Cell Stem Cell
Review

New Opportunities: The Use of Nanotechnologies to Manipulate and Track Stem Cells

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Nanotechnologies are emerging platforms that could be useful in measuring, understanding, and manipulating stem cells. Examples include magnetic nanoparticles and quantum dots for stem cell labeling and in vivo tracking; nanoparticles, carbon nanotubes, and polyplexes for the intracellular delivery of genes/oligonucleotides and protein/peptides; and engineered nanometer-scale scaffolds for stem cell differentiation and transplantation. This review examines the use of nanotechnologies for stem cell tracking, differentiation, and transplantation. We further discuss their utility and the potential concerns regarding their cytotoxicity.

The wide spectrum of nanotechnologies (referred to as nanomedicine by the National Institutes of Health for applications in the biomedical area) holds great promise for the study of stem cell biology and the development of new approaches to stem cell expansion, differentiation, and transplantation (Chen et al., 2007; Silva et al., 2004; Sniadecki et al., 2006). The term "nanotechnologies" in the title of this article reflects nanoscale (on the scale of approximately 1–1000 nm) or nanostructured materials used for medical diagnosis, drug delivery, and implants, which require novel and demanding chemical and manufacturing techniques. Therefore, the concept implies either the improvement of current materials or the advent of new materials with modified fundamental properties and bioactivity.

Examples of nanotechnologies in stem cell research are organic and inorganic nanoparticles (Corsi et al., 2003; Huang et al., 2005; Kutsuzawa et al., 2008), quantum dots (Chen et al., 2007; Shah et al., 2007; Slotkin et al., 2007), carbon nanotubes (Zhu et al., 2007), nanofibers (Dang and Leong, 2007; Silva et al., 2004; Yang et al., 2005), and nanoscale-engineered substrates (Bettinger et al., 2008; Dalby et al., 2007; Derda et al., 2007; Jan and Kotov, 2007) (Figure 1). Potential applications of nanotechnologies in stem cell research include (1) tracking of stem cell surface molecules and detailed examination of molecular motion without photobleaching, (2) noninvasive tracking of stem cells and progenitor cells transplanted in vivo, (3) stem cell delivery systems that enhance the survival of transplanted cells by releasing prosurvival biomolecules, (4) nanopatterned substrates that present covalently tethered biologically active molecules (adhesion sites, growth factors, and synthetic peptides) for stem cell differentiation and transplantation, and (5) intracellular delivery of DNA, RNAi, proteins, peptides, and small drugs for stem cell differentiation (Moghimi et al., 2005; Muschler et al., 2004). Though nanotechnologies are very powerful with respect to micro- and macroenvironmental control, they may have harmful drawbacks. Systematic studies must assess their toxicological profiles and evaluate potential interference with the self-renewal and differentiation programs of stem cells.

Historical Perspective on Nanotechnologies in the Stem Cell Field

Although some of the nanotechnologies described herein have already been applied to cell biology, their use in stem cell biology and regenerative medicine is more recent. This is likely due to (1) advances in the preparation of safer and more effective nanomaterials for biomedical applications, (2) growing awareness of material science and tissue engineering researchers regarding the potential of stem cells for regenerative medicine, (3) notable success in the application of nanotechnologies to medicine, and (4) developments in stem cell biology and the isolation of novel sources of stem cells.

Recent developments in the use of nanotechnologies with stem cells have been motivated by the continuous introduction of novel nanotechnology platforms during the last few years. Some of the nanomaterials reviewed here were discovered in the 1990s through technological developments such as carbon nanotubes, quantum dots, and nanowires. In some cases, the use of nanotechnologies in the stem cell field was propelled by research performed initially on somatic cells. For example, although the use of quantum dots for cell labeling was described in 1998 (Bruchez et al., 1998; Chan and Nie, 1998), their use for labeling stem cells is recent (Hsieh et al., 2006b; Seleverstov et al., 2006). Similarly, though the use of magnetic nanoparticles for intracellular labeling and detection by MRI was reported in the early 1990s (Yeh et al., 1993), only in 2000 were they applied to stem and progenitor cells (Lewin et al., 2000). In other cases, some established nanotechnologies were only recently used in the biomedical arena. The first patent in the preparation of electrospun nanofibers was awarded in 1934; however, they received little interest from biomedical researchers until the mid 1990s (Dzenis, 2004), and only in the 21st century were nanoscaffolds prepared for the culture and transplantation of stem cells (Li et al., 2005).

As necessity is often considered the mother of invention, new techniques are adapted when the need is recognized as a general problem. For example, as exogenous cell therapy undergoes

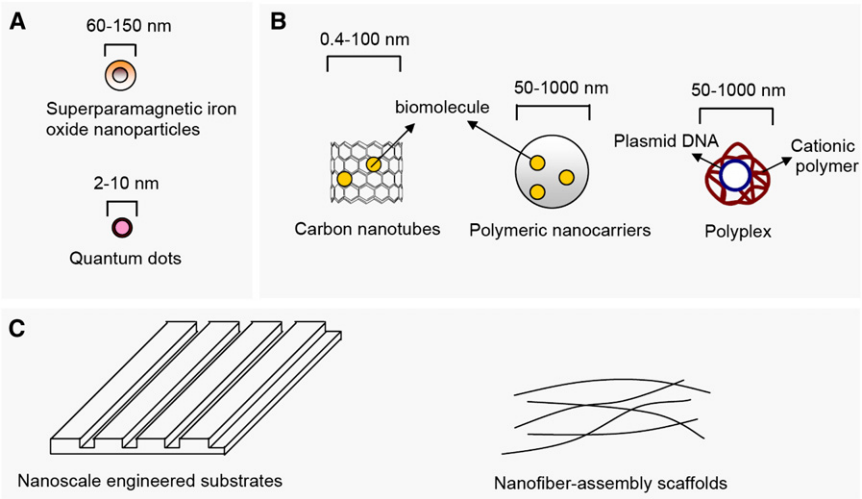

Figure 1. Current Nanotechnologies for Stem Cell Research

(A) Nanomaterials for stem cell labeling and tracking in vivo. Stem cells labeled by superparamagnetic iron oxide nanoparticles or fluorescent quantum dots might be tracked by magnetic resonance imaging or cell imaging systems, respectively, either in vitro or in vivo. In most cases, stem cells are loaded with nanomaterials at concentrations that do not exert cytotoxic effects.

