in the production of exosomes initially presenting some similar characteristics, while the number of exosomes generated increased. However, further analysis showed that the serum-free exosomes contained different constituent component levels. The serum-free medium appeared to cause a shift in exosomal biology [15].

Exosomes secreted by specific human T-cell clones have been employed to modulate immune cells' activity, including subsets of other T cell. Ceramide, tetraspanins and MAL protein are reported to be critical in T-cell exosome biogenesis. Such molecules may be functional in modulating both exosome production as well as the immune response in therapeutic applications [16]. Many studies have shown that stem cells secrete several important growth factors, and stem cell conditioned medium is often still currently required for efficient stem cell culture *in vitro*. Another complicating factor in the scale-up of stem cell cultures for exosome manufacturing is the lack of source for large volumes of conditioned medium. It has also been demonstrated that the expression of certain exogenous proteins in cultured cells can influence the type and characteristics of exosomes secreted [17].

Where FBS is required, contamination with bovine exosomes is a concern. To avoid this, exosome-depleted versions of bovine serum are now commercially available. These sera are produced by various exosome-reduction means, with each inducing their own effect upon the serum constituents and characteristics. Furthermore, these sera are affected by the standard source, product, lot specifics and other disparities of normal serum. They do exhibit significant reductions in exosome-sized vesicles resulting in very low levels of CD63-positive cow exosomes or cow miRNAs and vendors provide assay results for such properties as cell culture performance, sterility, mycoplasmas, secretion performance and endotoxins.

Small-scale culture production typically begins with a culture of adherent cells in a flask or plate for a defined period in a standard culture medium. Then, the medium is exchanged for either medium devoid of serum, or medium containing exosome-depleted serum. Many serum-dependent cell cultures will perform relatively normally for a day or two with no serum supplementation. These cultures are then maintained for a defined number of hours or days while they secrete exosomes into this fresh ambient medium. The medium is then decanted and processed for any required vesicle isolation, concentration, characterization and purification.

Production formats & modes

Because many of the desired production platforms involve adherent cell culture, scaling-up activities have focused on technologies that maximize culture surface area, such as microcarriers in stirred-tank reactors or culture in fixed-bed or hollow-fiber bioreactors [18]. As mentioned, beyond cell-mass expansion requirements, it is imperative to control identified production environmental parameters such that the cell's phenotype, and therefore secreted exosome characteristics, do not change [19].

As the field of *in vitro* exosome production is in its infancy, much research and many creative approaches are being reported. Quite a number of the aspects of culture have been examined, including the use of feeder cells, conditioned media, exogenous extracellular matrices and engineered producer-cell transduction. Such approaches may be valuable in some formats for large-scale exosome production. The rational design of biomimetic exosomes is also being addressed.

Methods exist to produce exosome-mimetic vesicles, overcoming such natural limitations as low loading, efficiency and low exosome production yields. One group has developed hybrid exosomes produced through their fusion with liposomes using a freeze–thaw processes [20]. Others describe a virus-mimetic fusogenic exosome demonstrating enhanced fusion efficiency through employing a vascular stomatitis virus viral fusogen within the exosomal membrane [21]. Such methods either produce *de novo* modified exosomes from engineered cells, or support secondary modification through the fusion of exosomes with liposomes harboring desired proteins, lipids or synthetic polymers. Some have even proposed such creative manufacturing approaches as the *in vitro* mass production of

exosome-mimicking nanovesicles using a mini-extruder, and found that they were similar to the native exosomes in some respects [13].

Large-scale production

Larger-scale batch exosome production is accomplished in such formats as simply employing dozens of large (e.g., T-225) flasks, multiple stacked array multilayer culture flasks, large fixed-bed bioreactors, in stirred-tank bioreactors employing microcarriers or continuous production in perfusion reactors [22]. First, it should be mentioned that all the issues of modern technical transfer will exist for development of a large-scale, and especially a clinically relevant, exosome manufacturing format. This includes all the steps involved in shifting from an investigational product to a commercial product, moving from laboratory-scale production to commercial scale and implementing a fileable quality control testing program. It involves such steps as process optimization, confirmation, validation and characterization – as well as process performance qualification activities. Also, pertinent is that many of the newer and higher industry goals in such transfer will apply. These goals include heightened process control, establishing as many closed operations as possible, moving to more automated and 'digital' processes, employing single-use systems, and implementing as many *in situ* monitoring devices and sample analytics as may be desirable.

Due to the poor efficiency of *in vitro* exosome production, the current scale-up of standard batch-mode manufacturing can involve hundreds of flasks or a significant investment in the more expensive and complicated multilayer flask systems. Challenges to these approaches include the cost of culture expansion prior to the actual production phase (sometimes involving, e.g., stem cell-conditioned medium) or the additional cost and timing of beginning of the production phase in serum-adjusted or specialty serum-modified medium. Robust production of lot-consistent populations of exosomes necessitates verification of somewhat reproducible culture conditions, which can take time to establish in the scale-up or scale-out of such systems.

Stirred-tank bioreactor cultures employing microcarriers have been reported, as well as a few challenges related to this upstream production approach [19]. There are many scale factors and basic environmental conditions differentiating small-scale flask culture and impeller-based bioreactor culture. Culture progression and production efficiency concerns arise from such factors as cell-to-microcarrier binding, gas mass transfer differences and the hydrodynamic forces generated in sparging and agitation. The effect of these factors upon other such culture characteristics as differentiation, apoptosis potential and exosome product quality and quantity have been reported as well.

Perfusion-based production can alleviate some of these concerns and avoid the added process and limitations associated with microcarriers. Many membrane-based flasks or bioreactors as perfusion-capable technologies have been employed and avoid, or at least reduce, such issues as those associated with hydrodynamic (shear) forces. These systems can not only support culture over an extended period (establishing a continuous biomanufacturing) but can concentrate exosomes within a membrane segregated compartment, facilitating feeding and harvest. In some reactors, the cell-containing side of the perfusion apparatus also supports the segregation of growth factors, allowing a severe reduction in added serum, added factors or conditioned medium. Some platforms have been reported to display entirely new culture characteristics under such high-density culture. These include cell-to-cell contact inhibition of division and a reduction (or elimination) of what had been a constitutive requirement for certain added growth factors or other surface-active agents in the culture medium. However, these systems do not ameliorate all issues of scale-up, -out or of high-density culture effects. For even larger-scale production in a related mode, many porous 3D scaffolds and fiber-based packed- and fixed-bed bioreactors are now available, and some with published applications data.

Hollow-fiber perfusion bioreactors have been employed in cell culture for many years, and their use in large-scale production of exosomes reported. These reactors can support large numbers of cells at high densities in a continuous culture mode without splitting and subculturing of the cells (Figure 2). Cells are cultured on either the inside or outside of the reactor fibers which allow nutrients and waste products to pass through but retain such larger secreted product as exosomes (Figure 3).

In some applications, collection of exosomes can be maintained over several months. In one example, the phenotype of cells in continuous culture remained constant over the 10-week period of production. It resulted in continuously harvested exosomes which, by orthogonal measure, did not vary in monitored characteristics [23]. So, while many of the large-scale culture methods and technical transfer approaches are similar to protein biological or cells-as-product manufacturing, we see that there are a few exosome-manufacturing-specific phenomena to be considered.

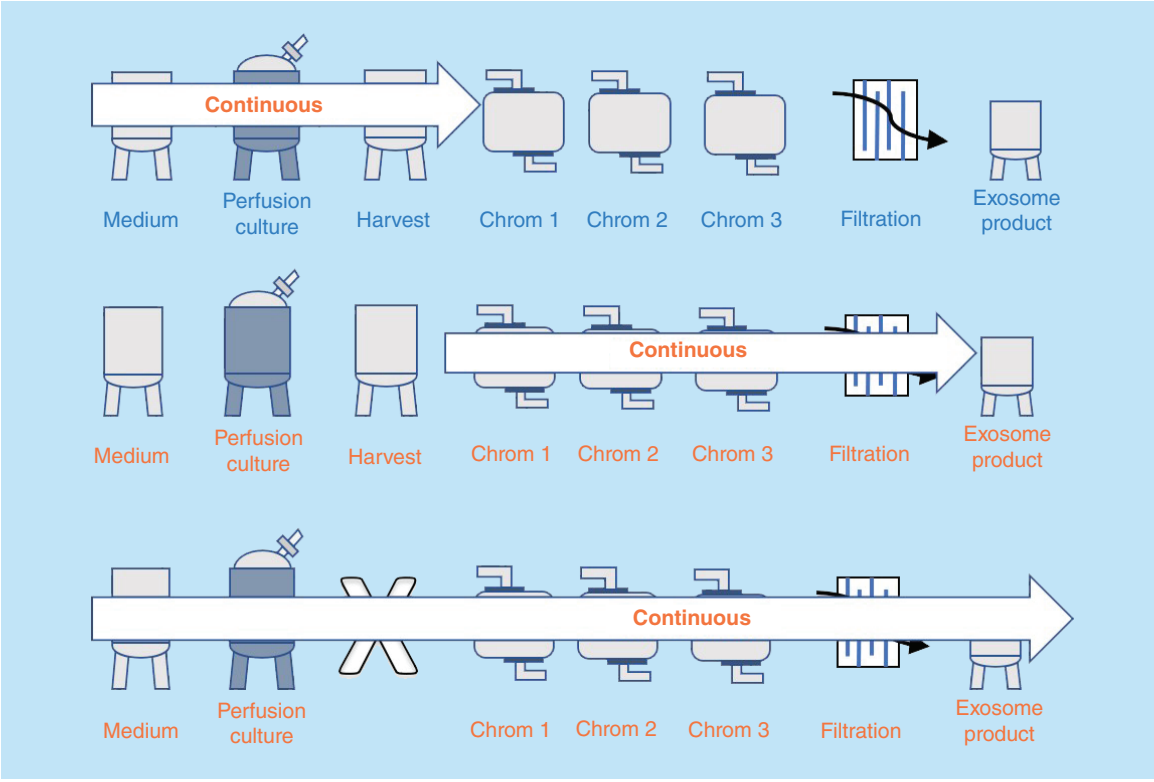

Figure 2. Schematic of model continuous manufacturing approaches.

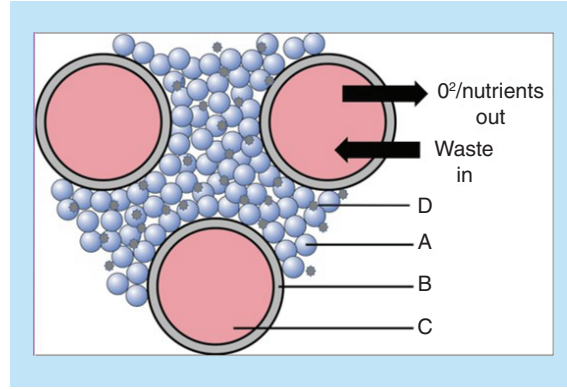

Figure 3. Cross-section of a hollow fiber reactor. Cells **(A)** and exosomes **(D)** in the extracapillary space receive nutrients contained inside **(C)** the semipermeable hollow fiber wall **(B)**.

Finally, it should be mentioned that not only are native or innate exosomes employed in biotechnology and medicine, but more recently populations specifically engineered for purpose are being devised. The type of engineering employed ranges from the design of production methods generating exosomes harboring-specific pharmaceutical content, to those producing exosome populations presenting synthetic surface targeting moieties. Generation of exosomes harboring exogenous therapeutic or imaging agents can be accomplished either *in vivo* or after secretion via several chemical or physical means. Functionalization of the exosome surface ligands for engineered targeting purposes can be accomplished by such approaches as the transfection of the donor cells prior to exosome secretion, or post exosome synthesis through the conjugation of biologically active molecules to harvested exosomes. This latter can be accomplished by click-reactions, such noncovalent chemistries as electrostatic binding, as well as exosome-native transferrin receptor binding [24,25].

Isolation & purification

When isolating exosomes for therapeutic applications, it is advantageous to have a good understanding of specific characteristics of the target exosome subpopulation or target composition of exosomes. This in combination with robust and consistent upstream feed-stream facilitates the downstream process development. As mentioned in the introduction, three major groups of EVs have been described according to mechanism of generation: microvesicles, apoptotic bodies and exosomes. The latter are often assumed to represent a homogenous population of EVs. However, recent work has revealed heterogeneities, and the presence of distinct subpopulations of differing biological properties and such molecular compositions as proteomic and nucleic acid repertoires [26]. Such subpopulations have been reported to mediate differential effects on the gene expression programs in recipient cells. Therefore, it must be considered that differing types and scales of isolation techniques may produce different populations of exosome types. For example, separation based on size solely cannot separate the three major EV groups since these three groups are overlapping in size.

Impurities to remove in the production of exosome-based pharmaceutical ingredients are primarily proteins and host cell DNA but also include cell debris, including nonexosome EVs, in other words, apoptotic bodies and microvesicles. Viral clearance and endotoxin removal must also be considered.

Ultracentrifugation is a commonly used approach for isolating EVs [27] but displays such limitations as low yield and modulation or disruption of the target vesicles and it is not suitable for large-scale exosome manufacturing due to technical and practical limitations. The GMP setup processes with several sequential ultracentrifugation steps, generating exceptionally high centrifugal forces (up to $10^6 \times g$) have been described [28]. A slow-speed centrifugation may, however, be considered for mid-scale manufacturing scales, ranging up to a few hundred liters of upstream batch feed. Exosome precipitation is an alternative approach, but such procedures indicate the addition of components that may not be suitable in a large-scale manufacturing setup and imply subsequent removal and associated regulatory and analytical burden [29,30].

Midstream removal of cell debris can be performed based on a sole normal flow filtration (~5 μm) step and if needed, be complemented with an initial slow-speed centrifugation. These type of midstream steps are usually robust and effective but have an impact on the yield and on the production cost. After removal of large impurities, it is usually appropriate to concentrate the feed-stream using, for example, ultrafiltration with a 500–750 kD cut-off filter and simultaneously remove remaining large impurities.

Ideally, a specific capture step would be based on a technology that is independent of midstream debris removal. Such capture technologies, based on a very porous base matrix or magnetic beads, however, require specific affinity ligands. Such ligands shall preferably be suitable for sanitization to easily fit in a GMP manufacturing process. With good characterization and analytics of the target exosomes, midstream independent affinity capture opportunities are within reach. Candidates for target exosomes epitopes are CD63, CD9, Tim4. Affinity ligands can also be designed to target recombinantly introduced tags such as the Fc-receptor [31]. Externally displayed epitopes, regardless of whether the origin is endogenous or not, are not only subjects for isolation established upon affinity-based purification, but also subjects for vesicle analysis. Antibodies to such externally displayed epitopes can be immobilized and used as analytical tool, ideally employing surface plasmon resonance-based technologies such as Biacore (GE Healthcare, Uppsala, Sweden) for detection of the target exosome population. Proteins associated with the endosomal sorting complex required for transport (Figure 1), such as ALIX, TSG101 [32] and miRNA, are found in the endosome interior and are therefore candidates for employment as analytical markers of the target exosome subpopulation using other traditional analytical techniques such as mass spectrometry, sequencing, electrophoresis – among others.

In the absence of exosome affinity ligands, separation based on a combination of size and charge are recommended to isolate the target exosome population. Ion-exchange resin, primarily anion exchange, offers a scalable method to isolate the target exosomes from impurities with deviating charge density [33,34]. Variation in the density of glycans present on the exosome surface can impact their separation when using ion-exchange chromatography [34]. Utilizing strong ion-exchange in a bind and elute mode determines elution of the target exosomes at relatively high conductivity. Such a process can, for especially enveloped viruses, have detrimental effect on the exosome yield due to modulation of the vesicle integrity. To minimize yield losses it is therefore important to minimize the process time, especially the critical elution phase of the chromatographic step [35,36]. Chromatographic techniques based on resins that rely upon diffusion of the target entity into the beads are associated with relatively long contact times, due to the limited flow that can be employed. An attractive approach to support higher flow rates and

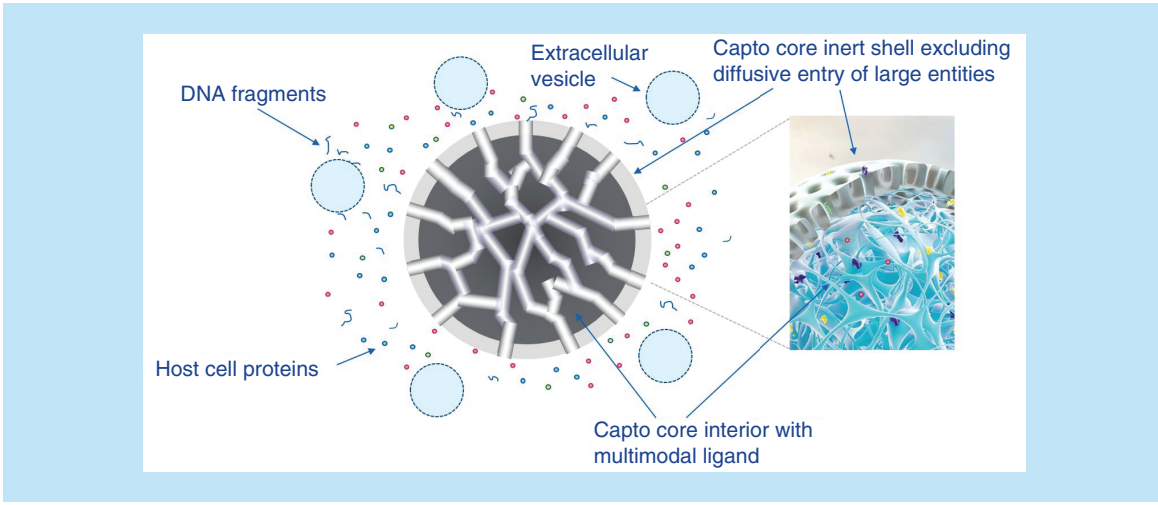

Figure 4. Capto core bead technology principle. Small impurities such as DNA residuals and host cells protein can enter the bead interior by passing the inert shell which has small pore size. The impurities that enter the bead bind almost irreversibly to an octylamine ligand with multimodal functionality. Extracellular vesicles are too big to pass the inert shell and pass in the flow-through.
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thereby shorter contact time and lower yield losses is to use a monolith, membrane or fiber-based chromatographic technique. These allow higher flow as they are independent of allowing the target entity to diffuse into beads [35,37].

Separation solely by charge of the target entity is, in most cases, not satisfactory to generate the desired purity of the target exosome population, and therefore separation based upon size or other properties can be employed to complement separation based on charge [22]. Size separation can be employed before or after an ion-exchange step. The order of these steps must be carefully considered to minimize the need for buffer exchange steps, usually based on ultrafiltration. Another consideration affecting the order of the separation steps is that an ion-exchange step is a good means to reduce potential viral contamination and can provide for endotoxin removal [38].

