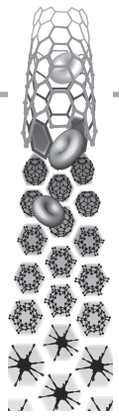

Nanotechnology that drives stem cell commitment

Stem cells (SCs) are undifferentiated cells responsible for the growth, homeostasis and repair of many tissues. The maintenance and survival of SCs is strongly influenced by several stimuli from the local microenvironment. The majority of signaling molecules interact with SCs at the nanoscale level. Therefore, scaffolds with surface nanostructures have potential applications for SCs and in the field of regenerative medicine. Although some strategies have already reached the field of cell biology, strategies based on modification at nanoscale level are new players in the fields of SCs and tissue regeneration. The introduction of the possibility to perform such modifications to these fields is probably due to increasing improvements in nanomaterials for biomedical applications, as well as new insights into SC biology. The aim of the present review is to exhibit the most recent applications of nanostructured materials that drive the commitment of adult SCs for potential clinical applications.

KEYWORDS: adipogenesis ▪ adipose-derived stem cells ▪ adult stem cells ▪ differentiation ▪ mesenchymal stem cell ▪ nanoparticles ▪ nanotechnology ▪ nanotubes ▪ neurogenesis ▪ osteogenesis

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Nanotechnology represents a fascinating new outlook on regenerative medicine that could promote extensive research and lead to the realization of interesting and innovative tools to improve and restore tissue function [1,2]. Worldwide interest in both adult stem cells (SCs) and embryonic SCs in the fields of tissue engineering and regenerative medicine has grown tremendously in recent years [3,4]. SCs have been identified as having the potential capacity to replace cells that are damaged or diseased and to restore vital functions, therefore, making SCs key players in tissue regeneration. Furthermore, the decreased immunogenicity and potential 'immunomodulatory' properties that have been observed in various populations of adult SCs may also facilitate allogenic transplantation, providing advantageous sources for cell-based therapies [3–5]. Recent insights into the multilineage potential and inherited plasticity of progenitor cells have also created opportunities, dictated by an increased need for new cell-based therapies, to enable the regulation of cell growth, differentiation and phenotypic expression through the modulation of SCs [6,7]. A SC is defined as a cell that can continuously produce unaltered daughters and, furthermore, has the ability to generate cells with different and more restricted properties. SCs can divide either symmetrically (allowing the increase of SC number) or asymmetrically. Asymmetric divisions maintain the number of unaltered SCs and are responsible for the generation of cells with different properties. These cells can either multiply (progenitors or transit amplifying cells) or be committed to terminal differentiation. Progenitors and transit amplifying cells have a limited lifespan and, therefore, can only reconstitute a tissue for a short period of time when transplanted. By contrast, SCs are self-renewing and, therefore, can generate any tissue for a lifetime. This is a key property for a successful therapy. The capacity to expand SCs in culture is an indispensable step for regenerative medicine, and a considerable effort has been made to evaluate the consequences of the cultivation on SC behavior [8]. Classically, the control of SC fate either *in vivo* or *in vitro* has been attributed to genetic and molecular mediators (e.g., growth factors or transcription factors). However, increasing evidence has revealed that a different array of additional environmental factors, which belong to the 'nanodimension,' may contribute to the overall control of the activity of SCs; this may herald the advent of new perspectives in biomedical research [9–11]. The integration of nanotechnological biomimetic materials and translational medicine could provide the chance to produce surfaces (e.g., bone, vasculature, heart tissue, cartilage, bladder tissue and brain tissue), structures and systems with nanoscale features that can mimic the natural cellular environment and quickly promote cellular events, such as

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adhesion, mobility and differentiation [12–14]. Further improvements, stemming from the optimization of nanomaterials by the continuous introduction of nanotechnology platforms, will boost the development of innovative cell-based therapeutics. The aim of this review is to evaluate the current strategies and the emerging applications of nanotechnology and its goals in SCs and regenerative medicine research.

Commitment by nanopatterned substrates

Extracellular environment & cell adhesion

The selection of a good-quality scaffold is an essential strategy for tissue engineering. Ideally, the scaffold should be a functional and structural platform able to mimic the native extracellular matrix (ECM) and support the morphogenesis of multiple tissues; on this basis, 3D nanofibrous scaffolds appear to be the most capable of influencing cellular behavior. Nanotechnology, as defined by the US National Nanotechnology Initiative (US NNI 2010), involves "structures with dimensions between approximately 1 and 100 nanometers." We can assume that all studies focused on the interactions between cells and nanoscale materials are nanotechnology, and their further *in vivo* applications can be consequently called nanomedicine [15].

The interactions between cells and ECM components strongly influence cell growth, guide cell mobility and differentiation, and affect general cellular behavior; as a consequence, cell–substratum interaction maintains a central role in many biological phenomena (Figure 1). Knowledge of these interactions is crucial to the understanding of many fundamental biological questions and to the design of medical devices. The complex structures of soluble and immobilized biomacromolecules in the ECM including collagens, glycoproteins and glycosaminoglycans range from several to a hundreds of nanometers in size. For example, collagens have a hierarchical structure composed of fibrils ranging from 10 to 300 nm in size to collagen fibers that can be up to several microns in size. At the other end of the spectrum, the basement membrane includes a complex mixture of pores, ridges and fibers that are nanometers in size [16]. Cell adhesion to the ECM is mediated by important transmembrane proteins called integrins. Cell spreading determines the clustering of integrins into focal adhesion complexes and the activation of intracellular signaling cascades [17]. In turn, focal adhesion complexes recruit numerous proteins such as FAK, vinculin, paxillin, talin and p130Cas among others [18]. The process of this concentration and the topography of cell-adhesion sites in the ECM are critical to integrin clustering and activation (Figures 2 & 3). For this reason, parameters including size, lateral spacing, surface chemistry and the geometry of nanofeatures are important variables that guide SC behavior [19–21].

Moreover, Seo *et al.* demonstrated after culturing bone marrow murine mesenchymal SCs (MSCs) on both flat and micro- or nano-scale patterned topographies that the formation and maturation of FAs is highly dependent on the topography of the substrate, while the shape, morphology and spreading of cells on different substrates were not significantly different [22].

The preconditioning of cellular function at nanostructured interfaces may result from direct influence on cellular responses or an altered ECM layer deposited on the surface and a consequent change in the availability of binding sites [23,24].