(B) Many powerful strategies for the differentiation of stem cells require the delivery of bioactive molecules (e.g., plasmid DNA, siRNA, proteins, peptides, and small molecules) into the cytosolic or nuclear compartments of these cells. Polymeric nanocarriers, carbon nanotubes, and polyplexes are examples of nanomaterials used to deliver biomolecules within stem cells. In the case of polymeric nanocarriers and polyplexes, the material might degrade over time within the cell.

(C) Nanoscale-engineered substrates and scaffolds to create biomimetic cellular environments. Stem cell adhesion to substrates or scaffolds with nanoscale resolution can cause clustering of cell integrins into focal adhesion complexes, and the concomitant activation of intracellular signaling cascades and guidance of stem cell behavior.

rigorous testing in animal and human trials, it has become increasingly important to track the movement of transplanted cells to assess toxicity and therapeutic efficacy.

Nanomaterials for Stem Cell Labeling and Tracking In Vivo

Magnetic Nanoparticles

The history and fate of transplanted stem cells or progenitor cells is generally assessed by labeling them in vitro with a fluorescent dye, thymidine analog (e.g., BrdU), or a transfected gene such as LacZ or green fluorescent protein (GFP) and visualization by immunohistochemistry after the removal of tissues or organs. One of the main goals in stem cell research is long-term noninvasive imaging of transplanted cells in vivo to monitor their survival, migration, differentiation, and regenerative impact. Magnetic resonance imaging (MRI) provides a noninvasive, in vivo method for studying the fate of transplanted cells labeled with magnetic nanoparticles. MRI offers several advantages over other techniques such as positron emission tomography including greater speed, higher spatial resolution, more direct anatomical correlation, and lower cost (Stroh et al., 2005). In vivo images with a spatial resolution of 50 × 50 × 500 μm can be acquired over 2 to 3 hr (Allport and Weissleder, 2001).

Superparamagnetic iron oxide (SPIO) nanoparticles have been used as a feasible means to enhance the contrast of cellular targets in MRI. Among several types of nanoparticles described, some (e.g., Feridex/Endorem and Ferucarbotran) have been approved for human use by the U.S. Food and Drug Administration (FDA) as MRI contrast agents (Reimer and Balzer, 2003; Wang et al., 2001) (Table S1 available online). Generally, a SPIO nanoparticle is composed of an iron oxide core coated with dextran (ferumoxides, commercialized by Guerbert and Berlex Laboratories under the trademarks Endorem and Feridex, respectively) or carboxydextran (Ferucarbotran, commercialized by Schering) that ensures aqueous solubility and prevents nanoparticle aggregation (Reimer and Balzer, 2003; Wang et al., 2001). The iron oxide core is normally formed by magnetite Fe_3O_4. The overall hydrodynamic diameters of Ferucarbotran and Feridex/Endorem are 80–150 nm and 62 nm, respectively (Wang et al., 2001).

Most labeling techniques currently use one of two approaches: (1) attaching magnetic nanoparticles to the stem cell surface or (2) internalizing biocompatible magnetic nanoparticles by endocytosis or phagocytosis. Surface labeling has some limitations, including iron content that is generally lower by an order of magnitude than intracellular labeling procedures using SPIO nanoparticles (Sykova and Jendelova, 2005). In addition, although surface labeling is efficient for in vitro cell separation, it is generally unsuitable for in vivo use because of rapid reticuloendothelial recognition and clearance of labeled cells (Lewin et al., 2000). For in vivo tracking using magnetic resonance cell imaging, SPIO nanoparticles are generally taken up through stem cell endocytosis during in vitro cultivation (Figure 2). Human mesenchymal stem cells (hMSCs) internalize SPIO nanoparticles in the absence of transfection agents at a concentration up to 23.4 pg Fe/cell (Hsiao et al., 2007). However, in most cases, internalization of SPIO nanoparticles requires the use of excipient (Table S1). For example, for improved cellular magnetic labeling, nanoparticles have been derivatized with a short HIV-1 *trans*-activating transcriptional activator (TAT) peptide (Lewin et al., 2000), which mediates nanoparticle internalization by membrane receptor binding or by coating iron oxide nanoparticles with dendrimers (Bulte et al., 2001). Others have used protamine sulfate, a small cationic transfection agent approved by the FDA within certain products, to facilitate the uptake of SPIO nanoparticles into stem cells (Arbab et al., 2004). The average iron content per cell after labeling varied from 1.47 pg to 17.90 pg Fe, depending on the incubation time, cell type, and cell culture methodology used (Arbab et al., 2004).

A number of factors affect the MRI detection threshold of SPIO-labeled cells, such as the SPIO concentration per cell, and intrinsic MRI parameters, such as field strength, signal-to-noise ratio, pulse sequence, and acquisition parameters (Guzman et al., 2007; Heyn et al., 2005). Some studies have shown that as little as 1.4–3.0 pg of iron per cell is sufficient for detection

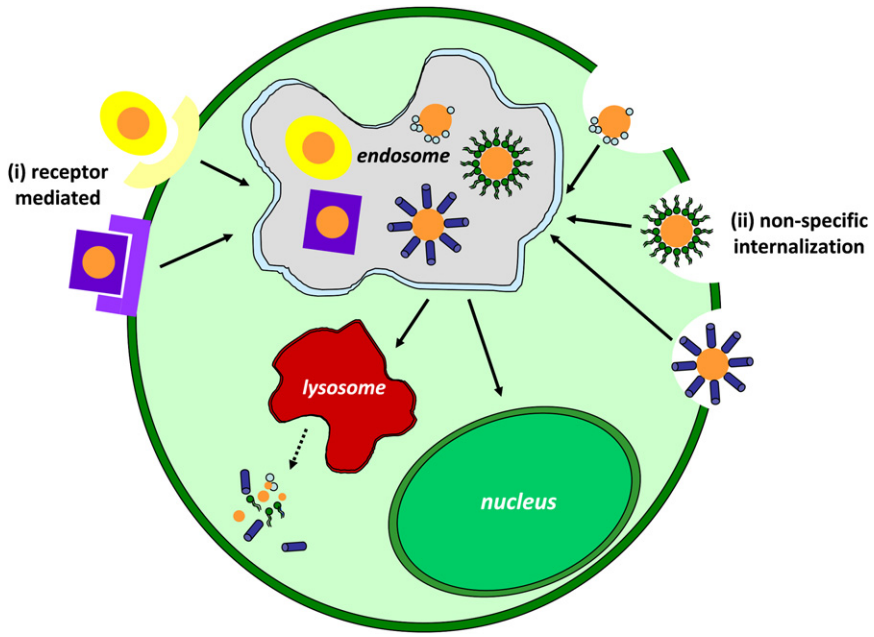

Figure 2. Strategies to Deliver Nanomaterials within Stem Cells
Schematic representation of steps involved in cytosolic and nuclear delivery of nanomaterials into stem cells. Nanomaterials can enter the stem cell either by (i) receptor-mediated interactions or (ii) nonspecific internalization pathways. In both cases, the nanomaterials become entrapped within endosomes and are then released in the cytoplasm or trafficked to the acidic environments of lysosomes for degradation. Cytoplasm-released nanomaterials might then be transported to the nucleus of the cell.

with MRI (Heyn et al., 2005). In vitro single cell detection by MRI has been described (Hoehn et al., 2002); however, in most cases MRI detection requires clusters of thousands of labeled cells (Guzman et al., 2007).