As the field of exosome manufacturing is so new, a number of creative approaches are appearing. Field-flow fractionation combined with multiangle and dynamic light scattering is being considered a as potentially powerful tool. It combines true size-based separation using field-flow fractionation with an independent determination of size and structure via a light scattering instrument. It can determine highly accurate size distributions and particle concentrations in native solutions. The size fractions may be isolated and automatically collected for further off-line investigation using a standard fraction collector [39].

Size-separation can be effectively performed using chromatographic size-exclusion based on a resin with a pore size that is tailored for the size-interval of the target exosome population. Size-exclusion chromatography is performed with an isocratic flow over a packed resin bed. The bed height must be high, up to 60 cm, to achieve desired resolution. The high bed height in combination with the low sample volume that can be applied in each chromatographic cycle make this size separation technology nonsuitable for large manufacturing. In addition, each chromatographic cycle is long and therefore contributes negatively from a process economy point of view [40].

A more attractive way to remove impurities smaller than the target exosome population is to separate by size, employing a core bead resin. Such resin, for example, Capto Core, is composed of an inactive shell enclosing an activated-ligand core. The inactive shell prohibits large entities from penetrating the smaller pores to the core (Figure 4). Larger entities, in other words, the exosomes, are collected in the flow-through fraction while smaller molecules (such undesired material as DNA, residual host cell proteins and process-related impurities) bind to the resin's active interior ligands. The functionalized core of each bead presents both hydrophobic and positively charged ligands that bind various small contaminants through a highly efficient multimodal chemistry. Different pore size distributions of the inactive shell in combination with flow optimization allow tailoring the size of large entities collected in the flow-through fraction. The beauty of core bead technology is that the bed height can be

reduced as compared with size-exclusion chromatography and a significantly increased flow rate can be employed. These features are valuable in the manufacturing of exosomes at large scale.

The concentration of intermediate or final preparations often requires variation. This can sometimes be accomplished via adjustment of final steps in the means of collection, isolation or reduced through simple dilution. Concentration of an intermediate or final preparation can be accomplished through a variety of means, including ultracentrifugation to a pellet and resuspension or by density gradient centrifugation in sucrose and band collection, and are reviewed in recent publications [41].

The downstream component of an exosome manufacturing process is usually finalized by a buffer-exchange step and virus filtration. The exact final formulation depends on the application area. Exosomes for therapeutic use may have additional and different process steps. Exosomes with paracrine functionality do not need additional processing, while exosomes used as drug delivery vectors may undergo various loading process steps after the chromatographic isolation [24].

Standards, analytics & regulatory

Despite 20 years of research, not only are the basics of exosome biology, occurrence, diversity and identity in their infancy, but also the roles they play in normal cellular and organismal physiology are far from fully understood. Currently, many different analytical techniques have been applied to the identification, quantification and potency evaluation of exosomes – with each measuring one or more of the properties relevant for their detection and isolation mentioned in the Introduction. The field is now populated with a number of immature and empirical descriptions and definitions for a number of reasons. These include an incomplete understanding of the specific characteristics and molecular composition each microvesicle type and subtype, any exosome impurity profile *per se*, as well as the product and impurity consequences of each exosome harvest source, manufacturing and isolation platform. Standardization and regulatory positions are complicated by the number of proposals for the use of purified preparations of exosomes including diverse research, diagnostic, analytic and product implementation modes and formats (e.g., microfluidic). There is also a wide range of contemplated and proposed therapeutic applications and clinical indications. A further consideration here is that, unlike many other biological entities within the medical applications arena, there exist many specific and distinct tissue and cellular sources (e.g., from cultured cells to autologous or allogeneic primary cells) for exosomes or exosome-containing products, with many having a current set of applicable quality and regulatory considerations. Considering many of upcoming *in vitro* or companion diagnostic exosome products, it is not clear to the authors which would be properly filed under, for example, a pre-market approval (PMA), 510(k) or humanitarian device exemption (HDE). Regarding the variety of proposed therapeutic products without precedent: depending upon the particular exosome source, isolation approach, described mode of action and route of administration, in the USA it is not intuitively obvious if one would properly file a new drug application (NDA) or biologic license application (BLA) with Center for Drug Evaluation and Research (CDER) or Center for Biologics Evaluation and Research (CBER).

Manufacturers currently work from previously published approaches or design techniques suitable for their current needs. We know of no publications or guidance by national compendial, standards setting or regulatory agencies on the topic. However, as so many highly regulated applications are being pursued for exosomes, these matters have been the topic of significant discussion during international and professional society meetings – and some few have published initial guidelines, suggestions and considerations on the matter [42–44]. Finally, as over a dozen clinical trials of exosome application are ongoing in the USA alone, sufficient evidence of control for investigational new drugs (INDs) have been established, and no doubt NDA filings for these are in final stages of development. For example, the US FDA has recently cleared a stem cell-based IND application for mesenchymal stem cell-derived exosomes as a therapeutic entity for burn victims [45].

Conclusion

There exist many different production platforms and modes, designed to produce several different types of exosomes, for many divergent applications. Neither standard analytic and isolation techniques been established, nor have standard exosome characterization and subtype definition been achieved. No generic platform or production modes for either the up- or downstream manufacturing of exosomes for any application has been described at this point. Equipment and consumables suitable for exosome manufacturing under GMP are available, but to a great extent these products have been developed for other purposes. Further tailoring and optimization for specific exosome subtypes and needs, within the broader exosome manufacturing arena, is desired. For example, we may

see perfusion-based upstream manufacturing, harvesting and capture based on midstream independent affinity technologies, and closed processing to remove the need for final virus filtration and associated yield losses.

Future perspective

Bioproduction is going through a number of revolutions. Uses for exosomes in research, analytic, preclinical testing, diagnostic and therapeutic applications continue to be discovered. Stem and other primary cells are being produced for several US FDA approved trials in clinical applications. As approvals are established, high efficiency, low-risk methods of production will have to be finalized. Both upstream and downstream manufacturing of protein biologicals, vaccines and cells for cell-based therapies are being transformed by such concepts and technologies as single-use, *Industry 4.0*, factory-of-the-future, modular, continuous and intensified manufacturing initiatives. There is no reason to believe that each of these advances will not influence approaches to both the culture of the cell platforms employed in, as well as the actual, upstream and downstream, production of exosomes themselves.

Executive summary

- Exosomes are a type of extracellular vesicle of unique generation.
- Their contents are quite diverse and can vary following many factors.
- They naturally occur in nearly every bodily fluid and many cultured cells.
- Interest is due to potential in research, diagnostics, analytics and therapeutics.
- Characterized by morphology, size, density, ζ potential, composition/properties.
- Large-scale production procedures are influenced by the exosome's application.
- Isolation and purification of exosomes has in general proven to be difficult.
- Pure populations are only achieved through a combination of techniques.

Production of exosomes

- Many cultured cells will secrete exosomes using standard T-flasks and cell culture media.
- It is often necessary to produce exosomes from less established primary or stem cell lines.
- Alterations in culture environmental parameters can affect exosome yield/characteristics.
- Scaling-up from T-flasks can cause issues that have often been addressed in the literature.
- Serum-free culture is discouraged for reasons of attachment and exosome characteristics.
- Successful serum-reduced (or free) culture reported in some cell types/production modes.
- When serum is employed in actual production, reduced-exosome serum is recommended.
- Many factors, metabolites, surface-active proteins, factors implicated in quality production.
- Large-scale: mainly stirred-tank reactor/microcarriers, fixed bed or hollow fiber bioreactors.
- Culture methods: feeder cells, conditioned media, extracellular matrices, engineered cells.
- Exosomes method: vesicle and virus-mimetic, various hybrid-fusion, mini-extruder effected.

Large-scale production

- Formats: T-225 flasks, multilayer flasks, large fixed-bed reactors, microcarriers in stirred-tank reactor.
- All issues of modern technical transfer will exist for development of large-scale manufacturing format.
- Concerns arise from, for example, cells-to-microcarrier binding, gas mass-transfer and the shear forces.
- Perfusion-based bioproduction can alleviate many bioreactor culture and technology transfer concerns.
- Many membrane-based flasks or bioreactors as perfusion-capable technologies have been employed.
- Perfusion supports culture over an extended period of time and yields concentrated lots of exosomes.
- Many porous 3D scaffolds, fiber-based packed- and fixed-bed bioreactors have been adapted for use.
- Hollow-fiber perfusion bioreactors are now being successfully used in large-scale exosome production.
- Perfusion supports high density, continuous culture, reduced or eliminated serum/factor requirement.
- Exosomes specifically engineered for research, diagnostic or therapeutic purposes are being designed.
- From harboring pharmaceutical content, to populations presenting synthetic surface targeting moieties.
- Exosomes with exogenous therapeutic or imaging agent cargo produced via chemical or physical ways.

Isolation & purification

- Scalable solutions exist that support regulated therapeutic applications.
- Support for bioreactor harvest from batch- or perfusion-based upstream manufacturing.
- Filter-based removal of cell debris and large-sized process impurities.
- Isolation and purification based on bind/elute chromatography and size-specific separation.
- Polishing methods to control impurities.

Standards, analytic & regulatory

- Affected by that exosome biology, occurrence, diversity and identity are in their infancy.
- Many analytical techniques have been applied to the identification, quantification and potency.
- Immature and empirical descriptions, definitions because of ill-defined characteristics, molecular composition.
- Standardization and regulatory positions are complicated by the number and diversity of exosome considerations.
- Including sources, isolation and processing approaches, described mode of action and route of administration.
- Many diverse research, diagnostic, analytic and therapeutic product implementation modes/formats exist.
- No publications or guidance by national compendial, standards setting or regulatory agencies on the topic.
- US FDA has cleared stem cell-based investigational new drug applications for mesenchymal stem cell-derived exosomes as therapeutic entities.

Conclusion

- Neither standard analytic nor isolation techniques have been established.
- No standard exosome characterization and subtype definition have been achieved.
- No generic platform or production modes for either up- or downstream manufacturing.
- Equipment and consumables suitable for exosome manufacturing under GMP are available.
- Different production platforms and modes are designed to produce different types of exosomes.

Future perspective

- More research, analytic, preclinical testing, diagnostic and therapeutic applications are yet to be discovered.
- Future seeing single-use, *Industry 4.0*, factory-of-the-future, modular, continuous and intensified approaches.

Financial & competing interests disclosure

Whitford W and Guterstam P are both employed by GE Healthcare a supplier of equipment and consumables for the manufacturing of biological therapeutics. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

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Mesenchymal stem cell exosome: a novel stem cell-based therapy for cardiovascular disease

Cardiovascular disease is a major target for many experimental stem cell-based therapies and mesenchymal stem cells (MSCs) are widely used in these therapies. Transplantation of MSCs to treat cardiac disease has always been predicated on the hypothesis that these cells would engraft, differentiate and replace damaged cardiac tissues. However, experimental or clinical observations so far have failed to demonstrate a therapeutically relevant level of transplanted MSC engraftment or differentiation. Instead, they indicate that transplanted MSCs secrete factors to reduce tissue injury and/or enhance tissue repair. Here we review the evidences supporting this hypothesis including the recent identification of exosome as a therapeutic agent in MSC secretion. In particular, we will discuss the potential and practicality of using this relatively novel entity as a therapeutic modality for the treatment of cardiac disease, particularly acute myocardial infarction.

KEYWORDS: acute myocardial infarction ■ exosome ■ mesenchymal stem cell ■ paracrine secretion

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Stem cells in the treatment of acute myocardial infarction

Acute myocardial infarction (AMI) is the primary cause of disease-related death in the world [1–3]. It is characterized by the disruption of blood supply to the heart muscle cells, which lead to myocardial infarction or death of cardiomyocytes. Reperfusion therapy or the restoration of blood flow by thrombolytic therapy, bypass surgery or percutaneous coronary intervention (PCI) is currently the mainstay of treatment for AMI and is responsible for the significant reduction in AMI mortality [4]. The efficacy of reperfusion therapy has led to increasing survival of patients with severe AMI who would not otherwise survive. However, many (23%) of these survivors progress to fatal heart failure within 30 days [5]. This phenomenon of an increasing number of severe AMI survivors contributes to an ever growing epidemic of heart failures [6–8].

Heart failure is characterized by dilatation and hypertrophy with fibrosis within the myocardium. The progression of an AMI survivor to heart failure is a multifactorial process that has been hypothesized to include the development of myocardial stunning and hibernation, remodeling and chronic neuroendocrine activation [9], and is dependent on the extent of the AMI suffered by the patient [10–15]. The development of reperfusion therapy and its subsequent improvements have significantly increased the salvage of ischemic myocardium from infarction and reduced infarct size, but further substantive improvement to reperfusion therapy is likely to require adjunctive therapies.

Although it was recognized as early as 1960 that reperfusion of severely ischemic tissue causes lethal injury [16], the concept that reperfusion causes *de novo* lethal injury became more widely accepted only when infarct size was shown to be reduced by interventions applied at the onset of reperfusion (reviewed in [10]). Such interventions, also known as postconditioning, involve ischemic conditioning or application of pharmacological agents before the onset of reperfusion, and have demonstrated some protection against reperfusion injury in animals and in small clinical trials. However, none of these agents have proven to be efficacious in large clinical trials and this has led to speculations that reducing reperfusion injury may not be tractable to pharmaceutical interventions [17].

With the emergence of stem cells as potential therapeutic agents, attempts to use stem cells to reduce infarct size and enhance cardiac function in animal models and patients have increased exponentially. To date, stem cell therapy for the heart accounts for a third of publications in the regenerative medicine field [18]. Mummery *et al.* have recently reviewed the use of both adult and embryonic stem cells, such as bone marrow-derived stem cells, which include hematopoietic stem cells (HSCs) and mesenchymal stem cells (MSCs), endogenous cardiac progenitor cells (CPCs), human embryonic stem cells (hESCs),

future medicine part of fsg

induced pluripotent stem cells, and hESC-derived cardiomyocytes [18]. The use of bone marrow-derived stem cells such as HSCs and MSCs to repair cardiac tissues was predicated on the hypothesis that these cells could differentiate into cardiomyocytes and supporting cell types. However, careful rodent experimentation has demonstrated that few of the transplanted bone marrow cells engraft and survive, and fewer cells differentiate into cardiomyocytes or supporting cells [18]. In spite of this, transplantation of bone marrow stem cells improves some cardiac functions in animal models and patients, and this has been attributed to a paracrine effect [18]. Although the presence of CPCs in fetal hearts is well established, the presence of CPCs in postnatal or adult heart remains controversial, and the possibility that the so-called CPCs from postnatal hearts are bone marrow cells has remained unresolved. Transplanted cardiomyocytes isolated from *in vitro* differentiation of hESCs and induced pluripotent stem cells could engraft in the heart to form a synctium with each other, but not with the recipient heart. This failure to couple with the recipient cardiomyocytes could cause arrhythmia, a potentially fatal condition.

Despite our still evolving understanding of stem cell transplantation in treating cardiac disease, stem cell transplantation has already being tested in clinical trials. In a recent review of more than 20 clinical trials that primarily used adult stem cells, such as bone marrow stem cells, mobilized peripheral blood stem cells and skeletal myoblasts to treat heart disease [19], the trends favored such transplantations to treat cardiac disease when measured using clinical end points of death, recurrence of AMI or hospitalization for heart failure. The failure to elicit a more robust therapeutic response has been attributed to low engraftment of cells and poor survival of engrafted cells with an untested caveat that improved engraftment and survival will enhance the therapeutical efficacy. A general consensus from these clinical trials is that bone marrow- or blood-derived stem cells do not replenish lost cardiomyocytes or vascular cells to any meaningful extent. Instead, circumstantial evidence suggests that these stem cells secrete factors that exert a paracrine effect on the heart tissues [19].

MSCs & the treatment of cardiovascular disease

Among the stem cells currently being tested in clinical trials for the heart, MSCs are the most widely used stem cells. Part of the reason for this is their easy availability in accessible tissues, such as bone marrow aspirate and fat tissue [20], and their large capacity for *ex vivo* expansion [21]. MSCs are also known to have immunosuppressive properties [22] and, therefore, could be used in allogeneic transplantation. They are also reported to have highly plastic differentiation potential that included not only adipogenesis, osteogenesis and chondrogenesis [23–28], but also endothelial, cardiovascular [29], neurogenic [30–32] and neovascular differentiation [33–35]. MSCs transplantation in most animal models of AMI generally resulted in reduced infarct size, improved left ventricular ejection fraction, increased vascular density and myocardial perfusion [36–40]. In a recent Phase I, randomized, double-blind, placebo-controlled dose-escalation clinical trial, single infusion of allogeneic MSCs in patients with AMI was documented to be safe with some provisional indications that the MSC infusion improved outcomes with regard to cardiac arrhythmias, pulmonary function, left ventricular function and symptomatic global assessment [41].

Despite numerous studies on the transplantation of MSCs in patients and animal models, insight into the mechanistic issues underlying the effect of MSC transplantation remains vague. An often cited hypothesis is that transplanted MSCs differentiate into cardiomyocytes and supporting cell types to repair cardiac tissues. However, contrary to this differentiation hypothesis, most transplanted MSCs are entrapped in the lungs and the capillary beds of tissues other than the heart [42,43]. Furthermore, depending on the method of infusion, 6% or less of the transplanted MSCs persist in the heart 2 weeks after transplantation [44]. In addition, transplanted MSCs were observed to differentiate inefficiently into cardiomyocytes [45] while ventricular function was rapidly restored less than 72 h after transplantation [46]. All these observations are physically and temporally incompatible with the differentiation hypothesis and have thus prompted an alternative hypothesis that the transplanted MSCs mediate their therapeutic effect through secretion of paracrine factors that promote survival and tissue repair [47].

Paracrine secretion of MSCs

Paracrine secretion of MSCs was reported more than 15 years ago when Haynesworth *et al.* [48] reported that MSCs synthesize and secrete a broad spectrum of growth factors and cytokines such as VEGF, FGF, MCP-1, HGF, IGF-I, SDF-1 and thrombopoietin [49–53], which exert effects on cells in their vicinity. These factors have been postulated to promote arteriogenesis [51]; support the

stem cell crypt in the intestine [54]; protect against ischemic renal [49,50] and limb tissue injury [52]; support and maintain hematopoiesis [53]; and support the formation of megakaryocytes and proplatelets [55]. Many of these factors have also been demonstrated to exert beneficial effects on the heart, including neovascularization [56], attenuation of ventricular wall thinning [39] and increased angiogenesis [57,58].

In 2006, Gnecchi *et al.* demonstrated that intramyocardial injection of culture medium conditioned by MSCs overexpressing the Akt gene (Akt-MSCs) or Akt-MSCs reduced infarct size in a rodent model of AMI to the same extent [46]. This provided the first direct evidence that cellular secretion could be cardioprotective [46,59]. The authors subsequently attributed the cardioprotective effect of the conditioned medium to the culturing of the cells under hypoxia and the overexpression of *AKT*, which induced secretion of Sfrp2. siRNA mediated-silencing of *Sfrp2* expression in Akt-MSCs abrogated the cytoprotective effect of their secretion [60].

