

Stem cell research meets nanotechnology

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SUMMARY

The recent application of nanotechnologies into the stem cell field promises to open new avenues in regenerative medicine. Nanotechnologies can be a valuable tool to track and image stem cells, to drive their differentiation into specific cell lineages, and ultimately to understand their biology. This will hopefully lead to stem cell-based therapeutics for the prevention, diagnosis, and treatment of human diseases. Despite these opportunities, nanotechnologies also pose several risks since they can be cytotoxic and affect the differentiation program of stem cells. Here, we discuss the future opportunities and challenges that face this young field of research.

INTRODUCTION

The existence of a multipotent hematopoietic stem cell was demonstrated for the first time by Till and McCulloch in 1961. They demonstrated that a single hematopoietic stem cell could (i) give rise to a mixed population of blood cells (granulocytes, macrophages, red blood cells, etc...) and (ii) had the ability to self-renew [1]. The isolation of mouse embryonic stem cells by Martin Evans in 1981, human embryonic stem cells by James Thomson in 1998, and inducible pluripotent stem cells by Shinya Yamanaka in 2006, propelled the scientific community to understand the properties of these cells and evaluate their therapeutic effect in the context of the regenerative medicine.

The first observation of nanomaterials was made by Richard Adolf Asigmondy in 1914. He performed a detailed study of gold sols

and other nanomaterials with sizes down to 10 nm. In 1959, Richard Feynman launched the foundation of the nanotechnology field. Since then, several extraordinary discoveries have been made: Richard Smalley discovered fullerenes in 1985, Sumio Iijima discovered carbon nanotubes in 1991, and Louis Brus the quantum dots in 1996.

The intersection of nanotechnologies with stem cell research is recent and has been reviewed by us elsewhere [2, 3]. In this work we will review the current research topics in this area: **stem cell microenvironment and tissue engineering, stem cell tracking and imaging, stem cell transfection, isolation and sorting, and molecular detection** (Fig. 1). When appropriate, we will describe some examples about the research that we are conducting at Centre for Neuroscience and Cell Biology (CNC) and Biocant in this research area.

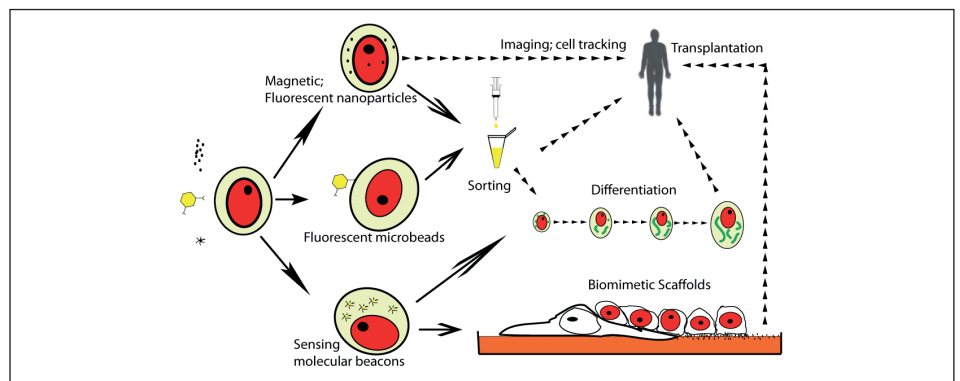
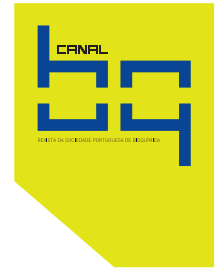


Figure 1. Nanotechnology applications in Stem Cell Biology and Medicine. Nanodevices can be used in stem cell tracking and imaging but also in isolation and sorting of stem cells, both for basic science and translational medicine. Stem cell fate can be modulated by internalization of nanocarriers with biological molecules or by external cues given by biomimetic scaffolds. Stem cell transfection and molecular detection make use of nanodevices for intracellular access but also for intelligent delivery and sensing of biomolecules. These technologies have a great impact in stem cell microenvironment and tissue engineering studies and have a great potential for biomedical applications.