SPIO-labeled stem cells/progenitor cells might contribute to our understanding of cell migration processes in the context of numerous diseases, such as neurologic diseases (Guzman et al., 2007), myocardial infarction (Arai et al., 2006; Kraitchman et al., 2005), and cancer (Arbab et al., 2006). For example, magnetically labeled mouse embryonic stem cells (mESCs), injected into the nonischemic side of the brain of a rat with partial brain ischemia, migrate along the corpus callosum, populating the border zone of the ischemic area of the contralateral hemisphere (Hoehn et al., 2002). In addition, the use of SPIO-labeled stem cells in animal models of disease can help determine optimal timing and location of transplantation. A recent study has demonstrated that human central nervous system stem cells that are transplanted into a mature rodent brain migrate only after cerebral injury (cerebral stroke) (Guzman et al., 2007). In this case, stem cells transplanted into the cortical region of the brain migrate through an ipsilateral transcortical migration pathway; the extent of transcortical migration depends upon the distance between the graft site and the lesion. In addition to the information obtained from cell migration studies, SPIO technology might yield important information about the differentiation process of stem cells/progenitor cells. SPIO-labeled $CD34^+$ progenitor cells injected into rodents can be isolated by magnetic separation after in vivo migration to study the differentiation of these cells exposed to a biological environment (Lewin et al., 2000).

A clinical study using stem cells labeled with SPIO in patients with neurological disease has recently been reported (Zhu et al., 2006). This approach can be adapted to evaluate the therapeutic effects of stem cells in the context of other diseases, including myocardial infarction. SPIO nanoparticles have not been yet approved by the FDA specifically as intracellular contrast agents. The unclear framework for approving new nanomaterials as medical products (Helmus, 2007) might delay the clinical use of SPIO nanoparticles.

In clinical trials involving bone marrow-derived stem cells and hematopoietic stem cells that are used in patients within 24 hr after their isolation, the labeling of stem cells with SPIO nanoparticles should be performed in less than one day. A rapid method to label stem cells has recently been reported based in the electroporation of cells ("magnetoelectroporation") (Walczak et al., 2005). This technique involves low-voltage pulses to induce endocytosis of contrast agents in a matter of minutes. In addition to the advantage of rapid labeling of cells, this technique does not require transfection agents for the internalization of SPIO nanoparticles, which simplifies the regulatory pathway required for approval by regulatory agencies.

Despite the unique ability of MRI to track SPIO-labeled stem cells after their in vivo transplantation, this technique has some limitations. First, long-term observation of SPIO-labeled stem cells might be limited because of dilution of SPIO by cell division. For example, the initial SPIO concentration in neural stem cells (NSCs) was shown to decrease by 50% every 3 days in vitro (Guzman et al., 2007). Nonetheless, neural stem cells were tracked in vivo for up to 18 weeks (Guzman et al., 2007). Second, due to the in vivo migration of SPIO-labeled stem cells, the density of cells is reduced considerably over time, leading to a gradual loss of MRI cell signal (Guzman et al., 2007). Third, it is important to note that MRI cannot determine whether the SPIO-labeled stem/progenitor cells differentiate into a specific cell type, but cell function may be inferred from complementary imaging studies, such as positron emission tomography or optical imaging (Arbab et al., 2006). Fourth, in most cases, MRI studies are conducted with a 1.5 T MRI unit, which has limited spatial resolution. Improvement in spatial resolution requires stronger magnetic fields; however, the potential hazards of these magnetic fields are still unknown (Hsiao et al., 2007; Stroh et al., 2005). Fifth, SPIO nanoparticles are not detected directly, but indirectly through microscopic disturbances of the magnetic field that misalign the orientation of water protons from which the magnetic resonance signal is derived (Bulte, 2005). Therefore, it is difficult to correlate the magnetic resonance signal to the number of cells. However, perfluoropolyether nanoparticles, which can be detected directly by ^{19}F imaging, offer an alternative to SPIO nanoparticles for accurate counting of local cells

(Ahrens et al., 2005). Future studies are needed to evaluate the cytotoxicity of these nanoparticles after internalization by stem cells (Bulte, 2005).

Cytotoxicity of Magnetic Nanoparticles

SPIO nanoparticles are composed of biodegradable iron that might be reused/recycled by cells using biochemical pathways for iron metabolism. Potential mechanisms of iron-mediated toxicity include generation of iron-catalyzed reactive oxygen species (Hoepken et al., 2004). The cytotoxicity of SPIO nanoparticles has been evaluated in hMSCs (Hsiao et al., 2007; Lu et al., 2007), mESCs (Arai et al., 2006; Stroh et al., 2005; Sykova and Jendelova, 2005), and NSCs (Bulte et al., 2001; Guzman et al., 2007; Wang et al., 2006). In most cases, the internalization of these nanoparticles by stem cells did not affect cell viability, growth, or differentiation. So far, only one study has demonstrated that the internalization of SPIO nanoparticles impaired the differentiation of stem cells. Bulte et al. reported that the uptake of SPIO by hMSCs (intracellular iron incorporation of 13–16 pg Fe/cell), in the presence of the transfection agent poly-L-lysine, impaired their chondrogenic differentiation (Bulte et al., 2004). Although the results of this study suggest that the inhibition effect was mediated by the Fe itself and not the transfection agent (Bulte et al., 2004), a study carried out by Arbab et al. suggests that impairment was caused by the transfection agent and not the Fe (Bulte et al., 2004). Subsequent studies confirmed that the internalization of SPIO nanoparticles by hMSCs using a liposome transfection agent did not affect their chondrogenic, adipogenic, or osteogenic differentiation (Song and Ku, 2007).

When choosing appropriate tracking agents, it is important to consider their respective imaging requirements. Use of magnetic nanoparticles often requires complex imaging systems, such as MRI, whereas use of quantum dots relies on optical imaging, which may be more accessible to the majority of researchers.

