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MINIREVIEWS

- 43 Stem cells as delivery vehicles for regenerative medicine-challenges and perspectives

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Stem cells as delivery vehicles for regenerative medicine- challenges and perspectives

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Abstract

The use of stem cells as carriers for therapeutic agents is an appealing modality for targeting tissues or organs of interest. Combined delivery of cells together with various information molecules as therapeutic agents has the potential to enhance, modulate or even initiate local or systemic repair processes, increasing stem cell efficiency for regenerative medicine applications. Stem-cell-mediated delivery of genes, proteins or small molecules takes advantage of the innate capability of stem cells to migrate and home to injury sites. As the native migratory properties are affected by *in vitro* expansion, the existent methods for enhancing stem cell targeting capabilities (modified culture methods, genetic modification, cell surface engineering) are described. The role of various nanoparticles in equipping stem cells with therapeutic small molecules is revised together with their class-specific advantages and shortcomings. Modalities to circumvent common challenges when designing a stem-cell-mediated targeted delivery system are described as well as future prospects in using this approach for regenerative medicine applications.

Key words: Stem cells; Delivery agents; Regenerative medicine; Nanoparticles; Targeted delivery

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Core tip: The capability of stem cells to mobilize, home and target to inflammatory sites justifies their use as delivery agents for regenerative medicine purposes. Cell

and membrane engineering techniques can be used to increase the selective targeting potential of stem cells. Gene therapy and nanoparticle-mediated small-molecule delivery of informational cues have the potential to increase the efficiency of clinically relevant stem-cell-based regenerative therapies.

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INTRODUCTION

Stem cells, the natural reservoir for growth, development and repair in all multicellular organisms, have been the object of intense scrutiny in the last several decades for scientific reasons, but most of all, for their potential applications in biology and medicine. Stem cells are defined by their capability to self-renew and to differentiate into more specialized progeny according to their degree of potency^[1]. The emerging domain of regenerative medicine (RM) aims to make possible the complete functional and structural restoration of tissues, organs or even bodily systems. Due to their unique properties in modulating local and systemic reparatory processes, stem cells are employed by RM as main the therapeutic tool^[2]. Basic and preclinical research involving (stem) cell therapy to fight acute or chronic degenerative disease in various medical fields is currently underway. Stem-cell-based tissue engineering applications aim to produce implantable bio-substitutes that could waive the need for costly tissue and organ transplantation^[3,4]. Some of the products of this new, research-intensive medical field are already in the stage of clinical trials, which promises to deliver revolutionary therapies in the near future.

Different types of stem cells are currently tested for RM applications: Embryonic stem cells (ESCs)^[5] adult stem cells (ASCs)^[6] or induced pluripotent stem cells (iPSCs)^[7], each of them with specific advantages and risks. An outline of stem cell type-dependent advantages and shortcomings relative to their use as therapeutic agents is beyond the scope of this review; the reader is referred to excellent works in this respect^[8,9].

Dependent on their type and therapeutic application, stem cells are expected to exert a certain effect, be it in recomposing the anatomy and/or function of a given structure, in restoring dysfunctional biological pathways or in modulating the local or systemic immune response. Stem-cell-based TE of various tissues (such as bone, cartilage, neural, cardiac or skeletal muscle and so on) is based on the cell capability to generate tissue specific progenies that directly recombine the given structure. TE strategies are largely based on

directed differentiation and structural participation of ESCs, iPSCs or mesenchymal stem cells (MSCs). Due to their large availability, MSCs are the most commonly used ASCs for RM applications. MSCs were shown to release cytokines and growth factors (GFs) that exert paracrine effects upon transplantation within an injured structure, contributing to tissue repair by recruiting local cells to induce repair^[10]. The expression of bioactive molecules with multiple trophic (regenerative) and immunomodulatory effects has prompted a call for changing the MSC denomination in medicinal signalling cells to more accurately reflect their potential for acting as therapeutic drugs^[11]. Stem-cell-derived microvesicles containing mRNA, microRNA and proteins and released as exosomes into the intercellular milieu as a modality of cell-cell communication are increasingly recognized as potent therapeutic agents. ESC- and MSC-derived exosomes that contribute to restraining tissue injury and induce cell cycle re-entry of resident cells, leading to tissue self-repair, are being tested for different applications in cardiac, neural or musculoskeletal repair^[12,13].

ESCs and MSCs were shown to home and engraft to the site of injury, facilitating tissue and organ repair following traumatic, degenerative, ischaemic or inflammatory processes^[14,15]. The stem cell migratory potential enables the choice for systemic delivery whenever the direct, intra-lesion administration is invasive, implies associated morbidity or involves the situation of multiple and/or remote lesions^[16]. In particular, MSCs are in focus as delivery vehicles due to their homing capabilities, low immunogenicity (not expressing class II histocompatibility molecules) and immune-modulatory capabilities. Recently, the use of stem cells as delivery agents for artificially produced molecules that exert a local or systemic therapeutic effect has emerged as another field of cell therapy application. In the following, a brief outline of MSC native and enhanced homing mechanisms, methods of loading cells with therapeutic agents and several current applications of stem-cell-based delivery will be presented.

HOMING OF STEM CELLS

Tissues and organs possess, at least to a certain extent, the capability to repair or regenerate. The natural repair mechanisms are based on activation of the intrinsic progenitor population, trans-differentiation of local adult elements and the capability to recruit and home circulating stem cells^[17]. Homing has been defined as the cell capability to arrest in the blood stream and localize and migrate through the endothelial walls of blood vessels within the tissue. This spontaneously occurring process enhances reparatory mechanisms, adding both cellular stock as well as signalling molecules, and normally leads to restoration of local structural and physiological parameters. The capability of self-repair can be overwhelmed by the severity of the injury combined with local or systemic particularities such as

inflammation, disease or ageing.