Modulation of stem cell-fate by the microenvironment

Stem-cell biology has been studied mainly *in vitro* with cells cultured on flat substrates coated, for example, with collagen or laminin, or in co-culture systems where feeder-cell layers are used to support stem cell growth. These culture conditions are very different from the environment that stem cells experience in the body. For example, the **extracellular matrix** (ECM) is difficult to mimic in plastic dishes; most frequently stem cells are cultured in rigid polystyrene tissue-culture plastic where cells are exposed to soluble factors in liquid media. This is different in the body where the ECM creates a soft microenvironment where these molecules are anchored in close proximity to cell surfaces. This much more constrained **three-dimensional (3D) niche** is a unique microenvironment that has a prominent role in the maintenance and differentiation of stem cells. This micro-environment is formed by different components including cell-cell interactions, extracellular matrix, mechanical properties and secreted factors. Collectively, they constitute a complex microenvironment that is difficult to recapitulate *in vitro*. Stem cell niche research uses nanotechnologies to mimic this microenvironment in order to determine what are the mechanisms underlying the conversion of a stem cell into different cell types. On the other hand, these **biomimetic** approaches to create synthetic microenvironments are very challenging because there is much we do not understand about the natural stem cell niche. Several researchers believe that it may be possible to create synthetic stem cell niches that are more bioinspired than biomimetic and potentially more efficient than those observed in nature. Therapeutically, it may be more useful to take this **bioinspired** approach in the design of the synthetic niche so that it acts on the stem cells in an unnatural way to achieve a therapeutic goal [4]. Current research efforts in both biomimetic and bioinspired strategies are focussing in

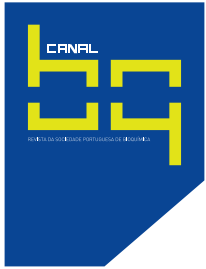
the following aspects: (i) Laser fabricated nanogrooves to study cell-cell interactions; (ii) nanowires to study intra- and intercellular biological processes; (iii) nanophase thin film to study cell adhesion and proliferation; (iv) Lab-on-a-chip with nanoreservoir to study environmental cues; (v) Self-assembly peptides and nanofibers to mimic ECM; (vi) Nanoliter-scale synthesis of arrayed biomaterials; (vii) Micro/nanopatterned surface to study stem cell response to topography and mechano-transduction; and (viii) Nanoparticles to control release growth factors and biochemicals.

It is clear from this previous list that biomaterial design for stem cell applications is progressively abandoning the strategy of developing an inert mechanical support and adopting the notion that this type of cells need a more dynamic substrate capable of directing interactions at the cell-material interface and may stimulate and commit cell behaviour through physical forces, biochemical interactions or topography. This interaction of biomaterials with the chemical and physical features of stem cells occurs at the micro- and nano scales.

Cell-cell interaction studies generally rely on co-culture strategies where the effects of particular molecules are hidden in the great complexity of the culturing system. It is therefore difficult to discern the role of soluble or tethered molecules in terms of cell-cell interactions. In tissues, the ECM contains many macromolecules such as proteoglycans, collagens, laminins, fibronectin and sequestered growth factors. This molecular repertoire is responsible for the bioactivity of the ECM. For example, the sequences of many ECM proteins or receptor ligands are presented to stem cells and are recognized by dimeric cell-surface receptors known as integrins. Binding of integrins to these molecules can trigger a cascade of signalling events that will impact the gene expression pattern of the stem cell. Therefore, the type of ECM molecules that a stem cell encoun-

ters in a given tissue is critical in determining how cells behave within that tissue. The ECM can be reproduced *in vitro* by the use of 3D scaffolds. For that purpose, several natural (fibrin, collagen, hyaluronic acid, etc...) or synthetic (polyethylene glycol, poly(lactic acid-co-glycolic acid), poly(glycerol sebacate) etc...) biomaterials can be used. Recently, we have prepared a hyaluronic acid-based gel to create a 3D microenvironment for the self-renewal of human embryonic stem cells [5].

When a greater control over the properties of the material is required the best option is to produce synthetic bioactive scaffolds. Issues like immunogenicity, pathogen transmission and purification difficulties have encouraged this option. An example of a synthetic scaffold is (polyethylene glycol) (PEG) gels which can be chemically modified to incorporate a compendium of bioactive molecules [6]. Immobilization seems to increase the stability of the molecules, promote persistent signalling and induce receptor clustering [7]. It was recently shown that the covalent attachment of fibroblast growth factor 2 (FGF2) to a synthetic nanofibrillar surface composed of a network of polyamide nanofibers resulted in the stabilization of the growth factor and increased its potency 100-fold relative to FGF2 in solution. In response to the tethered FGF2, embryonic stem cells exhibited increased proliferation through activation of mitogenic pathways [8]. Another example that illustrates the importance of ligand presentation in stem-cell fate and function is the immobilization of leukaemia inhibitory factor (LIF), which led to more efficient and prolonged activation of LIF targets and maintenance of embryonic stem cells in an undifferentiated state when compared with soluble LIF [9].