Quantum Dots

Quantum dots (qdots) are another class of nanomaterials used for the long-term labeling of stem cells and monitoring fate and regenerative potential. Qdots are light-emitting nanocrystals, typically in the size range of 2–10 nm, composed of atoms from groups II–VI (e.g., CdSc, CdTe, CdS, and ZnSe) or III–V (e.g., InP and InAs) of the periodic table. Since the first studies in 1998 reporting the use of colloidal qdots for cell labeling, many researchers have used them in biolabeling (Bruchez et al., 1998; Chan and Nie, 1998). Colloidal qdots with a wide range of bioconjugation are now available commercially. Qdots are superior to organic dyes or fluorescent proteins for long-term cell labeling because of their photostability and durable fluorescence intensity (up to a few hours using confocal microscopy) (Alivisatos, 2004; Michalet et al., 2005). The long fluorescence lifetime of qdots allows their signal to be separated from background autofluorescence in cells or tissues. In addition, the narrow emission spectrum and broad excitation spectrum of qdots allow simultaneous analysis of multiple cell targets using a single wavelength activation (Alivisatos, 2004; Michalet et al., 2005).

One of the potential applications of these nanomaterials is to follow the dynamics of cellular components in real time. For example, qdots have been used to study the membrane diffusion of integrins during the differentiation of bone marrow-derived progenitor cells (BMPC) (Chen et al., 2007). A similar approach could be adopted to study the participation and clustering of multiple integrins involved in the differentiation of stem cells and progenitor cells over time, either ex vivo or in vivo. Since integrins are crucial for transmitting many extracellular signals to stem cells, their study could lead to the design of more effective tissue-engineered scaffolds for stem cell transplantation.

Another advantageous application of qdots is for immunolabeling of proteins in sections of tissue with inherent autofluorescence such as the heart (Rota et al., 2007), which can lead to misleading artifacts during conventional immunostaining. After heart injury, autofluorescence increases due to accumulated lipofuscin, blood-derived pigments and other fluor-based molecules (Laflamme and Murry, 2005). The photostability of qdots allows preservation of the fluorescence signal of the stained biomolecule over time while eliminating the autofluorescence.

Qdots are also attractive nanomaterials for monitoring stem cell survival, location, and differentiation either in vitro or in vivo due to their inherent long-term fluorescence intensity (Chakraborty et al., 2007; Lei et al., 2008; Lin et al., 2007; Shah et al., 2007; Slotkin et al., 2007). Stem cells are exposed to qdots suspended in culture media, followed by their cellular internalization and quantification of fluorescence signal by cell imaging systems. In most cases, efficient internalization of qdots requires the use of specific peptides such as cholera toxin (Chakraborty et al., 2007), TAT-peptide (Lei et al., 2008), RGD (Shah et al., 2007), or phospholipids (Slotkin et al., 2007) (Figure 2). mESCs labeled with qdots with a high extinction coefficient and wide emission spectra within the near-infrared region (800 nm) could be detected in animals up to 14 days postinjection using an optical imaging system (Lin et al., 2007). However, qdots were not detected in animals 28 days postinjection, likely due to dilution and diffusion.

Qdots' long-term fluorescence make them an important new class of nanomaterials available for advancing the stem cell field. Although qdots can be optically imaged, in vivo tracking typically requires access to whole animal imaging, which may limit progress in this area. Whole animal imaging systems such as Caliper's IVIS have been widely used for imaging stem cells in vivo (Cao et al., 2006; Duan et al., 2007), yet none of these studies have so far have employed qdot labeling as a tool to track stem cells in vivo. Given the significantly higher quantum yield with qdot labeling and minimal autofluorescence at near infrared fluorescence wavelengths, it is anticipated that a qdot approach would dramatically enhance the sensitivity and even reduce detection thresholds. It is expected that in the short term, qdots will be used primarily as in vitro tools, since MRI and SPIO nanoparticle technology has a longer track record for imaging stem cells in vivo. There are also concerns over the toxicity of qdots, as outlined below.

Cytotoxicity of Qdots

The effects of qdots on stem cell self-renewal and differentiation are largely unknown, particularly in embryonic stem cells. Some studies have reported no adverse effects on stem cell morphology, viability, proliferation, or differentiation over the duration of the experiments (from several hours to several days) at qdot concentrations optimized for labeling efficiency (Chakraborty et al., 2007; Shah et al., 2007); while others have noticed alterations in the differentiation profile of stem cells (Hsieh et al., 2006a, 2006b) and abnormalities during embryo development

(Dubertret et al., 2002). Therefore, qdots are not completely innocuous, but there is likely to be a safe range within which they can accomplish their task without major interference in the processes under study (Michalet et al., 2005).

Perhaps the broadest effort to study the cytotoxicity of qdots in stem cells has been made using hMSCs. Some studies have reported that qdots do not affect cellular proliferation or cell-cycle distribution but do affect chondrogenic and osteogenic differentiation potential (Hsieh et al., 2006a, 2006b). However, recent findings indicate that qdots do not interfere with the differentiation program of stem cells (Chakraborty et al., 2007; Shah et al., 2007). hMSCs labeled with a range of external doses of qdots conjugated with a cell-penetrating peptide (cholera toxin) from 250 pM to 16 nM maintained their osteogenic differentiation potential. The cells showed upregulation of alkaline phosphatase activity, an early osteogenic marker, when cultured in osteogenic media and expressed the osteogenic gene *Osterix* after exposure to BMP-2 (Chakraborty et al., 2007). In a another study, hMSCs labeled with an exogenous concentration of 30 nM qdots were viable and continued to proliferate for at least 22 days while retaining qdots in their cytoplasm (Shah et al., 2007). No interference of qdots was detected in the differentiation of hMSCs into osteogenic, chondrogenic, and adipogenic cell lineages.