With the inherent plasticity and multilineage potential provided by SCs comes an increased need for regulating cell differentiation, growth and phenotypic expression. Classically, the control of SC fate, either *in vivo* or *in vitro*, has been attributed principally to genetic and molecular mediators (e.g., growth factors and transcription factors). However, increasing evidence has revealed that a diverse array of additional environmental factors contribute to the overall control of SC activity. In particular, fascinating data continues to mount on the important influence of the 'solid-state' environment, in other words, the influence ECM has on SC fate, with particular emphasis on the interactions of ECM ligands with cell surface receptors [25]. However, it is now clear that ECM-based control of the cell may also occur through multiple physical mechanisms, such as ECM geometry at the micro- and nano-scale, and ECM elasticity or mechanical signals transmitted from the ECM to the cells. In addition to the influence that an artificial ECM may have on cell shape, there is significant evidence that other physical properties of the ECM may also contribute to SC fate or lineage commitment [26–28]. Cells that attach to a substrate have been shown to exert contractile forces, resulting in tensile stresses in the cytoskeleton [29]. Interestingly, the relationship between these forces and the mechanical stiffness, or elasticity, of

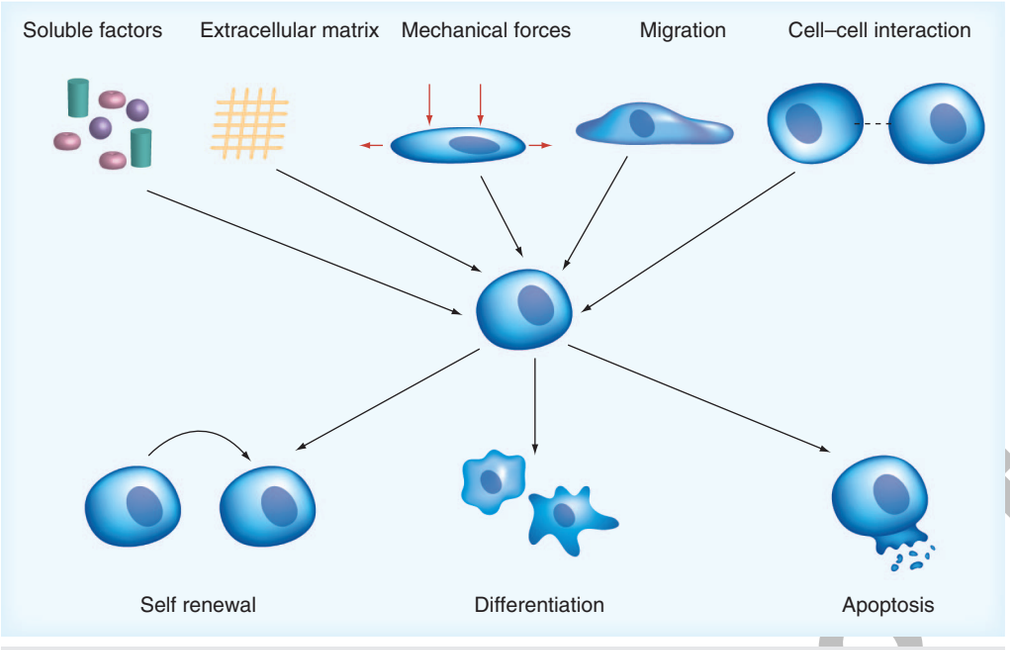

Figure 1. Interactions between stem cells and the extracellular environment. Soluble factors (e.g., growth factors and hormones) and extracellular matrix components (e.g., collagen, fibronectin and laminin) mechanically stimulate and influence cell growth, apoptosis and differentiation, and affect the general behavior of cells.

the ECM can have a major influence on cell behaviors, such as migration [30,31], apoptosis [32] and proliferation [33]. The mechanisms by which nanotopographic cues influence SC proliferation and differentiation are not well studied, but appear to involve changes in cytoskeletal organization and structure, potentially in response to the geometry and size of the underlying features of the ECM [34]. That is, changes in the feature size of the substrate may influence the clustering of integrins and other cell adhesion molecules, thus altering the number and distribution of FAs. For example, previous studies have demonstrated that the precise spacing between nanoscale adhesive islands on a substrate can modulate the clustering of the associated integrins, and the formation of focal adhesion and actin stress fibers, and can, therefore, control the adhesion and spreading of cells. These studies and others clearly demonstrate that physical interactions with the ECM significantly influence SC behavior, and can interact with chemical (i.e., composition), molecular (i.e., soluble mediators) or genetic (cell-type) factors to regulate cell fate. Importantly, the ability to engineer artificial ECMs that, through physical as well as molecular interactions, enable directed control of SC behavior may further extend our capabilities in engineering functional tissue substitutes [34–38].

Nanofibers

Nanotechnology is also capable of enhancing the reparative potential of tissue without direct manipulation of SCs [39–43]. Typically, SCs cultured on nanofiber scaffolds differ in morphology, viability and migration behavior compared with cultures grown on conventional substrates. For example, human MSCs (hMSCs) grown on 500–1000-nm nanofibers are flatter and demonstrate significantly higher cell viability and lower cell mobility than control cells grown on tissue culture polystyrene [44]. Nanofiber scaffolds offer great potential for SC applications, a fact that is also supported by recent studies demonstrating the responses of mammalian cells to nanoscale surface stimuli [45,46]. An application of such approaches, in other words, on the studies of haircells. The biological haircell is a modular building block of a rich variety of biological sensors. Liu *et al.* using micro- and nano-fabrication technology, developed an equivalent artificial haircell sensor, imitating the structure and transfer function of the biological haircell. The artificial haircells can be made of hybrid semiconductor, metal and polymers [47]. The paper discusses a number of strategies, using representative material systems, for building artificial haircell sensors and briefly outlines the fabrication method and performance. The motivation for imitating the biological haircell is also discussed to provide

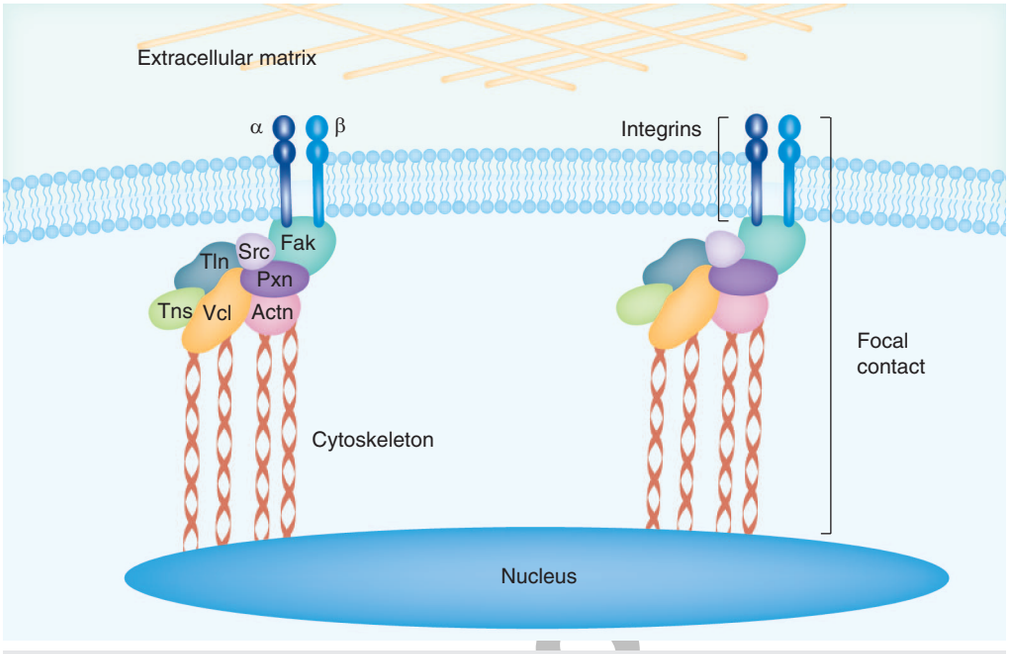

Figure 2. Integrin signaling. Following the integration of integrins and extracellular matrix components, the intracellular signaling pathways triggered by integrins are directed to several functions: for example, organization of the actin cytoskeleton; regulation of the fate of the cell mechanosensing; adhesion; migration; and tissue invasion. Integrins are catalytically inactive and translate positional cues into biochemical signals by direct and/or functional association with intracellular adaptors, cytosolic tyrosine kinases or growth factors and cytokine receptors. The attachment of the cell takes place through the formation of cell-adhesion complexes, which consist of integrins and many cytoplasmic proteins, such as TLN, VCL, PXN and ACTN. These act by directing kinases, such as FAK and Src kinase family members to phosphorylate substrates such as p130CAS. These adhesion complexes attach to the actin cytoskeleton. The integrins thereforeserve to link two networks across the plasma membrane: the extracellular matrix and the intracellular actin filamentous system.

a background for this work. In this context, Schroeder *et al.* implemented artificial cilia on giant-magneto-resistive multilayer sensors for a biomimetic sensing approach by means the use of polymeric nanowires of polypyrrole as [48]. The arrays were tagged with a magnetic material, the stray field of which changes relative to the underlying sensor as a consequence of mechanical stimuli that are delivered by a piezoactuator. The principle resembles balance sensing in mammals. Measurements of the sensor output voltage suggest a proof of concept at frequencies of approximately 190 kHz and a tag thickness of approximately 300 nm. Characterization was performed by scanning electron microscopy and magnetic force microscopy, and micromagnetic and finite-element simulations were conducted to assess basic sensing aspects.