A major challenge in tissue engineering is to vascularise the transplanted tissue constructs to meet the metabolic demands of recovery and integration into the organism. Therapeutic application of the main vascular signalling molecules (e.g. vascular endothelial growth factors (VEGFs), FGFs, TGFs, angiopoietins, ephrins and various chemokines) can be a promising approach to enhance blood supply and neovascularisation processes around the transplanted tissue. For example, the immobilization of VEGF onto a metal substrate using a biomimetic polymer film was able to promote the survival and proliferation of endothelial cells and to induce the differentiation of hMSCs into endothelial cells [10].

In order to discover novel biomaterials that have effects on stem cells, high-throughput approaches are likely needed. Recent efforts have used acrylate-based polymers spotted in arrays composed of hundreds of different polymer combinations and found several platforms that could promote embryonic stem cell attachment, proliferation and differentiation [11]. Similar studies must be conducted this time aiming at incorporating many other biophysical and biochemical parameters in this type of high throughput approaches. Different matrices, natural and/ or synthetic, can be produced to generate cell-culture substrates with defined physical characteristics like rigidity (stiffness) and topography. Unlike regular tissue culture plastic substrates, they provide diffusion of soluble molecules to the basal surface, as well as the apical surface. They are especially interesting in the context of studies of homeostatic and disease-related matrix stiffness impact on stem-cell behaviour. A groundbreaking study by Engler and collaborators [12] found that matrix stiff-

ness has a primary role in stem cell lineage specification. This study reported that human mesenchymal stem cells (MSCs) were able to differentiate into tissues that had their mechanical properties more closely mimicked by the polyacrylamide substrate upon which they were cultured. Thus, MSCs that were cultured on rigid (bonelike) gels differentiated into osteoblasts, those that were cultured on medium stiffness (muscle-like) gels differentiated into muscle cells, and those that were cultured on more elastic gels (neural-like) differentiated into neural cells [12]. The acknowledgment that matrix mechanical properties impact on stem-cell fate led to the exploration of further links between stem cell behaviour and matrix elasticity. Since then, several studies have reported that substrate stiffness modulates the proliferation and differentiation of embryonic stem cells and certain types of adult stem cells. For example, adult neural stem cells cultured on a relatively soft matrix to mimic brain tissue gave origin to more neurons than cells grown on a stiffer synthetic matrix, where glial cells were predominant [13]. Another study found that the rate of adult skeletal-muscle stem-cell proliferation increased with substrate stiffness [14]. We predict that more studies will show that the physical properties of culture substrate have a major impact on stem-cell fate. With time different culture platforms based on soft biomaterials are likely to largely replace those made of the standard, rigid, tissue-culture plastic in order to specifically modulate differentiation into different fates.

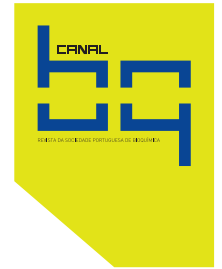
Usually stem cell cultures are presented with soluble growth factors and biochemicals in their culture media. This approach may not always be possible due to specific chemical properties of the molecules to be delivered. Instead, it may be more beneficial to deliver these molecules directly inside the cell to better control their bio-availability. Nanoparticles that can carry molecular payloads of proteins, growth

factors, and small chemicals present an excellent tool to control the differentiation of stem cells. Some of these biomolecules/chemicals have (i) poor solubility, (ii) can be quickly cleaved by cellular enzymes, (iii) and have side effects when administered systemically. Therefore, biodegradable and biocompatible nanoparticles able to target stem cells and release the payload in their cytoplasm with consequent activation of signalling cascades will be of great interest. Recently, we have reported the successful delivery of vascular growth factors into hESCs, by incorporating growth factor-release particles in human **embryoid bodies** (EBs) [15]. These biodegradable nanoparticles are compatible with cell viability and proliferation and are extremely effective in terms of differentiation. In some cases, the effect on vascular differentiation of particles containing growth factors was superior to the one observed by exposing EBs to large extrinsic doses of the same growth factors. Moreover, nanoparticles were taken up by human embryonic stem cells and accumulated in the perinuclear region indicating that they could constitute a delivery platform not only for growth factors but also for other type of biomolecules [15].

Stem Cell Engineering

Various micro-/nanofabrication technologies have been used to design scaffolds able to drive the differentiation of stem cells into specific cell lineages. For example, nanofibers are able to provide an in vivo-like extracellular scaffolding to promote regeneration of specific tissues. Nanopatterned or nanostructured scaffolds are designed to trigger stem cells to become specific cell types comprising the tissues and organs in the body.