The conflicting results of these studies might be related to different sources of qdots (inherent chemical composition [Derfus et al., 2004], size [Lovric et al., 2005a], and surface coating [Derfus et al., 2004]), mode of qdot internalization, and intrinsic characteristics of the target cells used. In most studies, the cytotoxicity of qdots is based on extracellular nanoparticle exposure concentrations; however, qdot size and surface coating might affect their cellular internalization and, consequently, their intracellular concentration. This variability presents a significant challenge to performing comparative studies of mechanisms of utility or toxicity. A recent study demonstrated the relevance of intracellular levels of qdots (Chang et al., 2006). The study correlated cellular toxicity with the intracellular exposure of modified qdots with a variety of surfaces and demonstrated that biocompatibility of surface coatings improves as cellular uptake of qdots via endocytosis decreases (Chang et al., 2006). Moreover, the cytotoxicity of qdots might arise from their intracellular degradation. Qdots are generally taken up by cellular endocytosis into vesicles called endosomes. (Figure 2) These endosomes are then trafficked to various cellular compartments, in particular the perinuclear region (Hsieh et al., 2006a; Seleverstov et al., 2006). The accumulation of qdots in cell nuclei has been reported (Dubertret et al., 2002); however, this process depends on the cell type and the surface coating of qdots (Shah et al., 2007). Nanomaterials that cannot be used by the cell are trafficked to acidic and oxidative environments of lysosomes and peroxisomes for degradation. (Figure 2) The oxidative degradation of qdots releases Cd^{2+} (Derfus et al., 2004), which can bind to the sulfhydryl groups of critical mitochondria proteins, leading to mitochondria dysfunction and, ultimately, cell poisoning (Rikans and Yamano, 2000). The release of reactive oxygen species during the degradation of qdots also contributes to cytotoxicity (Lovric et al., 2005a; Lovric et al., 2005b).

In addition to the need for tracking stem cells, there is a great need to exercise control over cell function, which can also be addressed with nanotechnologies.

Nanomaterials for the Intracellular Delivery of Genetic or Proteic Material

Intracellular Delivery of Genetic Material with Nanomaterials

Gene delivery [DNA or RNA interference (RNAi)] can be a powerful strategy to study the basic biology of stem cells or to direct their differentiation into specific cell types (Hough et al., 2006; Meinel et al., 2006). Genes can be delivered by viral and nonviral vectors. Viral vectors including retroviruses, lentiviruses, and adenoviruses have been extensively used for cellular transfection because of their efficient gene delivery and durable gene expression (Clements et al., 2006; Gropp and Reubinoff, 2006). However, these viral carriers have a number of disadvantages, including risk of toxicity, immunogenicity, insertional mutagenesis, and high manufacturing costs (Glover et al., 2005; Pack et al., 2005). In contrast, nonviral vectors, such as polymers (Clements et al., 2007; Corsi et al., 2003; Incani et al., 2007), lipids (Clements et al., 2007), and physical delivery methods such as electroporation (Zwaka and Thomson, 2003) and nucleofection (Aluigi et al., 2006; Lakshmipathy et al., 2004) offer several advantages over viral vectors, including high gene-carrying capacity, low risk of immunogenicity, as well as low cost and ease of production (Glover et al., 2005). In the case of nonviral vectors, most efforts are focused on the development of polymeric approaches for gene delivery, mainly due to low cost, ease of production, and controllable toxicity based on parameters including ionic charge, chemistry, and chain length.

Polymers are typically cationic and interact electrostatically with negatively charged DNA/RNA molecules, condense the genetic material into particles to several hundred nanometers in diameter, protect the genes from enzymes, and mediate cellular entry. Complexes of plasmid DNA with cationic polymers are known as polyplexes (Pack et al., 2005). Polymers that have been used for stem cell transfection include poly(L-lysine)-palmitic acid (Clements et al., 2007), chitosan (Corsi et al., 2003), polyethylenimine (Incani et al., 2007) (PEI), and poly(L-lysine) (Incani et al., 2007). For example, a polyplex formed by plasmid DNA and poly(L-lysine)-palmitic acid transiently transfected embryonic stem cells with a maximum efficiency of 22%. This method was significantly more effective than lipofectamine TM 2000-mediated transfection (liposome-based delivery system), which achieved 11% transduction under optimal conditions (Clements et al., 2007). These polyplexes have transfection efficiency equivalent to that of adenoviral vectors (35% after 24 hr) for the delivery of plasmid DNA in bone marrow stromal cells (Incani et al., 2007).

Nanoparticles have also been used for gene transfection. For example, apatite nanoparticles coated electrostatically with fibronectin and E-cadherin have been reported to be efficient gene delivery systems for embryonic stem cells (Kutsuzawa et al., 2006). Specific binding to cell surface integrin and E-cadherin molecules through double-ligand-created nanoparticles resulted in synergistic acceleration of gene delivery and subsequent expression into embryonic stem cells (59% of the cells expressed the gene) (Kutsuzawa et al., 2006, 2008). Gene expression was almost three times higher for this system than that typical of commercially available lipofectamine TM 2000.

Genetic material can be delivered within stem cells using a nanoscale-engineered cell substratum (Kim et al., 2007). Gene

transfection was achieved in mESCs cultured on silicon nanowire arrays, although at low levels (~1%). The ability of the arrays to penetrate the living cells did not impair cell viability when the diameter of the nanowires was relatively small compared with the diameter of the cells.

Another promising strategy for cell transfection is the use of carbon nanotubes. Nanotubes incorporating different functional groups are transported toward the perinuclear region a few hours after initial contact with cells, even under endocytosis-inhibiting conditions (Kostarelos et al., 2007). Although these nanomaterials have been used as delivery systems for nucleic acids, proteins, and drug molecules into mammalian cells (Bianco et al., 2005), their use in stem cells has not been significantly explored. Initial studies have focused on the cytotoxicity of these nanomaterials, and more research is needed to further evaluate their potential as biomolecule delivery vectors.

Intracellular Delivery of Peptide- or Protein-Based Molecules with Nanomaterials

Intracellular delivery of biomolecules, including proteins, growth factors, and small chemicals, is powerful method by which to control the differentiation of stem cells. We recently reported a new approach for the delivery of vascular growth factors into hESCs by incorporating growth factor-release particles into embryoid bodies (EBs) (Ferreira et al., 2008). We demonstrated that the incorporation of these polymeric biodegradable particles has a minimal effect on cell viability and proliferation but a great impact on differentiation. In some cases, the effect on vascular differentiation of incorporation of particles containing growth factors was superior to that produced by exposing EBs to large extrinsic doses of the same growth factors. In addition, we studied the intracellular trafficking of particles of different sizes within hESCs. We demonstrated that nanoparticles (diameter ~240 nm) could be taken up by hESCs and accumulate in the perinuclear region (Ferreira et al., 2008). These nanoparticles could serve as a platform to deliver growth factors and other biomolecules within stem cells. However, it will be crucial to ensure cytocompatibility of each formulation, likely on a case-by-case basis.