The use of stem cells as therapeutic agents is expected to increase or substitute regenerative processes. The use of stem cells as “biological regenerative supplementation” is largely based on their natural capability to mobilize, migrate and home. The mechanisms for cell migration and “nesting” to the sites of injuries have been described mostly in relation to MSCs, particularly regarding their migration to the bone marrow and other tissues. When mobilized from the host or therapeutically administered, similarly to leucocytes, MSCs first reach the blood stream, entering the systemic venous or arterial circulation. Reaching the target, the cells decelerate and come in contact with the endothelial walls by rolling and tethering. In this second step, MSC G-coupled protein receptors are activated followed by their arrest on the endothelial membrane within the target tissue by means of integrin receptor activation (third step)^[18]. Transmigration through both the endothelium and the underlying basal extracellular matrix (ECM) membrane represent the fourth and final step of homing. The process normally takes a few hours and results in transient retention of stem cells within the tissue. The navigation process of homing only implies cell anchorage and transmigration from the blood stream to the tissue. Depending on the stem cell type and tissue characteristics, this process can be followed by division of the migrated cells, having as a consequence their engraftment and, eventually, tissue repopulation^[19]. Homing is based on several constitutive abilities of stem cells to respond to external cues that enable their trafficking, guidance and migration. For therapeutic purposes, both stem cells as well as the external cues that govern homing can be modulated to enhance the targeting process and optimize the expected results.

MECHANISMS INVOLVED IN STEM CELL MIGRATION AND HOMING

Stem cells sense and are able to move according to a chemoattractant gradient by means of amoeboid cell migration. This highly conserved process, termed “chemotaxis” or “interstitial migration”, can take place independently of blood flow. Chemoattractant gradients within the extracellular space or mechanical signals guide cell movement by directing cellular actin polymerization. Different cell types can migrate (such as leucocytes, progenitor and stem cells or metastatic cancer cells) displaying specific changes in their focal adhesion, myosin-based contractility and actin polymerization^[20]. In naturally occurring circumstances, the first steps of the migration of progenitor cells require their mobilization by disruption of the ECM as well as cell adhesion to matrix proteins by means of proteolytic enzymes released by the injury and/or local inflammatory processes^[21]. In turn, stem cells release

proteases that contribute to ECM remodelling, also facilitating the migratory process. MSCs were shown to express several key components of the fibrinolytic cascade (including urokinase plasminogen activator receptor (uPAR) and plasminogen activator inhibitor (PAI-10) of the fibrinolytic system) known to exert crucial roles in cell migration, growth factor bioavailability during inflammation, tissue regeneration and cancer^[22]. The involvement of an autocrine mechanism based on heat shock protein 70 (HSP70) released by murine mesangioblast interaction with Toll-like receptor 4 (TLR4) and CD91 has been proposed as another mechanism responsible for the stem cell ability to transverse the ECM and stimulate migration^[23]. Adhesion molecules (such as E and P selectins) play an important role in the homing of haematopoietic stem cells (HSCs), being involved in the first step of tethering and rolling of those cells along the endothelial wall. Integrins such as CD49d/CD29 ($\alpha 4\beta 1$ or VLA-4) and CD11a/CD18 ($\alpha L\beta 2$ or LFA-1) are involved in adhesion of HSCs to the endothelium and trans-endothelial migration^[24]. VLA-4 binds to vascular cell adhesion molecule 1 (VCAM-1) within the endothelium and is functionally involved in MSC homing. MSCs express a large variety of chemokine membrane receptors that enable their migration towards wounds, inflammation and malignant-site-released chemokine gradients. It should be noted that chemokine receptors were shown to be species dependent, which is of importance when translating results from animal studies to clinical applications. For cultured MSCs, isolation and expansion methods are known to strongly influence the chemokine receptor repertoire inviting a thorough assessment when such cells are sought for targeting therapies^[25]. Several chemokine axes are known to regulate the homing of a large variety of stem cells. The CXCL12 (also known as stromal derived factor 1 α , SDF-1 α)-CXCR4 axis may represent a general mechanism for the chemoattraction of MSCs, haematopoietic stem cells, and neural and endothelial progenitors to injury sites. CXCL12-CXCR4 is constitutively expressed in several cell populations such as dermal fibroblasts, endothelial cells or pericytes, increasing consistently during tissue injury. CXCR4 and CXCR7 receptors that bind SDF-1 α have been detected on the surface of bone-marrow-derived MSCs and adipose-derived stem cells (ASDCs) that use an SDF1- α gradient to migrate to the site of acute and chronic injuries^[26]. Constitutively expressed on ESCs, CXCR4 is a major contributor to organogenesis during development. SDF-1 α /CXCR4 is also involved in pathological processes such as cancer stem cell migration and cancer metastasis^[27]. Other chemokine axes such as CCL27-CCR10, CCL5-CCR5 and the more skin-specific CCL21-CCR7 are involved in natural processes of stem cell migration and homing. Different methods of enhancing chemokine-based gradient targeting are currently under scrutiny for regenerative purposes (see below)^[28].

METHODS TO ENHANCE STEM CELL HOMING

Targeting injured or diseased tissues by stem cells largely depends on cell characteristics as well as on the existence of a chemoattractant gradient to guide their trafficking and homing. Stem cells and particularly MSCs are shown to possess migratory and targeting capabilities; however, only a small portion of therapeutically administered cells can home and engraft within the injured tissue^[29]. Several strategies addressing cells such as modified culture conditions, specific preconditioning or manipulation before transplantation are currently being tested with the purpose of increasing their migratory capabilities. Methods that modify external conditions such as improved delivery methods or modified route of administration contribute to increasing the number of cells that reach their target.

Improving homing by manipulating stem cell characteristics

Depending on their tissue of origin and donor age and in the absence of natural stimuli, MSCs express low amounts of chemokine receptors^[30]. *In vitro* expansion, which is required for obtaining a clinically relevant number of cells, further decreases the number of these receptors. Culturing stem cells under hypoxic conditions was shown to increase CXCR4, CXCR7 and SDF-1 expression by means of a hypoxia inducible factor-1 α (HIF-1 α) mechanism^[31]. Hypoxia can increase chemokine receptor expression, induce the differential expression of MMPs, reduce reactive oxygen species by reduced mitochondrial respiration, and induce Notch signalling pathway—all factors known to be involved in sustaining proliferative and migratory capabilities in stem cells^[32]. Some studies have raised concerns about the safety of MSCs cultured under hypoxic conditions^[33], although other reports show that the method results in culture-expanded cells that grow faster and display an enhanced migratory capability while being non-oncogenic and retaining multipotency^[34]. MSCs cultured at lower confluence were shown to possess superior migratory capabilities. It has been reported that highly confluent MSCs adapt to this condition by expressing higher amounts of tissue inhibitor of metalloproteinase-3 (TIMP-3), resulting in a decreased migratory potential^[35].