Current research efforts in nanotechnology applications in tissue engineering are focussing in the following aspects: (i) Micro/nano structured scaffolds for tissue engineering; (ii) magnetic nanoparticles for magnetic force-based tissue engineer-



ing; (iii) nanocomposites for bone tissue engineering; and (iv) micro/nanoencapsulation for cell therapy

The ultimate goal of tissue engineering is to recreate the right conditions to support the massive growth, physical folds and twists and cellular and molecular events of great complexity that occur during regeneration or replacement of a tissue. The general strategy is to grow cells in a scaffold engineered to define the geometry of the replacement tissue and provide the right environmental cues that promote tissue regeneration.

Stem cell research has been showing that stem cells or at least progenitor cells can be isolated from almost every tissue in the body. With the appropriate conditions it may be possible to stimulate these cells to form new tissue. Several studies have tried to use this biologic intrinsic regenerative potential. Stevens and collaborators have injected alginate gels or modified hyaluronic acid gels into an artificial space between the tibia and the periosteum (the outer lining of the bone). This stimulated bone and cartilage formation from resident progenitor cells in the inner layer of the periosteum [16]. This is an example of how simple biomaterials can support the generation of complex tissue by using the body as a bioreactor and without the need of exogenous cell transplants. In situations where the regenerative potential is low due to different factors like age, trauma, scarring or inflammation like the ones that follow myocardial infarction or brain stroke for example, biomaterial interventions that include cells of external origins must be included.

Several clinical studies with stem cell-based therapies are currently being performed worldwide. Despite the considerable knowledge gathered in the last years in stem cells biology, further pre-clinical and clinical studies are needed to clarify what is the best stem cell source for certain medical applications, the mechanism underlying their regenerative effect, the

timing and delivery methods, etc...It is of utmost importance to demonstrate the long-term safety of these cell-based therapies. For example, studies in mice have showed that stem cells injected into the heart following myocardial infarction gave origin to mineralized tissue [17]. This was possibly due to the reaction of the transplanted cells to the stiffer mechanical environment of the scar tissue that was not appropriate to induce cardiogenesis.

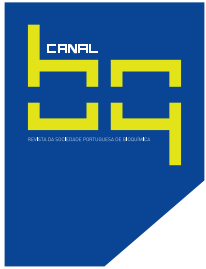
Stem cell based therapy is in hand when compared with the major challenge that is replacing an entire organ with a complex repertoire of cell types carefully organized to maximize its functional output.

Self-organization seems to be an intrinsic characteristic of cells; cells will cluster and communicate with cells that express the same cellular adhesion molecules and under the right conditions can form complex structures like the sprouting tubular networks formed by endothelial cells lining blood vessels. Simple artificial cell adhesions have been engineered using biotin conjugated to cell surfaces and the addition of avidin to trigger the assembly of multicellular clusters due to the biotin-avidin interaction in order to aid in the development of more complex cellular interactions [18].

Communication between cells in the tissue is essential but a lot of information is also coming to cells from their extracellular environment; the scaffold that surrounds and separates cells within a tissue is a complex material called the Extracellular Matrix (ECM). Tissue engineering takes lessons from the characterization of natural bioactive scaffolds in order to construct artificial ones. When possible, a very efficacious strategy is to use cadaver- or animal-derived decellularized ECM because these products have an inherent bioactivity to induce regeneration. This type of approach has found clinical applications in routine medical procedures and in life-saving scenarios. Products derived from the small intestinal submucosa of pigs are used routinely in reconstructive surgery,

and ECM derived from the pericardium of horses can be used as a reconstructive material in the dura mater layer of the brain meninges following a craniotomy. In a recent development, it was possible to engineer a bioartificial heart through a decellularization process with detergents to produce a biocompatible cardiac ECM scaffold with a perfusable vascular tree, patent valves and a four-chamber-geometry template for biomimetic tissue engineering. These researchers managed to populate this ECM scaffold with an appropriate cell composition, and the maturation of this construct developed a nascent pump function [19]. Almost at the same time another group reported the transplant of a tissue engineered airway confirming that this approach can in fact produce whole-organ tissue engineering products that are clinically relevant [20]. The scaffold in this case was a decellularized human donor trachea that was seeded with the patient's own bone marrow cells that had been differentiated into cartilage cells. In contrast with traditional transplant surgery, the decellularization process solved the problem of tissue rejection because it removed human leukocyte antigen traces that are major determinants in tissue compatibility with the advantage that the patient did not need any immunosuppressive drugs [20].