With regard tonanofibers, there are currently three manufacturing approaches to fabricating nanofibrous scaffolds: electrospinning [58], phase separation [59] and self-assembly [60]. Structures created by each of these approaches are quite different and, therefore, have their own unique advantages. For example, the phase separation technique allows for control of pore architectures [61]. The most common method for fabricating nanofibers is electrospinning. In this process, nanofibers are produced from polymer solutions via the application of a high electric field and the presentation of a grounded region some distance away [62–65].

Nanopatterned surfaces

A crucial element of tissue engineering is to create a favorable extracellular microenvironment, mainly the ECM, to guide cell differentiation and tissue regeneration [50,51].

In addition to topography, the extracellular microenvironment may also provide signaling cues to the anchorage-dependent cells via a feedback of local matrix stiffness [52]. Matrix elasticity can direct hMSCs to differentiate into specific lineages: a soft matrix induces a neurogenic phenotype, while increasingly stiffer matrices induce myogenic and osteogenic phenotypes accordingly [53]. Taken together, the observations of nanotopography-induced and stiffness-directed differentiation suggest that physical interactions between the

cells and the extracellular environment, either in the form of topography or stiffness, or a combination of these can, therefore, modulate cell function and SC differentiation [53]. The application of nanotechnology to cell surfaces involves a number of different arrangements. Most of all, a great variety of techniques are used to produce nanotopographies on biomaterial surfaces. Methods leading to ordered topographies with regular, controlled patterns and methods leading to unordered topographies with random orientations and organization have both been developed. These in turn can be divided into chemical and physical processes.

Chemical modifications involve chemical reactions where parameters such as temperature, duration and composition of solutes can be adjusted to improve upon the number and depth of nanopits produced. Nanosurfaces are obtained through anodic oxidation or a combination of acids (and bases) and oxidants. Physical methods generate porous layers through collisions with microscopic particles (blasting) or by coating with small particles (plasma spray). In some cases, a combination of chemical and physical methods has also been used [54–56]. For example, the combination of particle blasting and HF acid treatment has been used to create a commercial endosseous Ti implant with microrough surfaces and superimposed uncharacterized features ranging in size from 50 to 200 nm.

Nanoscale features are able to orient cells, control cell spreading by limiting the surface area available for cell attachment, and modulate FA patterns and resultant stress fiber organization. For example, Teixeira *et al.* demonstrated that epithelial cell morphology was dictated by precisely controlled nanogroove and nanoridge patterns [57]. The nanotopographical surface was created with 400–4000-nm wide pitches and 150–600-nm deep grooves, and coated with silicon oxide to eliminate any effect from surface chemistry. The authors found that epithelial cells aligned and elongated along the nanoridges (Figure 1A), while cells on smooth surface substrates remained predominantly round. Furthermore, a greater percentage of aligned cells were observed in deeper grooves. In addition, cells extended lamellipodia and filopodia primarily along ridges and down to groove floors (Figure 1B). Finally, the size of the FAs was dependent on the ridge width, with wider ridges allowing for larger FAs to form. Together, these data suggest that nanoscale surface features can have profound effects on cell morphology.

While 2D surfaces are valuable tools for studying basic cellular response to nanotopography, translation of these findings towards clinical application will require 3D structures. In this review, the authors describe three such structures: nanotubes, nanoparticles and nanofibers, and their effect on biological regulation.

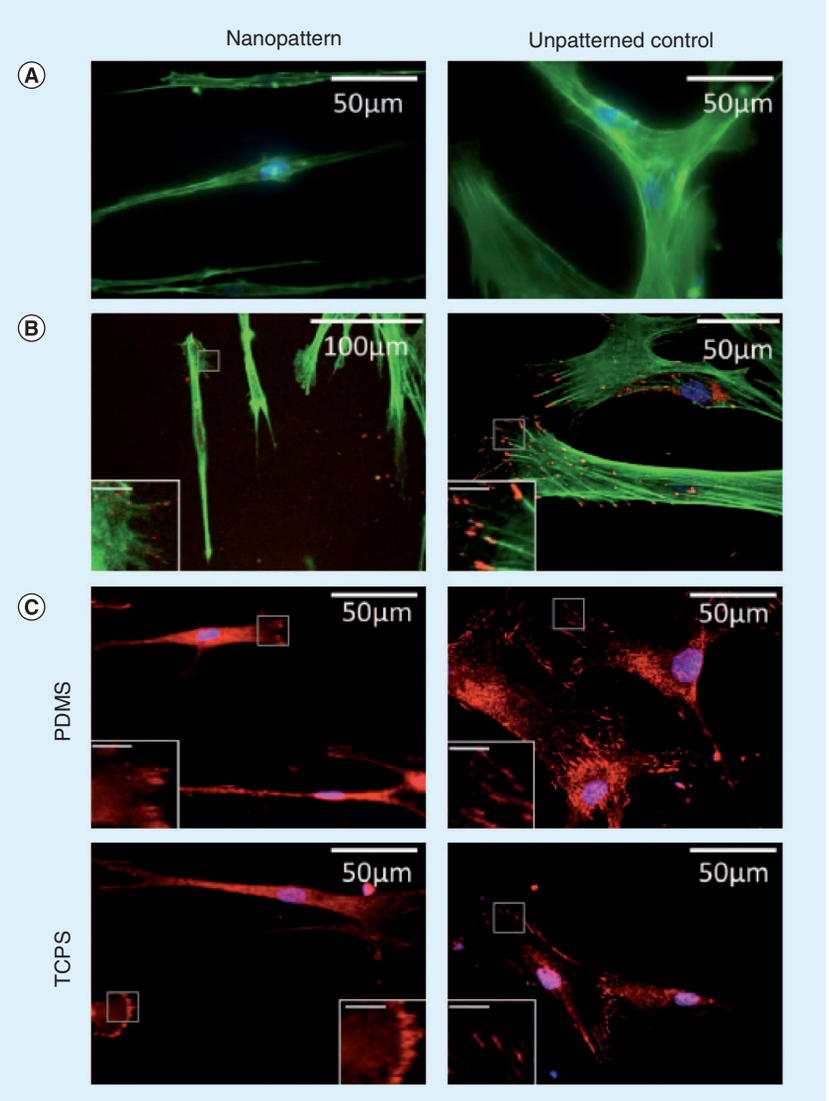

Figure 3. TITLE. (A) F-actin cytoskeleton visualized by Oregon®-green-labeled phallodin in human mesenchymal stem cells on PDMS with 350-nm gratings or unpatterned substrates. **(B)** Distribution of focal adhesions visualized by immunofluorescence staining of tyrosine-397 phosphorylated FAK (red) and F-actin (green). **(C)** Distribution of focal adhesions visualized by immunofluorescence staining of vinculin (red). Images of **(A)** are taken with fluorescence microscopy; images of **(B & C)** are taken with confocal microscopy. Boxes indicate the area of the magnified views shown in the insert figures; scale bar is 10 µm in the insert figures.
PDMS: Polydimethylsiloxane; TCPS: Tissue culture polystyrene.
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Commitment based on SC surface interaction