Three-dimensional (3D) culture conditions were shown to increase differentiation and surface antigen expression, increasing their therapeutic potential in terms of cell viability and targeting capabilities^[36]. Increased cell survival after transplantation and reduced replicative senescence was observed in spheroid cultured MSCs; however, a direct impact on the cell migratory potential and how this correlates with the drastic modification in the cell cytoskeleton under such conditions, needs to be further analysed^[37].

An interesting approach exists for increasing CXCR4

expression in mouse bone marrow MSCs that have internalized magnetic nanoparticles (MNPs). MNP-loaded cells were shown to possess improved cell homing efficiency. MNP payload enabled cell tracking *in vivo* using magnetic resonance imaging (MRI), demonstrating this could be an efficient strategy for enhancing cell targeting and tracking capability^[38].

Cell preconditioning

performed by exposing cultured cells to various soluble molecules is used to improve stem cell homing. Concern exists about the gradual decrease of chemokine receptors in cultivated MSCs; therefore, preconditioning could not only counteract this phenomenon but also enhance their innate targeting capability. Increased expression of cytokine membrane receptors (CXCR4) could be obtained using a cocktail of factors added to the culture media. Fms-like tyrosine kinase (Flt-3) ligand, stem cell factor (SCF), interleukin (IL) and hepatocyte growth factor (HGF) were shown to rapidly increase CXCR4 expression in human foetal-derived bone marrow stem cells and to increase their homing potential within the bone marrow of sub-lethally irradiated NOD/SCID mice^[39]. Conditioned medium from tumour necrosis factor alpha (TNF α) pre-stimulated cord-blood-derived stem cells were shown to enhance intravenously and intramuscularly administered epithelial progenitor cells (EPCs) in a model of hind limb ischaemia by a mechanism involving interleukin-6 (IL-6) and interleukin 8 (IL-8)^[40].

Preconditioning stem cells with insulin growth factor-1 (IGF-1) or with SDF-1 were shown to improve their migratory and homing capability *in vitro* and *in vivo*^[41]. Other small molecules such as inhibitors of glycogen synthase kinase-3 β (GSK-3 β) or erythropoietin combined with granulocyte-colony stimulating factor (G-CSF) were shown to enhance MSC migratory capability by enhancing CXCR4 and MMP expression or by stimulating the extracellular signal related kinase (ERK)1/2 pathway, respectively^[42,43]. MSC preconditioning with oxytocin was shown to increase the expression of protein kinase B (PKB or Akt) and phospho-ERK1/2 together with other proteins and genes such as vascular endothelial growth factor, thrombospondin, tissue inhibitor of metalloproteinase (TIMP-) 1, TIMP-2, TIMP-3, and MMP-2, increasing their therapeutic potential^[44,45]. Histone deacetylase (HDAC) inhibitor valproic acid combined with lithium was shown to be effective in enhancing the MSC migratory ability *in vitro* by means of a HDAC-CXCR4, GSK-3 β -MMP9 stimulatory mechanism^[46].

Another strategy to improve stem cell homing is to increase the expression of targeting molecules in therapeutic cells by gene manipulation. CXCR4 overexpression has been reported by several groups to show a variable efficiency in increasing the targeting potential of MSCs. Non-viral methods are preferred, especially considering potential clinical applications, but are notorious for having a low transfection efficiency.

The use of several cationic liposomal agents (such as IBAfect, a polycationic liposomal transfection reagent) was shown to yield an improved transfection efficiency compared to that of adenoviral methods, resulting in a superior chemotactic index in transfected cord-blood-derived MSCs^[47]. Overexpression of other chemokine receptors, such as CXCR7 and CXCR1, was shown to enhance the migratory and targeting properties in various stem cell populations^[48].

Stem cell surface modification with rapid incorporation of recombinant CXCR4 protein on the membrane was shown to enhance stem cell migration towards an SDF-1 gradient^[49]. Surface engineering aims to transiently modify cell membrane in order to improve their adhesion or endothelial transmigration. Several ingenious methods such as CD44 fucosylation to obtain P-selectin glycoprotein ligand-1 (PSGL-1) or HCELL on the surface of MSCs, biotinylation of MSC membranes or conjugating various antibodies against adhesion molecules [intercellular adhesion molecule (ICAM) or vascular adhesion molecule (VCAM-1)] were shown to increase the homing in surface-engineered cells^[50].

Mode and route of administration is an important factor that influences therapeutic cell survival, migration and homing potential. Intravenous administration, the most commonly used modality of systemic cell delivery, poses the inconvenience of cell trapping within organs such as the lung, spleen or liver. This results in an important quantity of capillary-arrested cells and a decreased therapeutic effect if the target is located elsewhere. Pre-treatment of the host with vasodilatory substances or heparin administration pre-procedure was shown to diminish ADSC lung trapping and to increase hepatic targeting in a rat model of liver failure^[51].

Genetically modified stem cells as therapeutic agents

Stem cells, and particularly MSCs, can be genetically engineered to release therapeutic proteins for regenerative purposes, with various applications in treating monogenic diseases (such as muscular dystrophy or haemophilia) or even degenerative diseases (such as inflammatory arthritis). Even if viral transduction methods were preferred in the beginning for their superior transfection rate, the improved efficiency of newer non-viral methods support their use for therapeutic purposes. Lower immunogenicity, increased scalability, decreased toxicity and considerable versatility of non-viral methods compared to viral methods recommend the use of the former when considering clinical applications^[52]. Physical methods such as electroporation and nucleofection, which use an electric pulse to ferry nucleic acids through the cell membrane or nuclear membrane, respectively, as well as ultrasound-based gene delivery are methods that require intensive protocol optimization due to decreased cell viability^[53]. Chemical non-viral methods employ liposomes, dendrimers, inorganic nanoparticles, magnetic nanoparticles (MNPs) or polymers as transfection agents. High transfection efficiency was

reported using poly (ethylenimine) (PEI) in combination with gold or silica nanoparticles^[54,55].