Both decellularized tissues and synthetic scaffolds offer distinct and important benefits for tissue engineering. Typically, biomaterials-engineering approaches focus on chemical and/ or physical mechanisms by which the ECM influences cells and try to reproduce those effectively for a given tissue. For instance, it may be sometimes necessary to work the **anisotropic** features of the culturing system to better



mimic the tissue. **Nanogrooves** induced by laser irradiation are an example of this type of approach in bone differentiation studies. The alignment of bone cells and collagen matrix is closely related to the mechanical properties of bone. Scaffolds that are able to promote osteoblast differentiation and modulate their orientation to generate mineralization in a preferred direction are essential for the generation of biomimetic bone tissue. Bangshang Zhu and collaborators, used nanogrooves to induce alignment of rabbit mesenchymal stem cell (MSC)-derived osteoblast-like cells and collagen fibres. Nanoscale groove-ridge patterns (300 nm in periodicity, 60–70 nm in depth) on the surface of polystyrene were made by polarized laser irradiation. The cells and actin stress fibers were aligned and elongated along the direction of the nanogrooves. The results suggested that nanoscale fibrous cues in the longitudinal direction might contribute to the aligned formation of bone tissue [21]. A recent study has shown that osteoblasts are responsive to nanopatterns down to 75 nm in width and 33 nm in depth. Nanotexture-driven mineral deposition is induced and responsive to even smaller nanopatterns of 50 nm in width and 17 nm in depth. In addition, gene expression of osteoblast specific markers is upregulated by nanogrooves [22]. These studies indicate that nanogrooves can be a very promising tool to direct the bone response at the interface between an implant and the bone tissue, which can benefit the installation of implants in compromised patients. Although various models have been proposed for how this alignment of cells in response to nanopatterns occurs, much remains to be clarified. Studies with fixed cells do not lend themselves to answering these questions. The dynamics of the in-

teraction of cells with these nanogrooved surfaces was recently analysed by live cell imaging [23]. These studies have shown that cells acquire elongated morphologies on a surface with nanogrooved patterns and align along that pattern. In this study, the dynamic behaviours of living mesenchymal stem cells on a nanogroove substrate with a 200 nm groove depth, an 870 nm ridge width and a 670 nm groove width were observed using time-lapse microscopy. These researchers found that **filopodia** moved as if they were probing the surroundings of the cell protrusion, and then some cell protrusions invaded the probed areas. Cell protrusions that extended perpendicular to the nanogroove direction tended to retract more rapidly than those that were parallel to it. From these observations, the authors hypothesize that the retracting phase of cell protrusions play a role in cell alignment along the nanogroove patterns. Further studies using similar live cell imaging strategies are required to clearly elucidate the role of filopodia-mediated cell alignment in these nanopatterned substrates.

Stem Cell Tracking and Imaging

To better understand stem cell biology and realize the full potential of stem cell therapy, it is essential to monitor the trafficking of labelled stem cells by molecular and cellular imaging. Monitorization and tracking of these cells inside an organism is a difficult task. This is why stem cells are usually tracked invasively by immunohistochemistry after removal of tissues or organs from small animals. On the other hand, for pre-clinical and clinical trials, it will be fundamental to track stem cells noninvasively in order to assess their grafting and therapeutic effect. Research in this area is focussing on the development of the following nanotechnologic approaches: (i) Superparamagnetic iron oxide nanoparticles for stem cell labelling and diagnostics; (ii) quantum dots and fluorophore nanocrystals for stem cell tracking and imaging; (iii) nanoprobes for stem cell detection and

electrophysiological application; and (iv) photothermal nanospectroscopy to identify stem cells in the body.