Cytotoxicity of Nanomaterials Used for Intracellular Drug Delivery

The cytotoxicity of polyplexes and nanoparticles has been evaluated in mESCs (Tran et al., 2007), hESCs (Ferreira et al., 2008), hMSCs (Corsi et al., 2003), and rat bone marrow stromal cells (Incani et al., 2007). In general, the cytotoxicity profile correlates with nanoparticle chemistry (Corsi et al., 2003; Incani et al., 2007; Kutsuzawa et al., 2006) and concentration (Corsi et al., 2003; Incani et al., 2007). The toxicity of carbon nanotubes has been recently evaluated in stem cells. There is evidence that many factors contribute to the toxicity of carbon nanotubes, including their concentration, size, shape, and surface coating (Magrez et al., 2006). In a recent study, the genotoxicity of multiwalled carbon nanotubes (MWNTs) in mESCs was reported (Zhu et al., 2007). The results show that MWNTs accumulate and induce apoptosis in mESCs and activate the tumor suppressor protein p53 within 2 hr of exposure. A mutagenesis study using an endogenous molecular marker, adenine phosphoribosyltransferase, showed that MWNTs increased the mutation frequency 2-fold compared with spontaneous mutation in mESCs (Zhu et al., 2007). The results indicate that, under the conditions tested, carbon nanotubes are genotoxic, and further surface chemical modification might be required before they can be used safely in stem cell research. The mechanism responsible for the cytotoxicity of MWNTs is unclear. Recent studies indicate that carbon nanotubes might generate reactive oxygen species when exposed to irradiation, and this might dramatically affect cellular behavior (Joshi et al., 2008). It would be interesting to study whether these reactive oxygen species are generated in cells that accumulate MWNTs.

In addition to delivering agents into the cell to affect cell function, nanotechnologies can also be employed to create extracellular microenvironments for directing stem cell function from the outside.

Nanoscale Engineering to Create Biomimetic Cellular Environments

In living organisms, stem cells are prevented from exiting the mitotic cycle by specific environments, called niches (Scadden, 2006). These niches are formed by cellular and noncellular elements. The noncellular elements include instructive extracellular matrix (ECM) molecules (e.g., collagen, elastin, proteoglycan, fibronectin, and laminin) secreted by cells in the vicinity of stem cells. These ECM molecules provide stem cells with biophysical clues, including specific surface chemistry and topography with nanoscale features. For example, collagen (the most abundant ECM protein) forms triple-helical structures that pack together, producing fibrils with a diameter ranging from 15 to 300 nm (Bruns, 1976). The nanoscale structure of the ECM provides cellular anchorage points and presents instructive clues to guide cell behavior. The ECM also creates sites for cell adhesion molecules and the immobilization of soluble factors. Cells are in contact with the ECM via integrins, transmembrane proteins that interact with specific amino acid sequences found in ECM proteins. These receptors tether the cell cytoskeleton to the fibers of the ECM and activate a cascade of intracellular signaling pathways, affecting cellular behavior at different levels including adhesion, proliferation, migration, and differentiation (Sniadecki et al., 2006).

The ability to engineer materials to resemble the structural complexity of ECM, including its nanotextured topography, has made large contributions to our understanding of several cellular processes including stem cell-matrix interactions, stem cell differentiation in response to different nanoscale topographies, and stem cell migration. Several patterning techniques have been used for this purpose, as thoroughly reviewed elsewhere (Curtis and Wilkinson, 2001; Dzenis, 2004; Geissler and Xia, 2004; Norman and Desai, 2006; Sniadecki et al., 2006).

Nanoscale Topography

Cell adhesion to the ECM causes clustering of integrins into focal adhesion complexes and activation of intracellular signaling cascades (Sniadecki et al., 2006). Focal adhesion complexes recruit numerous proteins such as focal adhesion kinase (FAK), vinculin, paxillin, talin, p130Cas, and others (Mitra et al., 2005). The concentration and topography of cell adhesion sites in the ECM are critical for integrin clustering and activation (Figure 3). Parameters including size (Park et al., 2007), lateral spacing (Park et al., 2007), surface chemistry (Chua et al., 2006), and geometry (Dalby et al., 2007) of the nanofeatures are important to guide stem cell behavior. For example, adhesion, spreading, and differentiation of rat mesenchymal stem cells into the osteogenic lineage was highest on TiO_2 nanotubes vertically aligned with a diameter of

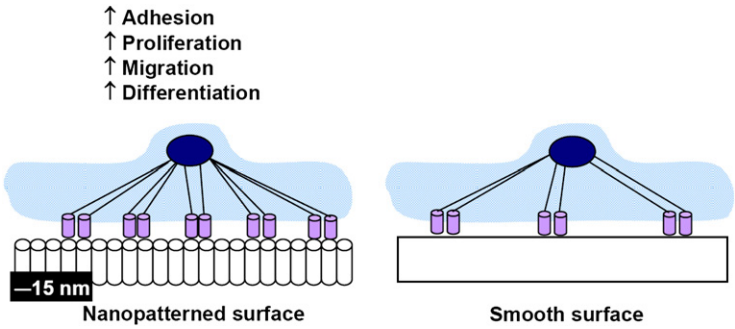

Figure 3. Response of Stem Cells to Nanopatterned Substrates

Focal adhesion complexes transduce external signals from the ECM to the cell interior. These complexes are formed by numerous structural and regulatory proteins, and their composition varies constantly depending on external cues and cellular responses (reviewed in Mitra et al., 2005). A spacing of 15 nm seems optimal for integrin assembly into focal contacts and the induction of assembly of actin filaments and signaling to the nucleus. Mesenchymal stem cells attached to these substrates have higher adhesion, proliferation, migration, and differentiation than cells attached to smooth surfaces (Park et al., 2007).

15 nm, which roughly corresponds to the predicted lateral spacing of integrin receptors in focal contacts on the extracellular matrix, and declined significantly with increasing nanotube diameter (Park et al., 2007). The phosphorylation of FAK and the extracellular signal-regulated kinase (ERK), which is a target of the FAK signaling pathway, was highest in stem cells growing on 15 nm tubes but low on 100 nm nanotubes (Park et al., 2007). Our research group has also shown that although the culture of hESCs in a nanometer-scale line grating enhances the alignment and elongation of cells and also organizes and polarizes cytoskeleton proteins, cell proliferation is reduced (Gerecht et al., 2007). Furthermore, in a different study, we showed that endothelial progenitor cells respond to ridge-groove grating of 1200 nm in period and 600 nm in depth with alignment, elongation, reduced proliferation, and enhanced migration (Bettinger et al., 2008). Although endothelial cell-specific markers were not significantly altered, endothelial progenitor cells cultured on substrate nanotopography formed supercellular band structures after 6 days (Bettinger et al., 2008).