Genetically engineered stem cells are under scrutiny for the treatment of several monogenic diseases. Using systemically delivered MSCs, a high level of therapeutic protein production can be obtained using stem-cell-mediated gene delivery, which can substitute for the abnormal gene function at the tissue and organ level. Hydroxyapatite- polylactic-polyglycolic acid (PLGA) composites can be coated with biomineralized collagen 1 in combination with autologous gene-engineered factor IX (hFIX). MSCs were shown to deliver a consistent amount of hFIX in a mouse model of haemophilia B^[56]. Autologous HSCs transduced with a viral vector containing a healthy copy of the mutated gene were shown to improve lysosomal storage diseases such as metachromatic leukodystrophy (MLD) or mucopolysaccharidosis type I (MPS-I). The method allows production of the defective enzyme and cross correction of target cells in multiple target tissues^[57].

Osteogenesis imperfecta (OI) represents a group of genetic disorders characterized by bone disease produced by missing or abnormal synthesis of type I collagen, with different levels of severity^[58]. Adeno-associated virus vector MSCs from OI patients were shown to disrupt dominant-negative mutant COL1A1 collagen genes and to produce normal collagen similar to that of wild-type cells—a fact that could be used to develop a therapeutic platform for addressing this incurable and disabling disease^[59].

A particular field of interest is to use genetically modified stem cells for inducing immune tolerance against antigens of interest in several autoimmune diseases (such as diabetes type I) or after organ or cell transplantation. Several strategies include transfection of a particular gene into HSCs or immunological precursor cells by educating the host immune system to recognize the therapeutic protein as “self”. Another possibility is to induce the expression of a therapeutic protein in antigen-presenting cells such as immature dendritic cells or B cells that will induce immune tolerance for the respective antigen^[60].

A large portion of genetic engineering strategies aim to improve stem cell survival and homing potential during cell transplantation for various cell therapy applications. A particular field of therapeutic protein delivery intends, however, to use genetic manipulation for providing repair and regeneration enhancers or to substitute for congenitally absent or abnormal protein production. Stem-cell-mediated gene delivery for RM is sought as a method for enhancing stem cell survival and/or targeting capabilities (see above), to increase local or systemic reparative processes or for steering immunomodulatory processes. Musculoskeletal tissue regeneration, cardiac diseases and brain traumatic injury are among the several domains in which this method is currently being tested.

Autologous or allogeneic MSCs engineered to overexpress bone morphogenetic protein (BMP) was

found to enable the repair of large bone defects in several animal models^[61]. Systemically administered, viral transduced MSCs encoding BMP and vascular endothelial growth factor (VEGF) were able to promote the repair of large segmental bone defects in mice^[62]. Scaffold-mediated locally delivered ADSCs overexpressing runx-2 were able to induce healing in large rat calvaria defects^[63]. Bone marrow derived MSCs (BMSCs) cell sheets transfected with Lipofectamine 200 to overexpress anti-miR-138 (a miRNA precursor) were shown to significantly increase osteogenesis *in vitro*. Forced expression of the transcription factor early growth response protein (Egr1)-programmed MSCs towards the tendon lineage promoted the formation of *in vitro*-engineered tendons, while Smad 8/BMP overexpressing MSCs locally delivered to injured Achilles tendons were shown to improve the histology and biomechanical properties^[64,65]. However, caution needs to be exerted when assessing the therapeutic efficiency, as long-term results might differ from those of the immediate evaluation. MSCs engineered to overexpress basic fibroblast growth factor (bFGF) did not cause improvement in the histological appearance and biomechanical properties of the Achilles tendon 12 mo after transplantation in a rat model of tendon defect, questioning the overall impact of such therapies^[66].

Cell sheets are versatile structures that can be easily manoeuvrable and accommodate a large variety of clinical defects^[67]. MSCs overexpressing IL-8 binding protein (IL-BP8) were shown to decrease inflammation and enhance repair by means of enhanced vascularization in a rat model of global cardiac ischaemia^[68]. ADSC sheets overexpressing VEGF were shown to reduce the infarct size and to improve cardiac functions to non-diseased levels in a rabbit model of cardiac infraction^[69].

Adeno-associated virus (AAV)-transfected MSCs overexpressing IL-10 were proved to provide neuroprotection and enhance intravenously administered stem cell engraftment, improving brain recovery in a rat model of acute ischaemic stroke^[70]. Human umbilical cord blood-derived mesenchymal stem cells (HUMBSCs) overexpressing VEGF were shown to reduce the clinical manifestation and the loss of dopaminergic neurons in the lesioned substantia nigra in hemi-parkinsonian rats. Enhanced HUMBSC differentiation to dopaminergic neurons was observed during this experiment, offering an improved modality for cell transplantation in Parkinson's disease^[71].

Equipping stem cells with therapeutic agents: The role of nanoparticles

Genetic engineering of therapeutic cell populations can result in the release of only proteins that are intended to contribute to cell viability, proliferation, homing potential or to influence the regenerative process. Stem cells can also be engineered to deliver a larger variety of therapeutic molecules, making them veritable drug-delivery tools. Delivery of bioactive molecules is

an important chapter for RM purposes. A group of information mediators and signalling molecules are grouped in three overlapping categories: (1) Mitogens (stimulate cell division); (2) growth factors (GFs) with multiple biological actions; and (3) morphogens (control the generation of tissue form) are crucial factors for initializing and sustaining regeneration and repair; therefore, the control of signalling molecules may potentially allow the control and modulation of regenerative processes. GFs are proteins or steroid hormones that stimulate cell growth, differentiation and healing^[72]. Functional particularities of GF consist in their short-range diffusion through the extracellular matrix. To orchestrate regeneration, controlled local delivery coupled with precise timing is needed in order to obtain the desired action on target cells. Nanoparticles (NP) of different composition, shapes and physical properties are currently used to load native, primed or modified stem cells with information molecules intended to modulate regenerative processes. NP-drug complexes can be internalized by the cells or attached to their surface to be released at the target tissue following cell migration and targeted homing^[73]. Such a procedure could be extended for enabling information-molecule-equipped stem cells to target regenerative sites (Figure 1).

