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Stem Cells and Nanostructures for Advanced Tissue Regeneration

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Abstract Stem cells are a promising alternative for cell therapy applications because of their self-renewing capability and potential to differentiate into a wide range of specialized cell lineages, including osteoblasts, chondrocytes, cardiomyocytes, and neuronal cells. Different kinds of stem cells are considered for cell-based tissue engineering approaches, of which bone-marrow-derived mesenchymal stem cells (BM-MSCs), adipose-derived stem cells (ADSCs) and embryonic stem cells (ESCs) are frequently utilized for advancement of new tissue engineering strategies. Nanostructures created by natural, synthetic, or composite polymeric biomaterials provide artificial templates of the extracellular matrix (ECM). They possess a high surface area to volume ratio, are porous with good mechanical properties, are sufficient to serve as a biomimetic platform to attract stem cells, cause differentiation, and provide functioning of the tissues. Nanoscale features such as fibers, pits, and grooves modulate cell behavior and might even stimulate the differentiation of stem cells to specific lineages; for example, the nanotopographical cues combined with chemical cues influence the neuronal induction of MSCs, resulting in high microtubule-associated protein expressions. Scaffolds can also be impregnated with several ligands for adhesion of receptors to the cells, or with other regulatory molecules and growth

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factors for designed cell behavior or controlled cell differentiations. Different polymeric blends or fibers incorporated with nanoparticles (such as hydroxyapatite) in aligned or custom-made patterns can induce specific differentiation of stem cells for bone, cartilage, cardiac, nerve, and skin tissue regeneration. In this review, we will discuss the current state of the art and future perspectives on stem cells and their differentiation on nanoengineered substrates for advanced tissue regeneration.

Keywords Bone · Cardiac · Cartilage · Electrospinning · Mesenchymal stem cells · Nerve

Contents

1 Introduction

The basic functional units of cells and tissues are of nanoscale dimensions, and nanotechnology promises an important area of study in regenerative medicine. Cells are typically of tens of micrometers in diameter, and cellular structures such as the cytoskeletal elements and transmembrane proteins too exist in nanoscale proportions [1]. The supporting tissues form an intricate network of cues on the nanoscale (5–200 nm), and this network consists of a complex mixture of pores, pits, projections, etc., showing the complexity of the structures present in vivo [2]. Nanostructures are therefore important cues at the cellular level, and cells regulate and function depending on the topographical features and the type of surface molecules. The nanotopographical features are known to induce or disrupt the focal adhesion and spreading of cellular populations, which is mediated via the perturbation of integrin activation and clustering [1]. An ideal strategy in tissue engineering (TE) or regenerative medicine is to identify and utilize the right combination of biomaterial scaffolds, cells, and biological factors that assist cells to adhere, organize, and behave similarly to native tissue [3]. Polymeric biomaterials fabricated in various

structures, designs, and forms serve as provisional scaffolds for cells to attach, grow, and maintain differentiated functions.

This review provides an introduction to the different methods utilized for the fabrication of nanostructures, the different kinds of nanotopographies, and the major stem cell types studied in the field of tissue regeneration. Emphasis is given to the attachment and differentiation of mesenchymal stem cells (MSCs), embryonic stem cells (ESCs), and adipose-derived stem cells (ADSCs) on biomimetic nanomaterials for advancing the field of bone, cartilage, cardiac, nerve, and skin tissue regeneration.

1.1 Nanotechnology in Tissue Regeneration

Nanotechnology refers to one of the rapidly growing scientific disciplines studying and developing objects and materials with characteristic dimensions to resolve disease-related organ damage using tissue engineering. Nanotechnology could be a powerful tool for deciding the cell–biomaterial communications, inducing stem cell differentiation in a desired fashion (tissue). Nanotechnology aims to create structures at the atomic and molecular levels with a size range of 10–500 nm. It is also known that highly organized fibers (specific spatial organization) are present in native tissues such as ligaments and bone, providing sufficient mechanical strength. For example, collagen fiber bundles are aligned in parallel in native tendons and ligaments and are found as concentric waves in bone [4, 5]. Stem cells contribute to the organized tissue architecture of bone and cartilage to a certain extent, and aligned extracellular matrix (ECM) might be crucial for their differentiation behavior [6]. Cells come in contact with the ECM via integrins and transmembrane proteins, which interact with certain amino acid sequences found in the ECM proteins. The cell cytoskeleton becomes tethered to the fibrous structure of the ECM through these receptors and it activate a series of intracellular signaling pathways that affect cellular behavior such as cell adhesion, proliferation, and differentiations [7]. The fate of stem cells is decided by the signals and biophysical clues found in the cellular microenvironment. These signals are mainly the ECM molecules, soluble factors, biophysical factors, and intercellular contacts. Scaffolds can be engineered with suitable substrate moieties (high density epitopes) to assist or control the stem cell behavior in tissue assembly.