Osteogenic commitment

There is currently an unmet need for the supply of autologous, patient-specific SCs for regenerative therapies in the clinic. MSC differentiation can be driven by the material–cell interface suggesting a unique strategy to manipulate SCs in the absence of complex soluble chemistries or cellular reprogramming. However, so far the derivation and identification of surfaces that allow retention of multipotency of this key regenerative cell type have remained elusive. Adult SCs spontaneously differentiate in culture, resulting in a rapid diminution of the multipotent cell population and their regenerative capacity. Bone fractures, healing critically sized segmental defects and regeneration of articular cartilage in degenerative joint diseases owing to various traumas or natural ageing represent typical aspects of tissue malfunction. Surgical treatment frequently requires the implantation of a temporary or permanent prosthesis, which represents a challenge for orthopedic surgeons, especially in the case of large bone defects [66]. An understanding of how mechanics influences tissue differentiation during repair and regeneration crucially requires spatial and temporal knowledge of the local mechanical environment; it is now evident that mechanical boundary conditions influence the regeneration of bone. Mimicking the structures of natural ECM may lead to the successful regeneration of damaged tissue [67]. As mentioned, cell adhesion to the ECM strongly influences cellular events; a recapitulation of bone topography enriched with nanoadhesion sites might guide SC fates and prove useful for regenerative treatments for bone healing [68]. The literature offers some examples. Chitosan, a natural polymer (a linear polysaccharide composed of randomly distributed β-(1–4)-linked D-glucosamine (deacetylated unit) and *N*-acetyl-D-glucosamine (acetylated unit) obtained from chitin, has played a major role in bone tissue engineering (BTE) over the last two decades. Chitosan's interesting characteristics that make it suitable for tissue reconstruction are its minimal foreign body reaction, its intrinsic antibacterial nature and its ability to be molded into various geometries and forms, including porous structures suitable for in in cell growth and osteoconduction [69]. Owing to its favorable gelling properties, chitosan can even deliver morphogenic factors and pharmaceutical agents in a controlled fashion, presenting an ideal method for gene delivery strategies (owing to its additional capacity to complex with DNA molecules) [70]. Composites of chitosan, including hydroxyapatite (HA), are also very popular because of their biodegradability and biocompatibility with nature. Recently, a material composed of grafted chitosan polymers and carbon nanotubes was incorporated into composites for bone regeneration to increase the mechanical strength of these composites [70].

A key tenet of BTE is the development of scaffold materials that can stimulate SC differentiation in the absence of chemical treatment to become osteoblasts without compromising material properties. At present, conventional implant materials fail owing to encapsulation by soft tissue, rather than direct bone bonding. Indeed Dalby *et al.* in 2007 demonstrated that the use of nanoscale disorder to stimulate hMSCs to produce bone mineral *in vitro*, in the absence of osteogenic supplements [143]. This approach has similar efficiency to that of cells cultured with osteogenic media. In addition, the current studies demonstrate that topographically treated MSCs have a distinct differentiation profile compared with those treated with osteogenic media, which has implications for cell therapies. A few years after the same group performed a more detailed analysis that identified a nanostructured surface that retains stem-cell phenotype and maintains SC growth over 8 weeks. Furthermore, the study implicates a role for small RNAs in repressing key cell signaling and metabolomic pathways, demonstrating the potential of surfaces as noninvasive tools with which to address the SC niche [142].

The use of HA nanocrystals has been extensively studied by Bigi *et al.* [71]. This group coated a biocompatible, nanostructured titanium alloy, $Ti_{13}Nb_{13}Zr$, with a thin layer of HA nanocrystals and investigated the response of cultured human bone-marrow-derived mesenchymal cells to this material. Supersaturated solutions with ionic compositions more or less similar to that of human plasma have been widely employed with the aim of mimicking the mineralization process of bone. With this view, a slightly supersaturated CaP solution was used for coating, resulting in a fast deposition of nanocrystalline HA that reached thicknesses of 1–2 µm after 3 h of soaking. The same coating, deposited on Ti_6Al_4V, was examined for comparison after a culture period of up to 35 days. Although the presence of a HA coating slightly reduces cell proliferation, it also strongly induces the differentiation of

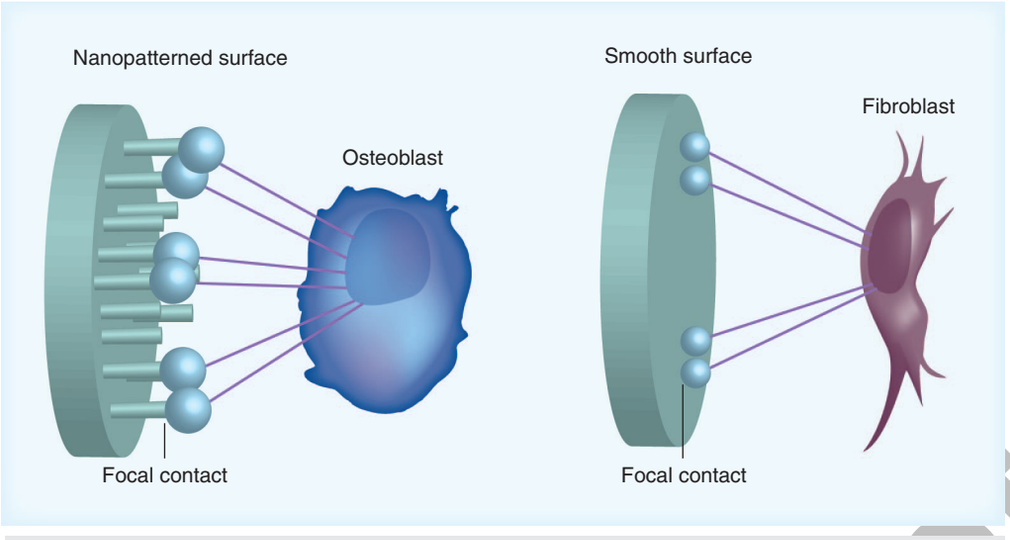

Figure 4. The topographies of nanoadhesion sites in tissue engineering scaffolds are able to guide stem cell fate. The original study compared different diameter TiO_2 nanotubes, ranging from 15 to 100 nm. On the 100 nm nanotube, diameter adhesion is significantly reduced and cell death by anoikis (lack of adhesion) was observed.
Reproduced from [72].