One of the most straightforward procedures is to load stem cells with NPs by simply adding them within the cell culture media, taking advantage of cell membrane mechanisms responsible for foreign body internalization (Figure 2). NP complexes are internalized by cells *via* endocytosis, a largely polymorphic process that depends on the cell type, particle size, composition, culture media formulation, electrical charge, and surface modifications-to name only some of the factors. NPs intended to be used for regenerative purposes must possess several obligatory characteristics such as (1) Biocompatibility, *i.e.*, not being toxic to cells, tissues and organs and not eliciting an immune response at the local or systemic level; (2) biodegradability-capable of being decomposed by means of normal cellular metabolic pathways; and (3) stability of physical properties after surface modification; efficacy at therapeutic doses; chemical stability in physiological conditions^[74]. Moreover, NP-loaded stem cells need to preserve critical stem cell characteristics such as proliferative, differentiation, and immune-modulatory as well as migratory and homing capabilities. Each type of NP-drug complex must be thoroughly characterized with regard to chemical and physical properties, effect on cell viability, phenotypic features, and migratory and homing potential after particle internalization.

Several classes of NPs, of which the most commonly used are briefly outlined below, can be employed for loading stem cells with small molecules, each of them with particular advantages and shortcomings (Table 1).

Polymeric NPs

Poly (D, L-lactide) (PLA) and poly (D, L-lactic acid-

Table 1 Advantages and disadvantages of the different types of nanoparticles used for stem cell loading

| Composition | Type | Advantages | Disadvantages | Payload | Homing to | Ref. |
|------------------------|---|---|--|--|----------------------------------|------------|
| Polymeric NP | Poly(D,L-lactide), poly(D,L-lactic acid-co- α , β -malic acid), Poly-L-lactic acid | Biocompatible, FDA approved, versatility, efficient upload by stem cells, human HSCs, MSCs retain differentiation potential | Biphasic and uncontrolled payload release | Antitumour drugs, | Glioma tumours | [77-79] |
| Silica NP | Mesoporous silica, amorphous silica | Fast uptake, negligible toxicity, long retention inside cells, lysosomal activation not associated with oxidative stress | Long term remanence within cells/tissues | Antitumour drug doxorubicin, fluorescent dye, paclitaxel | Mammary tumours, infarcted heart | [80-82,92] |
| Liposomal NP | Liposomes | Relatively facile manufacturing, versatility for drug delivery | Less-efficient uptake process, higher concentrations needed, which can be toxic to cells | 6-coumarin | Glioma tumours | [83] |
| Magnetic nanoparticles | Iron oxide NPs, magnetite, maghemite | Cellular tracking potential, reduced cell toxicity, high loading efficiency | Can induce oxidative stress in carrier cells | NGF, FGF | Dorsal root ganglia, HUVECs | [84-86] |

NP: Nanoparticle; NGF: Nerve growth factor; FGF: Fibroblast growth factor; HUVECs: Human umbilical endothelial vein cells.

co- α , β -malic acid) (PLMA) have the advantage of being already in clinical use and FDA approved, can be tuned in variable sizes (ranging from micrometres to several nanometres). Polymeric NPs are uploaded by cells by means of pinocytosis or clathrin-mediated endocytosis^[75]. Particle upload is time and concentration dependent, with good loading efficiency, viability and phenotype preservation demonstrated in various cell types^[76]. Polymeric NPs have a hydrophobic core with an important loading capability and a hydrophilic shell that provides stability and can be designed to encapsulate hydrophobic or hydrophilic molecules, as well as proteins and nucleic acid. After internalization by the cell, polymeric NPs such as PLA and polyglycolide (PLGA) are able to disrupt the lysosomal membrane, where they initially accumulate due to modifications in the surface charge, and escape into the cytoplasm^[77]. While the majority of studies involving stem cell polymeric nanoparticle loaded drug delivery have aimed to develop anticancer therapies, the potential use for regenerative medicine purposes is promising^[78]. Human HSCs and MSCs loaded with Poly-L-lactic acid (PLLA) and PLLA-Fe complexes were shown to retain viability and main phenotypic characteristics as well as differentiation potential, qualifying as a method for drug-enhanced cell therapy^[79].

Negatively charged silica NPs are shown to be uptaken by clathrin-mediated endocytosis and to co-localize with the cell lysosomal system. Drug-loaded silica NPs were shown to be uploaded by decidual stem cells without affecting their viability and differentiation potential^[80]. Human bone marrow MSCs loaded with amorphous silica NPs were shown to retain viability and differentiation and to engraft within the beating heart in a mouse model of cardiac ischaemia, offering a promising tool for cell tracking and potential drug delivery^[81]. Mesoporous silica NPs conjugated with

a fluorescent dye (cyanine) could be internalized by human MSCs, making possible the discrimination between viable and early apoptotic cells and pointing towards their potential role as cell-tracking agents in preclinical studies^[82].

Liposomal NPs are uptaken by stem cells *via* endocytosis and release their cargo into the intracellular space after fusing with the endosomal membrane. Human MSCs loaded with lipid nano-capsules retained their viability and differentiation as well as migratory and targeting potential. Liposomal NPs can be manufactured to carry a large variety of small molecules. The loading process is, however, less efficient than in the case of polymeric nanoparticles. The higher concentration of liposomes required for efficient loading may interfere with cell viability, a process that is cell dependent^[83].

Magnetic NPs (MNPs) have been used as such or in combination with other biomaterials for targeted delivery as well as controlled release of information molecules. MNPs incorporated into poly-L-lactic acid (PLLA) containing nerve growth factor (NGF) were shown to direct the extension of neurite outgrowth from the dorsal root ganglia (DRG) *in vitro* as a model for controlled axonal regeneration^[84].