Stem cell nanotechnology has gained huge momentum and it is currently being applied for the treatment, repair, and regeneration of cells and tissues. Cells can interact with features as small as 5 nm and, hence, nanotopographies influence the cell behavior in addition to the type of substrates used for cell growth [8]. Nanoscale topographies are created by various fabrication methods such as electrospinning, polymer phase separation, photolithography, chemical vapor deposition, electron beam lithography etc. [9]. The morphological similarity of electrospun nanofibers to native ECM signifies the application of these scaffolds as a supportive matrix for stem cell attachment and differentiations. Nanofibrous scaffolds mimic the fibrous ECM of native tissue and could provide mechanical, biological, and

chemical cues to cells. Studies on the controlled release of basic-fibroblast growth factor (bFGF) from nanofibrous scaffolds and induced angiogenesis indicate the role of environmental factors required for the survival of cells in enhancing tissue regeneration [10]. By providing the appropriate biological cues, cell receptors can bind to signaling biomolecules and transmit the signals intracellularly by activating signaling cascades, which further modulate gene expression and decide the cell fate or the differentiation module. Cell alignment could be important for the treatment of nerve, cardiac, and even muscle tissue regeneration. Cells probe the topographical features of the ECM to proliferate and differentiate, whereby the "filapodia" gather the topographical, spatial, and chemical information from the material surfaces [11]. When engineering tissues such as ligaments, articular cartilage, and blood vessel walls, the principle of contact guidance dominates, mainly because these tissues possess highly anisotropic cellular organization [12]. The formation of elongated focal adhesion points is important in osteospecific differentiation of MSC, and the adhesion elongation relies on enhanced integrin clustering [1]. With focal adhesion corroborations, increased focal adhesion kinase (FAK) is recruited and subsequently activated to initiate the downstream signaling cascades. Moreover, limited focal adhesion points can also cause reduced mechanosensitive signaling events [1]. Substrate topography together with electrical conductivity has also been shown to contribute to nerve stem cell differentiation to specific neuronal lineages [13]. Among the various ECM-derived cell binding motifs, the scaffolds used in TE are mainly modified with peptides such as RGD, IKVAV, YIGSR, PHSRN and GFOGER [14]. There is an urgent need to evaluate and understand the particular cell behavior of stem cells on nanostructures, which might eventually help us to design a scaffold for enhanced tissue regeneration.

1.1.1 Nanostructure Fabrication Methods

Electrospinning is a simple, versatile, and affordable method for producing nanofibers with defined geometry by electrically charging a suspended droplet of polymer melt or solution. Natural, synthetic, and composite polymeric materials are electrospun to obtain fibers, and the diameter of the fibers can be adjusted by varying the polymer concentrations, solvents used, or by modulating the spinning conditions [15, 16]. Electrospun nanofibers provide a high surface area for cell attachment, and functionalization of fibers is possible by chemical conjugation of ECM molecules or by protein coatings (composite scaffolds). A variety of topographical patterns such random, aligned, porous, and core–shell nanofibers have been studied extensively for various TE applications. Figure 1 shows the nanofibrous architectures created by various electrospinning methods. Advanced and modified technologies such as multilayered electrospinning and simultaneous electrospin–electrospray methods have also been studied recently for obtaining scaffolds with multifunctional properties [17].

Phase separation is utilized for the fabrication of porous membranes and it allows for the generation of three-dimensional (3D) porous networks within the scaffolds. Considering the relationship of specific cells and pore sizes, polymeric

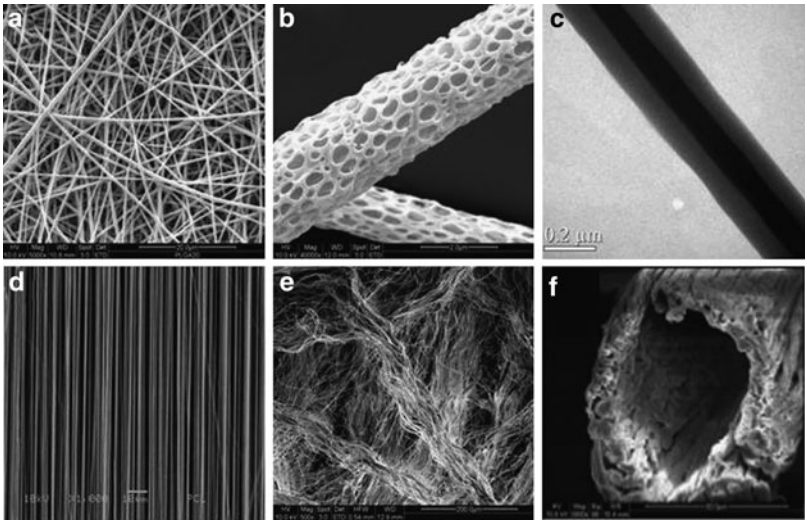

Fig. 1 Nanofibrous architectures created by electrospinning: (**a**) random nanofibers, (**b**) porous nanofibers, (**c**) core–shell nanofibers, (**d**) aligned nanofibers (**e**) nano-yarn, (**f**) hollow nanotubes

scaffolds should be designed not only with high degree of porosity but also with greater control over the pore size and morphology [18, 19]. The phase separation method is based on the thermodynamic de-mixing of a homogenous solution of polymer in solvent into polymer-rich and polymer-poor phases by exposure to another immiscible solvent or by cooling the solution below the glass transition temperature (*T*g) of the polymer. Processing variables such as the polymer type, concentration, solvent, and temperature are critical parameters that affect the morphology of the fabricated scaffolds.