MSCs toward a phenotypic osteoblastic lineage, in agreement with the increased expression of osteopontin, osteonectin and collagen type I, as evaluated by reverse transcription-PCR. Type I collagen expression has been demonstrated to be higher in $Ti_{13}Nb_{13}Zr$ MSC culture as compared with Ti_6Al_4V, representing a more efficient ECM deposition. In terms of topography, Park *et al.* demonstrated that the differentiation of rat MSCs (RMSCs) into an osteogenic lineage occurs most frequently on vertically aligned TiO_2 nanotubes with diameters of 15 nm; this size approximates the predicted lateral spacing of integrin receptors in contact with the ECM (FIGURE 4). The phosphorylation of FAK and ERK, which is a target of the FAK signaling pathway, confirmed these data, while significantly poorer results were obtained with increased nanotube diameters (100 nm) [72]. Compared with flat substrates, structures with nanoscale features and different chemistries (e.g., silica, alumina and poly[methyl methacrylate]) have been reported to enhance adhesion, growth and osteogenic differentiation of hMSCs and marrow stromal cells, and could have potential applications as osteogenic coatings for orthopedic implants [72–75].

Electrospun nanofiber-based synthetic and natural polymer scaffolds are being explored as scaffolds similar to natural ECM for tissue engineering applications. Nanostructured materials for bone scaffolds are smaller in size, in the 1–100 nm range, and have specific properties and functions related to the sizes of natural materials (e.g., HA). Bone contains considerable amounts of minerals and proteins: HA $Ca_{10}(PO_4)_6(OH)_2$ is one of the most stable forms of calcium phosphate, and it occurs in bones as a major component (60–65%), along with other materials, including collagen, chondroitin sulfate, keratin sulfate and lipids.

The development of nanofibers with nano-HA (n-HA) has enhanced the scope of scaffold fabrication that mimics the architecture of natural bone tissue. Nanofibrous substrates supporting the adhesion, proliferation and differentiation of cells and the incorporation of HA induce cells to form organic mineralized and non-mineralized matrices [76]. Biocomposite polymeric nanofibers containing n-HA fabricated by electrospinning could be promising scaffolds for bone reconstruction: nanofibrous scaffolds of poly-L-lactide (PLLA; 860 ± 110 nm), PLLA/HA (845 ± 140 nm) and PLLA/collagen/HA (310 ± 125 nm) were proposed by Prabhakaran *et al.* [77]. For this purpose. Prabhakaran *et al.* evaluated the morphology, chemical and mechanical characteristics of the nanofibers using scanning electron microscopy, Fourier transform infrared spectroscopy and tensile testing, respectively [77]. The synergistic effect of the presence of an ECM protein, collagen and HA in PLLA/collagen/HA nanofibers allows these nanofibers to act as temporary templates for bone regeneration that actively stimulate the attachment of osteoprogenitor cells. This approach, providing cell recognition sites together with apatite, significantly improves

cell proliferation and osteoconduction, as well as optimizing the mineralization process and bone formation [77].

A combination of several biomaterials has also been used also by Ravichandran *et al.* who fabricated nanofibers based on PLLA/poly-benzyl-L-glutamate (PBLG)/collagen by electrospinning and deposited n-HA by the calcium phosphate dipping method for BTE [78]. As source of SCs has been put on adipose-derived SCs (ADSC) ADSCs were cultured on these scaffolds and were induced to undergo osteogenic differentiation in the presence of PBLG/n-HA for BTE. The cell-biomaterial interactions were analyzed using cell proliferation, scanning electron microscopy and 5-chloromethylfluorescein diacetate dye-extraction techniques. Osteogenic differentiation of ADSC was confirmed using alkaline phosphatase activity, mineralization and dual immunofluorescent staining using both an ADSC marker protein and osteocalcin, which is a bone-specific protein. The utmost significance of this study is the bioactive PBLG/n-HA biomolecule introduced on the polymeric nanofibers to regulate and improve specific biological functions, such as adhesion, proliferation and differentiation of ADSC into osteogenic lineage. The observed results proved that the PLLA/PBLG/collagen/n-HA scaffolds promoted greater osteogenic differentiation of ADSC as evident from the enzyme activity and mineralization profiles for BTE. The authors conclude that the importance of this study is the application of bioactive macromolecules PBLG/n-HA that have been introduced on the surface of polymeric nanofibers, to regulate and improve specific biological functions, such as adhesion, proliferation and differentiation. Smart materials such as PLLA/PBLG/collagen/n-HA scaffolds that can also elicit therapeutic effects by incorporating biosignaling molecules within the nanofibers, such as proteins and genes, hold great promise as scaffolds for BTE with drug delivery application. Owing to their abundance and accessibility, ADSC cells may prove to be desirable cell therapeutics for bone repair and regeneration.

An innovative tool using magnetic nanoparticles was recently developed: Kanczker *et al.* have investigated remote magnetic field activation of magnetic nanoparticle-tagged mechanosensitive receptors on the cell membrane of human bone-marrow stromal cells for use in osteoprogenitor cell-delivery systems and for the activation of differentiation, *in vitro* and *in vivo*, toward an osteochondral lineage [79]. Other strategies take advantage of the ability of bioactive glass to bond directly with bone. Clinical applications of these materials are likely to use their particulate form. Microstructured apatite-forming bioactive glass particle scaffolds with nanoscale or non-nanoscale surface features have been investigated for bioactivity and cellular responses; microstructures and micronanoscale surface morphology have been controlled by adding a hydroxycarboxylic acid (citric acid) in the sol-gel process [80]. Results have demonstrated that the addition of citric acid induces the formation of nanoscale surface structures and increases the specific surface areas, pore volumes and pore sizes of the particles. In particular, the use of citric acid with low-concentration-derived sol-gel bioactive glass resulted in enhanced apatite formation in simulated body fluids, as compared with normal bioactive glass [81,82].

Combinations of nanostructures with growth factors are alternative strategies adopted by Schofer *et al.* who evaluated the influence of 3D PLLA nanofiber scaffolds on bone formation *in vivo* and analyzed whether incorporated BMP-2 could enhance their efficacy [83]. PLLA nanofiber scaffolds were demonstrated to facilitate cell immigration and, therefore, to achieve high cell densities. However, they lacked adequate osteogenic stimuli to allow further differentiation of those cells. The incorporation of recombinant human BMP-2 into PLLA nanofibers could overcome this problem. Therefore, PLLA/BMP-2 implants were able to close critical-size calvarial defects within 8 weeks. Increased expression of osteocalcin, BMP-2 and Smad5 suggests a subsequent activation of the osteoblast lineage. Therefore, PLLA/BMP-2 nanofiber scaffolds combine a suitable matrix for cell migration with an osteoinductive stimulus.

In the field of osteogenic commitment, dental pulp represents another highly specialized mesenchymal tissue that has a limited regeneration capacity due to anatomical arrangement and the post-mitotic nature of odontoblastic cells. Dental caries remain one of the most prevalent infectious diseases in the world, with a demonstratable pharmaceutical impact. Available treatment methods rely on the replacement of decayed soft and mineralized tissue with inert biomaterials. Regenerative endodontics promises innovative results using SCs associated with scaffolds and responsive molecules. Wang *et al.* investigated the odontogenic differentiation of human

dental-pulp SCs on nanofibrous PLLA scaffolds *in vitro* and *in vivo* [84]. The combination of a phase-separation technique and a porogen-leaching method recapitulated the architecture of collagen type I fibers in the design of a highly porous nanofibrous PLLA scaffold [84,85]. Positive immunohistochemical staining for dentin sialoprotein, together with other assays confirmed the more effective differentiation of dental-pulp SCs into odontoblast-like cells with the capacity to regenerate dental pulp and dentin.