Our group recently demonstrated that culture medium conditioned by human ESC-derived MSCs (hESC-MSCs) significantly reduced infarct size by approximately 50% in a pig and mouse model of myocardial ischemia/reperfusion (MI/R) injury when administered intravenously in a single bolus just before reperfusion [61]. However, these MSCs were derived from hESCs instead of rat bone marrow and were not genetically modified to overexpress Akt. The conditioned medium was prepared using a chemically defined medium without hypoxia treatment.

We further demonstrated through size fractionation studies that the active component was a large complex 50–200 nm in size. Using electron microscopy, ultracentrifugation studies, mass spectrometry and biochemical assays, we identified this complex as an exosome, a secreted bi-lipid membrane vesicle of endosomal origin (FIGURE 1). When purified by size exclusion using high-performance liquid chromatography, hESC-MSC exosomes also reduced infarct size, but at a tenth of the protein dosage used for conditioned medium [62]. We subsequently showed that exosomes constitute about 10% of the conditioned medium in terms of protein amount [LAI RC, LIM SK, UNPUBLISHED DATA]. Therefore, the therapeutic activity in the hESC-MSC conditioned medium could be attributed primarily to the exosome [62]. The secretion of cardioprotective exosomes was not unique to hESC-MSCs and was also found to be produced under nonhypoxic culture conditions by MSCs derived from aborted fetal tissues [63]. Therefore, these observations suggest that the secretion of protective exosomes is a characteristic of MSCs and may be a reflection of the stromal support role of MSCs in maintaining a microenvironmental niche for other cells such as hematopoietic stem cells. The secretion of exosomes may also be a dominant function of MSCs. We recently observed that when GFP-labeled exosome-associated protein CD81 is expressed in hESC-MSCs (FIGURE 2A), they exhibit a punctate cytosolic distribution and these labeled proteins were secreted (FIGURE 2C). CD81 is a classical tetraspanin membrane protein usually found localized to the plasma membrane (as typified by their distribution in HEK 293 cells) (FIGURE 2B). The cellular distribution of the labeled CD81 in hESC-MSCs and its cellular secretion suggest that MSCs are prolific producers of exosomes, and that exosome, whose main function is to mediate intercellular communication (as discussed later), is also MSCs' vehicle of choice for intercellular communication.

What are exosomes?

Exosomes are one of several groups of secreted vesicles, which also include microvesicles, ectosomes, membrane particles, exosome-like vesicles or apoptotic bodies (reviewed in [64]). Exosomes were first found to be secreted by sheep reticulocytes approximately 50 years ago [65,66]. They have since been shown to be secreted by many cell types, including B cells [67], dendritic cells [68], mast cells [69], T cells [70], platelets [71], Schwann cells [72], tumor cells [73] and sperm [74]. They are also found in physiological fluids such as normal urine [75], plasma [76] and bronchial lavage fluid [77].

Compared with other secreted vesicles, exosomes have much better defined biophysical and biochemical properties(reviewed in [64]). They have a diameter of 40–100 nm, with a density in sucrose of 1.13–1.19 g/ml, and can be sedimented at 100,000 g. Their membranes are enriched in cholesterol, sphingomyelin and ceramide, and are known to contain lipid rafts. The presence of exposed phosphatidylserine was reported to be present on the membrane of some exosomes [78,79] and absent from others [80,81]. Exosomes contain both proteins and RNAs. Most exosomes have an evolutionarily conserved set of proteins, including tetraspanins (CD81, CD63 and CD9), Alix and Tsg101, but they also have unique tissue/cell type-specific proteins that reflect their cellular source. Mathivanan and Simpson have set up ExoCarta, a freely accessible web-based compendium of proteins and RNAs found in exosomes [82,201].

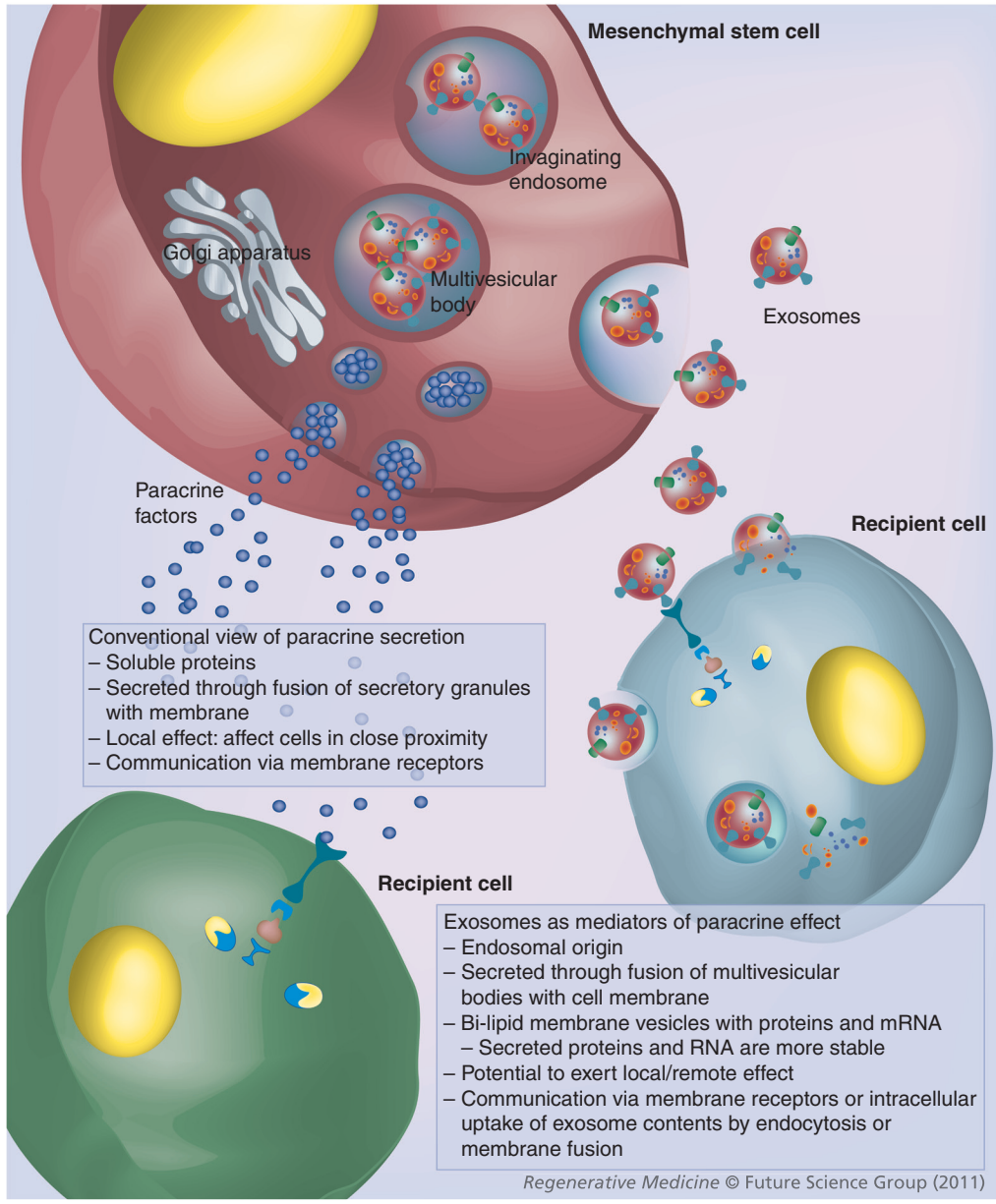

Figure 1. Paracrine effects of mesenchymal stem cells.

The functions of exosomes are not known, but they are believed to be important for intercellular communications. Exosomes were first documented in 1996 to mediate immune communication when it was observed that, when secreted by antigen-presenting cells (APCs), they bear functional peptide–MHC complexes [67]. This also provides the implication that exosomes could be used therapeutically. The therapeutic potential of exosomes was subsequently illustrated by the use of exosomes secreted by tumor peptide-pulsed dendritic cells to suppress tumor growth [68]. Ironically, exosomes are also implicated in tumorigenesis, with the observation that microvesicles mediate intercellular transfer of the oncogenic receptor EGFRvIII [83]. Exosomes have also been reported to have the potential to protect against tissue injury such as MI/R injury [62] or acute tubular injury [84].

In recent years, exosomes have also been implicated in neuronal communication or pathogenesis. For example, exosomes have been found to be released by neurons [85], astrocytes [86] and glial cells [87] to facilitate diverse functions that include removal of unwanted stress proteins [88] and amyloid fibril formation [89,90]. Exosomes containing α-synuclein have been demonstrated to cause cell death in neuronal cells, suggesting that exosomes may amplify and propagate Parkinson's disease-related pathology [91,92]. It was also reported that, in Alzheimer's disease,

β-amyloid is released in association with exosome [93]. More recently, oligodendrocytes were demonstrated to secrete exosomes to coordinate myelin membrane biogenesis [94]. Besides neuronal communication, exosomes secreted by cardiomyocyte progenitor cells were reported

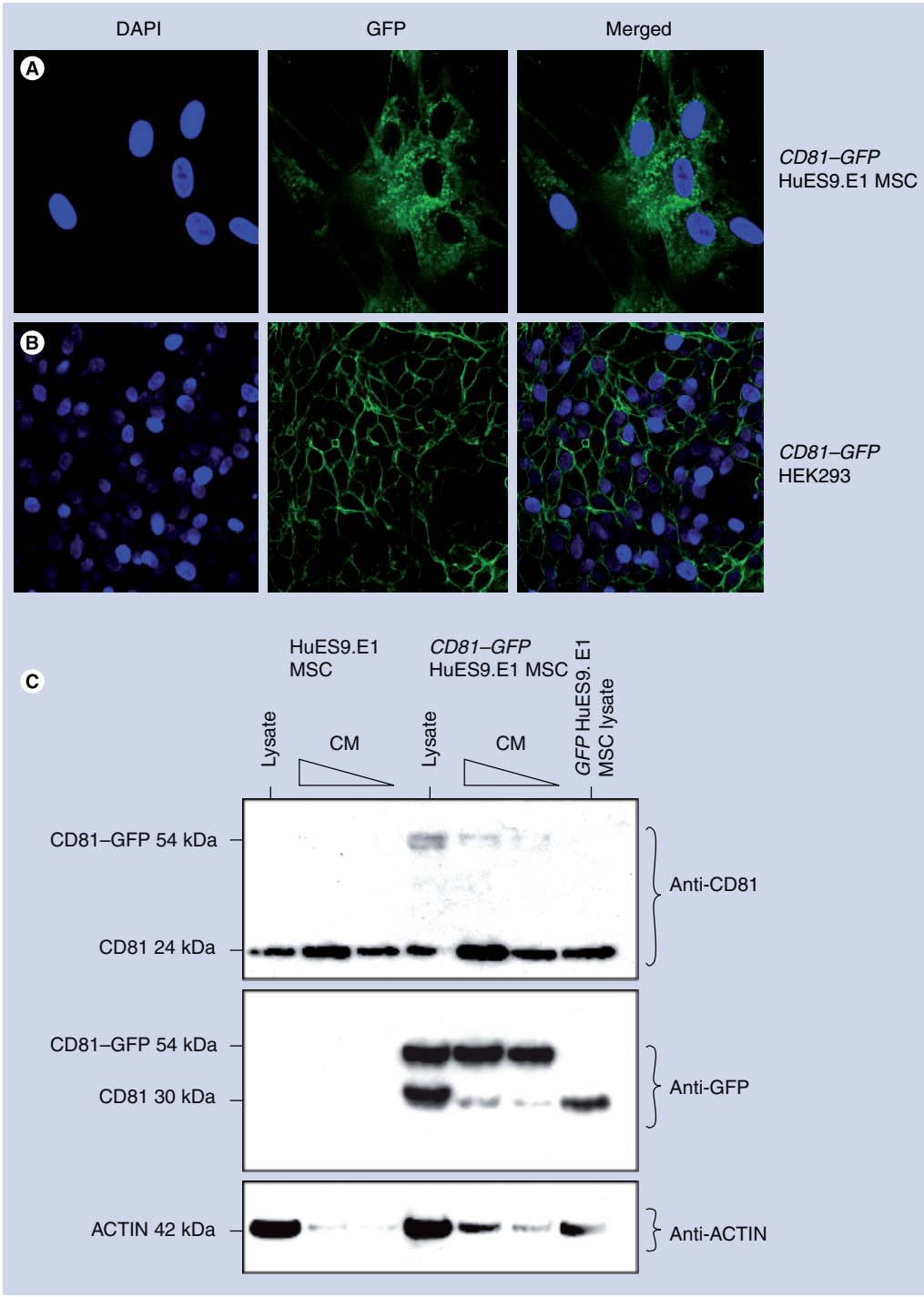

Figure 2. Expression and detection of CD81–green fluorescent fusion protein in cell lines.
(A & B) Expression of *CD81–GFP* in HuES9.E1 MSC and HEK293 cells. A *CD81–GFP* fusion gene was cloned into a pLVX-puro lentiviral expression vector to generate a CD81–GFP lentivirus. After infecting HuES9.E1 MSCs and HEK293 cells with the virus followed by drug selection, the cells were seeded onto a glass chamber slide and stained for DAPI. **(C)** Secretion of CD81–GFP fusion protein. Cell lysate and CM were prepared from HuES9.E1 MSCs and *CD81–GFP*-transfected HuES9.E1 MSCs. The cell lysate and CM were analyzed by western blot hybridization using antibodies against CD81 (top panel), GFP (middle panel) and ACTIN (bottom panel).
CM: Conditioned media; GFP: Green fluorescent protein; MSC: Mesenchymal stem cell.

to stimulate the migration of the endothelial cells [95], while those secreted by the egg facilitate the fusion of the sperm and egg [96].

Exosomes have also been implicated as a vehicle for viral and bacterial infection (reviewed in [97]), including the assembly and release of HIV [98–100] and intercellular spreading of infectious prions in transmissible spongiform encephalopathies. The association of exosomes with disease or pathological conditions makes exosomes good sentinels for diseases. It was reported that the miRNA profile of circulating exosomes could be indicative or diagnostic of ovarian cancer [101]. Similarly, the proteins in the urinary exosome have been demonstrated to reflect acute kidney injury and are candidate diagnostic markers [102]. More recently, the function of exosomes as vehicles for intercellular communication has been exploited for the delivery of therapeutic siRNAs to the brain and to provide for alternative drug delivery systems [103].

Exosome as an alternative therapeutic of MSCs?

The paracrine hypothesis introduces a radically different dimension to the therapeutic applications of MSCs in regenerative medicine. By replacing transplantation of MSCs with administration of their secreted exosomes, many of the safety concerns and limitations associated with the transplantation of viable replicating cells could be mitigated. For example, the use of viable replicating cells as therapeutic agents carries the risk that the biological potency of the agent may persist or be amplified over time when the need has been resolved, and cannot be attenuated after treatment is terminated. This could lead to dire consequences, especially if treatment was terminated as a recult of adverse outcomes. Although repeated direct endomyocardial transplantation of MSCs has been demonstrated to be relatively safe [104], intravascular administration could lead to occlusion in the distal microvasculature as a consequence of the relatively large cell size [105]. Transplantation of MSCs has been reported to induce proarrhythmic effects [106–108]. Their potential to differentiate into osteocytes and chondrocytes has also raised long-term safety concerns regarding ossification and/or calcification in tissues as reported in some animal studies [109].

Besides mitigating the risks associated with cell transplantation, exosomes can also circumvent some of the challenges associated with the use of small soluble biological factors such as growth factors, chemokines, cytokines, transcription factors, genes and RNAs [110]. The delivery of these factors to the right cell type and, in the case of those factors that work intracellularly, the delivery into the right cellular compartments, while maintaining the stability, integrity and biological potency of these factors during manufacture, storage and subsequent administration remains a costly challenge. As a bi-lipid membrane vesicle, exosomes not only have the capacity to carry a large cargo load, but also protect the contents from degradative enzymes or chemicals. For example, protein and RNA in MSC exosomes were protected from degradation by trypsin and RNase as long as the lipid membrane was not compromised [62,111]. We also found that storage without potentially toxic cryopreservatives at -20°C for 6 months did not compromise the cardioprotective effects of MSC exosomes or their biochemical activities [Lai RC, Lim SK, Unpublished Data].

Exosomes are known to bear numerous membrane proteins that have binding affinity to other ligands on cell membranes or the extracellular matrix, such as transferrin receptor, tumor necrosis factor receptors, lactadherin, integrins and tetraspanin proteins (e.g., CD9, CD63 and CD81) [82]. These membrane bound molecules provide a potential mechanism for the homing of exosomes to a specific tissue or microenvironment. For example, integrins on exosomes could home exosomes to cardiomyocytes that express ICAM1, a ligand of integrins after MI/R injury [112], or to VCAM-1 on endothelial cells [113]. Tetraspanin proteins, which function primarily to mediate cellular penetration, invasion and fusion events [114], could facilitate cellular uptake of exosomes by specific cell types.

Exosomes may also facilitate the uptake of therapeutic proteins or RNAs into injured cells. Although cellular uptake of exosomes has been demonstrated to occur through endocytosis, phagocytosis and membrane fusion [115–117], the mechanism by which these processes are regulated remains to be determined. It was observed that the efficiency of exosome uptake correlated directly with intracellular and microenvironmental acidity [117]. This may be a mechanism by which MSC exosomes exert their cardioprotective effects on ischemic cardiomyoctyes that have a low intracellular pH [118].

Despite being smaller than a cell, exosomes are relatively complex biological entities that contain a range of biological molecules, including proteins and RNA, making them an ideal

therapeutic candidate to treat complex injuries such as MI/R injury. It is well established that MI/R injury occurs paradoxically in response to a therapy that is highly effective in resolving the disease precipitating problem of no flow and ischemia. During MI/R injury, the restoration of blood and oxygen to ischemic myocardium paradoxically exacerbates the ischemia-induced cellular insults. This is because the biochemical cascades required for cell survival that are initiated by cells during no flow and ischemia [119] are not compatible with the rapid restoration of flow and oxygen supply, and at the same time, cells cannot alter their biochemical activities expeditiously enough to adapt to this restoration. This latter phenomenon was best evidenced by the reduction of MI/R through postreperfusion conditioning or postconditioning where cells were exposed to repeated short nonlethal cycles of reperfusion/ischemia to facilitate biochemical adaptation to reperfusion [120–131].

We postulate that with their complex cargo, exosomes would have adequate potential to participate in a wide spectrum of biochemical and cellular activities, and simultaneously target and correct the various ischemia-induced cascades, and prevent occurrence of the paradoxical reactions induced by reperfusion. In addition, many of the proteins in the exosomes are enzymes. Since enzyme activities are catalytic rather than stoichiometric, and are dictated by their microenvironment (e.g., substrate concentration or pH), the enzyme-based therapeutic activities of exosomes could be activated or attenuated according to the release of injury-associated substrates, which in turn, is proportional to the severity of disease-precipitating microenvironment. Resolution of the disease-precipitating microenvironment would reduce the release of injury-associated substrates and also the activity of exosome enzymes. Consequently, the efficacy of exosome-based therapeutics could be highly responsive to, but also limited by, the disease-precipitating microenvironment.