Self-assembly involves the autonomous organization of individual components in an ordered structure or pattern without human intervention. It occurs through non covalent forces such as hydrogen bonding, electrostatic forces, or hydrophobic forces. Control of self-assembly in the design of peptide systems is carried out by switching the pH, or by varying the temperature, concentration etc. [20]. Cell-adhesive peptide ligands are commonly exposed to the self-assembled nanofibrous structures to enhance the bioactivity of the scaffolds for convenient applications in tissue regeneration [21].

Physical patterning techniques such as reactive ion etching, polymer molding etc. create microgrooves for designated cellular orientations [22, 23]. Patterned surfaces provide cues for cellular attachment, migration, orientation, and function. Soft lithographic techniques have been used to generate exquisite control over protein and cells in spatially defined patterns. Cell shape has been regulated at the microscale, and even the temporal and spatial distribution of biomolecules has been performed to direct explicit cell behavior and functions. Methods such as imprint lithography, photo or electron beam lithography, or microcontact printing are also carried out for patterning biological molecules or for constructing geometrically

designed substrates suitable for cellular interaction on a nanoscale [24, 25]. The Imprint lithography method utilizes a silicone rubber stamp (made by casting silicone rubber into a patterned die) inked with molecules to transfer the agent to a prepared surface. It is possible to provide specific cues, potentially suitable for a particular cell type or for specific differentiation modulation by this method. Grids, honeycomb networks, dots etc. are a few patterns created using the microcontact printing method to mimic the basement membrane structures of nanometer-sized pores and ridges [26]. Microcontact printing also has advantageous over other patterning methods as it does not involve the usage of harsh solvents and high temperatures [27]. Nanoimprint lithography is capable of creating patterns of sub-10 nm features and uses very simple equipment with convenient processing steps [28]. On the other hand, atomic force microscopic etching involves scratching the material surface with a nanosized pyramidal tip, but has limitations in application over a large surface area [29]. Design of nanotopographical patterns based on the specific needs of a particular tissue regeneration is therefore possible by application of the above-mentioned methods.

1.1.2 Substrate Stiffness and Effect of Mechanical Stretch

Stiffness of substrate plays an important role in cell adhesion, especially towards the lineage-specific differentiation of stem cells [30]. Hydrogels have the ability to simulate the nature of soft tissues and are highly attractive materials for developing synthetic ECM analogs [31]. Three-dimensional biomaterial constructs not only act as a mechanical support, but also provide a suitable structure and well-defined array of macromolecular signals to direct the de novo tissue development [32]. Moreover, cell infiltration can be improved using 3D structures, whereas the mechanical properties of the gels, release of biomolecules, transport and degradation kinetics can also be tuned for improving the cytocompatibility of encapsulated cells or towards stem cell differentiation [33, 34]. Engler and coworkers demonstrated that the differentiation of human mesenchymal stem cells (hMSCs) was dependent on the mechanical stiffness of 2D culture platforms [35]. Soft gels were demonstrated to support MSC differentiation to neuronal cells, while stiffer gels supported an osteoblast-like phenotype. Figure 2 shows the differentiation of stem cells on substrates with varying elasticity. Moreover, it is also possible to incorporate other inducing factors during matrix design to bestow lineage specificity [35]. Control of the biochemical and mechanical properties of the scaffolds will help us to understand the effect of specific cell–ECM interactions in 3D tissue models, and could used to dictate that cells behave in a desired fashion in vivo for tissue development [31].

Mechanical stretching of cell membranes can be achieved by nanotechnological approaches and it can direct and control the intracellular signaling and differentiation of stem cells [36]. Modifications made to the spatial distribution or cytoskeletal re-organization provide physical impetus, whereby the mechanical deformation becomes translated into biochemical responses [37]. When the cells encounter