Nanotechnology enables labelling stem cells using magnetic, genetic or fluorescent probes which can be monitored by **magnetic resonance imaging** (MRI) or fluorescence imaging. For example, superparamagnetic iron oxide (SPIO) nanoparticles can be used to label stem cells and analyse their fate in transplantation assays by MRI. In fact, several SPIO nanoparticle formulations (e.g., Feridex/ Endorem and Ferucarbotran) have FDA (United States Food and Drug Administration) approval for human use as MRI contrast agents. The development of nanoparticles for cell tracking is a multidisciplinary task that needs highly skilled biological, physical and chemical expertise. In most cell types, the nanoparticles are taken up through endocytosis during *in vitro* cell cultivation and accumulate in the endosomes. Although, some cell types are easier to label than others, one has to take into account the biological features of the cells to be labelled and sometimes use chemical tricks to promote the internalization of the nanoparticles; e.g. mononuclear blood cells are easier to label because by their nature they are primed for internalization of other cells or molecules by phagocytosis. Also, quite often the internalization of nanoparticles requires the use of **excipients**, which may include peptides and cationic agents [2]. The labelling of stem/progenitor cells and their transplantation and tracking inside the organism may enlighten the dynamics of stem cell differentiation, migration and therapeutic benefit in several disease scenarios like myocardial infarction, cancer and neurological conditions. In fact, not so long ago, Lewis and collaborators succeeded in demonstrating that stem/progenitor cells labelled with magnetic nanoparticles when injected in the blood stream of small animals can later be isolated by magnetic separation after *in vivo* migration to study the differentiation of the cells exposed to a



biological environment [24]. Although feasible these type of studies are still limited by technical challenges. In some cases, it is difficult to distinguish SPIO-labelled cells from other hypointense regions on MRI images. Such signals can arise from regions containing blood hemoglobin, or blood clots/trombi [25]. The development of new nanoparticle formulations based on probes other than iron oxide will be of great interest for stem cell applications. Some examples have been recently reported based on nanoparticles containing fluorine or manganese [26].

Stem cell differentiation programs are highly regulated processes that may be sensitive to nanoparticle internalization. Therefore, it will be essential to evaluate the long-term effects of these nanomaterials in the biology of stem cells. It is possible that the intracellular degradation of the nanoparticles produces molecules that are bioactive and have potential to activate signalling cascades that can change the differentiation program of the stem cells. The prospect of tracking stem cells with nanoparticle labelling technologies is dependent on a careful evaluation of their impact on stem cell biology and solving issues like dilution of nanoparticle content (and consequent decrease of signal) during cell division and release by exocytosis. Therefore, complementary techniques like fluorescence must be developed to validate the MRI results. Our group is developing nanoparticle formulations that escape the endosome and combine fluorescent and magnetic labelling to circumvent these issues.

Other nanoparticles that are increasingly used in cell biology are **quantum dots** (qdots). These are another class of nanomaterials usually in the size range of 2-10 nm that can be used for long-term labelling of stem cells. Qdots have become a commercial success because they exhibit a brighter fluorescent signal, have higher photostability (hours) and large Stokes shift (difference between excitation and emission wavelengths) than organic dyes and fluorescent proteins. They have nar-

row emission and broad excitation spectrum which allows simultaneous analysis of multiple cell targets by using a single wavelength activation [27]. Qdot conjugation has been used to follow biomolecules like growth factor receptors, integrins, phospholipids, and enzymes among others, when stem cells are exposed to different environments or soluble factors [28].

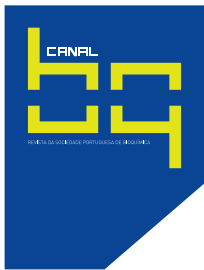
In vivo, small animal-tracking of delivered stem cells has been difficult due to technical limitations in terms of labelling but also due to the autofluorescent nature of animal tissues. With imaging platforms like Caliper's IVIS it is now possible to do qdot-tracking in whole animals. Rosen and collaborators (2007) have reported the optimization and validation of a qdot long-term tracking technique of labelled mesenchymal stem cells (MSCs) in the mammalian heart. These researchers found that bright qdot crystals were able to illuminate MSCs in histological sections for at least 8 weeks following delivery enabling the complete three-dimensional reconstruction of the locations of all stem cells following injection into the heart [29]. The use of these nanocrystals for stem cell-labelling depends on their origin and surface modification, mode of internalization and type of stem cells used [30]. Stem cells are labelled with qdots in several ways, including receptor-mediated uptake, lipofection, electroporation, or passive loading. Under appropriate conditions, qdots are effective at labelling stem cells without affecting their self-renewal and differentiation potentials. For example, hMSCs labelled with qdots (0.250 to 16 nM) maintained their osteogenic differentiation potential [30]. Also, intravenous injection of Qdots-labelled mesenchymal stem cells into NOD/SCID mice (1×10^6 cells) showed an accumulation after 24 h in the lungs, liver and spleen, but not in the heart, brain or kidneys [31]. At the moment, most studies were dedicated to labelling multipotent mesenchymal stem cells. Therefore, it will be important to extend these studies

to pluripotent embryonic stem cells. Also, the long-term effects of these nanoparticles and their degradation products on stem cells should be also assessed at gene and protein level. Indeed, qdots may induce cytotoxic effects due to release of cadmium triggered by their oxidative degradation [32]. This metal can bind to the sulfhydryl groups of critical mitochondrial proteins and induce the production of reactive oxygen species, leading to mitochondrial dysfunction and ultimately cell death [33]. However, it might be possible to coat qdots in a way that circumvents their *in vivo* degradation.