Together, the features discussed here render exosomes a highly efficacious therapeutic in neutralizing the complexity of MI/R and an effective adjuvant to complement current reperfusion therapy.

Translating hESC-MSC exosomes into therapeutics

The translation of cardioprotective MSC exosomes into a therapeutic agent presents several unique challenges. The first major challenge would be to manufacture Good Manufacturing Practices (GMP) grade exosomes from nonautologous cell sources. Although exosomes as therapeutics have already been tested as a form of cancer vaccine in the clinic [132–134], these tests were limited to exosomes produced during short-term *ex vivo* culture of autologous dendritic cells. These exosomes, also known as dexosomes, were found to be safe in the small clinical trials [132]. Unfortunately, the manufacture of these exosomes cannot provide guidance for the large-scale GMP production of exosomes from nonautologous cell sources such as exosomes from hESC-MSCs. This manufacturing process faces many unique challenges, including ethical, legal, technical and regulatory/safety issues.

The use of hESCs for the derivation of MSCs presents both ethical and legal challenges. While ethical objections to the derivation and use of hESCs have initially hindered hESC research, they have abated. Instead, the use and applications of hESCs is now being hindered by complex and widespread patenting in some countries [135] and the ban on stem cell-related patents in other countries [136]. To encourage the development of hESC-based therapeutic applications, the need for freedom to use and share hESC resources and knowledge must be balanced with a need to incentivize commercial development of stem cells by protecting the intellectual property generated from research and development efforts. Unfortunately, this balance has not yet been reached.

One of the major technical hurdles to the use of hESC-MSCs is their large but finite expansion capacity, resulting in the need for constant costly re-derivation from hESC and re-validation of each of the derived cell batch. Therefore, a robust scalable and highly renewable cell source will be central to the development of a commercially viable manufacturing process for the production of MSC exosomes in sufficient quantity and quality to support clinical testing or applications. To address this issue, we demonstrated that immortalization of the ESC-MSC by Myc did not compromise the quality or yield of exosomes [137]. Therefore, this provides a potentially inexhaustible cell source for MSC exosome production. The translation of MSC exosomes into clinical applications is also complicated by the relative novelty of exosomes with few precedents in the regulatory and safety space of biopharmaceuticals. This will require the formulation of new standards for manufacture, safety and quality control.

Future perspective

The discovery of stem cells and their potential in regenerative medicine has evoked much excitement and hope in treating some of today's most intractable diseases, including cardiac disease. However, much of the euphoria has dissipated as animal experimentation revealed and identified potential problems in translating the use of stem cells to treat cardiac disease. Although the reproducible large-scale preparation of homogenous clinically compliant 'normal' healthy cells has been a major preoccupation in the development of stem cell-based therapies in general, this has proven not to be an impediment in the development of such therapies for cardiac disease, as evidenced by the large number of stem cell-based clinical trials that are already being conducted. Instead, the problems facing stem cell-based therapies for cardiac disease are potentially more insidious. At present, most of the stem cells used in clinical trials are MSCs and bone marrow mononuclear cells that are generally considered to be safe. However, despite eliciting a sometimes positive therapeutic response, these cells often do not integrate or persist in the heart tissues. By contrast, the use of myogenic cells, such as

Executive summary

Stem cells in the treatment of acute myocardial infarction

- Advances in reperfusion therapy have increased survival of patients with severe acute myocardial infarction and contributed to a growing epidemic of heart failure.
- As reperfusion therapy itself causes lethal injury and has been demonstrated to be intractable to pharmaceutical intervention, stem cells are being scrutinized as alternative therapeutic agents.
- Attempts using stem cells to treat heart disease have generated mixed outcomes.
- Transplantation of bone marrow stem cells generally improved cardiac functions with little evidence of engraftment and differentiation of the transplanted stem cells.
- Effects of stem cell transplantation have been attributed to secretion of paracrine factors by the transplanted stem cells.

Mesenchymal stem cells & the treatment of cardiovascular disease

- Animal studies and early clinical trials demonstrated that mesenchymal stem cell (MSC) transplantation improved cardiac function after myocardial infarction.
- Inefficient MSC engraftment and differentiation, and their rapid cardioprotective effects suggested that MSCs act via a secretion-based paracrine effect rather than a cell replacement effect.

Paracrine secretion of MSCs

- MSCs synthesize a broad spectrum of growth factors and cytokines that exert paracrine effects.
- Gnecchi *et al*. produced the first evidence that cellular secretion alone improved cardiac function in an animal model of acute myocardial infarction.
- Culture medium conditioned under nonhypoxic conditions by untransformed MSCs derived from human embryonic stem cells or aborted fetal tissues reduce infarct size in animal models of myocardial ischemia/reperfusion.
- Exosome is the primary mediator of MSCs' paracrine effect.

What are exosomes?

- Exosomes are bi-lipid membrane vesicles secreted by many cell types into culture medium and other bodily fluids such as blood and urine.
- They function as mediators of intercellular communication.

Exosome as an alternative therapeutic for MSC?

- Exosome-based therapy circumvents some of the concerns and limitations in using viable replicating cells and does not compromise some of the advantages associated with using complex therapeutic agents such as cells.
- Exosomes are ideal therapeutic agents because their complex cargo of proteins and genetic materials has the diversity and biochemical potential to participate in multiple biochemical and cellular processes, an important attribute in the treatment of complex disease.
- Exosomes home to specific tissue or microenvironment.
- Their bi-lipid membranes can protect their biologically active cargo allowing for easier storage of exosomes, which allows a longer shelf-life and half-life in patients.
- Their biological activities are mainly enzyme-driven and, therefore, their effects are catalytic and not stoichiometric.
- Having enzyme-driven biological activities, they are dependent on the microenvironment (e.g., substrate concentration or pH) and could be activated or attenuated in proportion to the severity of disease-precipitating microenvironment.
- Exosome-based therapy cannot replace lost myocardium but can prevent or delay loss of myocardium.

Challenges for translating embryonic stem cell-MSC exosomes into therapeutics

- Ethical issues exist, especially with the derivation and use of human embryonic stem cells for generating MSCs.
- Legal issues include excessive intellectual property protection in some countries, which hinder research and development. A ban on embryonic stem cell-related intellectual property in other countries de-incentivize research and development.
- Technical limitations include the need for a robust scalable and highly renewable cell source embryonic stem cell-MSCs to support large scale, commercially viable manufacturing process.
- Exosomes are relatively novel biological entities with few precedents to establish safety and manufacturing guidance.

skeletal myoblasts, cardiac progenitors or stem cell-derived cardiomyocytes, to replace lost myocardium has been demonstrated to increase the risk of arrhythmias when the donor cells failed to couple with the host tissues, in early clinical trials and animal studies. Resolution of these problems would require the development of cell delivery or cell engraftment techniques that can facilitate proper mechanistic integration of the donor cells into the recipient tissues to enable coordinated heart functions. Other potential problems include problems that are generally universal in cell-based therapy, such as host rejection and risk of tumor formation. We anticipate that aside from the issue of proper integration of donor cells into the recipient heart, many of these problems will be resolved or partially resolved in the next 5–10 years. However, without the resolution of the poor coupling between donor and recipient cells, it is unlikely that cell-based therapy using stem cells to replace lost myocardium will evolve into a standard therapy for the treatment of cardiac disease. Although paracrine secretion of stem cells provides an alternative approach for the development of stem cell-based therapies, it does not replace the need for cell-based therapy to replace lost myocardium. However, it may reduce cardiac injury and delay the loss of myocardium to the extent that replacement of lost myocardium does not become critical. The identification of exosomes as the cardioprotective factor in MSC secretion reduces the paracrine secretion to a single biological entity that is more amenable to the stringent criteria for clinically compliant preparation and use. As a bi-lipid membrane vesicle with many membrane-bound proteins and a diverse cargo, exosome represents an ideal therapeutic agent that has the potential to home to target tissues and treat complicated diseases such as MI/R injury. With the advance of new bioengineering and cellular modification techniques, engineering or modification of the exosome surface antigen and internal content will enable it to target other more complex diseases with even more specificity.

Financial & competing interests disclosure

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

No writing assistance was utilized in the production of this manuscript.

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Review

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Regenerative Medicine

Microtechnology applied to stem cells research and development

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Microfabrication and microfluidics contribute to the research of cellular functions of cells and their interaction with their environment. Previously, it has been shown that microfluidics can contribute to the isolation, selection, characterization and migration of cells. This review aims to provide stem cell researchers with a toolkit of microtechnology (mT) instruments for elucidating complex stem cells functions which are challenging to decipher with traditional assays and animal models. These microdevices are able to investigate about the differentiation and niche interaction, stem cells transcriptomics, therapeutic functions and the capture of their secreted microvesicles. In conclusion, microtechnology will allow a more realistic assessment of stem cells properties, driving and accelerating the translation of regenerative medicine approaches to the clinic.

First draft submitted: 22 August 2017; Accepted for publication: 5 January 2018; Published online: 20 March 2018

Keywords: cancer • differentiation • microfabrication • microfluidics • microtechnology • regenerative medicine • stem cells • translational medicine

Stem cells are divided into two major cell types: pluripotent embryonic cells and the multipotent adult stem cells (e.g., mesenchymal stem cells, hematopoietic stem cells). Embryonic stem cells are devoid of problems related to teratomas formation in patients and important ethical controversy, whereas adult stem cells have a reduced expansion and self-renewal capacity. The health-threatening disadvantages of embryonic stem cells leave adult stem cells as one of the most feasible and potent alternative for cell therapy treatments in many different pathologies, however there are many drawbacks that require intensive research and development to bring the therapeutic potential of adult stem cells to the bedside. Some of the major challenges that developer of stem cell-based therapies need to solve are the following: heterogeneity of cell population after tissue-derived isolation; exhaustion of functionality of intensively expanded cells in culture; low understanding and control of cell renewal and differentiation; lack of tools for therapeutic quality control of cell doses; and deficient strategies for cell storage and delivery. These problems redound into inconsistent cell products, lower quality and ultimately less efficient cell therapies. Microtechnology, and more specifically microbioreactors and microfluidics, can contribute to a deeper understanding of stem cell functions and their interaction with the environment. Microtechnology offers the tool to precisely fabricate the physical microenvironment of stem cells, while microfluidics and microtechnology give the ability to create well-defined chemical and physical cues. As such, it can capture the complex mechanical and biochemical nature of cell-to-cell interaction or between the cell and its extracellular matrix (ECM). It is the complex interaction of the biochemical, physical and mechanical aspects of the microenvironment that determines cellular decisions such as cell proliferation, differentiation and migration. Microfluidics allow for continuous perfusion, meaning that stem cells do not have to be cultured on a feeder cells, but will need the required soluble factors added to the culture media to maintain the optimal soluble microenvironment [1]. In this manner, microfluidics-based cell cultures can be maintained for several weeks. In other areas of cell studies, microtechnology has already been used with success. Indeed, 3D-cell cultures using microfluidic perfusion systems showed improved cell functions over 2D-plated cultures [2–5]. Drug studies on plated cells have little relevance to *in vivo* clinical studies [6–8]. The dimensions

Future Medicine

of a microfluidic system are similar to those in *in vivo* systems, providing a more physiological environment, which cannot be captured by Petri dish, batch cultures or other culturing methods. Micro- and nano-fabrication has also found their way into the mechanical interrogation of cells in the field of mechanobiology [9]. Cell force measurements have been measured with the aid of micropillars, which typically have a diameter of 2 μm, while cell binding events have been observed on various micro- and nano-structures and fibronectin patterns. These surface patterns will determine how cells see the property of the surface, depending on the spacing between features, feature heights and material [10], which would in effect, determine stem cell fate. This review highlights some applications of microtechnology and microfluidics for the isolation, differentiation, transcriptomics studies, capture of microvesicles and microenvironment studies of stem cells. Additionally, these devices can contribute in elucidating the specific understanding of stem cells, the control over stem cell fate, their use as disease biomarkers and to define better strategies to translate the use of stem cells into clinic.

Why microfluidics?

Microfluidics is a generic name for microflow channels fabricated on a chip, principally in the same manner as electronic devices are fabricated, manipulating fluids in the order of 10^{-9} to 10^{-18} l [11]. These channels are typically in the range of 50–200 μm. A typical cell is around 15 μm. The advantage of microfluidics is the reduced amount of substrate and reagents needed. Also, due to the small size, reaction rates are faster and more in line with physiological reaction rates. Another advantage of microfluidics systems is the possibility of high throughput screening [12], already performed with stem cells to screen their interaction with their extracellular matrix [13], while it can be fully automated with integrated valves, mixers and pumps, termed microfluidic large scale integration, similar to integrated circuits in electronics [14]. Microfluidics give the possibility to construct cell specific microenvironments due to the precise control of microscale structures. The ability to control, for example, flow patterns gives control over the transport of soluble factors, reagents, oxygen and the hydrodynamic stress [15]. The size of the cell chamber will influence the behavior of the cells, altering transport due to diffusion distances and cell-to-cell interaction. The perfusion flow rate can also have an effect on the growth dynamics of the cell [16]. For example, embryonic stem cells showed no proliferation in either no or little flow, while for higher flow conditions they did proliferate [17]. The flow also affects cell viability [5]. Cells can be in direct contact with a flow, or shielded by a microfabricated barrier [5,18], microgroove substrate [19], internal membrane oxygenators [20] or hydrogels [4]. There are several ways to fabricate optical transparent microsystems; transparent, so the microfluidic devices can be used under microscopes for life cell imaging. These include etching (chemical and reactive ion etching), hot embossing, laser machining and soft-lithography. Laser machining includes the commercial laser cutters which can make microchannels in poly(methyl methacrylate), known as acrylic and does not need a cleanroom, while soft-lithography uses a heat curable polymer, poly(dimethylsiloxane), cast against a mold to replicate the features (Figure 1) [21,22]. The latter method is the preferred method in biological labs, due to the fast time of prototyping and the low cost of materials, while a simple lithography system could be run in laminar flow hood (with Hepa filters). Sensors can be integrated on chip as well. For example, pH sensitive ions of the ion sensitive field effect transistors [23] or nanosensors based on aluminum gallium nitrides (AlGaN/GaN) [24] give good pH readings. Other methods included optical sensors for pH and O_2 using fluorescence spots (optodes) [25] or a fluorescent poly(ethylene glycol) hydrogel microarray sensor using 2′,7′-Bis-(2-Carboxyethyl)-5-(and-6)-carboxyfluorescein (BCECF)-dextran [26]. Also biosensors can be incorporated on chip, such as captor probes immobilized to the surface [27] or beads to detect certain biomarkers or metabolites [28]. All points considered, microfluidic systems can present, control and mimic cell natural environment for cell cultures or tissues at much cheaper cost, shorter time and in a high throughput screening. These technologies provide systems adaptable to common imaging technology in biology and different microaccessories permit high quality quantification of biologically relevant parameters measured with excellent resolution in space and time. The use of such technology would allow a deeper understanding of biological phenomenon. Microfluidics and microtechnology have allowed stem cells research to get into a new level and many advances have been reached in various aspects of enormous value in the field. There is still great potential in which microfluidics and microtechnology will benefit the progress of stem cells and regenerative medicine.

Efficient isolation of stem cells

Adult stem cells such as mesenchymal stem cells (MSCs) are showing promising results in numerous clinical trials for the treatment of various pathologies. However, low standardization criteria, inconsistent therapeutic quality

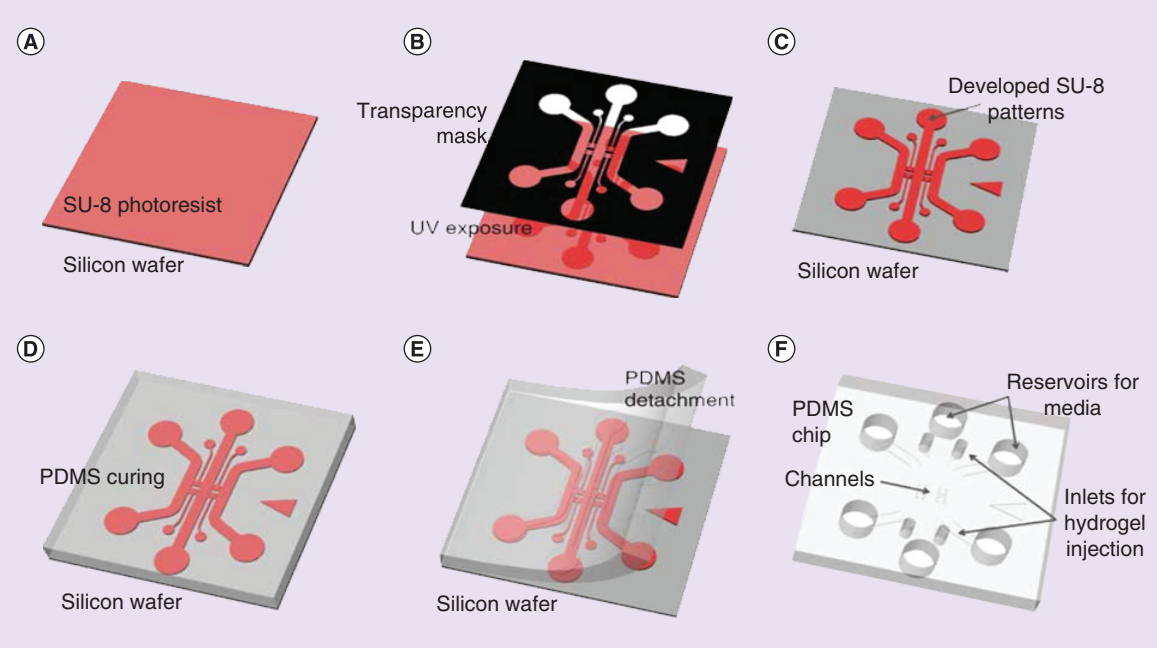

Figure 1. Schematics of the photolithography (A–C) and soft lithography (D–F) procedures. (A) The photoresist SU-8 is spin-coated and prebaked on a bare wafer. **(B)** With a transparency photomask (black), UV light is exposed on the SU-8. **(C)** Exposed SU-8 is then baked after exposure and developed to define channel patterns. **(D)** The two component elastomer poly(dimethylsiloxane) mixed solution is poured on the wafer and cured. **(E)** Cured poly(dimethylsiloxane) is then peeled from the wafer. **(F)** The device is trimmed, punched and autoclaved ready for assembly.
PDMS: Poly(dimethylsiloxane).
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and high heterogeneity of cell populations among different donors and cell batches are the main bottlenecks facing the cell therapy field [29]. To overcome these hurdles, new efficient isolation technologies of stem cells products with good quality and therapeutic properties need to be developed. MSCs are commonly isolated from different tissue sources such as bone marrow, adipose tissue, menstrual fluid, umbilical cord and placental tissue, and the process comprises either tissue explants or mononuclear fraction culturing [29]. Most of the obtained cell cultures present a portion of cell contaminant subpopulations that are usually indistinguishable from the MSCs. Indeed, fibroblasts, for example, are one of the main contaminants of MSC cultures. Additionally, isolated MSCs are considered to be a heterogeneous group of cells having diverse MSCs subpopulation with different therapeutic potentials. Wei *et al.* showed that only eight out of 24 MSC clones that were randomly selected using single-cell monoclonal technology showed a differential potential toward cardiomyocytes [30]. In fact, different microfluidics-based technologies have been tested aiming to increase MSCs isolation yield, homogeneity and therapeutic quality through tissue dissociation, cell type selection or sorting and expansion. Unlike isolation and expansion of MSCs through explants culturing, previous tissue dissociation could allow an increased number of viable cells to initiate stem cells cultures. Trypsin treatment of biopsies in small bioreactors within perfused microfluidic devices has been able to reduce processing time and allowed control enzymatic exposure of detached cells by immediate extraction through the output channels [31]. Usually, subsequent analysis of isolated stem cells population requires integrity of biomarkers or surface proteins, which are known to be affected by prolonged trypsin treatments. New microfluidic devices use specific microfluidic designs and hydrodynamic forces for an automated mechanical dissociation, avoiding the need for enzymatic disaggregation [32]. Although higher yield is obtained through tissue dissociation, this process is unable to remove contaminating cells, such as fibroblasts, and could also favor the outgrowth of undesired cell population. As such, a selection or cell sorting step is required to enrich the first culture with high quality and homogeneous stem cells. Since preparative cell isolation using fluorescence activated cell sorting (FACS) has certain limitations in a production context, such as limiting capacity of sorted cells per day, high number of lost cells after sorting, presence of fluorescent antibodies that may alter cell activities; several