Chondrogenic commitment

One of the most important application fields of cartilage tissue engineering is for osteoarthritis treatments. Osteoarthritis is the most prevalent musculoskeletal disease in humans, causing pain, loss of joint motility and function, and severely reducing the standard of living of patients. Cartilage tissue engineering attempts to repair the damaged tissue of individuals suffering from osteoarthritis by providing mechanical support to the joint as new tissue regenerates. The combination of SC biology with nanotechnology has been used by Fong *et al.* who used human umbilical cord Wharton's jelly on poly(ε-caprolactone) (PCL)/collagen nanoscaffolds in the presence of a two-stage sequential complex/chondrogenic medium for 21 days [86]. In separate experiments the authors demonstrated that the 16 ng/ml of bFGF present in the complex medium may have contributed to driving chondrogenesis. Chondrogenic commitment of SCs could be driven by nanotubes as reported by Erisken *et al.* who obtained chondrogenic differentiation of human adipose-derived stromal cells using a the combination of twin-screw extrusion and electrospinning generated a nanofibrous scaffold suitable for inducing SC commitment [87,88]. The results of the implementation of this scaffold were selective differentiation of human adipose-derived stromal cells toward a chondrogenic lineage.

Adipogenic commitment

Adipose tissue pathologies and defects have always represented a reconstructive challenge for plastic surgeons. Contour defects resulting from resections of tumors, trauma and congenital abnormalities not only affect patients cosmetically but may also impair function, making adipose tissue restoration a clinical need [89,90].

The emerging field of adipose tissue engineering aims to develop biological substitutes that promote regeneration and restore function, through the application of the principles and methods of engineering and the life sciences [91]. The development of adipose tissue-engineering strategies requires investigation of all key aspects of the tissue engineering process, including the selection of a cell source, scaffold biomaterial and microenvironment to provide the appropriate cues and signals for cell growth and adipose tissue formation [92]. For many years, bone marrow-derived SCs (BMSCs) were the primary source of SCs for tissue engineering applications [93]. Recent studies have demonstrated that subcutaneous adipose tissue provides a clear advantage over other SC sources owing to the ease with which adipose tissue can be accessed (under local anesthesia and with a minimum of patient discomfort), as well as the ease of isolating SCs from the harvested tissue [94]. Moreover, the SC frequency is significantly higher in adipose tissue than in bone marrow and the maintenance proliferative ability in culture seems to be superior in ADSCs as compared with BMSCs [95].

Adipogenic differentiation of SCs can also be efficiently induced by physical factors and modulation of ECM nanostructures. The literature offers several different methods to induce such differentiation through the use of high-quality nanoparticles of various chemical compositions. Miyagawa *et al.* reported the highly efficient *in vitro* differentiation of human bone marrow-derived mesenchymal stem/progenitor cells using a novel nanotechnology-based culture plate, called the NanoCulture® Plate (Infinite Bio, Inc., CA, USA) [96]. The NanoCulture Plate is composed of uneven microfabricated elements (with diameters of ~2–3 μm) arranged in a honeycomb pattern on the surface. At first, human mesenchymal stem/progenitor cells cultured in 3D culture using the NanoCulture Plate system rapidly form adhesive spheroids. These spheroidal clusters demonstrate enhanced adipogenic differentiation characterized by a more rapid accumulation of triglycerides than in 2D culture.

An alternative strategy to achieve commitment to an adipogenic lineate is laser-assisted bioprinting, which permits the production of computer-generated 3D tissue grafts. In a recent study, laser-assisted bioprinted human ADSCs embedded in a hydrogel environment in a free-scalable 3D grid pattern [97]. The authors demonstrated that the biological behavior of the SCs was affected by the nanotechnological procedure; in fact, after 10 days of enhancing the

adipogenic lineage, quantitative assessments of adipogenic markers demonstrated that the 3D grafts resembled cell lineages of natural adipose tissue.

Recent advances have resulted in an increasing interest in the development of bioconjugated carriers for the delivery of bioactive molecules to SCs. The novel properties of these nanoparticles are intended to favor and modulate SC differentiation. Liu *et al.* used this method to promote the adipogenic differentiation of RMSCs *in vitro*, reporting biocompatible silica nanoparticle (SiNP)–insulin conjugates [98]. After the biocompatibility of SiNPs with RMSCs was tested, a cell viability assay was performed to screen the SiNP concentration for its cytotoxicity toward RMSCs. After the optimized SiNP concentration with minor cytotoxicity on RMSCs and the resultant absence of effects on the RMSC phenotype, SiNP–insulin conjugates were used for RMSC adipogenic differentiation, resulting in the prompt differentiation of RMSCs into adipocytes when cultured in the presence of insulin-conjugated SiNPs.

Neuronal commitment

Biotechnology is being increasingly used to recapitulate specific aspects of brain niches able to promote regeneration and repair damaged neuronal pathways with SC therapies. Many of these approaches are gaining momentum because nanotechnology allows for greater control over material–cell interactions. This, in turn, allows for the induction of specific developmental processes and cellular responses including differentiation, migration and outgrowth.

Many studies have examined the importance of exogenous soluble factors in promoting cell fate specification. Soleimani *et al.* experimented with a 3D nanofibrous scaffold fabricated from aligned PLLA, studying its ability to support neurogenic and hinder dopaminergic differentiation of conjunctiva MSCs *in vitro* [99]. Neurogenic lineages were induced by culturing cells in specific differentiation media. The tested nanofibrous PLLA scaffold has been demonstrated to be a potential cell carrier in neural tissue engineering applications with the partial inhibition of the dopaminergic differentiation of conjunctiva MSCs [99].

Alternative strategies have been proposed by Cho *et al.* who developed a NGF-conjugated aligned nanofibrous mesh-based method for neuronal differentiation of MSCs [100]. Amine-terminated poly(ethylene glycol) was conjugated to (PCL) to produce amine-functionalized copolymers, which were then electrospun in a rotating drum to obtain aligned nanofibrous meshes. NGF was chemically linked to the amine groups of the nanofibrous meshes in the aqueous phase. *In vitro* release profiles of the NGF were then investigated; the growth factor physically adsorbed on the nanofibrous meshes and demonstrated an initial burst release in MSCs cultured for 5 days [100].

Regarding CNS tissue repair strategies, the achievement depends on the restoration of appropriate neuronal connectivity. In this light modification of 3D electrospun PCL nanofiber scaffolds by fiber alignment and aminolyzation is superior to classical 2D culture-ware in promoting the *in vitro* proliferation and differentiation of cortical cells. Horne *et al.* demonstrated that tethering the BDNF onto modified nanofibers is superior to culturing in the presence of soluble BDNF [101]. Functional immobilization of BDNF onto polymer nanofibers enhances neural SC proliferation and directs cell fate toward neuronal and oligodendrocyte specifications, essential for neural tissue repair. These findings indicate that modified PCL nanofibrous 3D scaffolds are capable of supporting neural SCs and their derivatives and may present a new avenue for encouraging neural repair in the future. Carbon nanotubes have electrical, mechanical and chemical properties that make them one of the most promising materials for applications in neuroscience. Single- and multi-walled carbon nanotubes have been increasingly used as scaffolds for neuronal growth and, more recently, for neural SC growth and differentiation. They are also used in interfaces with neurons, where they can detect neuronal electrical activity and also deliver electrical stimulation to these cells. Therefore, in the near future, they could be used in brain–machine interfaces [102].