Fluorescently labelled heparin mesoporous silica MNPs were shown to being able to deliver bFGF to human umbilical endothelial vein cells (HUVECs) up to 6 d in culture conditions, suggesting their potential use as multimodal carriers^[85]. Stem cells loaded with iron oxide MNPs can achieve various degrees of magnetization, being traceable *in vivo* using clinically approved methods such as magnetic resonance imaging (MRI). High-resolution tracking of MNP-loaded stem cells using MRI allows cellular imaging, which is an important advantage for therapeutic application as it allows observing the cell fate after implantation. MSCs loaded with ferucarbotran could be observed using MRI while differentiating to

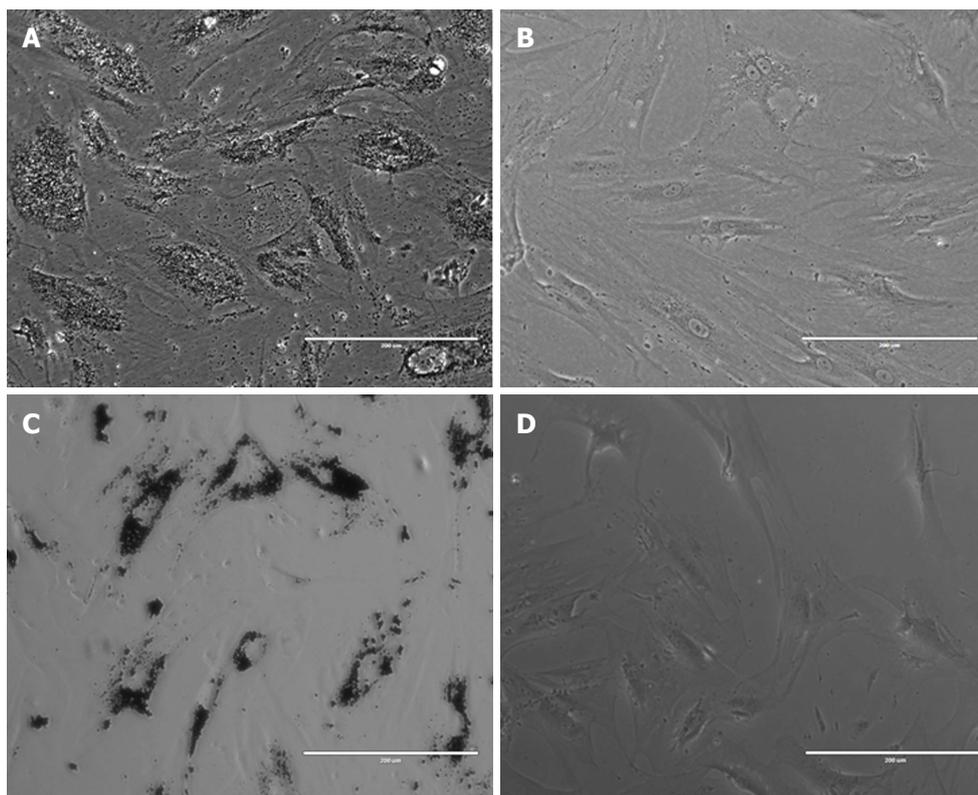


Figure 1 Stem and progenitor cells loaded with magnetic nanoparticles added to culture media. A: human osteoblasts loaded with Fe-Cr-Nb-B magnetic nanoparticles (MNPs); B: Non-loaded human osteoblasts; C: Human bone-marrow-derived mesenchymal stem cells loaded with Fe-Cr-Nb-B MNPs; D: Non-loaded human bone-marrow-derived mesenchymal stem cells.

functional neurons, a potential method for cellular tracking in stem-cell-based therapies^[86].

Depending on the particle type, the upload process can extend from hours to several days of NP cell co-incubation. When considering potential clinical applications, the cell culture time needs to be decreased to a maximum. NP-dependent parameters such as NP colloidal stability, tendency to aggregate, surface charge or cell characteristics (*e.g.*, phagocytic or non-phagocytic cells, logarithmic growth phase in culture) are known to influence particle upload. Several methods to increase the efficiency of NP uptake have been proposed with variable efficiency depending on the cell and NP type. NP functionalization with antibodies against MSC surface antigens (receptor-mediated endocytosis) was shown to speed up the upload process in cultured cells^[87]. Several transfection agents, commercially available poly-cationic polymer poly-L-lysine, protamine sulphate or lipofectamine are available for enhancing NP upload in therapeutic cells. Physical methods that produce a temporary disruption of the cell membrane, such as electroporation, pulsed ultrasonication or application of a magnetic field, the later applicable for MNPs (magnetoporation), have variable efficiency in increasing specific NP upload. However, such methods impose supplementary cell manipulation procedures and can affect the cell viability and phenotypic stability; therefore, their use needs to be carefully poised when designing clinical applications^[88].

Attaching NP complexes to the cell membrane is another possibility to endow stem cells with therapeutic molecules. Cell surface engineering, a complex area in cell engineering, can rely on a large variety of procedures such as chemical modification based on native membrane functional molecules, metabolic or gene engineering of the cell membrane to express functional groups, adsorption, or insertion of hydrophobic coupling groups or ligand/receptor interaction at the membrane interface are currently exploited to attach NPs to various cell types^[89]. Several reports exist regarding surface modifications that could allow the trafficking of NP-conjugated molecules using stem cells. Liposome-based non-covalent membrane modifications allow attaching a cargo to engineered binding sites on the cell surface. Liposome fusion was obtained by co-incubating MSCs with lipid vesicles containing ketones and oxyamine molecules. The liposomes underwent spontaneous membrane fusion to present the respective molecules from the cell surfaces serving as sites for chemo-selective ligation with oxyamine-conjugated molecules *in vitro*^[90]. The majority of anchoring techniques aim to modify membrane characteristics for improved targeting capabilities (see above) and fewer NP delivery processes.