Stem Cell Transfection

Efficient gene delivery systems are required to fully manipulate stem cell behaviour. This ability is essential for studies of gene function, control of stem cell differentiation, cellular labelling and purification, and cellular secretion of therapeutic drugs. Viral methods have been widely used and have good transduction efficiencies; however they integrate into the genome of the host cell. Because of safety issues, non-viral gene delivery systems are preferred for stem cell transfection. The key challenge in this case is to deliver genes to stem cells with high efficiency and low cytotoxicity. Nanotechnology provides invaluable tools for stem cell transfection. The main efforts in this area are focussing on: (i) Nanomaterials for *in vivo* gene delivery; (ii) nanowires for gene delivery to stem cells; and (iii) micro/nanofluidic devices for stem cell electroporation.

Nanoparticles have been shown to be effective vectors for gene transfection. Green and collaborators developed a class of polymers (poly(B-amino esters)) that are able to condense DNA into nanoparticles that



facilitate cellular uptake and endosomal escape. These particles can be coated for ligand-specific delivery, are biodegradable and have low toxicity [34]. Another approach used specific recognition of cell surface molecules coupled to an organic-inorganic hybrid carrier where carbonate apatite nanoparticles were coated electrostatically with fibronectin and E-cadherin producing an efficient gene delivery system for embryonic stem cells [35, 36]. These studies with nanoparticles reported higher efficiencies for gene delivery and expression than the ones obtained with the leading commercially available transfection agent, Lipofectamine 2000 [34-36].

Nucleic acids (DNA and RNA) can be delivered in the cytoplasm by the nanoparticles in a gradual release profile or suddenly, depending if the genetic modulation is intended to be sustained in time or not. This is of great advantage when compared with the commercially available options. Indeed, nanoparticles with covalently immobilized DNA or siRNA were shown to be a very effective strategy to regulate gene expression [37,38]. Rosi and collaborators have shown that DNA-gold nanoparticles can have effective intracellular target recognition and binding and can be used for antisense gene regulation on stem cells [37]. For somatic cells, it has been reported that these systems have high resistance to nuclease degradation and high cellular uptake as a result of their oligonucleotide functionalization. These nanoparticle systems offer exciting opportunities for gene expression regulation and the control of stem cell fate. Our research group has several projects in this area aiming to modulate the differentiation of pluripotent stem cells by the use of nanomaterials.

Other good delivery strategies to transfect stem cells are **carbon nanotubes** (CNTs).

These nanodevices are helical structures of approximately 1–30 nm in diameter with lengths \rightarrow 100 nm [39], that are able to encapsulate drugs and genetic material. These CNTs are internalized by an endocytosis independent way and reach the perinuclear region after a few hours of contact with the cells [40]. After 24 h, a significant number of CNTs have been observed at the cell nucleus of mesenchymal stem cells [41]. Recent advances on this type of strategy have produced a novel platform for intracellular delivery of genetic material and nanoparticles, based on vertically aligned carbon nanosyringe arrays of controllable height. Using this technology, Park and collaborators have shown that plasmid and quantum dots can be efficiently delivered to the cytoplasm of cancer cells and human mesenchymal stem cells [42].

Stem Cell Isolation and Sorting

A key challenge in stem cell research is to identify and isolate stem cells from a heterogeneous cell population by a low cost, fast and easy procedure. Magnetic or fluorescent nanoparticles can be used to label stem cells followed by magnetic force or flow cytometry sorting. In the stem cell biology research field the MACS® technology, briefly described below, is the leading commercial brand and has made the separation of certain stem and progenitor cells a routine procedure.

The MACS® System is characterized by the use of nano-sized superparamagnetic particles (approx. 50 nm in diameter), cell separation columns, and MACS Separators which provide the required strong magnetic field [43]. Magnetic cell separation is performed in three steps:

1) Labelling: cell preparation and labelling methods are similar to those used in flow cytometry. Each target cell in a cell suspension is immunomagnetically labelled using MACS MicroBeads, which typically are covalently conjugated to a monoclonal antibody (mAb) or to a ligand specific for a certain cell type.