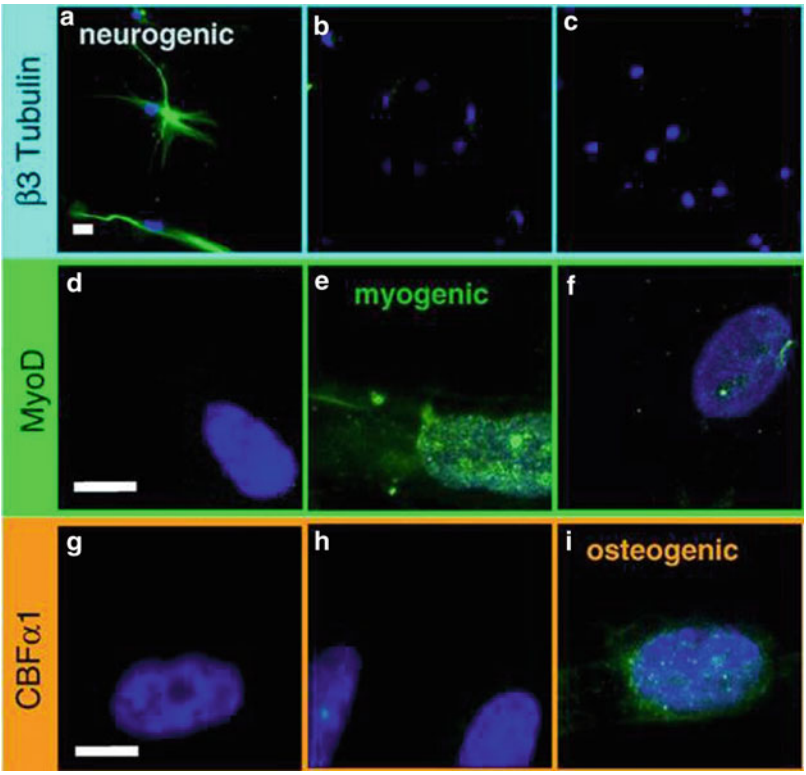

Fig. 2 Mesenchymal stem cell differentiation on substrates with different elasticity: (**a–c**) neurogenic differentiation on soft matrices; (**d–f**) myogenic matrices showing upregulated muscle transcription factor; (**g–i**) osteogenic differentiation on stiffer matrices. Reproduced with permission from Engler et al. [35]

topographic discontinuity, such as a groove or a cliff, re-organization of the microfilament occurs and cell activation can be further related to specific gene expressions compared to its growth on flat surfaces [38]. Studies using defined arrays of bound peptide fragments showed that the protein recruitment to focal adhesions requires an integrin spacing of less than 60 nm. A higher spacing between the integrins (70–300 nm) facilitates integrin clustering and focal adhesion formation [39]. Grooved substrates can provide an elongated morphology and alignment of cells, thus influencing cells including the MSCs to respond and upregulate the expression of ECM components and proteins pertaining to cellular adhesions [40]. On grooved surfaces, the cell microfilament bundles align predominately along the edges of the grooves [24]. It is known that the FAK-mediated ERK1/ERK2 signaling pathway is an important modulator of osteo- and adipo-specific differentiation of MSCs. Topographical information of a tissue-engineered graft regulates cellular adhesion and differentiation because an increase in integrin–substratum

interaction has been shown to upregulate the expression of FAK and ERK1/ERK2 in osteoprogenitor cells [41].

1.2 Stem Cells: Origin and Multipotency

Stem cells are present in every type of tissue, and smart engineered scaffolds loaded with stem cells can differentiate into specific cell lineages for effective tissue regeneration. "Stem" refers to the ability of progenitor cells to self-renew, meaning to divide and retain a daughter cell that does not differentiate, while also producing another daughter cell that differentiates or divides and then differentiates. Various organs, including the peripheral blood, bone marrow, pancreas, muscle, skin, fat, and neuronal system contain stem cells [42, 43]. "Differentiation" is the major cellular event that forms the basis for utilization of stem cells in regenerative medicine, and it is the microenvironmental cues that define the kind of differentiation [44]. Control of cell behavior by ligand density and/or specificity, nanopatterning, material architecture, mechanical properties of the material etc. are possible, aiming towards specific lineage differentiations. Distinct cell culture conditions or nanoenvironments, in conjunction with ECM molecules such as laminin, collagen, gelatin, cytokines or other growth factors, are utilized for the differentiation of stem cells to specific functional progeny [45].

Stem cells are mainly classified as adult stem cells and ESCs. ESCs are obtained from embryonic blastocysts, and adult stem cells are derived from various tissues of developed (adult) or developing individuals. The most commonly studied adult stem cells are the bone-marrow-derived mesenchymal stem cells (BM-MSCs) and the adipose-tissue-derived stem cells (ADSCs), discussed in this review.

1.2.1 Mesenchymal Stem Cells

MSCs are non-hematopoietic progenitor cells that have the potential to differentiate into various lineages of mesenchymal origin. They are attractive cell types for TE applications, relatively privileged in terms of immune compatibility, and can be easily isolated [46]. They are isolated from bone marrow (BM), immobilized blood, umbilical blood, cord blood, deciduous teeth, and even placenta. However, BM is relatively accessible and it serves as the main source of MSC. The advantage of using BM-MSC in regenerative medicine is because they are naturally poised to generate a particular tissue, which might consist of several cell types such as adipocytes, chondrocytes, osteoblasts, tenocytes, myoblasts, neurocytes etc. MSCs constitute approximately 2–3% of the total nuclear cell fraction of the BM [47]. Isolation of these cells involves seeding the mononuclear cell layers obtained from Ficoll density gradient centrifugations. MSCs have a spindle-shaped fibroblast-type morphology and adhere to the base of the culture flask, while the non-adherent hematopoietic cells are washed off. MSCs are positive to a variety of

surface markers such as CD44, CD105, and CD29 and are negative to markers such as CD45, CD34, and CD14 [48, 49]. However, the specificity of these markers is relatively weak, which means that they are not exclusively markers for MSCs. Under in vitro conditions, MSCs do not expand indefinitely unlike ESCs, and several agents including hormones, ECM molecules, and growth factors tightly determine the differentiation possibility of MSCs.