Release of therapeutic cargo from NP-equipped stem cells takes place mainly by passive diffusion or exocytosis. Release is dependent on the particle size, cell type and modality of cytoplasmic NP storage. Diffusion

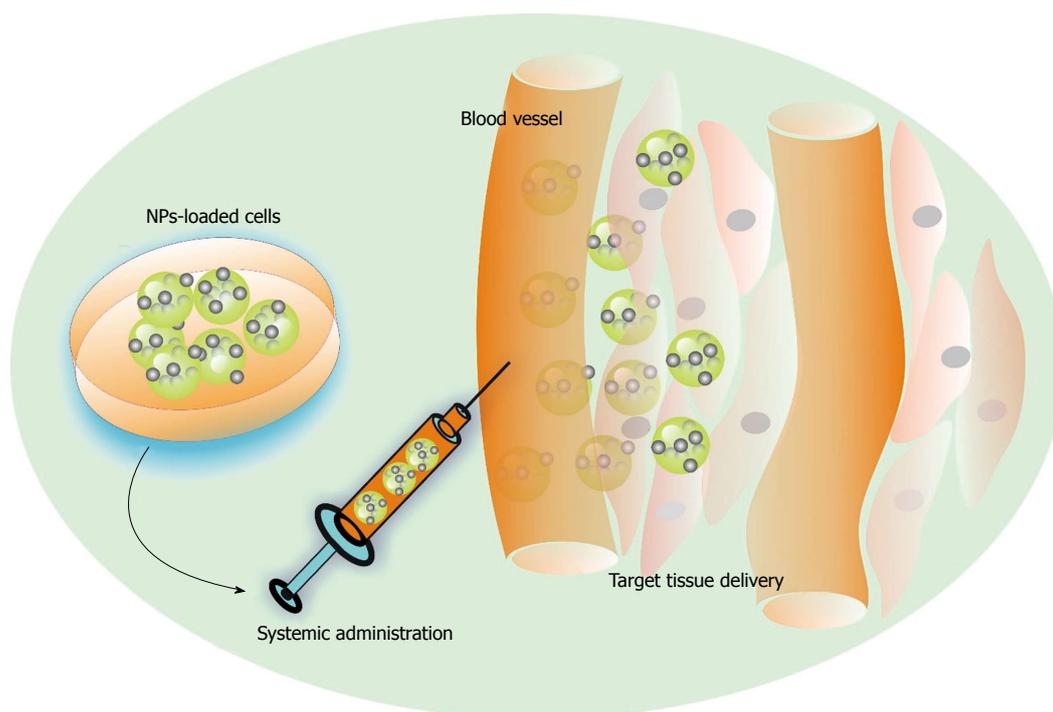


Figure 2 Possible scenario for nanoparticle-loaded stem cell delivery of bioactive molecules towards target organs. Stem cells loaded or decorated with nanoparticle attached to their membrane can be delivered via systemic infusion (intravenous or intraarterial) to migrate by means of blood flow, homing to regenerative sites.

and exocytosis can take place in time intervals ranging from minutes to several hours, while release from the cellular endosomal compartment, an energy consuming process, can take several days^[73]. Ideally, a combination of the two mechanisms would be needed in order to enable uniform particle release. Pulsed and non-uniform payload release can impose hazards due to sudden drug or particle accumulation, not only compromising the therapeutic effect but also introducing supplementary hazards. In an ischaemic rat model subjected to MNP-loaded MSC injection, magnetic targeting resulted in vascular embolism and an inhomogeneous distribution of loaded cells, which prevented the intended targeted cell therapy from translating into a functional benefit^[91]. Ingenious platforms for externally controlled release were shown to enhance the targeted release of a drug payload from decidual stem cells. Ultrasound-responsive mesoporous silica nanoparticles internalized by human decidual cells were shown to deliver their cargo under clinically available ultrasound frequencies both *in vitro* and *in vivo*, a promising strategy that could potentially be extended to the delivery of regenerative molecules^[92].

For NP complexes attached to the cell membrane, the release profile can be engineered dependently on the type of receptor used for its conjugation to the cell membrane. A proposed solution is the design of slow-release systems by means of surface engineering as cell membrane biotinylation to obtain "synthetic biotin-avidin"-based cargo coupling^[93].

Challenges for using stem cells as delivery agents in RM

Due to their targeting capabilities, stem cells are sought as biological "carriers" for the delivery of therapeutic molecules. Many research groups are proposing stem-cell-mediated drug delivery for targeting tumour tissues or for cancer theranostic applications. The interest in combining the intrinsic role of stem cells with their potential use as drug carriers is increasing; however, the procedure is not yet a road much travelled. Even though the possibility of delivering a supplement of growth factors or immune modulating agents together with various cell populations for either cell and/or gene therapy or for tissue engineering is appealing, and several challenges need to be addressed. Issues that challenge regenerative medicine product development generally apply to the field of stem-cell-mediated drug delivery. Improved scalable methods for stem cell isolation, expansion and culture combined with the use of serum-free media formulations are mandatory for large-scale clinical applications. Innovative methods for stem cell characterization and automated sorting for cell-based product characterization are expected to provide improved quality control tools. Particularly for cell-based carriers, a basic requirement is to ascertain that cargo loading and presence not only is non-toxic to cells but also preserves their phenotypic features. Stem cell type, origin, and culture method as well as NP size, surface, electric charge, and coating determine the modality of the interaction, impact the cell biological potential. Every (stem) cell type and NP-drug cargo