2) Separation: the cell suspension is passed through the separation column that contains

a ferromagnetic matrix and is placed in a MACS Separator. The separator contains a strong permanent magnet creating a high-gradient magnetic field in the magnetisable column matrix. Labelled target cells are retained in the column via magnetic force, whereas unlabeled cells flow through. By simply rinsing the column with buffer, the entire untouched cell fraction can be eluted.

3) Elution of the labelled cell fraction: after removing the column from the magnetic field of the MACS Separator, the retained labelled cells can easily be eluted with buffer.

The entire procedure can be performed in less than 30 min, and both cell fractions, magnetically labelled and untouched cells, are immediately ready for further use, such as flow cytometry, molecular analysis, cell culture, transfer into animals, or clinical cell therapy applications.

MACS MicroBeads are superparamagnetic particles made of an iron oxide core and a dextran coating. They are nano-sized, ranging between 20 and 150 nm in diameter, and form colloidal solutions, i.e., they remain dispersed [43]. Superparamagnetism means that in a magnetic field the iron oxide cores magnetize strongly like ferromagnetic material, but when removed from the magnetic field the particles do not retain any residual magnetism. The dextran coating of the MicroBeads permits chemical conjugation of biomolecules. Numerous highly specific mAb, fluorochromes, oligonucleotides and various other moieties have all been covalently linked to MicroBeads, thereby transferring additional biochemical and physical properties to them [43]. The nano-sized iron-dextran particles confer several unique features on MACS Technology. MACS MicroBeads are biodegradable and do not alter cell function. Effects on the functional status of cells by magnetic labelling with MicroBeads are primarily dependent on the target cell surface antigen and on the degree of crosslinking by mAb or ligands conjugated to the MicroBeads, but not on the MicroBeads themselves. Cells labelled with MicroBeads have been used for numerous functional in vitro assays, experi-



mental transfers into animals, and therapeutic transplantations in humans.

Molecular Detection and Biosensors

In addition to detect labelled stem cells, it is of paramount importance to detect particular molecules in the stem cell pathway at the cellular level. Nanotechnology provides advanced probes and devices for molecular detection. For example, (i) carbon nanotube optical probes for single molecule detection in living cells; (ii) carbon nanotube nanoelectrode array for deep brain stimulation; (iii) nanoparticles for neurochemical detection and biosensors; (iv) nanowires for molecular detection in stem cells; (v) self-assembly polymeric micelle-based bioassays; (vi) nanoarrays in mass spectrometry for proteomic and metabolomic applications; (vii) nanofluidic device for single cell genomic analysis on a chip.

The aim of these tools is to monitor biomolecules in real time without using invasive or endpoint procedures. Currently, most strategies to analyse intracellular biochemical processes rely on several steps of cell-processing like fixation, permeabilization and labelling, which are time consuming and expensive when scale-up or high throughput screening is needed. Nanoparticles can be an appropriate solution for "bio-sensing" inside stem cells. **Sensors** are usually composed of two parts: one that recognizes and binds the target molecule and another that signals the binding event. One way of doing this is to immobilize the recognition molecule to the surface of a nanoparticle. This type of approach was used by Hwang and collaborators to monitor neuronal differentiation *in vivo* using a molecular beacon [44]. They have generated a quencher-based fluorescent beacon system to sense the neuron-specific miR124a expression. Moreover this beacon was built upon a cobalt ferrite magnetic core which enables the dual-imaging nanoparticle beacon system to be used for *in vivo* cellular tracking by magnetic resonance as well as for monitoring the changes in the

expression of intracellular targets with the fluorescence-quenching beacon [44]. Other examples include pH nano-sensors [45] and nanoparticles able to quantify enzymatic activities [46]. A recent study reported the preparation of polymeric nanoparticles bearing a kinase peptide substrate and near-infrared fluorophore chemically coupled to the nanoparticle. In the nonphosphorylated state, these nanoparticles have low levels of fluorescence because of the short distance between each fluorescence probe in the nanoparticle. Upon kinase phosphorylation of the phosphate groups that are incorporated into the peptide substrate the polymeric nanoparticles dissolve due to charge unbalance and the fluorescence is recovered [46].

CONCLUSION

This report identifies challenges and opportunities where nanotechnology can be utilized to advance stem cell research. Although stem cell nanotechnology is still a young discipline, it is already contributing for new discoveries in stem cell research and the development of better stem cell technology. This survey of research topics in stem cell nanotechnology will allow non-nano-experts to realize the impact that nanotechnology is having in both basic stem cell biology and in translational applications of stem cell research into medicine.

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