groups have been working on developing high-throughput microfluidic systems capable of isolating therapeutically relevant subpopulations of multipotent stem cells. Different mechanical properties such as cell size, stiffness, area fluctuation of the nuclear membrane [33] and plasma membrane capacitance [34] have proven to correlate with stem cells multipotency or specific progenitor types. The property of laminar flows, inherent to microfluidics, can take advantages of the differences of the cell's mechanical properties to drive cell sorting. CT Lim's group have developed a spiral microfluidic system that uses inertial forces to sort cell subpopulation based on size, which, for instance, allows spatial clustering of cell synchronized in a particular cell cycle phase [35]. On the other hand, Prieto *et al.* designed a microfluidic device including arrays of dielectrophoretic electrodes capable of immobilizing neural stem cells from a sample of heterogeneous cells depending on the applied dielectrophoretic frequency, which is correlated to the difference in cell membrane capacitance. There are several other examples in the literature where microdevices are designed for cell-size separation in a continuous flow systems utilizing internal pillar-based separation structures, which is known as deterministic lateral displacement [36,37]. Karabacak *et al.* developed a microfluidic device that uses a continuous deterministic lateral displacement for size-based sorting to separate white blood cells and circulating tumor cells (CTCs) from red blood cells and platelets, inertial focusing for the positioning of cells and magnetophoresis to separate cancer cells from white blood cells linked to magnetic beads coated with antibodies anti-CD44 and -CD66b [28] (Figure 2A). Although this system uses antibodies, the loss of cell is highly reduced in this system compared with FACS technology. Sorting in a FACS system is based on the generation of individual cell-containing droplets, which are derived to different collecting tubes under the control of an electrical field. Formed droplets could very often contain more than one cell, generating detection conflicts and drop discarding. Karabacak's device performs the sorting under continuous microflows in presence of a magnetic field, which avoids the cell loss by sorting directly the cell and not cell-containing droplets. Transient cellular adhesion to surfaces occurring during cell rolling is a key cellular response for trafficking and homing of different cells types such as leucocytes, progenitor stem cells and cancer cells. Researchers have developed a feasible way to sort rolling cells from cells that do not present transient cell–surface interactions using microfluidic designs. This technique has been called deterministic cell rolling, where rolling cells drift from nonadherent cells through parallel grooves at the bottom of the devices, which are oriented obliquely to the main flow vector [38–40]. As such, researchers have been able to observe different cell rolling behaviors of MSCs in this chip and correlate these responses with their state of differentiation (Figure 2B) [41]. Typical versatility of microfluidic devices originated mainly from their sharp spatial and temporal control of added elements, making possible much broader applications within this type of devices. Using this device, it is possible, for example, to explore more mechanistic elements of the rolling ability of MSCs, such as adhesion molecules involved in the process, regulation effects of extracellular or intracellular elements, mechanical parameters, among others. Comparatively, other previously described nonmicrofabricated adhesion assays, which are capable to determine the adhesion and rolling behavior of cells, are based on perfused chambers coated with cell-adhesion substrates [42]. However, those systems are unable to sort between rolling and nonadherent cells at the output of the perfused chamber, limiting the system to a just visual inspection of the cell response.

Mimicking the *in vivo* stem cells microenvironment

Although the heterogeneity issue of adult stem cells can be bypassed by smart and deterministic sorting techniques, live cells are highly sensitive to the applied isolation and expansion procedures. Multipotency and therapeutic quality maintenance of expanding cells is very challenging, since mimicking the physiological conditions of a cell's natural microenvironment is highly complicated, and requires a precise spatial and temporal coordination of extracellular component, biochemical gradients and cellular effectors in the stem cells niche. Every cell type needs its own microenvironment. The microenvironment refers to a specific set of chemical and mechanical conditions which influence and regulate cell fate. Under these conditions, a cell constantly senses numerous inputs: soluble factors, cell-matrix interactions and cell-to-cell contacts as well as physical parameters (pH, oxygen concentration, shear stress, etc.) [43]. Upon sensing these inputs, cells process the information and adapt themselves to the environment. Therefore, understanding the key inputs of the cellular environment is necessary to recreate a physiologically acceptable cellular microenvironment in a microfluidic device. Given the complexity of stem cells niche, reconstitution or design requires high-throughput capacity and spatiotemporal control of elements, which are some of the intrinsic abilities of microfluidic devices. In 2007, Stroock and co-workers designed perfused microchannel structures within a 3D-cell culture system, controlling solutes and metabolites concentration, and creating steady-state gradients within the cellular microenvironment. An embedded channel for reactive and

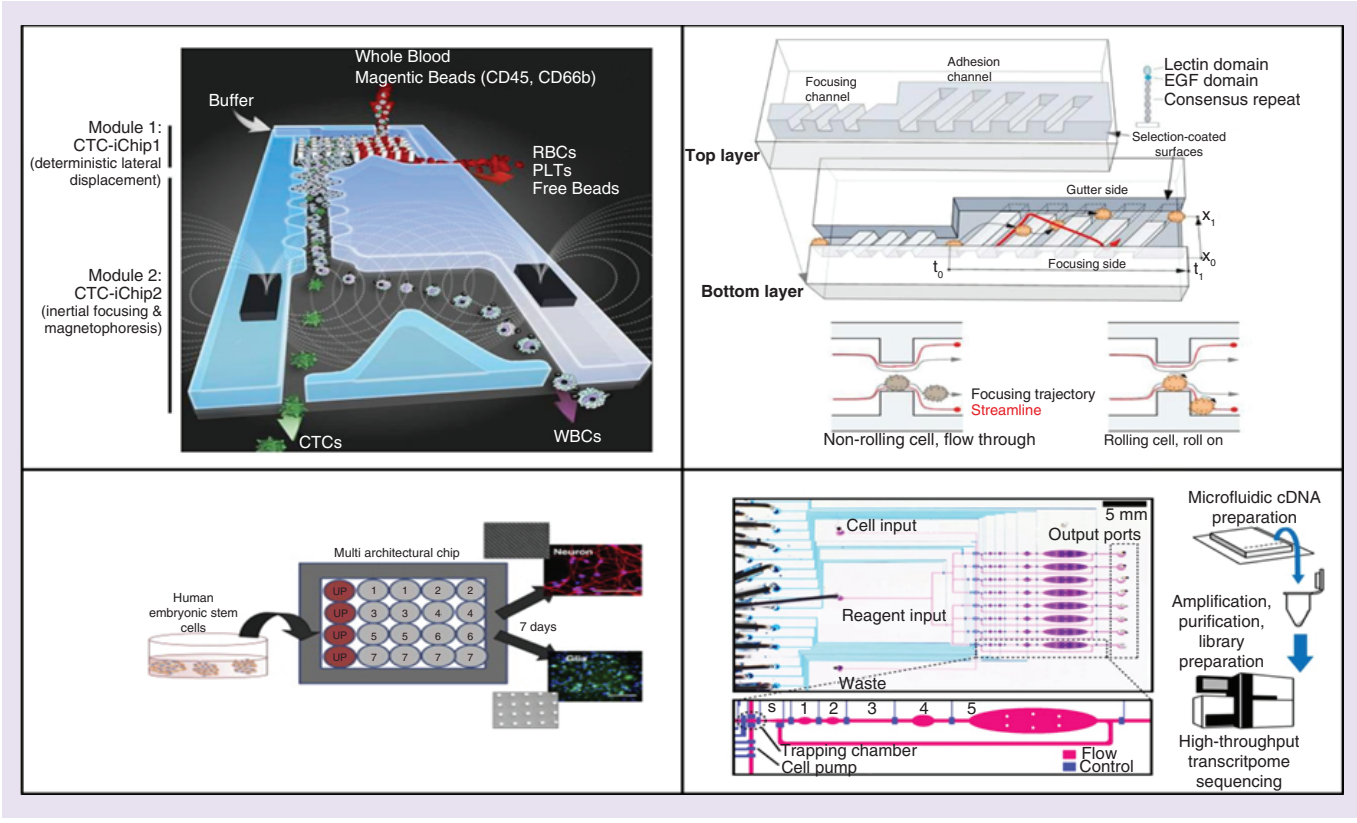

Figure 2. Microfluidic devices. (A) CTC-iChip scheme. Two separated devices make the complete CTC-iChip. The first microdevice constitutes a deterministic lateral displacement to remove nucleated cells from red blood cells and platelets, using a specially designed array of pillars. Second device uses an inertial focusing system to line up cells for later magnetic separation of bead-labeled white blood cells and unlabeled CTCs through h magnetophoresis. **(B)** Cell rolling cytometer (Top). Scheme of the cell rolling cytometer. Cells are pushed to get in direct contact with ridges coated with adhesion molecules (Bottom). Cross-section views of the cell rolling cytometer. When cells do not generate transient cell–surface interactions, it quickly follows focusing trajectory, otherwise rolling cells start drifting through the parallel grooves. **(C)** Optional neuron or glial differentiation of human embryonic stem cells trigged by surface topography. On the multiarchitecture chip, numbered circles represent different patterned surfaces and red circles unpatterned control. **(D)** Microfluidic device for single cell whole-transcriptome sequencing. The device comprises a cell input for injection of a cell suspension, and a reagent input. From the output ports, cDNA is collected. The single-reaction pipeline is shown in the figure. Single cells are actively trapped in the trapping chamber to be then pushed into the sorting chamber. Afterward cells are subjected to lysis, reverse transcription, polyA tailing, primer digestion and cDNA synthesis.
CTC: Circulating tumor cell.
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nonreactive solutes and sink channels working in unison enabled spatial and temporal control of soluble factor [44]. While this system has great control over soluble factors, it has low screening capacity. Therefore, Yang *et al.* created a 3D culture array to analyze the effect of multiple mixtures of ECM on the self-renewal and differentiation of stem cells within one chip and one experiment. The authors were able to observe altered oxygen diffusion among the different combinations of ECM elements, which were affecting stem-cell fates [45]. The stem cell's microenvironment is not only defined by the identity of mixed elements, but also by the relative abundance of components. Mixing in microfluidic devices can create opposite gradients of ECM, soluble factors or cell components embedded in a 3D hydrogels. Mahadik *et al.* simulated the reconstitution of a hematopoietic stem cells (HSCs) niche, combining opposite gradients of stem cells and one or more putative niche cells in the same 3D hydrogel. This 3D microenvironment assay enabled the evaluation of HSCs fate at different cell ratios or in the presence of different niche components. This made possible the determination of an optimized concentration of elements for a HSC niche reconstitution [46]. In the attempt to develop high-throughput systems capable of generating new hydrogel

formulation from multiple components, thereby allowing thorough screening of important niche diversity, Lutolf's group created a droplet microfluidic system to potentially generate microhydrogels with a variety of mechanical and biochemical properties. In this particular work, authors fabricated microsized poly(ethylene glycol) hydrogels with different tethered biomolecules to modify the bioactivity upon MSCs culturing [47]. In a different study, a 3D microfluidic device was developed to study the molecular regulation of perivascular stem cell niches. Endothelial cells (ECs) were cultured with bone marrow mesenchymal stem cells forming a primitive vascular plexus and maturing into a robust capillary network with hollow and well-defined lumens. The results have shown that MSCs formed pericytic associations with the ECs. The authors concluded that this *ex vivo* model could be used to study how proximity to blood vessels may influence stem cell multipotency [48]. The identification and reconstitution of *in vitro* cell niche would signify a key advance in the understanding of stem cells fate and their control, and by this mean generating optimized protocol of cell expansion and cell transplant with high therapeutic efficiency for regenerative medicine. Handling of many elements (gradient of soluble factor, extracellular matrix, cell-to-cell contacts), concentrations and spatial location of those comprises a highly complex system that orchestrates stem cell fate. Due to the inability of standard cell culture systems to either decipher or mimic those conditions *in vitro*, most of the actual knowledge about cell niche comes from *in vivo* studies focused on individualized elements, still lacking the information about their functional relation with other important elements [49]. In this context, microtechnology and microfluidic devices appear as the only feasible option to provide precise control of elements and high-throughput combinatorial screening to unveil the real nature of the stem cells niche.

Differentiation on a chip

About a decade ago microfluidic technology started being used to create a concentration gradients of various growth factors. Creating a concentration gradient in microfluidics is not challenging especially when compared to the less efficient and laborious step-wise gradients made in multiwell plates. The differentiation of neural stem cells (NSCs) into astrocytes, monitored by time-lapse microscopy, was proportional to the concentration of the growth factor [50]. Differentiation has also been shown in 3D microfluidic cell cultures. Bone marrow MSCs maintained differentiation potency for up to a week before triggering the differentiation to osteoblast by means of a chemical cue at a defined time point [4,5]. Differentiating stem cells will play an important role in personalized medicine. Functional *in vitro* human disease models can be fabricated combining differentiation strategies with organ-on-a-chip. These tools can potentially be used as a platform to test drugs, toxicity and determine IC_{50} values [51]. For example, induced pluripotent stem cells of patients with Barth syndrome, a rare genetic disorder, have been differentiated on a chip to produce an *in vitro* heart disease model [52], or physiological meaningful micromodels of cardiac tissue have been developed on a chip and kept viable and functional for several weeks [53]. This represents an important milestone in personalized nanomedicine. Microfluidics has also proven to be a useful tool to study NSCs differentiation. It has been shown that C17.2 NSCs undergo neuronal cell differentiation under depletion of nutrients [54]. Other means of differentiation is by physical cues. Different topography will activate different differentiation cascades. A study using a single chip containing 49 different topographical features, ranging from concaves, lines, pillars to hierarchical features (called multi architectural chip), was able to control the differentiation fate of hESCs. Anisotropic features, like gratings, have a bias toward neural differentiation [55] while the grating size (nano vs microns) determines the number or neurons positive to mature neuronal marker (Figure 2C). Also, mechanical tension is capable of activating differentiation, which was easily demonstrated with a microfabricated cell stretching device [56] as well as changing the material of which the microfluidic device is fabricated [57]. Additionally, mechanical strain can be incorporated in a microfluidic system having an impact in the differentiation of cells too. For example, the differentiation of mouse Col1a1GFP MC-3T3 E1 osteoblastic cells after exposure to shear stress (0.07 dyne/cm^2 at a flow rate of 0.2 μl/min) was studied. The results have shown an increase in the expression of green fluorescent protein (GFP) and alkaline phosphatase, which is an enzyme marker of osteoblasts [58]. Therefore, control over physical and chemical cues can be reached combining micro or nano-features, biomaterials and microfluidics, and determine this way, and more precisely, the stem cell fate. This is particularly relevant for regenerative medicine when different diseases caused by a decrease in functional cells can be treated by transplant with new healthy cells. Differentiation to functional cells starting from pluripotent stem cells derived from patients, requires precise control of culture conditions and optimized substrates or extracellular matrix for cell adhesion. Proper differentiation is only achieved after the induction by a pool of different growth factors and small molecules presented sequentially at a very precise concentration and exposure time. This obliges researches to undertake a laborious experimental process of combinatorial testing using growth factors, concentration, sequential order and exposure time as parameters.

Although the accumulated knowledge in the study of embryo's development has been very helpful in determining the factors and sequential order of exposed elements, optimum protocol of differentiation *in vitro* still requires higher level of combinatorial analysis and control of culture conditions, which is unfeasible under standard culture systems [59]. Due to the high-throughput and control capability that microtechnology and microfluidics may offer, they have the potential to generate important progress in this field at a lower reagent cost and time consumption.

Stem cells transcriptomics

MSCs as a source of therapeutic stem cells have demonstrated to be a heterogeneous population. They are composed of phenotypically similar MCSs and different progenitor cells [30,60]. Distinguishing cellular heterogeneity is relevant for a better understanding of the stem cell activity in an indicated therapy, and for directing a better prescription in disease cases recommended to be treated through cell therapy. Thus far, it has not been possible to gather a sufficient pool of biomarkers to allow a thorough phenotypic classification and isolation of MSC subpopulations using flow cytometry or FACS, as it has been the case for leukocytes [61]. Single-cell transcriptomic studies arise as a suitable technique to evaluate such heterogeneity. However, it is hindered by the technical difficulties of single-cell measurements, which derives in a limiting number of cell sampling and high variability. Microfabrication techniques and microfluidic devices have the potential to control single cell localization and perform serial automatic single-reactions with high precision and sensitivity. Although there are many reports of microdevices capable to detect specific mRNA at a single-cell resolution with a screening capacity of less than 100 cells [62–65], RT-PCR reactions at femtoliter scale suffer from high variability. As such, more than a thousand single-cell measurements are required for significant statistical evaluation. Dimov *et al.* used a single microwell array of 60,000 reaction chamber [66], that was adapted to trap single cells. It was able to generate a RNA-cytometer analysis and identify RNA heterogeneity within a population of purified mouse hematopoietic stem cells. Also, the system could specify cell subpopulations showing high or low expression of aging-related genes such as Birc6, and how the relative abundance of a low or high-expressing subpopulation changes upon aging [67]. On the other hand, if whole-transcriptome sequencing at a single cell level is desired, it is required more sensitivity and precision in cell trapping, sorting, lysis and reverse-transcription of mRNA. Under closed microfluidics conditions, these processes are carried out free of exogenous RNA and RNase contaminants, and the improved amplification efficiency allows for higher detection sensitivity. A commercial device from Fluidigm™ has shown to have good performance using a passive cell trapping system allowing for high-throughput analysis [68]. A later development for single cell whole-transcriptome sequencing has incorporated an active cell trapping system, expanding the application to rare cells from a particular population, since active trapping could discard uninteresting cell types (Figure 2D) [69].