BM-MSCs possess high plasticity and have been differentiated into various cell lineages and contribute much to the regeneration of tissues at injured sites [50, 51]. It is reported that scaffolds loaded with different ratios of hydroxyapatite/tricalcium phosphate (HA/TCP) with MSCs showed different degrees of bone formation in vivo. Optimal composition of HA/TCP ratios within the composites were designed to match tissue deposition with scaffold degradation so as to promote the greatest ectopic bone formation [52]. Physical or mechanical forces are also suggested to regulate the stem cell differentiation process, and the latest trend is the "functional tissue engineering approach," where bioreactors are designed to reiterate certain segments of the in vivo and in vitro cell culture system [53].

1.2.2 Embryonic Stem Cells

ESCs are cells derived from the inner cell mass of blastocyst-stage embryos. ESCs have indefinite self-renewing capacity and possess pluripotency or the ability to differentiate to cells from three germ layers (endoderm, mesoderm, and ectoderm). ESCs differ from other tissue-specific stem cells in that they can be readily expanded in culture over an extensive period of time [54]. Their isolation involves treating day 5 blastocysts with pronase, transferring the zona-free blastocysts onto irradiated or mitomycin-C-treated mouse embryonic fibroblast (MEF) feeder cells or human adult fibroblasts, and then culturing in media containing bFGF and insulin-transferrin-selenium (ITS). The inner cell mass (ICM) lump is mechanically detached using capillary pipettes, cut into smaller pieces and transferred to fresh irradiated or mitomycin-C-treated MEFs and incubated with culture media [55]. OCT4 and telomerase are better-accepted markers for ESCs, but their expression in adult stem cells is less certain [56, 57]. ESC self-renewal versus differentiation is regulated via interactions with other cells, ECM components, soluble factors, and the physicochemical environment [58]. However, concerns exist regarding the immune reactions of ESCs along with the potential of undifferentiated ESCs towards teratoma development, which hamper the clinical applications of ESCs [59].

1.2.3 Adipose-Derived Stem Cells

Adipose tissue is the most abundant and accessible source of stem cells and ADSCs are demonstrated to possess multiple differentiation capacity. ADSCs originate from the stromal vascular fraction of adipose tissue. Liposuction is being undergone

by many people to remove excess adipose tissue, which could serve as a source of ADSCs. The contaminating hematopoietic cells are removed by washing the minced fat pads and the tissue fragments incubated with enzyme collagenase; then the contents are centrifuged and the mature adopicytes separated from the pelleted stromal vascular fraction [60]. ADSCs adhere to plastic surfaces and display fibroblastic characteristics, with abundant endoplasmic reticulum and large nucleus relative to the cytoplasmic volume [61]. Immunophenotypical studies suggest that the glycoprotein CD34 is present during early passage of human ADSCs and has not been found on MSCs [62]. At passage 0 (cells cultured for 72 h), less than 10% of human or murine ADSCs express CD31 [63]. Recent studies by Traktuev et al. [64] suggested that ADSCs can be defined by coexpression of CD34 and CD140b. ADSCs are suggested to contribute towards the angiogenic properties because they secrete angiogenic cytokines such as vascular endothelial growth factor (VEGF) and hepatocyte growth factor (HGF) [65]. Due to its angiogenic capabilities, ADSC-augmented nanostructures could be utilized for effective regeneration of vascular tissue. However, for clinical use, health care professionals should be educated with regard to the unique properties of ADSCs in order to avoid their inappropriate use (and ensure correct application).

2 Strategies for Advanced Tissue Regeneration

The efficiency of stem cell transplantation might be enhanced by encapsulating the cells in biocompatible scaffolds that provide a temporary 3D matrix for cell adhesion, migration, and differentiation. There is great interest in the ex vivo and in vivo differentiation capability of stem cells on nanomaterials for regeneration of injured tissues and organs. Here, we focus on recent developments in the mimicking of cell–matrix interactions representative of the stem cell niche, with an emphasis on nanostructures for modulating the stem cell differentiation for advancing the field of cardiac, bone, cartilage, nerve, and skin regeneration.

2.1 Osteogenesis

Orthopedics is a multibillion dollar industry and every year millions of people suffer from bone defects arising from cancer, fractures, periodontitis, osteoporosis, and infectious disease and many people die due to insufficient bone substitute. The functional treatment of fracture nonunion and bone loss associated with trauma and revision joint arthroplasty has become increasingly common and it remains a significant challenge to the field of musculoskeletal injury [66]. Bone grafts are increasingly used; however, they are plagued by high-failure rates of 16–50% [67]. The replacement of diseased bone tissue has taken a variety of forms: metals, ceramics, polymers, and bone itself, but none has proven ideal for TE. Biomaterials

can be either permanent or biodegradable, and need to be biocompatible, osteoinductive, osteoconductive, integrative, and mechanically compatible with native bone to fulfill the desired functionality in bone TE. These materials provide cell anchorage sites, mechanical stability, structural guidance, in vivo milieu, and an interface to respond to the physiological and biological changes in order to remodel ECM and integrate with the surrounding tissue [68]. Therefore, polymeric nanofibrous scaffolds applied for bone TE should be biocompatible with the surrounding biological fluids and tissues, biodegradable, and highly porous with interconnected spaces favorable for the diffusion of nutrients and the migration of a large number of cells.