Hepatocyte-like SCs

It is well known that tissue engineering proves to be a temporary treatment for patients suffering from hepatic failure. For successful tissue regeneration, the cells constituting the tissues to be regenerated are necessary. Considering the proliferation activity and differentiation potential of cells, SCs are practically promising. hBMSCs have great potential for liver tissue engineering because autologous BMSCs can be harvested, expanded extensively *ex vivo* and differentiated into a hepatic phenotype for transplantation back into the patient [102]. Differentiation of hBMSCs into hepatocyte-like

cells (HLCs) in standard monolayer or 2D cultures is now well established, however, the challenge remains to develop robust protocols to generate functional hepatocytes from hBMSCs suitable for transplantation [103]. In recent years, with respect to nanofibers for tissue engineering purposes, a wide variety of nanofibrous scaffolds have been produced [104,105]. The experimental results have demonstrated that, although synthetic biodegradable PCL supports cell growth, to increase proliferation and encourage cell ingrowth for better integration between cells and the scaffold, the biologically inert PCL nanofibers need effective hybridization with bioactive molecules. It has been reported that electrospinning of PCL with collagen gives encouraging results in improving the cell–scaffold interactions [105].

Moreover, polyethersulfone has many fascinating properties including favorable mechanical strength, thermal and chemical resistance, and excellent biocompatibility. Therefore, the polymer blend of PCL/collagen/polyethersulfone can overcome the shortcomings of natural and synthetic polymers, resulting in a new biomaterial with good biocompatibility and improved mechanical, physical and chemical properties [106].

In a study of Kazemnejad *et al.*, the potential of hBMSCs to differentiate into functional hepatocytes within designed PCL/collagen/polyethersulfone nanofibers has been investigated [107]. Cytochemical, biochemical and molecular features of HLCs differentiated from hBMSCs on the scaffold were used to show the role of nanofibrous structure to support differentiation. Using similar PCL nanofiber scaffolds, Hashemi *et al.* tested the *in vitro* differentiation of human cord blood-derived unrestricted somatic SCs into HLCs [108]. In their study, the authors tested the ability of PCL nanofiber scaffold to support and maintain hepatic differentiation of *in vitro*. Unrestricted somatic SCs and self-renewing pluripotent cells, were isolated from human cord blood. The electrospun PCL nanofiber porous scaffold was constructed of uniform, randomly oriented nanofibers. Unrestricted somatic SCs were seeded onto PCL nanofiber scaffolds, and were induced to differentiate into hepatogenic lineages by culturing with differentiation factors for 6 weeks.

Ultra-web® (Donaldson, Leuven, Belgium) nanofibers (nano$^+$ and nano$^-$) have indeed been used by Piryaei *et al.* to better differentiate and maintain the function and engraftment of differentiating MSCs both *in vitro* and *in vivo* [109]. MSCs, early and late HLCs in both nano- and nano$^+$ culture conditions that were transplanted by an intravenous route caused a decrease in liver fibrosis when engrafted in the recipient liver and were able to differentiate into functional hepatocytes (ALB$^+$), with the exception of late HLCs in the nano$^-$ group. Late HLCs transplanted in the nano$^+$ group were more effective in rescuing liver failure, enhancing serum ALB, homing transplanted cells and undergoing functional engraftment than the other groups. These results demonstrated that topographic properties of nanofibers enhance differentiation of HLCs from MSCs and maintain their function in long-term culture, which has implications for cell therapies. The authors in the end concluded that nanofibers have the capability not only to drive SC commitment into hepatocyte-like SCs but also to maintain stable differentiation, thereby achieving transplantable hepatocytes.

Other commitments

Shi *et al.* investigated the effects of substrate nanotopography on the endothelial differentiation of ADSCs [110]. The authors compared two nanograting substrates with periods (ridge and groove length) of approximately 250 and 500 nm, respectively, with a flat surface. Endothelial differentiation of ADSCs on both flat and nanograting substrates can be induced with VEGF. PCR analysis demonstrated significantly enhanced upregulation of vWF, PECAM-1 and VE-cadherin at the gene level in ADSCs grown on the nanograting substrates. *In vitro* angiogenesis assays on Matrigel™ (BD Biosciences, NJ, USA) showed that nanograting substrates enhanced capillary tube formation.

Another cellular lineage studied is myogenic differentiation. Tian *et al.* used growth factors to stimulate SCs into smooth muscle-like cells [111]. Growth factors alone or combined with either bladder ECM coatings or a dynamic culture system induced BMSCs to express smooth muscle-specific genes and proteins *in vitro*. A nanofibrous 3D PLLA polymer porous scaffold provides an optimal microenvironment for facilitating cell-matrix penetration and retention of myogenic-differentiated BMSCs, thereby promoting tissue remolding with rich capillary formation *in vivo*. Yu *et al.* stressed the important role of focal adhesion in driving SC commitment [112]. The authors postulated that differentiation outcomes can be controlled by modulating FA morphology and distribution.

Gekas *et al.* investigated the behavior of human SCs obtained from amniotic fluid [113]. When cultured *in vitro* in myogenic-specific induction media, these SCs were able to differentiate as expected. However, when transplanted into the skeletal muscle of mice, differentiation into tubular glandular-like tissue occurred.

Commitment through intracellular delivery of small particles

Commitment of SCs as reported above could be induced by either both ECM or soluble factors. The final results is the 'reprogrammations' of the genome of the cells and its reprogramming. The technique appointed to do this is gene therapy.

Gene therapy is a technique for correcting missing, defective or overexpressed genes that are responsible for disease development. Although viral vectors can efficiently transfect cells, their clinical application is limited by the related risks for patients [114–114]. Nonviral delivery systems are a safer approach, are easier to manufacture, are more versatile and are more cost effective. Nevertheless, their transfection efficiency is low compared with that of viral vectors. Many groups have dedicated considerable effort to improving the efficiency of nonviral gene delivery systems, and particular attention has been devoted to investigating complexes composed of DNA and soft materials, such as lipids, polymers, peptides, dendrimers and gemini surfactants. The theoretical approach in designing these nanoparticles considers different components essential for assuring high levels of transfection, biocompatibility and tissue-targeting ability [116].

Several advanced *in vitro* studies have proved the broad potential of cationic solid lipid nanoparticles as synthetic nucleic acid vectors that have been proposed as an alternative to liposomes. Certainly, results regarding their transfection performances [117,118]. Nanovectors already play a very important role in pharmaceutical applications for the delivery of drugs or other biologically active materials. Solid lipid nanoparticles are basically composed of a solid lipid core in nanometer ranges stabilized by a layer of emulsifier; they can be prepared by using lipids with a relatively high melting point (i.e., triglycerides, hard fat types, partial glycerides, steroids and waxes). Among these lipids, glycerides, which are composed of fatty acids, can be employed in injection form since they are already used in parenteral nutrition. The development of innovative DNA-based medicines for incurable disorders (gene therapy) is an important component of pharmaceutical advancement. Gene therapy vectors can be categorized into two groups, biological (viral) and nonbiological (nonviral) systems, and each group has its own advantages and limitations [119]. Synthetic nonviral vectors, although being less efficient in bringing about cellular transfection, enjoy several advantages over biological vehicles, such as immunological inertia and a large degree of flexibility in the design of their properties [120]. Nonviral vectors such as nanoparticle-mediated gene delivery systems have become a hot area of research both in academia and industry. Indeed, there is a large number of publications on the *in vitro* behavior of gene vector such as cationic polymer/nucleic acid complexes (lipoplexes), cationic liposomes or polymeric nanoparticles, and recently several studies on cationic solid lipid nanoparticles have been reported [121].