Microfluidic device for isolation, quantification, production & characterization of microvesicles

Microvesicles and exosomes are secreted membrane vesicles that carry proteins and RNAs for intercellular communication. They are increasingly seen as possible drug delivery vehicles due to their ability to traverse biological barriers. Human MSCs represent the ideal cell source of immunologically inert exosomes, as infinite and reproducible exosome production from single cell clones is possible through the immortalization of these cells without compromising their qualitative exosome production [70]. It is important to understand whether it is more advantageous to inject MSC or only their secreted vesicle product [71,72]. This will mostly depend on the intended applications. Also, it is important to take into consideration other parameters such as safety and regulatory issues. It is basically safer to inject a proliferation and immunological inert product rather than the cells, to bypass any undesired effects such as immune reactions. Furthermore, microvesicles and exosomes represent a precious biomedical tool as they can be isolated from different bodily fluids including blood and urine [73]. They have the potential to provide information about state and progression of a specific disease representing a great clinical importance as biomarkers. A common procedure to isolate microvesicles from bodily fluids requires series of centrifugation and filtration to remove dead cells, large debris and other cellular contaminants resulting from cell lysis, followed by a final high-speed ultracentrifugation. In order to take full advantage of the potential of microvesicles, less cumbersome and time consuming methods of isolation are needed. Microfluidic devices are attractive alternatives for an easier and faster isolation of these microvesicles. These devices have small dimensions, and minimal amounts of reagents are needed, allowing reduced purification times, and increased sensitivity, without requiring ultracentrifugation steps or resolution on sucrose gradients (Table 1). Chen *et al.* established an easy and rapid microfluidic immunoaffinity method to isolate microvesicles from small volumes of both serum from blood samples and conditioned medium from cells

Table 1. Comparative table between the currently used differential centrifugation and the proposed microfluidic methods.

	Differential centrifugation method	Microfluidic method
Centrifugation numbers	Multiple	Single
High-speed centrifugation step	Required	Not required
Cellular contamination (cell fragments and debris)	High level	Reduced level
Exosome morphology	Aggregated	Native
Marker specific exosome purification	Not possible	Possible
Isolation time	Long (4–5 h)	Short (1–2 h)

Data taken from [74,103,104].

in culture [74], where only one short, low-speed centrifugation step was applied to the serum sample. Cells under normal established culture conditions secrete only a small number of exosomes. While different treatments aiming at increasing their vesicles production are currently being tested, new microfluidic system harboring nanofabricated blades allows cell membrane slicing with the resulting generation of nanovesicles [75]. These artificially produced nanovesicles were shown to be able to engulf different cell contents such as protein and mRNAs, and by testing transfer of fluorescent polystyrene beads to recipient cells, they show the feasibility of using this nanovesicles as biological delivery systems. However, further studies are required to test whether this artificially produced exosomes using microfluidic technology preserve their molecular content and promote a similar biological effect of naturally produced exosomes.

Microfluidics for the assessment of metastatic activity of cancer & cancer stem cells

Given the broad range of possible microfluidic designs and the new technical improvements in the field, application for microfluidic devices can be found even in the study of complex natural phenomenon. The study of the metastatic activity of cancer cells and cancer stem cells has recently been explored within microfluidic device. Due to the extended control of parameters and the adapted analytical methods, it will become a key player in cancer research. Despite the advancement of anticancer treatments, many patients experience tumor recurrence and metastasis, which has led to the hypothesis that tumors may possess a stem cell-like subpopulation known as cancer stem cells (CSCs) [76]. CSCs are a subset of cancer cells which play a key role in predicting the biological aggressiveness of cancer due to its dynamic model including tumor initiation, heterogeneity, therapy resistance, recurrence and metastasis. Understanding these basic concepts of CSCs is important for translational applications and finding new potential targets for effectively treating patients [77]. A simple biomechanistic approach for cell sorting based on cell deformability proved that higher cell deformability is associated with a less differentiated cell with higher tumor initiating capacity. A hydrodynamic force pushes the cells against an array of micropost, separating deformable cells from a group of stiffer cells. After spatial clustering of deformable cell, gene-expression analysis revealed 30 upregulating genes relevant for tumor cell migration when compared with nondeformable population [78]. CTCs originated from CSCs or cancer cells that have undergone epithelial-to-mesenchymal transition are important targets for cancer biology studies. Nagrath *et al.* described the development of a unique microfluidic platform (the 'CTC-chip') capable of efficient and selective separation of viable CTCs from peripheral whole blood samples, mediated by the interaction of target CTCs with antibody (EpCAM)-coated microposts. The CTC-chip successfully identified CTCs in 99% of the peripheral blood of patients with metastatic lung, prostate, pancreatic, breast and colon cancer, with a range of 5–1,281 CTCs per milliliter and approximately 50% purity [79]. Following the high sensitivity of the chip, they also demonstrated its utility in monitoring response to anticancer therapy in patients with metastatic cancer undergoing systemic treatment where changes in CTC numbers correlated reasonably well with the clinical course of disease [80]. Recently, acoustic-based methods have been successfully developed to isolate rare CTCs from peripheral blood samples obtained from cancer patients. This method uses tilted-angle standing surface acoustic waves and was first validated for the separation of a variety of cancer cells with a recovery rate better than 83% [81]. Another strategy uses magnetic particles bearing tumor-cell specific antibodies, self-assembled in a regular array in a microfluidic flow cell. This device was capable of capturing epithelial cells with efficiency above 90% for concentrations as low as a few cells per milliliter. The scaling-up capacity and rapidity of the method is also important for this technology, if the purpose is to reach the clinic [82]. The authors also characterized a two-step blood processing method to allow for rapid processing of 10 ml blood tubes in less than 4 h, and

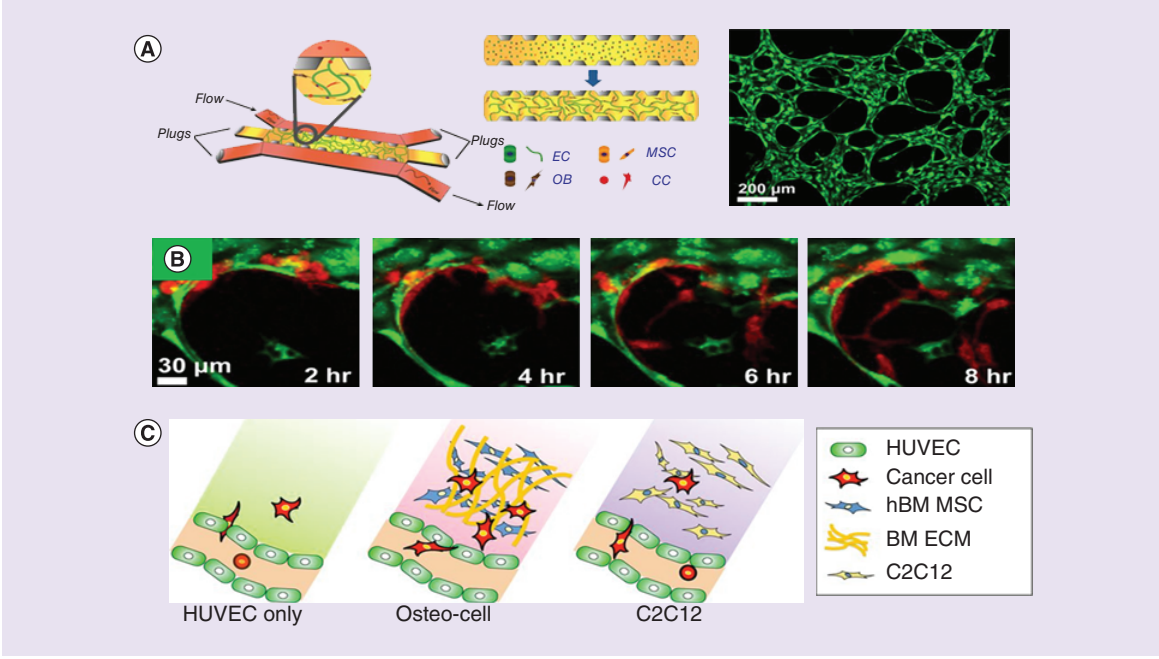

Figure 3. Extravasation model. (A) Two parallel channels allow addition of cancer cells, biochemical factors, and flow across the vasculature formed in the 3D gel channel. Before hydrogel polymerization, it already contains ECs, MSCs, and OBs. ECs form microvasculature, whereas MSCs and OBs form a bone marrow-like niche. Perfusion with CCs generates a vessel extravasation of CCs into the bone marrow-like gel. The microvascular network is characterized by highly branched structures. **(B)** CCs (red) introduced into the vascular network (HUVECs, green) is monitored in real time. **(C)** Schematic of HUVEC only, osteo-cell, and C2C12 cell added systems. HUVECs forming the vessels are in green, osteo-cells in blue are secreting the bone matrix (yellow), and C2C12 cells are depicted as yellow cells. CCs (red) are extravasated out from the vessels.
BM: Bone marrow; CC: Cancer cell; EC: Endothelial cell; ECM: Extracellular matrix; hBM: Human bone marrow; HUVEC: Human umbilical vein endothelial cell; MSC: Mesenchymal stem cell; OB: Osteoblast-differentiated cell.
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showed a capture rate of 70% for as low as 25 cells spiked in 10 ml blood tubes [82]. Thus, the CTC-chip offers the potential to serve as valuable tool in cancer research, diagnostics, drug efficacy assessment and, therefore, has broad implications in advancing both cancer biology research and clinical cancer management. Due to the multiple biological variables that governed the metastasis activity of tumor cells and the organ specificity for extravasation, which include organ-specific chemo-attractant molecules, cell–cell and ECM–cell interaction for transendothelial migration, *in vivo* models are unavoidable for replicating physiological conditions. However, these models offer limited imaging and low variables parameterization, therefore restricting the possibilities to analyze specific cell–cell interaction, ECM–cell interaction and effect of soluble factors. Microfluidic technology has been able to bridge many of the *in vivo* model limitations with the fabrication of hybrid artificial microchannels coupled to nature-like vascular network generated within cell-laden hydrogels [83]. Kamm's Lab has developed microfluidic devices with perfusable human and stable microvasculature structures embedded in controlled 3D microenvironment, which, for instances, can mimic bone-like niche. This *"microvasculature on a chip"* offers great possibilities to study mechanistic elements in the metastasis process of tumor cells with high resolution on a real time setting (Figure 3). In a different study, a microfluidic system was established in order to mimic a 3D physiological tumor model for cancer drug testing. Colo205 tumor spheroids were created by a modified hanging drop method and maintained inside poly(dimethylsiloxane) microbubble cavities in perfusion culture. The toxicity of the anticancer drug, doxorubicin, on Colo205 cells in spheroids was tested using this microfluidic system and results have shown a threefold increase in resistance to doxorubicin for Colo205 spheroids (cultured in flow) compared with Colo205 monolayer cells (cultured under static conditions) [84].

Translation of stem cells therapies into clinic & microtechnology

Cell therapy efficiency and standardization is highly dependent on the quantity of cell-doses. The isolation yield of a well-defined cell subpopulation from a tissue source determines the homogeneity, quality and quantity of the final product. In this sense, microfluidic technology has demonstrated enormous potential [31,32], representing a retrospective solution in a myriad of different cell therapy clinical trials failures cause for example by donor variations and cell product heterogeneity [85]. Likewise, it is now well accepted that at later passages of intensified cell expansion, stem cells change dramatically their stemness potential (proliferation, differentiation capacity, regenerative function and secretion profile) [86]. It was noted that late passages of MSCs subjected to differentiation agents only demonstrated a high degree of osteogenicity with no chondrogenicity and adipogenicity [87]. On the other hand, Rosland *et al.* [88] showed that long-term cultures of MSCs frequently undergo spontaneous malignant transformation. The drawn conclusion is that the proliferation rates and other properties of the cells gradually change during expansion and therefore, it is recommended that hMSCs should not be expanded beyond four or five passages. This is virtually impossible for cell therapy companies with allogenic strategies, as they will require, for example, daily basis extractions of bone marrow from donors to cover a worthily portion of the market. As a result, among the hundreds of trials none of them used this strategy for impractical reasons. This issue represents an important drawback in the translation of stem cell therapies, in which an ideal solution would be a new system of cell expansion that mimics for example the microenvironment of stem cells for cell renewal without losing their stemness. The high-throughput capacity and spatiotemporal control of elements of microtechnology and microfluidics systems are an invaluable tool to determine the combination and spatiotemporal control of factors and parameters for high quality stem cells expansion, and also to define an optimum bioreactor design for high yielded manufacturing of progenitor cells with unscathed regenerative functions [89–91]. However, up to now, most of the studies have focused primarily on engineering a microenvironment toward controlling the differentiation of stem cells into different cell types [92,93]. Therefore, there is still important research effort to be made to solve this fundamental problem for translation of stem cell therapies. Fortunately, in this regard, extensive work on the use of an advance nanofabrication technique called two-photon laser polymerization has been made to fabricate 3D 'nichoids' for embryonic stem cells expansion [94,95]. Results have shown that the physical constraint of the microenvironment maintains the expression of pluripotency makers (OCT4), while expression of differentiation markers (GATA4 and α-SMA) stays low. Exploring this 'nichoid' microtechnology could offer a good opportunity for large scale expansion of therapeutically relevant adult stem cells, at a reduced cost, better quality and in absence of feeder cells. However, the combination with other cell expansion strategies would probably be necessary due to the higher demand of control over different factors in the process of self-renewal of this type of cells compared with embryonic stem cells [96]. Stem cell translation therapy has been hindered by the lack of an appropriated potency test [97], especially to comply with regulatory requirements and therapeutic quality assessments of different batches of cell-dose production. An obvious consequence of not providing a control system of therapeutic quality, it is the manufacturing of inconsistent cell products, thus inconsistent clinical results should be expected too. In this regard, the major challenges to develop a quick potency test to help the standardization of stem cell products are: the lack is their poor definition of the mechanism of action and the difficulties in defining the group of most relevant biological activities involved in the therapeutic effect. Microtechnology and microfluidic systems are potentially relevant tools to integrate different elements and to perform quick evaluations of multiple biological activities of stem cells, increasing the chances to obtain an *in vitro* potency test with excellent correlation with *in vivo* therapeutic results. Currently, no reports of microfluidics-based potency test can be found in the literature. Finally, an important consideration that need to be taken into account is the cell-dose delivery, either by cryopreservation at -80°C or biopreservation at 4°C [98]. The feasibility of the cell therapy business and multicenter Phase III clinical trials need efficient systems of dose conservation and transport, which also needs to comply with regulatory requirements. Existing technologies of conservation are commercially available for cell therapy companies; however, they still generate certain deleterious effects in cell viability and cell function [99]. Microfluidic-based bioreactors have been created to enhance the survival of cryopreserved stem cells through previous induction of upregulated focal point of cell adhesion [100]. On the other hand, microfluidic systems have been developed to control the cell damage provoked by the cryoprotectant-derived osmotic shock and to control the cell thawing and DMSO removal [101].

Conclusion

Owing to their excellent biocompatibility, simple design and low cost, microtechnology and microfluidic devices will offer new and effective tools for accurate identification, measurement of stem cells potential, and the capture

of their secreted microvesicles. However, their use at a larger scale for stem cell manufacturing processes is still under development. For example, producing therapeutical doses of cells (about 100 millions/doses or more), as well as their application in the isolation of exosomes from large volumes (at the range of liters) of cell culture supernatants still requires more work in scaling up microfluidic platforms. This is coupled to the will of research centers to invest in this kind of translational research. The advances in nano and microtechnologies will contribute in eventuating new insights into the biology of stem cells, in particular, in the assessment of their potency. As such, it can greatly contribute in the establishment of the quality standards of MSCs prior to their therapeutic application. Merging the advanced fabrication technologies with high-throughput methods to systematically probe the role of different microenvironmental elements on stem cell fate, will permit the identification of novel key stem cell properties. Additionally, it will push toward an improved mimicking of their *in vivo* niche requirements, allowing the preservation of their important stem cell properties during their *in vitro* nonminimal manipulation, as well as replacing existing animal models. This will permit an improved assessment of stem cells properties and provide a basis for establishing the quality standards of stem cells, unlocking their clinical potential in tissue engineering and regenerative medicine. Well established designs of microfabricated devices and microfluidic technology have the potential to recapitulate in a control manner and simultaneously several variables that define cell fate and functionality of stem cells. Their high-throughput capability makes possible the titration of those variables, and collection of results using actual microscope technology is possible due to the level of transparency of devices. Since size matters significantly when expensive reagents such as recombinant protein are required, microtechnology and microfluidics enable cheaper and rapid experimentation. All points together make this technology especially attractive for stem cells research; however the use of this technology has been concentrated on research group dedicated mostly to design and fabricate biologically meaningful devices. New collaborative endeavors between expert in the field of microfluidics and stem cell researchers are necessary to make significant progress in the field. Associations of this kind have already resulted in very relevant discoveries, such as discrimination of changes in the cellular heterogeneity of mesenchymal stem cells samples upon aging [67].

Future perspective

Microtechnology and microfluidic devices have proved broadly their potential to mimic and control cellular microenvironment, and handle either cells or subcellular components. There is no doubt today that these abilities will permit profound insights into the cellular understanding at the level of their function and regulation. A drawback of miniaturization is the reduced yield of processing, which is usually solved by meaning of multiplying the individual units integrated in a unique process. Scaling-up becomes an intensive and complex endeavor if excessive increments in the number of liquid handling and control units are expected to be evaded. These problems need to be included and integrated during the very first design and evaluation of individual units, therefore scaling-up will become less challenging in the development and translation into the industry and Clinic. While new research in microtechnology and microfluidic devices focused on stem cells is still far from being exhausted, important research efforts need as well to be placed on efficient design for scaling-up, only then these technologies will reach application in the society.

Author's contributions

JP Acevedo, I Angelopoulos, D van Noort and M Khoury contributed equally to this review study. The final manuscript was read and approved by all authors.

Financial & competing interests disclosure

This work was supported by the Chilean Ministry of Economy with its CORFO grant 3IDL2–18745 and the PMI fund UAN1301 granted by the Chilean Ministry of Education. M Khoury is the chief scientific officer of Cells for Cells and REGENERO. JP Acevedo received stipends from Cells for Cells. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

Executive summary

- This review highlights several scientific advances where fruitful interaction converges between stem cells research and microfluidics and microtechnology, aiming to a better understanding of stem cells functions and improved tools toward the development of efficacious stem cells therapies.

Efficient isolation of stem cells

- Microfluidics-based technologies have been tested aiming to increase mesenchymal stem cells isolation yield, homogeneity and therapeutic quality through tissue dissociation, cell type selection or sorting and expansion.

Mimicking the *in vivo* stem cells microenvironment

- New microfluidic devices and microtechnology represent key advances in the understanding of stem cells fate and their control through the special placement and regulation of cell microenvironment.