Recently, 3D scaffold materials have become a crucial element for bone TE. These scaffold materials are designed to mimic one or more bone-forming components of autograft, in order to facilitate the growth of vasculature into material and provide an ideal environment for bone formation. The formation of bone can be roughly divided into three phases: (1) proliferative phase, during which collagenous matrix is deposited, (2) maturation phase, which is characterized by the activity of alkaline phosphate, and (3) mineralization phase, when the newly formed matrix begins to calcify. These phases are influenced by an increased amount of collagen type III coating, giving rise to an increase in cell proliferation and synthesis of collagen, both of which are characteristics of the early phase of bone formation. Collagen provides an inherently good biocompatibility with cells, and collagen-based implants are well known for their feasibility in promoting tissue regeneration. HA [$Ca_5(PO_4)_3OH$] is considered to be a structural template for the bone mineral phase. It is a major inorganic mineral component of bone and is commonly used as a bioceramic filler in polymer-based bone substitute because of its high bioactivity and biocompatibility [69]. Calcium phosphate biomaterials such as HA and TCP with appropriate 3D geometry are able to bind and concentrate endogenous bone morphogenetic proteins (BMP) in circulation, and may become osteoinductive [70] and effective carriers of bone cells.

2.1.1 Stem Cells and Nanomaterials for Bone Regeneration

Silk is an attractive material for osteochondral TE since its mechanical properties are favorable for engineering of load-bearing tissues and it displays a higher elastic modulus and tensile strength than other natural and synthetic biomaterials [71]. RGD-coupled silk films and scaffolds improved osteogenesis, stimulating increased osteogenic gene expression, calcification, and formation of bone-like trabeculae compared to unmodified silk materials [72]. Functionalization of silk films by covalent conjugation of bone morphogenetic protein-2 enhanced the osteogenic differentiation of MSCs compared to soluble delivery of bone morphogenetic protein-2 [73]. Another attractive ECM-mimicking biomaterial for creating tissue-specific microenvironments that facilitate synthetic cell–ligand interactions is composed of peptide amphiphiles (PAs). PAs are a broad class of molecules that self-assemble into nanofibrous supramolecular formations, emulating the native ECM architecture. These molecules consist of a peptide sequence covalently linked

to a hydrophobic alkyl chain [74]. The RGD ligand is prevalent in many ECM molecules, including fibronectin, laminin, and osteopontin, which have been well documented to increase cell adhesion [75]. Recent studies have shown that inclusion of RGD ligand can potentially increase osteogenic differentiation [76, 77]. Anderson et al. observed the osteoconductive potential on RGD-modified PA scaffolds independent of stimulatory aid, though not to the same phenotypic levels as the supplemented scaffolds [78]. However, PAs offer a promising regenerative tool for the continued development of bone TE applications.

Osteogenic activation of MSC requires the presence of β-glycerophosphate, ascorbic acid, and dexamethasone [79]. Yoshimoto et al. cultured rat MSC on poly(ε-caprolactone) (PCL) nanofibrous scaffolds of 400 nm diameter and observed ECM production (collagen) and multiple cell layer formation within a short span of one week [80]. Salerno et al. reported a novel approach for the design of porous PCL scaffolds with well-controlled microarchitectures, by combining gas formation and selective polymer extraction methods for osteogenic differentiation of hMSCs in vitro [81]. Hosseinkhani et al. investigated MSC behavior on self-assembled PA nanofibers and they found significantly enhanced osteogenic differentiation of MSCs in 3D PA scaffolds compared to 2D static tissue culture [82]. Dalby et al. [83] demonstrated the use of nanoporous topography to stimulate hMSCs to produce bone mineral in vitro, in the absence of osteogenic supplements. Their results demonstrated that highly ordered nanoporous topographies produce negligible cellular adhesion and osteoblastic differentiation. Dynamic bioreactor culture systems are essential for the in vitro cultivation and maturation of TE bone grafts, especially for larger grafts where the core of the scaffold is more than 200 μm [84]. The dynamic media flow causes mechanical stimulus to the cells, enhancing cellular osteogenesis and mineralization through triggering of mechanotransduction signaling pathways. In yet another study, the dynamic culture and osteogenic priming of hMSC-mediated macroporous PCL/TCP scaffolds in biaxial rotating bioreactors generated an effective TE bone graft for healing a critically sized defect [85].

Lyons et al. fabricated freeze-dried collagen/glycosaminioglycan (CGAG) and collagen/calcium phosphate scaffolds (CCP) for the culture of bone marrow stromal cells (BMSCs) for bone TE [86]. These researchers created a 7-mm transosseous calvarial defect in rats, and implanted it with either CGAG scaffold, TE-CGAG construct, CCP scaffold, TE-CCP construct, or with no scaffold (empty control), and the suture was closed. Animals were sacrificed after 4 and 8 weeks of follow-up study for the analysis of bone tissue regeneration. Results showed that the empty control group defects are filled with structurally and morphologically organized fibrous tissue consistent with failure to heal the bone defect (Fig. 3ai), demonstrating the critical size of the defect. Fibrous tissue was clearly seen extending from the defect margin (Fig. 3aii) with a few small areas of the new bone formation visible immediately adjacent to the host bone, indicating unsuccessful attempts at regeneration. CGAG scaffold-filled defects did exhibit signs of healing with woven bone formation visible around the defect periphery, with most found at the interface with the host bone (Fig. 3bii, biiii). Qualitative spectrum of newly formed bone was