The induction of SC differentiation by drugs and growth factors has been the objective of many studies aiming to optimize methods for the regeneration of new tissues or the repair of degenerated tissues via transplantation. Park *et al.*, for example, used drugs and growth factors with a high potential for tissue repair embedded in hMSCs; cell differentiation into chondrogenic, osteogenic and adipogenic lineages was subsequently enhanced [122]. This culture condition, enriched with microspheres coated and loaded with drugs and growth factors, demonstrated proliferation and, as expected, induced differentiation of transplanted hMSCs into the desired specific cell types.

In this context, polymeric nanoparticles are promising gene delivery systems because they offer stability and controlled release, have the capacity to encapsulate large amounts of genetic material, allow for codelivery and can readily be surface modified to enhance stability, transport properties, targeting or uptake. Polymers that are biodegradable, biocompatible and nontoxic make attractive candidates for constructing *in vivo* delivery vehicles. Chitosan, cyclodextrin, polyethyleneimine (PEI), poly(lactic-co-glycolic) acid, dendrimers and metallic-core nanoparticles have become popular for use in delivery systems, although none of these materials possess all of the desirable properties [128]. Chitosan is a natural, cationic polysaccharide harvestable from crustacean exoskeletons. It is an extensively studied biomaterial due to its biocompatibility, mucoadhesive properties and nuclease resistance [30]. Optimal cationic charge for maximal siRNA encapsulation in chitosan can be attained by tuning the ratio of amines to

phosphates. In two separate studies, optimized chitosan–siRNA nanoparticles have been successfully administered intranasally to silence *GAPDH* and *EGFP* in the lungs of mice [129].

Extensive branching and dense cationic charge gives synthetic polymer PEI the capacity to condense siRNAs, protect them from degradation by RNases, and facilitate their cellular uptake via endocytosis. An added feature is the ability of PEI to act as a proton sponge, because its extensive amine groups buffer the acidic inner compartment of an endosome causing water to swell the endosome to the point of rupture, thereby facilitating endosomal escape of its encapsulated siRNA. Some wariness surrounds PEI use *in vivo*, however, due to *in vitro* evidence of high cytotoxicity [130]. In an effort to reduce toxic effects of PEI, the polymer has been modified with polyethylene glycol (previously demonstrated to slow clearance and reduce toxicity) and the PEI–polyethylene glycol/siRNA complex was demonstrated to exhibit decreased toxicity, but drastically increased particle size [131–133].

Dendrimers are heavily branched polymeric molecules that can be engineered to form modular, nanosized, spherical structures for siRNA delivery. Packaging siRNAs in dendrimer structures can be accomplished by positively charging the core while abolishing surface charge [134]. Alternatively, siRNAs can be caged within dendrimer polyplexes via disulfide linkages, which incidentally also provide for controlled release in the reducing intracellular milieu. These structures can be additionally stabilized through the incorporation of polyethylene glycol [135–137]. The modularity of dendrimers allows for dendrimer–siRNA polyplexes to be further improved for siRNA delivery by combining them with targeting ligands and technologies that provide for endosomal release.

Another siRNA delivery strategy involves metallic core nanoparticles [138]. Metal cores of iron oxide, iron cobalt, iron gold or iron nickel are coated with a layer of sugars or other polymers generating a core shell structure to which siRNA can be externally conjugated through linking molecules, such as thiols [139], dextran [138], cationic polymers [135] or biotin–streptavadin [30]. Contingent upon the metal used, the cores of these particles can impart properties that allow for study of biodistribution upon injection using MRI or targeting to specific tissues by applying external magnets.

Andersen *et al.* presented a novel method involving adhering nanoparticles containing different siRNAs onto nanostructured scaffolds [140]. This allows for spatial retention of the siRNAs within nanopores until their cellular delivery. The released siRNAs were capable of silencing *BCL2L2* and *TRIB2* genes in MSCs and enhancing osteogenic and adipogenic differentiation, respectively. This approach for enhancing a single type of differentiation is immediately applicable to all areas of tissue engineering. Different nanoparticles localized to spatially distinct locations within a single implant allow for two different tissue types to develop in controllable areas of an implant. As a consequence of this, the authors predict that complex tissues and organs can be engineered by managing the *in situ* development of multiple cell types reprogrammed by spatially restricted nanoparticles.

In the end, an important finding is put on the combination of nanostructures to increase transfection efficiency.A well-written review by Adler *et al.* explains that the use of surface nanotechnology to modify particulate parameters has gained well-deserved attention in nonviral gene delivery, as these parameters are becoming increasingly well-understood modulators of uptake and transfection efficiency [141]. Another approach worth considering is the engineering of desirable endocytic cellular phenotypes through substrate surface nanotechnology. Optimization of nonviral gene delivery has so far mostly focused on design of particulate carriers that are endowed with desirable membrane targeting, internalization and endosomal escape properties.

Conclusion & future perspective

The creation of complex tissues and organs is the ultimate goal of tissue engineering, and engineered morphogenesis necessitates the spatially controlled development of multiple cell types within a biomaterial-based scaffold. The current scenario of directly transplanting adult SCs *in vivo* for the treatment of different diseases or for direct delivery to injured sites is increasingly changing. Recent progress in nanotechnology and a better understanding of the molecular pathways that control differentiation have led us to combine biocompatible scaffolds with adult SCs. As SC technologies transition from the research laboratory to clinical applications, there will be an increasing need for robust culture systems that consistently control SC growth and differentiation.

Concerns regarding the use of nanoparticles in commitment of SCs and the protection of

the bioactive molecules against environmental degradation are still valid; moreover, it is essential to better control the deliver factors in a dose- and time-correct manner. As discussed, particle delivery systems have been conceived to provide improvements in SC commitment such as the ability to enhance the differentiation and stability of SCs. However, further investigation is necessary to better determine therapeutic concentrations, combinations of molecules and methods for controlled release of factors. Recent advances in biotechnology, SC biology, polymer chemistry and nanotechnology are now opening up exciting possibilities for the improvement and restoration of tissue function, while minimizing adverse effects and improving patient compliance.

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Executive summary

Stem cell commitment through extracellular modification at the nano level

Osteogenic commitment

- Commitment of stem cells into the osteogenic lineage is highest on:
  - TiO_2 nanotubes vertically aligned with a diameter of 15 nm;
  - Nanofibers with nanohydroxyapatite fabricated by electrospinning;
  - Hydrogel scaffolds combined with peptide–amphiphile tailored by incorporation of the cell-adhesion sequence RGD and an enzyme-cleavable site;
  - Bioactive glass particles with nanoscale surface features;
  - Chitosan natural polymer with carbon nanotubes incorporated to increase the mechanical strength.
- Nanostructured scaffolds embedded with adhering nanoparticles containing different siRNAs or growth factors.

Adipose commitment

- Commitment of mesenchymal stem cells into adipose features is improved by the *in vitro* cultivations in:
  - Nanotechnology-based culture plate, composed by uneven microfabrications (diameters of ~2–3 μm) arranged in a honeycomb pattern on the cell surface;
  - Biocompatible silica nanoparticles–insulin conjugates;
  - Nanostructured scaffolds embedded with adhering nanoparticles containing different siRNAs or growth factors.

Neuronal commitment

- Neuronal commitment of adult stem cells is enhanced by the applications of:
  - Nanofibrous scaffold fabricated from aligned poly L-lactic acid;
  - NGF-conjugated aligned nanofibrous meshes;
  - Electrospun poly-ε-caprolactone nanofiber scaffolds.

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