Differentiation on a chip

- Microfluidics and diverse microfabrication techniques have made possible the spatiotemporal presentation of chemical and physical cues for optimized cell differentiation, leading, for example, to improved *in vitro* disease models for more realistic drug assessment.

Stem cells transcriptomics

- Developments of microtechnology-based devices are capable to perform high-throughput transcriptional analysis at single cell level, revealing the nature of stem cells heterogeneity. This requires more sensitivity and precision in cell trapping, sorting, lysis and reverse-transcription of mRNA.

Microfluidic device for isolation, quantification, production & characterization of microvesicles

- Microfluidic devices are attractive alternatives for an easier and faster isolation of microvesicles. These devices have small dimensions, and minimal amounts of reagents are needed, allowing reduced purification times, and increased sensitivity, without requiring ultracentrifugation steps or resolution on sucrose gradients.

Microfluidics for the assessment of metastatic activity of cancer & cancer stem cells

- The study of the metastatic activity of cancer cells and cancer stem cells has recently been explored within microfluidic device, allowing deep observation of dynamics and mechanism of cancer cells extravasation.
- Microfluidic platform (the circulating tumor cell '[CTC]-chip') capable of efficient and selective separation of viable CTCs from peripheral whole blood samples, mediated by the interaction of target CTCs with antibody (EpCAM)-coated microposts.

Translation of stem cells therapies into clinic & microtechnology

- Microtechnology and microfluidic systems are potentially relevant tools to integrate different elements and to perform quick evaluations of multiple biological activities of stem cells, increasing the chances to obtain an *in vitro* potency test with excellent correlation with *in vivo* therapeutic activities.

Conclusion

- Microfluidics has been concentrated on research group dedicated mostly to design and fabricate biologically meaningful devices.
- New collaborative endeavors between expert in the field of microfluidics and stem cell researchers are necessary to make significant progress in the field.

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Research Article

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Regenerative Medicine

Extracellular vesicles derived from mesenchymal cells: perspective treatment for cutaneous wound healing in pediatrics

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Aim: We evaluated the effects of the intradermal injection of extracellular vesicles (EVs) derived from adipose stem cells (ASC-EVs) and bone marrow cells (BM-EVs) in an experimental cutaneous wound repair model. **Methods:** Mesenchymal stem cells (MSCs) were *in vitro* expanded from adipose (ASC) or BM tissues (BM-MSC) of rabbits. EVs were separated from the supernatants of confluent ASC and BM-MSCs. Two skin wounds were induced in each animal and treated with MSC or EV injections. Histological examination was performed postinoculation. **Results:** EV-treated wounds exhibited a better restoration compared with the counterpart MSC treatment. ASC-EV-treated wounds were significantly better than BM-EVs ($p = 0.036$). **Conclusion:** EV topical inoculation provides restored architecture during cutaneous wound healing and represents a promising solution for regenerative medicine in children.

First draft submitted: 3 January 2018; Accepted for publication: 14 May 2018; Published online: 9 July 2018

Keywords: animal model • children • cutaneous wound repair • extracellular vesicles • mesenchymal stem cells

Wound healing is a complex dynamic response to a physical trauma that comprises three overlapping phases: inflammatory, proliferative and remodeling. Phase progression depends on a well-coordinated interplay of cell-signaling events at the wound site and surrounding tissues, in which endogenous stem cells are vital players [1].

Children have specific characteristics of wound healing. Tissue insult or injury in the fetus can heal without scarring. After 2 years of age, wound healing is usually very rapid with an important remodeling phase and can be associated with more hypertrophic scars, both in duration and in intensity, compared with younger infants [2]. Skin thickness and the limited extension of tissue is a serious negative factor in case of trauma, extensive damage or loss of tissue in the pediatric age, even though efficient growth tissue mechanisms exist [2]. Cutaneous wound healing remains a challenge in children with disfiguring giant melanocytic nevi, where complete surgical removal is difficult to achieve because of the lack of available skin to graft over the resultant defects [3,4], or following serious burns or due to tissue surgical destruction or trauma in which skin damage usually never fully recovers [5,6]. Stem cells offer promising possibilities for improved wound repair and tissue regeneration.

Preclinical studies have shown that bone or adipose tissue-derived mesenchymal stem cells (MSCs) have a competitive advantage over other types of stem cells due to their better defined multipotent differentiating potential, paracrine effects, immunomodulatory properties and better safety profile [1,7–9]. However, large controlled clinical

Future Medicine

trials are needed to examine MSC capabilities in humans and further assess their safety profile to support their potential use in safe regenerative medical approaches [7–9], particularly in pediatric patients [10].

The therapeutic effect of MSCs is increasingly attributed to their capacity to secrete soluble factors that influence tissue regeneration by inhibiting cell apoptosis, stimulating cell proliferation and promoting tissue vascularization [7]. Recent studies have indicated that beside soluble factors, MSCs also secrete extracellular vesicles (EVs) which are small, spherical membrane-bound fragments involved in cell-to-cell communication and are capable of altering the cell fate and phenotype of recipient cells [8]. EVs derived from MSCs (EV-MSCs) have been shown to mimic the proregenerative effects of the origin cells in different experimental models [9].

We previously demonstrated that topical inoculation of adipose stem cells (ASCs) restored skin architecture during cutaneous wound healing, more rapidly than bone marrow-MSCs (BM-MSCs) [10].

In the present study, we evaluated the effects of an intradermal injection of EVs derived from ASCs (ASC-EVs) or from bone marrow-mesenchymal stem cells (BM-MSCs-EVs) in an experimental cutaneous wound repair model. Results of autologous and allogeneic ASCs and BM-MSCs were also compared.

Methods

Adipose tissue & bone marrow harvest

Healthy young female New Zealand rabbits (n = 18, 3 months old, median weight 3.5 kg) [11], were used as the animal model. The experimental protocol was approved by the National Animal Care and Ethics Committee (reference number: DGSAF0009484-A-13/04/2015) and conducted in accordance with Italian and European legislation (D.lgs. 116/92, European Directives 86/609/EE for the protection of animals used in scientific and experimental studies and 2010–63 UE).

After overnight fasting, eight experimental animals were premedicated with an intramuscular midazolam injection (1 mg/kg). Under general anesthesia with Zoletil 0.4 ml/kg (Virbac, Milano, Italy), and after local anesthesia with levo-bupivacaine 0.25% or ropivacaine 0.2% (2 ml/cm wound), a 2 cm longitudinal incision was made in the inguinal area in order to harvest the adipose panicle (lipectomy), while BM was harvested by aspiration from femoral medullary cavities [10].

Local anesthesia with levo-bupivacaine 0.25% or ropivacaine 0.2% (2 ml/cm wound) were administered for pain management. Subcutaneous Enrofloxacin (0.1 ml/2 kg/day for 3 days; Bayer, Milano, Italy) and Meloxicam (0.3 mg/kg/day for 3 days; Boehringer Ingelheim, Milano, Italy) were subsequently administered [10].

The animals were housed until cells and EVs were ready for autologous injection, another group of eight rabbits was then used for the allogeneic setting with the same MSCs and EVs previously expanded.

Isolation, culture & characterization of ASCs & BM-MSCs

ASCs and BM-MSCs and respective EVs from eight rabbits were isolated, cultured and characterized as previously described [10,12]. Briefly, to obtain ASCs, inguinal fat pads were placed in sterile phosphate-buffered saline (PBS, Euroclone, Milan, Italy) with gentamicin, minced manually and digested with 0.0075% type II collagenase (3 mg/ml, Sigma-Aldrich, MO, USA) in Dulbecco's Modified Eagle Medium (DMEM, Gibco, Invitrogen, Monza, Italy) for 20 min at 37°C with gentle agitation. The stromal vascular fraction, containing ASCs, was suspended in DMEM +10% fetal bovine serum (FBS, Euroclone), in order to inhibit enzyme activity. The specimen was then filtered through a 100 mm sterile nylon mesh filter (Millipore, Darmstadt, Germany), and centrifuged at 1200 rpm for 10 min. The resultant pellet was suspended and counted with 0.4% Trypan blue (Sigma-Aldrich). Cells were subsequently plated in culture flasks (Corning Costar, Amsterdam, The Netherlands) at a density of 160,000/cm^2 in αMEM (Gibco, Invitrogen) containing 10% FBS (Euroclone) and 1% antibiotic–antimycotic (Sigma-Aldrich) at 37°C, 5% CO_2 in a humidified atmosphere.

To obtain BM-MSC, mononuclear cells were isolated from 1 ml of BM aspirate, by density gradient centrifugation (Ficoll 1.077 g/ml; Lympholyte, Cedarlane Laboratories Ltd, The Netherlands), counted and plated at the same density of 160,000/cm^2 in the same culture medium. Cultures were then maintained at 37°C, 5% CO_2 in a humidified atmosphere. After 48 h, nonadherent cells were removed and culture medium was replaced twice a week. After reaching ≥80% confluence, MSCs were harvested using Trypsin–EDTA (Lonza, Copenhagen, Denmark), and propagated at 4000 cells/cm^2. ASC were expanded until passage (P)4, while BM-MSC were expanded until P3 because of their lower rate of proliferation. At each passage, viable cells were counted using 0.1% eosin and culture supernatants were tested for sterility.

Table 1. Scheme of the injections.

Animal	First lesion	Second lesion
Rabbit 1	Allogeneic ASC	Control
Rabbit 2	Allogeneic BM-MSC	Control
Rabbit 3	Allogeneic ASC-EV	Control
Rabbit 4	Allogeneic BM-MSC-EV	Control
Rabbits 5 8	Autologous ASC-EV	Autologous ASC
Rabbits 9 12	Autologous BM-MSC-EV	Autologous BM-MSC
Rabbits 13 15	Allogeneic ASC-EV	Allogeneic ASC
Rabbits 16 18	Allogeneic BM-MSC-EV	Allogeneic BM-MSC

ASC: Adipose stem cell; ASC-EV: Adipose stem cell-extracellular vesicle; BM-MSC-EV: Bone marrow-mesenchymal stem cell-extracellular vesicle.

Isolation & characterization of ASC-EVs & BM-MSC-EVs

EVs were obtained from the supernatants of confluent ASC and BM-MSCs following a standard procedure, previously reported by Camussi *et al.* and validated in human MSC using specific exosome markers such as CD63, CD9 and CD81 [12]. Cells were cultured overnight in D-MEM deprived of FBS. To obtain EVs, the supernatants cells were centrifugated at 2000 $\times g$ for 20 min to remove debris, cell-free supernatants were then centrifuged at 100,000 g (Beckman Coulter Optima L-90K ultracentrifuge) for 1 h at 4°C, washed in serum-free medium and submitted to a second ultracentrifugation under the same conditions. EVs were characterized by flow cytometry by measuring their dimensions and expression of positive and negative MSC surface markers. Monoclonal antibodies specific for rabbit CD49e (as positive marker) and CD45 (as negative marker) were used [8,12].

Calibration beads (range 0.1–1 μm) were employed to gate MVs by dimension parameters (Mega Mix, Stago, Milan). Analysis of CD marker expression was performed by direct immunofluorescence with a Navios flow cytometer (Bekman Coulter) and data were calculated using the Kaluza software.

Rabbit cutaneous wound model

When an adequate number of MSCs and EVs were obtained, the skin wounds were surgically induced. After overnight fasting, experimental animals were premedicated with intramuscular midazolam (1 mg/kg). Under general anesthesia with Zoletil 0.4 ml/kg and after local anesthesia with levo-bupivacaine 0.25% or ropivacaine 0.2% (2 ml/cm wound), two identical full thickness 2 × 2 cm wounds were created on the back of each rabbit, at a distance of more than 2 cm from each other. After creation of the wounds, local anesthesia with levo-bupivacaine 0.25% or ropivacaine 0.2% (2 ml/cm wound) was administered. Subcutaneous enrofloxacin (0.1 ml/2 kg/day for 3 days) and meloxicam (0.3 mg/kg/day for 3 days) were subsequently administered [10].

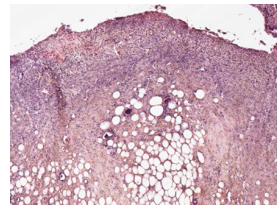
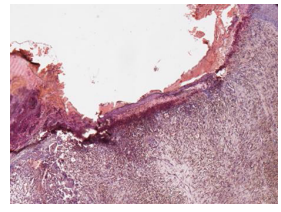
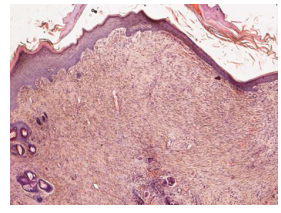
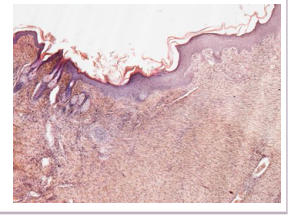
Intradermal injection

Rabbits were randomized as reported in Table 1. Within 5 min of wound lesion establishment, autologous or allogeneic ASC, BM-MSC, ASC-EVs or BM-MSC-EVs in 3 ml of saline + 2% rabbit albumin (Sigma-Aldrich) were injected (1.5 ml subcutaneously injected around the wounds at four injection sites and 1.5 ml directly applied to the wound beds of the first lesion). The protocol called for an injection of 10×10^6 cells into the wound bed [10]; while, an EVs volume obtained from the 10×10^6 of confluent ASC or BM-MSC was injected. As a control, 3 ml of saline, 2% rabbit albumin solution were injected. Rabbits did not receive any immune suppression. The general conditions of the animals and wound healing were monitored daily. The wounds were photographed using a digital camera.

Histological analyses

Rabbits were euthanized with a bolus of pentobarbital 100 mg/kg iv. For histological examination, wounds were harvested after 14 days of treatment (rabbits). The regenerated tissue biopsies were collected using dermal biopsy punches. The samples were bisected along the widest line of the wound, fixed in 4% neutral-buffered formalin for 48 h, dehydrated with a gradient alcohol series, cleared in xylene and eventually embedded in paraffin. Sections (8 μm) were obtained using a Leitz microtome and prepared for histology. All stained slides were examined under a Axiophot Zeiss light microscope (Oberkochen, Germany) equipped with a digital camera.

Table 2. Histological scoring system.

Score	Epidermal phenotypes	Dermal phenotypes	Histological phenotypes
1	Incomplete re-epithelialization; the surface is covered by fibrinous exudate infiltrated by polymorphs	High degree of inflammation in nonepithelized area; alterated collagen matrix organization, a layer of granulation tissue with dilated capillaries and edema, adipose tissue substitution	
2	Complete or nearly complete re-epithelialization; the epidermis has variable thickness and is linear	No dermal papillae. Mild infammatory cell infiltration, dilated vessels, granulated tissue	
3	Complete re-epithelialization; epidermis shows complete keratinization	Presence of dermal papillae. Remodeling of the granulation layer and collagen fibers	
4	Complete re-epithelialization; epidermis has normal thickness and is normally keratinized	Presence of dermal papillae and cutaneous annexes (roots of the hair, sebaceous glands). Well-formed connective matrix with thick collagen bundles in the reticular dermis and a network of thin fibers in the papillary dermis	

Histological scores were assigned as described by Galeano *et al.*, [13] considering the degree of epithelialization (absence or presence of epithelial covering, crusting and intraepithelial inflammatory cells), granulation tissue and collagen matrix organization (adipose tissue substitution as an index of impaired wound closure, dense eosinophilic collagen matrix, edema, hemorrhage and degree of inflammation), inflammatory infiltrates (neutrophils and lymphocytes) and angiogenesis. The histological scoring system ranged between 1 and 4 as described in Table 2.

The pathologist was blinded to the scheme of injections.

Statistical analysis

Quantitative data and the histological score were described as the median and interquartile range (IQR: 25–75th percentile) and compared by fitting multivariable ordinal logistic regression models. Groups (using controls as a reference) were included in the models as independent variables. Wald tests of simple and composite linear hypotheses regarding the parameters of the fitted model were performed to evaluate EV versus MSC and cells or EVs obtained from adipose tissue versus cells or EVs expanded from BM. Statistical significance was defined as a $p < 0.05$. Data analyses were performed with the STATA statistical package (release 14.2, 2012, Stata Corporation, TX, USA).

Results

ASC-EVs & BM-MSC-EVs

Following standard procedures, we were able to expand cells both from adipose tissue and BM in all eight animals. All of the MSC exhibited typical characteristics of plastic adherence, spindle-shape morphology and capacity to differentiate, as previously described [10]. We were also able to obtain and characterize EVs, in particular to precisely

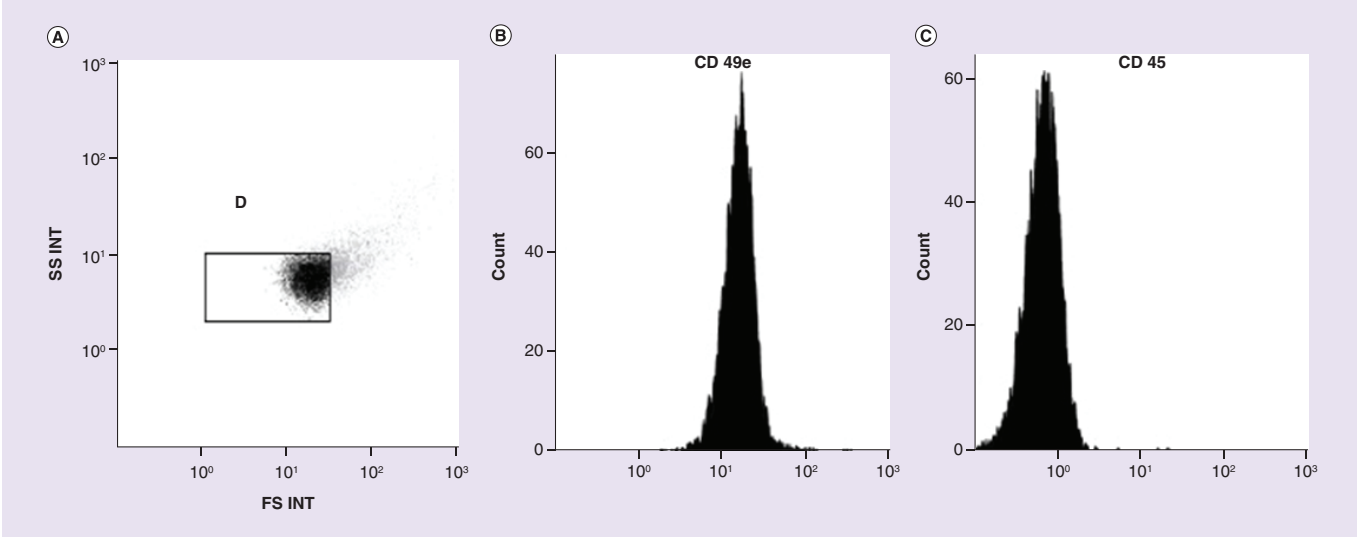


Figure 1. Morphological characterization of a representative extracellular vesicles (EVs) preparation (A), region D is defined using 0.1 μm calibration beads. EV characterization using a positive surface marker as anti-CD49e **(B)** and one negative as anti-CD45 **(C)**.