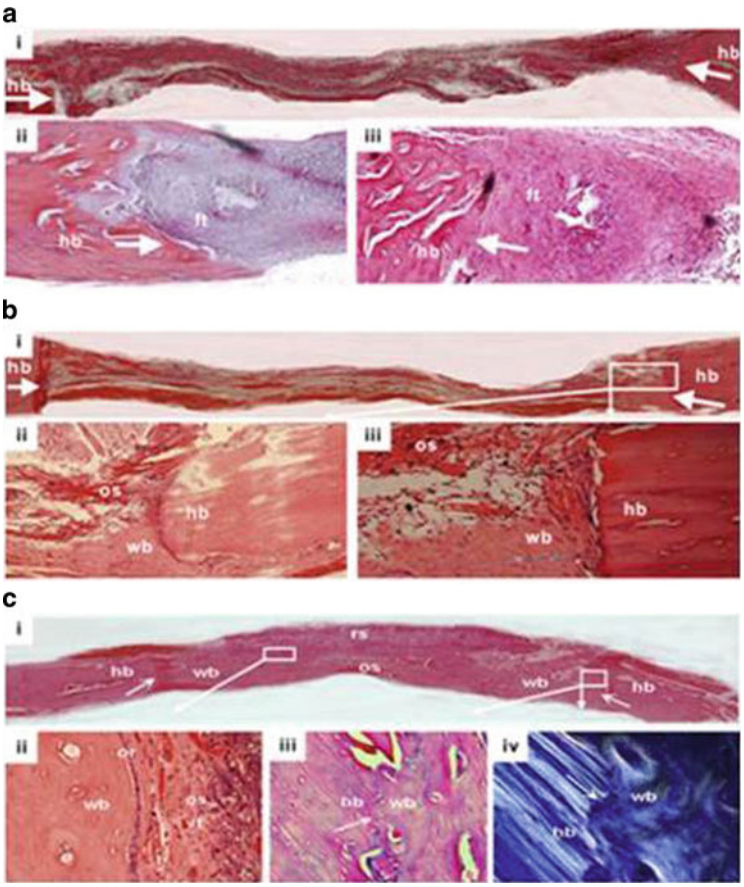

Fig. 3 Histological images (stained with hematoxylin and eosin) of bone tissue regeneration after implantation of a transosseous calvarial defect with CGAG or CCP scaffold. (**a**) Empty control group (no scaffold implant) at 8 weeks shows no healing of the defect: (**ai**) full-size image of the specimen, with the original host bone at the edges; higher magnifications (**aii** 20×, **aiii** 40×) shows fibrous tissue filling the defect and original host bone at the periphery. (**b**) CGAG group at 8 weeks post-surgery: (**bi**) full specimen; at higher magnifications (**bii, biii**), healing is seen extending from the host bone into the defect. (**c**) CCP group at 8 weeks post-surgery: (**ci**) shows progressive areas of healing across the defect and large areas of the defect filled in with healing woven bone and osteoid. The remnant of the scaffold can be seen heavily infiltrated with host cells. High magnification images (**cii**, **civ**) show a vertical rim of osteoblasts at the interface of new woven bone, and unmineralized osteoid with vascular tissue also present. (**ciii**) demonstrates the interface between the host bone and areas of new woven bone filling the defect. (**civ**) Epifluorescence microscopy image shows the interface between host bone and woven bone in the defect. *Arrows* indicate original host bone margins, *ft* fibrous tissue, *hb* host bone, *wb* woven bone, *os* unmineralized osteoid, *rs* remnanat of scaffold, *or* rim of osteoblasts, *vt* vascular tissue. Reproduced with permission from Lyons et al. [86]

visible bridging the defect in the CCP group at 8 weeks (Fig. 3ci), with woven bone advancing from the defect-host interface (Fig. 3ciii and civ). Staining of the immunomodulatory and tissue remodeling (M2 phenotype) marker CD163 at 4 weeks demonstrated active M2 mononuclear cell activity in the scaffold substance, particularly at the site of new bone formation and the host–scaffold interface in cell-free scaffolds (Fig. 4a). In the TE-CGAG and TE-CCP constructs, M2 phenotype mononuclear cells (MNC) were evident at 8 weeks, suggesting an advanced stage of remodeling process (Fig. 4a). In the TE-CGAG and TE-CCP constructs, M2 phenotype MNCs was evident at both 4 and 8 weeks predominantly at the periphery of the scaffold, especially in the fibrous/inflammatory capsule seen previously on