The simultaneous effect of surface topography with surface chemistry has been evaluated in the context of stem cell differentiation. Compared to flat substrates, substrates with nanoscale features and different chemistries (silica [Lipski et al., 2007], alumina [Popat et al., 2007], and poly[methyl methacrylate] [Dalby et al., 2006]) have been reported to enhance the adhesion, growth, and osteogenic differentiation of hMSCs (Lipski et al., 2007) and marrow stromal cells (Dalby et al., 2006; Popat et al., 2007), and could have potential application as osteogenic coatings for orthopedic implants. Moreover, the combination of substrate topography with substrate electrical conductivity has been shown to contribute to the differentiation of NSCs into specific neuronal lineages (Jan and Kotov, 2007).

In addition to the dimensions of nanotopographical features, their conformation (e.g., ridges, grooves, whorls, pits, and pores) and symmetry may be equally important. Surfaces with different levels of nanoscale order and symmetry have been created to stimulate the in vivo differentiation of osteogenic progenitor cells (Dalby et al., 2007). Strikingly, the disordered nanoscale features stimulated hMSCs to produce bone mineral in vitro, in the absence of osteogenic supplements at levels similar to stem cells grown on flat surfaces and exposed to osteogenic supplements. These nanofeatures might be incorporated into orthopedic repair material to influence osteogenic progenitor cells in vivo to produce mature osteoblasts leading to direct bone-material contact, rather than fibrous tissue, one of the problems associated with orthopedic biomaterials.

Since embryonic stem cells and adult stem cells differ in their properties and differentiation potentials, they will likely respond differently to nanoscale features. Unfortunately, the current lack of systematic studies assessing the effect of nanoscale features on multiple types of stem cells under the same conditions prevents any inferences or conclusions. Furthermore, the general mechanism underlying cellular response to nanofeatures remains undefined. The alteration of cellular function at nanostructured interfaces may result from (1) direct influence on cellular responses or (2) an altered extracellular layer matrix deposited on the surface and consequent change in the availability of binding sites. When cells adhere to a material, they interact not with the material itself but with proteins adsorbed to the material (Wilson et al., 2005). The mechanisms underlying the influence of nanostructured surfaces on cellular response likely involve the organization of integrins in the cellular membrane, which in turn can modify cytoskeleton organization and intracellular signaling mediated by FAK, vinculin, paxillin, and other proteins (Mitra et al., 2005). In some cases, stem cells grown on nanotextured surfaces are elongated, with a stretched nuclear morphology that might alter the internal nuclear matrix structure and hence affect the expression of silent genes (Dang and Leong, 2007).

Nanofiber-Assembly Scaffolds

The regenerative potential of stem cells has been demonstrated in vivo by injecting cells suspended in an appropriate medium into the desired tissue. However, this approach limits the spread of the cells from the injection site, and in some cases, the injected cells have poor engraftment. Specifically, one of the greatest challenges in traditional stem cell therapies involving direct injection of cells into local tissues is that fewer than 10% of cells engraft (Laflamme and Murry, 2005; Mooney and Vandenburgh, 2008). This likely results from a combination of different factors, including the loss of cells from the injection site into the circulation or other tissues, cell death due to the absence of an appropriate ECM for cell adhesion, and lack of efficient mass transport of oxygen and nutrients to the transplanted cells. Stem cell transplantation efficiency might be enhanced by placing the cells in biocompatible and biodegradable scaffolds that act as a temporary three-dimensional ECM for cell adhesion, survival, migration, differentiation, and organization. Specifically, scaffolds can be designed to prevent anoikis of the transplanted cells, improve cell survival and promote migration, guide stem cell differentiation and three-dimensional organization, and promote adequate responses from the host tissue for the survival of the cells (e.g., promoting the vascularization of the tissue constructs) (Mooney and Vandenburgh, 2008).

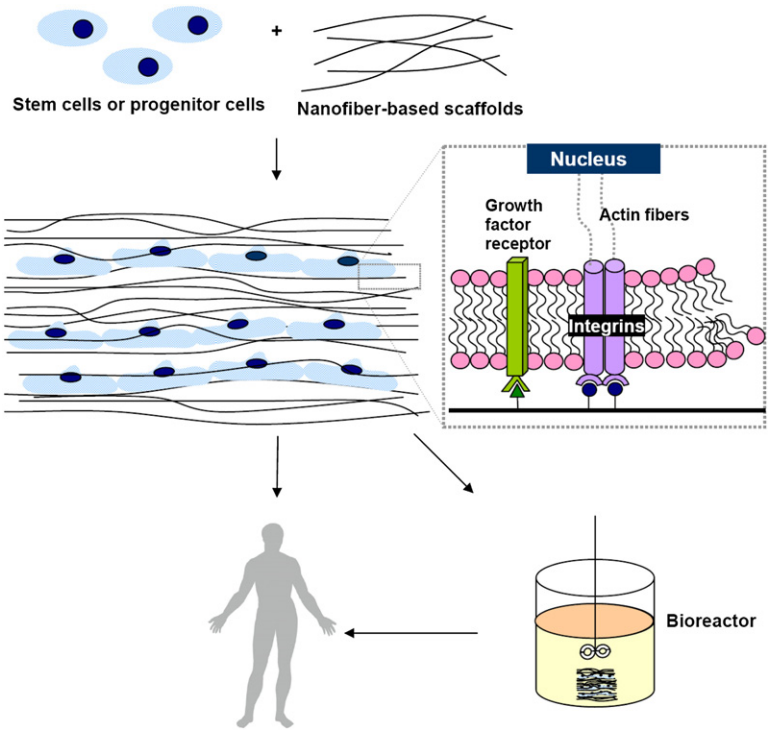

Figure 4. Nanofiber-Assembly Scaffolds for Tissue Engineering

Initially, stem cells or progenitor cells are seeded on three-dimensional scaffolds formed by nanofibers. These nanofibers may present a high density of ligands, including cell-adhesion epitopes or immobilized growth factors, for stem cell differentiation. The tissue constructs can be implanted immediately after incorporation of a cell source (<24 hr) into the defective tissue. Alternatively, the tissue constructs can be cultured in bioreactors to allow cell proliferation, differentiation, and three-dimensional organization before their final implantation. In both cases, the scaffold acts as a temporary 3D ECM for cell adhesion and tissue formation and typically is designed to degrade when new extracellular matrix is deposited.