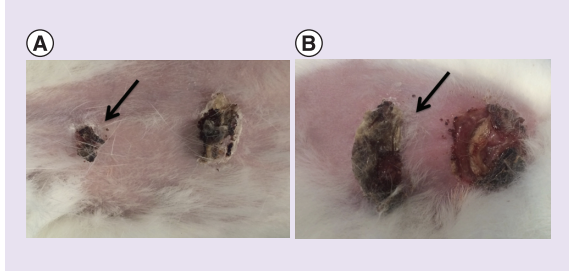


Figure 2. Skin regeneration at Day 14 after autologous ASC-EVs injection (arrow) vs ASCs (A), autologous BM-MSC-EVs (arrow) vs BM-MSCs (B).
ASC-EV: Adipose stem cell-derived extracellular vesicle; BM-EV: Bone marrow-derived extra-vesicle; BM-MSC: Bone marrow-derived mesenchymal stem cell.

gate them by morphological parameters, using 0.1–1 μm calibration beads. Cytofluorimetric analysis showed the presence of EVs positive for CD49e, a surface marker expressed on the rabbit MSC surface and negative for CD45 (Figure 1).

Macroscopic aspect of wound healing

No severe infections were reported for any of the skin wounds. Skin regeneration at Day 14, after EV injection was more rapid compared with MSCs- and/or saline-treated lesions. Lesions treated with cells or EVs derived from BM seem to exhibited slower wound restoration in comparison with wounds inoculated with sources from adipose tissue (Figure 2). ASC-EV treated wounds exhibited more complete wound healing when compared with the other experimental conditions.

Histological results

At 14 days, saline-treated wounds had extensive skin defects, necrosis and inflammatory cells in the cutaneous layer, as well as absence of hair follicles and sebaceous glands in the dermis.

Conversely, all of the treatment groups had accelerated reduction of inflammatory infiltration and partial restoration of the epidermal and dermal structure. The details of the hematoxylin and eosin-stained sections of wounds observed under the light microscopy are reported in Figure 3.

Histological scores for the different experimental conditions are provided in Figure 4. No differences were observed between the autologous or allogeneic setting (Figure 4). For this reason, data were statistically evaluated without this distinction.

EV-treated wounds exhibited better restoration compared with the MSC treatment ($p = 0.005$). In particular, BM-EVs achieved better regeneration than BM-MSC ($p = 0.048$) and ASC-EVs than ASC ($p = 0.017$) with

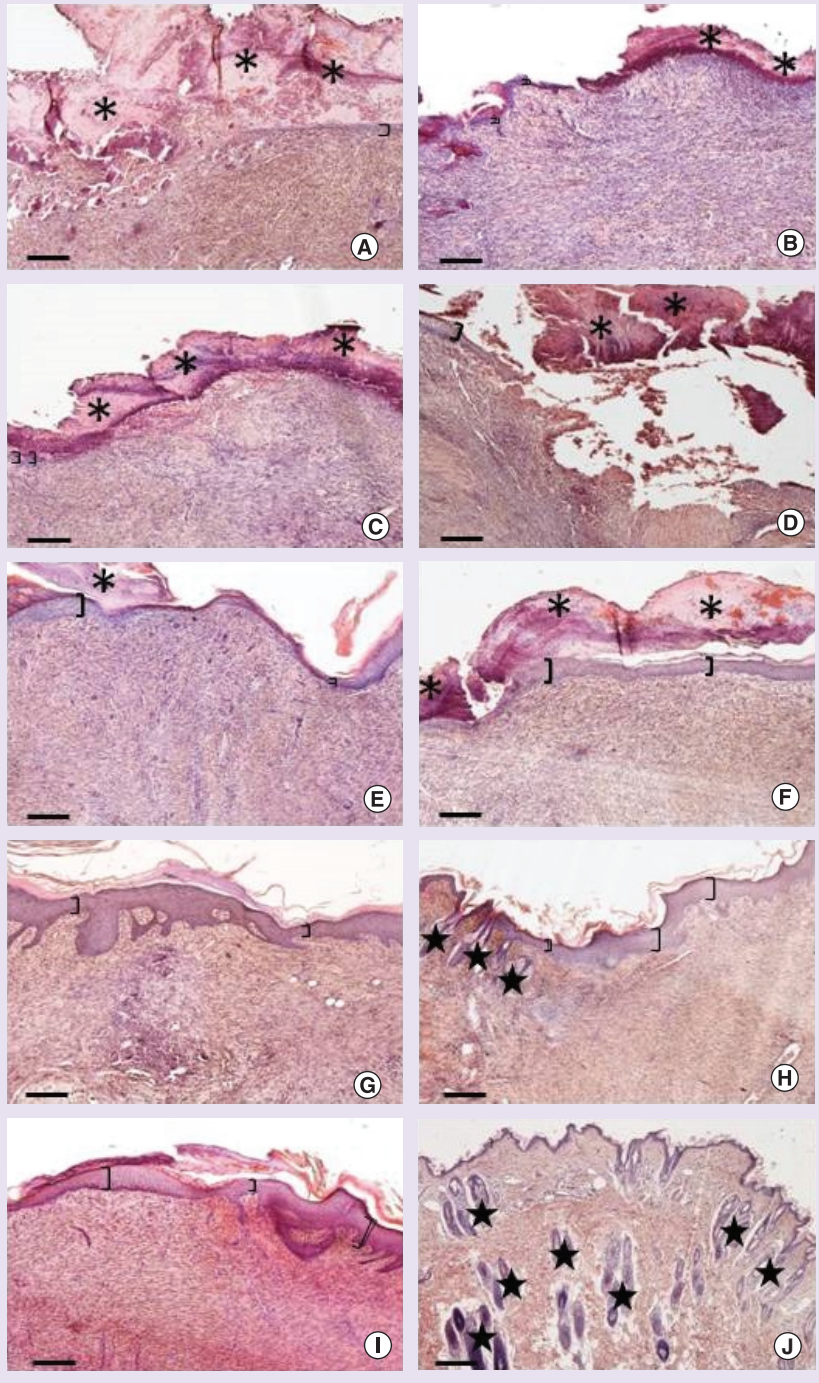


Figure 3. Haematoxylin and eosin stained sections of wounds at Day 14 observed under light microscopy. Original magnification 5× (Bar = 200 mm; Epidermis = Bracket; Necrotic material = Asterisk; Roots of the hair and sebaceous glands = Star; Empty dots = Allogeneic setting; Full dots = Autologous setting). Skin of saline-treated animals **(A)** shows extensive necrosis and inflammatory cells in the cutaneous layer and absence of hair follicles and skin glands. Autologous BM-MSCs **(B)** as well as allogenic BM-MSCs **(C)** treated wounds have an incomplete epithelial layer with mild inflammatory cell infiltration. The skin of autologous ASC-infused rabbits **(D)** exhibit nearly complete re-epithelialization, while in allogenic ASCs **(E)** and autologous BM-MSC-EVs **(F)** a complete epithelial layer as well as a partially organized derma is visible. Animals treated with allogenic BM-MSC-EVs **(G)** exhibit almost completely restored skin, with a keratin layer and reorganized dermal structures. Autologous **(H)** and allogenic ASC-EV **(I)** treated wounds exhibit well regenerated tissue with the presence of a complete epithelial layer, similar to that present in healthy skin **(J)**. Dermal papillae and cutaneous annexes (roots of the hair, sebaceous glands) are present. The connective matrix is also well restored, with thick collagen bundles in the reticular dermis and a network of thin fibers in the papillary dermis.
ASC: Adipose stem cell; ASC-EVs: Adipose stem cell-derived extracellular vesicles; BM-EV: Bone marrow-derived extra-vesicle; BM-MSC: Bone marrow-derived mesenchymal stem cell.

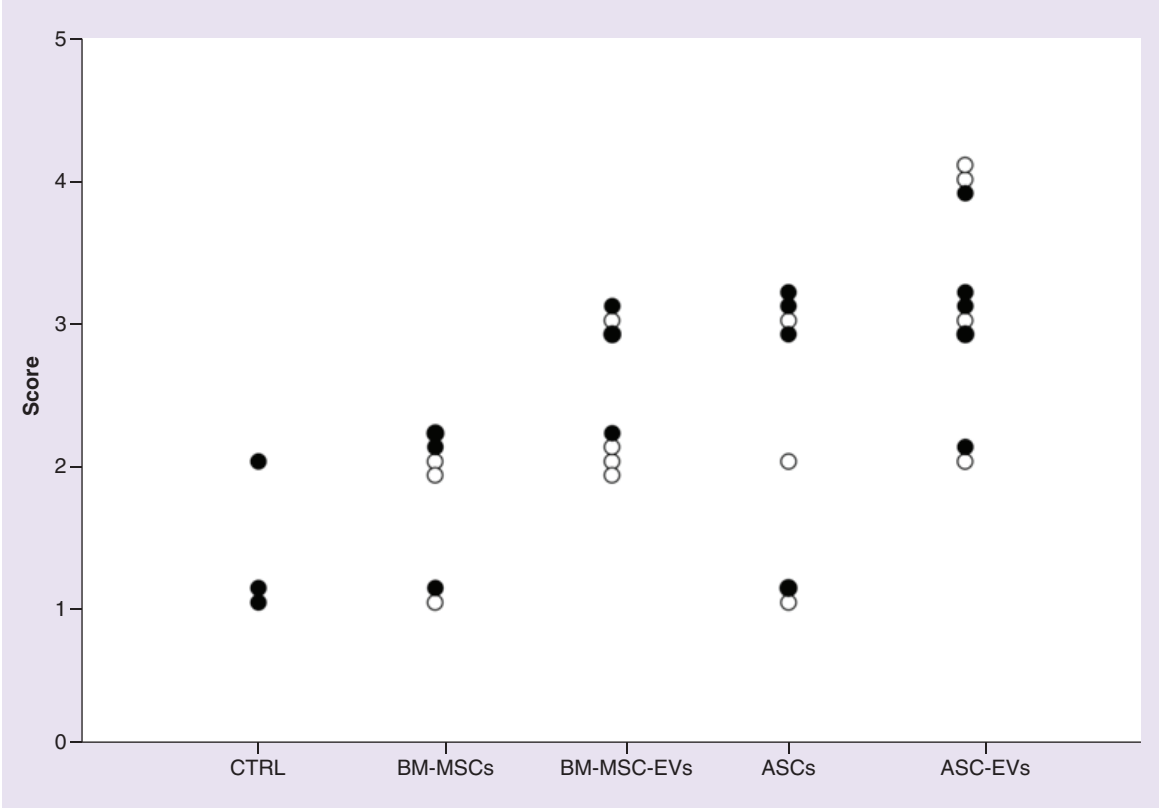


Figure 4. Histological scores of wounds according to Galeano's criteria [13], for the different experimental groups.
Empty dots = Allogeneic setting; Full dots = Autologous setting.
ASCs: Adipose stem cells; ASC-EVs: Adipose stem cell-derived extracellular vesicles; BM-MSCs: Bone marrow-derived mesenchymal stem cells; BM-MSC-EVs: Bone marrow-mesenchymal stem cell-derived extra-vesicles; CTRL: Saline treated control.

complete re-epithelialization and restored architecture between the dermis and epidermis with the presence of dermal papillae. Moreover, as previously reported, wound restoration was better when cells or EVs obtained from adipose tissue were used with respect to cells or EVs expanded from BM ($p = 0.036$).

Discussion

In the present study, we investigated whether the intradermal injection of ASC-EVs or BM-MSC-EVs exerts therapeutic effects in a cutaneous injury model. Our findings demonstrated for the first time that MSC-EV treated wounds promote tissue regeneration with the presence of a complete epithelial layer. Further, the results indicated that EVs obtained from adipose tissue promote better regeneration than EVs expanded from BM.

The integrity of healthy skin plays a crucial role in the maintenance of physiological homeostasis. Moreover, it provides protection against mechanical forces and infections, fluid imbalance and thermal dysregulation. At the same time, it allows for flexibility enabling joint function in some areas of the body and more rigid fixation to hinder shifting of the palm or foot sole.

Inadequate wound healing necessitates medical intervention; tissue destruction following surgery or acute trauma are followed by a loss of skin organ function rendering the organism vulnerable to infections, thermal dysregulation and fluid loss [14–18,20]. Currently, treatment outcomes are not always ideal because of the failure to achieve complete wound closure in around 60% of cases, as well as high rates of recurrence and scarring [19]. Therefore, there is a need for more effective therapies. Stem cells offer promising possibilities and they have been considered as an excellent material for regenerative medicine. Preclinical studies have shown that bone- or adipose tissue-derived MSCs have a competitive advantage over other types of stem cells due to their better defined multipotent differentiating potential, paracrine effects, immunomodulatory properties and safety profile [7–9].

Wound healing requires coordinated interplay among cells, growth factors and extracellular matrix proteins. Central to this process are endogenous MSC, which coordinate the repair response by recruiting other host cells and secreting growth factors and matrix proteins [9,14–18]. The beneficial effect of exogenous MSCs on wound healing has been observed in a variety of animal models and in reported clinical cases [7–10].

The therapeutic effects of MSCs might also be attributed to the secretion of a wide array of paracrine factors in order to stimulate the survival and functional recovery of resident cells, or to regulate the local microenviroment or niche and immune response [19,21]. In fact, stem cells are able to communicate with nearby and distant cells through soluble factors and direct cell–cell contact by long and thin tubular appendages such as cytonemes and cilia, as well as via detached, EVs.

Recently, on the basis of paracrine or endocrine MSC mechanisms, a novel strategy of 'cell-free therapy' which uses cell-derived EVs for tissue repair has been proposed [27–32]. EVs are membranous structures released by various cells; they can transfer bioactive molecular contents including proteins, mRNAs and miRNAs sequences to target injured tissue cells [12]. EVs are released from many cell types and recent studies have shown that EVs are one of the key secretory products of MSCs mediating cell-to-cell communication to enhance wound healing [28]. Hu *et al.* [33] reported that exosomes secreted by ASCs have a positive role in the promotion of skin tissue wound repair and can facilitate cutaneous wound healing by optimizing the characteristics of fibroblasts.

In the present study, we obtained EVs from adipose tissue and BM and confirmed the potential use of EVs in skin regeneration. The high abundance of MSCs found in adipose tissue makes it a very attractive source of adult stem cells. Additionally, adipose tissue-derived MSCs are expected to be a more reliable cell source for regenerative medicine because they can be isolated using minimally invasive techniques compared with other MSCs. We showed that MSC-EVs are superior in their ability to support wound healing and that the adipose tissue source induced better wound restoration compared with BM [10,33]. These data suggest that different therapeutic potentials of MSCs from various sources [20] may also correlate with different paracrine effects. Even though the autologous setting is generally recommended, the absence of differences in therapeutic effects between autologous or allogeneic sources may be considered a relevant factor in the translation of results to the pediatric population, particularly in neonates or young infants, in which technical difficulties in obtaining adipose tissue can occur.

Children with abnormal, extended or pathological wound healing should benefit from complementary treatments to minimize tissue scarring and to prevent specific complications related to their growth body rate, such as contractures, alopecia and scar intussusceptions [2,22]. Several reports support the promise of MSCs in dermal wound healing, nevertheless clinical translation to the pediatric age remains limited [21]. The translation of MSCs to the clinic should be cautiously considered, because of immune-mediated rejection, senescence-induced genetic instability or loss of function, limited cell survival and possibility of malignant MSC transformation [34]. Our preclinical study, using young animals, showed that EVs could be a promising alternative to treat wounds in children. After ASC-EV inoculation, well-regenerated tissue with the presence of a complete epithelial layer, dermal papillae and cutaneous annexes and restored connective matrix was obtained. The complete reparative properties observed supports the therapeutic role of EVs in both superficial and in full-thickness cutaneous lesions, such as third-degree burns, in which all layers of skin are involved and hair follicles and sebaceous glands are destroyed. Thus, MSC-EVs represent a novel 'cell-free therapy' for pediatric skin regeneration, which might overcome the obstacles and risks associated with the use of native or engineered stem cells. Moreover, further studies are mandatory to optimize the exact dose of MSC-EVs necessary to obtain the best results.

The evolutionary character of wound healing in children implies a need for further dedicated experimental and preclinical studies focusing on cell free extracellular vesicle approaches in order to validate their skin regeneration efficacy in the pediatric area.

Conclusion

Rabbit EVs can be obtained from *in vitro* ASCs and BM-MSCs. Topical inoculation of EVs restored skin architecture during cutaneous wound healing, with better results after ASC-EV treatment as compared with BM-MSC-EVs. The use of EVs may improve regenerative medicine in pediatric surgery.

Translational perspective

Our preclinical study, using young animals, showed that EVs could be a promising alternative to treat wounds in children. After ASC-EV inoculation, well-regenerated tissue with the presence of a complete epithelial layer, dermal papillae and cutaneous annexes and restored connective matrix was obtained. The complete reparative properties

observed support the therapeutic role of EVs in both superficial and in full thickness cutaneous lesions, such as third degree burns, in which all layers of the skin are involved and hair follicles and sebaceous glands are destroyed. These experimental results could also be translated to regenerative medicine for the treatment of congenital and acquired skin lesions occurring in the pediatric age, particularly in neonates or young infants with disfiguring lesions, in which the technical difficulties in obtaining adipose tissue preclude their use.

Thus, MSC-EVs represent a novel 'cell-free therapy' for pediatric skin regeneration, which might overcome the obstacles and risks associated with the use of native or engineered stem cells.

Summary points

- Based on the paracrine and endocrine mechanisms of mesenchymal stem cells (MSCs), extracellular vesicles (EVs) derived from MSCs have been used for tissue repair.
- We evaluated the effects of the intradermal injection of EVs derived from adipose stem cells (ASC-EVs) and bone marrow (BM-MSCs-EVs) in an experimental cutaneous wound repair model. A comparison of autologous and allogeneic ASCs versus BM-MSCs is also reported.
- MSCs were *in vitro* expanded from adipose (ASC) or BM tissues (BM-MSC) of young female New Zealand rabbits. EVs were obtained from the supernatants of confluent ASC and BM-MSCs.
- All treatments accelerated inflammatory infiltrate reduction and promoted partial epidermal and dermal restoration.
- EV treated wounds, exhibited better restoration compared with the counterpart MSC treatment.
- Better wound restoration was observed with cells or EVs obtained from adipose tissue in comparison with BM.
- EV topical inoculation provides restoration of the skin architecture during cutaneous wound healing, and significantly better results in comparison with those obtained from ASC.
- EVs represent a promising solution for regenerative medicine in pediatric surgery.

Acknowledgements

The authors thank L Kelly for English revision of the manuscript.

Financial & competing interests disclosure

This study was supported by grants from the project 'Fetal therapy for congenital malformations' and from Fondazione IRCCS Policlinico San Matteo (RC 08066110/13, project number 765).

No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Conflict of interest

The authors declare that they have no conflict of interest.

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