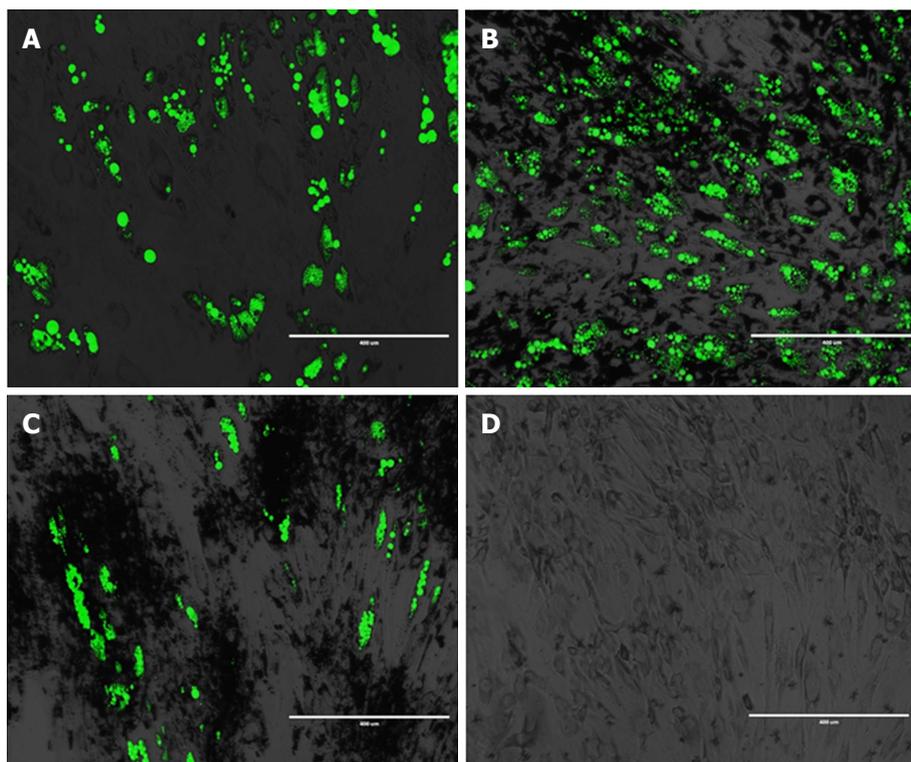


Figure 3 Adipogenic differentiation of human primary adipose-derived stem cells loaded with bare and chitosan-coated Fe-Cr-Nb-B magnetic nanoparticles. A: Adipose-derived stem cells (ADSCs) cells; B: ADSC-magnetic nanoparticles (MNPs); C: ADSC-C-MNPs; D: Control of differentiation (ADSCs grown in normal culture media).

combination needs to be tested in this respect. With several exceptions (such as titanium wear debris), biocompatible NP internalization or surface conjugation has been shown to preserve MSC proliferative, differentiation and immunomodulation capabilities in different cell types^[94] (Figure 3). Moreover, NPs can be engineered to increase their differentiation capability towards a desired lineage. Silver nanoparticles were shown to increase bone healing by increasing mouse MSC proliferation, chemo-attraction and osteogenic potential in a long bone fracture model^[95]. Internalized MNPs were shown to increase MSCs osteogenesis *via* a long non-coding RNA INZEB₂ mechanism^[96]. Superparamagnetic iron oxide nanoparticles (SPIOs) used as contrast agents were shown to transiently and reversibly affect chondrogenesis in some cell types but not in others, stressing the importance of testing each particular cell type for a specific application^[97]. Membrane structure and composition after particle internalization or conjugation needs to be assessed, as changes in surface antibodies could affect stem cell migratory capabilities. Interesting, various types of MNP internalization were shown to enhance the expression of chemokine receptor CXCR4 in MSCs and to improve their homing to a brain injury and glioblastoma model compared to the findings in non-loaded cells^[98]. Stem cells loaded with MNPs present a particular interest in drug delivery, as they may be remotely actuated using external magnetic fields in order to increase delivery to target tissues and/or organs. Challenges regarding

the intensity of the magnetic field, distance from the target organ (which influences the tracking capability), and risk of cell agglutination within the blood stream still need to be addressed. Magnetic force actuation of MNP-loaded stem cells has proved efficient for directed cell localization and consecutive repair of arterial injuries in small animal models or cartilage defects in larger animals (swine), paving the way for future clinical applications^[99].

For NP drug complexes attached to the cell membrane, enzymatic or hydrolytic drug degradation within the biological environment is a serious challenge that increases side effects and lowers the therapeutic efficiency. Relevant *in vivo* models must be employed for testing collateral particle release within the blood stream and the specific pharmacodynamics of these particles, especially the tendency to accumulate within non-target tissues such as lung, spleen or liver. Drug-induced immunogenicity needs to be ruled out as well as potential interactions with cellular and non-cellular immune effectors within the biological milieu.

It should be noted that both cargo and NP loading can affect cell migratory and homing capabilities. Genetic engineering can be used in this case to modify cell surface receptors, to improve their migratory capabilities and to increase survival. The efficiency of such cell manipulation in every phase of cell homing needs to be tested in relevant models *in vitro* as well as *in vivo*, in combination with the drug release efficiency.

FUTURE PROSPECTS

Stem cells are currently being tested by several groups as vehicles for targeting, imaging and treating tumours. For anticancer therapies, vehicle viability after successful cargo delivery is not an issue. In contrast, due to possible stem cell recruitment by tumours, it might prove safer to control cell proliferation and viability by means of cargo. Moreover, the demise of carrier cells triggers an immune response, contributing to tumour treatment^[100]. For RM purposes, it would be beneficial not only to enable survival of these cells but also to improve their direct contribution to the regeneration process.

Controlling the stem cell fate by means of cargo could represent an appealing modality in steering regenerative processes. An MNP-based GF delivery and activation strategy was reported to be able to release TGF- β from its latent complex by application of an external magnetic field. Latent TGF-bioactive molecules such as GF, cytokines, DNA or miRNA could be delivered to control stem cell fate using MNPs. Magnetic core-shell MNPs composed of a highly magnetic core surrounded by a thin uniform gold shell enabled the delivery of controlling genetic materials [small interfering RNA (siRNA) and plasmid DNA (pDNA)] used to direct neural stem cell differentiation to neurons and oligodendrocytes. MNP-mediated RNA interference suppressed two key "neural switch" genes CAVEOLIN-1 and SOX9 that are responsible for oligodendrocyte and neuron differentiation, respectively. When conjugated to the surface of poly (ethylene glycol)-coated MNPs, molecules could be activated and released in a controlled manner^[101]. Such a triggered GF-release strategy could be expanded to remote-control-delivered stem cell differentiation, increasing their participation in local healing.

Control of stem cell fate *via* the magnetic actuation of magnetic responsive cargo could be used to control the osteogenic differentiation of systemically delivered MSCs, preventing their adipogenic conversion for systemic osteoporosis treatment.

The large majority of current studies regarding stem-cell-mediated delivery of therapeutic agents are based on adult stem cells (MSCs or ESCs). MSCs and neural stem cells (NSCs) are promising targets for overcoming the blood-brain barrier to enable RNA and drug delivery for tumours or neurodegenerative disorders^[102].

As is the case with any other stem-cell-based therapy, improved methods for cell collection and culture protocol standardization combined with innovative methods for donor and cell selection are expected to accelerate the transition into safe and efficient therapies. The stem-cell-mediated delivery of therapeutic agents for targeting regenerative sites poses special challenges. Cell procurement and culture that enables preservation and/or methods for augmenting the homing potential are needed for both native and genetically modified cells. The dynamic and phenotypic modification of NP-

complex augmented cells needs to be described in detail as well as its effect in modifying the microenvironment of target tissues. Stem-cell-mediated delivery has the potential to consistently enhance the therapeutic effect of these cells for RM applications.

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