Nanofiber scaffolds have been prepared to recreate the fibrous network of ECM and to improve stem cell transplantation (Figure 4). Nanofiber-based scaffolds have high porosity and specific surface area and present nanometer-scale topographical cues that are potent effectors of cellular behavior (see above). Nanofibers with controlled diameter have been prepared by electrospinning (Dzenis, 2004; Murugan and Ramakrishna, 2007; Norman and Desai, 2006) or by self-assembly of peptide amphiphile molecules (Silva et al., 2004). Synthetic polymers such as poly(lactic acid) (PLA), poly(caprolactone), poly(amide), or natural polymers including collagen, silk protein, and chitosan have been electrospun into nanofibers. The electrospun fibers can be aligned by manipulating the electrical field or by collecting the fibers on a rotating target, producing three-dimensional scaffolds.

Typically, stem cells cultured on nanofiber scaffolds differ in morphology, viability, and migration from cells cultured on conventional substrates. For example, hMSCs grown on 500–1,000 nm nanofibers are flatter and demonstrate significantly higher cell viability and lower cell mobility than control cells grown on tissue culture polystyrene (Shih et al., 2006). Clearly nanofiber scaffolds offer great potential for stem cell applications, yet systematic studies are needed to define how specific scaffold properties affect cell function prior to creating ''designer'' scaffolds for particular applications.

One of the potential applications of nanofiber-based scaffolds is in the creation of synthetic niches for stem cell self-renewal. The chemical, mechanical, and three-dimensional features of these scaffolds can influence the activation of different signaling pathways, resulting in stem cell proliferation and self-renewal (Nur et al., 2006). Another application of these scaffolds is for stem cell differentiation. Studies have shown that the differentiation process correlates with the amplification of bioactive epitope presentation to cells by the nanofibers (Silva et al., 2004). The high density of epitopes on nanofibers is a direct consequence of their high surface area and tailored chemistry and can be several orders higher than the density of epitopes in substrates formed by ECM components like laminin (Silva et al., 2004). The nanofibers in the scaffold can be aligned to create complex guidance channels for the alignment of stem cells or progenitor cells (Yang et al., 2005). Cell alignment in tissue constructs might be particularly important for the treatment of nerve tissue after spinal cord injury and the regeneration of cardiac and muscle tissues.

Cells are generally seeded on top of the nanofiber-based scaffolds to repopulate the matrix. The small pore size of these scaffolds might prevent cell invasion into the interior of the matrix and, thus, the formation of a homogenous tissue construct (Li et al., 2008). Therefore, unless these nanofiber-based scaffolds are engineered to have enzyme-degradable links that can be rapidly degraded by advancing cells, they might provide more suitable substrates for the development of cell sheets that can subsequently be assembled to form tissues. In a recent study, scaffolds composed of thermally responsive hydroxybutyl chitosan nanofibers (diameter of 436 nm) were used to produce aligned cell sheets derived from hMSCs (Dang and Leong, 2007). hMSCs cultured on the surface of chitosan nanofiber scaffolds showed alignment and elongation in both cell body and nucleus and underwent myogenic differentiation. The thermal dissolution of the fiber-based scaffold produced aligned cell sheets that might be used in engineering of tissue constructs for cardiac and muscle regeneration (Dang and Leong, 2007).

One of the ultimate applications of nanofiber-based scaffolds is in vivo stem cell transplantation. In this case the scaffold would act as a temporary ECM to guide tissue formation and typically would degrade in concert with deposition of new ECM. Unfortunately, there are few in vivo studies of stem cells transplanted into these scaffolds (Hashi et al., 2007). In contrast to traditional scaffolds for cell transplantation, nanofiber-based scaffolds offer the opportunity to control stem cell behavior by incorporation of high-density epitopes and control of cell alignment. Moreover, the intrinsic properties of the scaffolds might contribute to the differentiation of endogenous stem cells in the vicinity of the implant.

Future Prospects

Currently, there are 2,045 clinical trials involving stem cells (www.clinicaltrials.gov, accessed March 24, 2008). For clinical efficacy, it is imperative to image stem cells and their final location in vivo. Detection by MRI of magnetic nanoparticle-labeled stem cells may serve as a suitable means to achieve this objective; however, clinical studies are needed. The gradual loss of MRI cell signal due to cell division should be addressed in future studies. Qdots might be an alternative for the long-term labeling of stem cells. However, the current cost of qdot labeling and accessibility of whole animal imaging is a barrier to large-scale studies; hopefully, this issue will be overcome soon. In the mean time, we believe that qdots will be particularly useful for ex vivo assays. Qdots offer a unique platform to study the dynamics of biomolecules and intracellular compartments in stem cells. Further studies will be required to demonstrate the effect of qdots on stem cell self-renewal and differentiation, particularly in embryonic stem cells.

Many powerful strategies for the differentiation of stem cells require the delivery of biomolecules into the cytosolic or nuclear compartments. In most cases, it is largely unknown how vectors are internalized by stem cells, information likely to be critical for the design of more efficient delivery systems. Carbon nanotubes that cross the cell membrane through mechanisms other than endocytosis might be an interesting approach to deliver biomolecules within stem cells (Kostarelos et al., 2007); however, further studies are needed to study cytotoxicity and effects on stem cell differentiation.

The successful in vivo engraftment of stem cells might require the use of biomaterials in the form of scaffolds, which would isolate the cells at the transplantation site and act as a temporary three-dimensional ECM to guide tissue formation. These scaffolds can incorporate nanocarriers to deliver biomolecules at specific times and places within the network, and the migration and differentiation of stem cells can be tracked by the imaging tools described above. Specifically, nanofiber-assembly scaffolds might improve stem cell differentiation and in vivo engraftment. The combined effect of nanofiber organization and immobilized bioactive factors will likely contribute to cell guidance and differentiation in vivo. Moreover, it is clear that surface nanotopography can influence stem cell adhesion, migration, differentiation, matrix production, and autocrine and paracrine signaling events. Approaches to incorporate nanotextures on implant or scaffold surfaces may be useful to guide tissue regeneration through direct influence on exogenous or endogenous stem or progenitor cells.

Nanotechnologies clearly have great potential to enhance stem cell research and stem cell-based therapeutics. They have already begun to advance our ability to understand and control stem cell-fate decisions and to engineer novel stem cell technologies, with the ultimate goal to create stem cell-based therapeutics for the prevention, diagnosis, and treatment of human diseases.

SUPPLEMENTAL DATA

The Supplemental Data include one table and can be found with this article online at http://www.cellstemcell.com/cgi/content/full/3/2/136/DC1/.

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