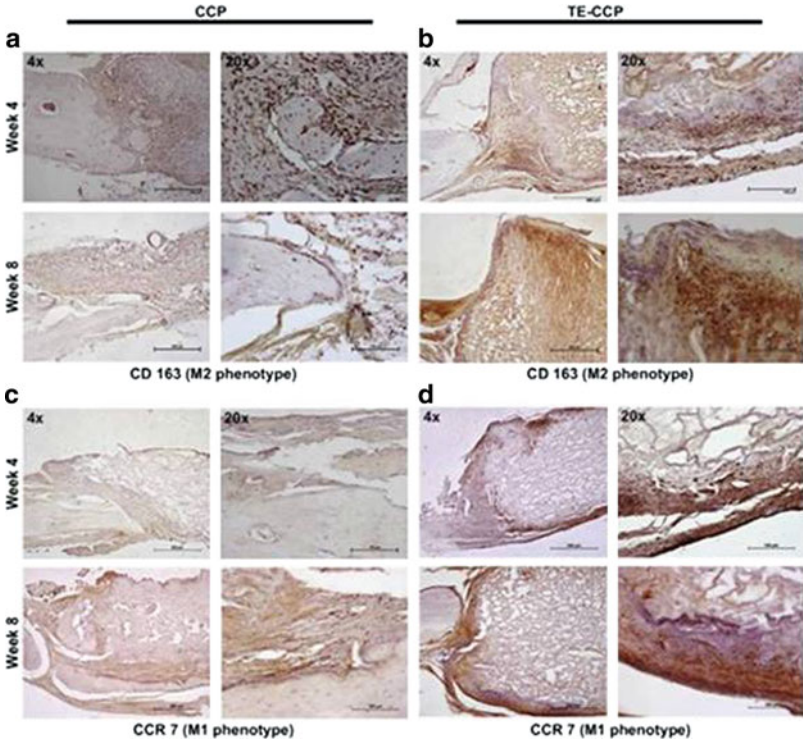

Fig. 4 Immunohistochemistry results for cell-free CCP and TE-CCP scaffolds at 4 and 8 weeks. (**a**) Staining for the immunomodulatory and tissue remodeling (M2 phenotype) marker CD163 at 4 weeks demonstrated active M2 macrophage activity in the scaffold substance, particularly at sites of new bone formation and the host–scaffold interface in cell-free scaffolds. These cells were less evident by 8 weeks, suggesting an advanced stage of the remodeling process. (**b**) In the TE scaffolds, M2 phenotype macrophages were evident at both 4 and 8 weeks predominantly at the periphery of the scaffold, especially in the fibrous/inflammatory capsule seen previously on histological examination. (**c**) The proinflammatory (CCR7) marker demonstrated little M1 macrophage cell activity in unseeded scaffolds at either 4 or 8 weeks. (**d**) There was marked population of these cells at the periphery of TE scaffolds at both 4 and 8 weeks. Reproduced with permission from Lyons et al. [86]

histological examination (Fig. 4b). The pro-inflammatory (CCR7) marker demonstrated little M1 phenotype MNC activity in unseeded scaffolds at either 4 or 8 weeks (Fig. 4c) but a marked population of such inflammatory cells at the periphery of the TE-CGAG constructs at both 4 and 8 weeks (Fig. 4d). These results proved that the collagen/calcium phosphate (CCP) scaffolds provided increased stiffness and accelerated new bone formation in the defect compared to a non-mineralized CGAG scaffold.

2.1.2 Embryonic Stem Cells on Nanostructures for Bone Regeneration

ESCs represent a potential advance in cell sourcing for TE because they proliferate for longer than other types of stem cells and possess the ability to differentiate to any tissue type within the body. The cell–cell interactions and BMPs secreted by primary bone-derived cells stimulated human ESCs (hESCs) into osteogenic lineages in a direct co-culture system [87]. Cell extracts derived from hESC-derived osteogenic cultures induced undifferentiated hESCs into osteogenic lineage [88]. Human ESC-derived embryoid body cells were cultured in the presence of osteogenic supplements such as ascorbic acid and β-glycerophosphate (BGP) for 14 days, and dexamethasone was added to this medium for another 24 h. The stimulated cells were further seeded onto poly(lactic acid) (PLA) scaffolds and implanted subcutaneously to the back of immunodeficient mice for 5 weeks. Discrete areas of mineralization were observed, and osteocalcin was expressed by the implanted cells [89]. Implantation in the peripheral cavity of osteogenic and control hESC-derived embryoid body cells in injection chambers for 11 weeks resulted in the formation of mineralized areas [90]. However, the osteogenic supplementation for 4 days before implantation was not sufficient to enhance osteogenic differentiation of hESCs to levels higher than spontaneous osteogenic differentiation.

Recent advances made in the field of nanotopography-mediated stem cell regeneration provide optimism that bone TE can create a permissive environment for bone regeneration. Progress has been made over the last few years for bone TE, but usage of ESCs still remains in its infancy. We are optimistic about the future perspectives in bone TE, though stem-cell-based therapy involves many legal and social questions that must be addressed before stem-cell-based therapies become clinically available.

2.2 Chondrogenesis

Developing artificial articular cartilage to repair cartilage has been an ongoing battle for scientists and surgeons for decades. Articular cartilage defects can be caused by congenital and metabolic diseases as well as trauma or injury, or from degeneration due to osteoarthritis. Articular cartilage damage leads to disability, morbidity, and dependence, consequently giving rise to healthcare expenditures

and loss of work [91]. Articular cartilage is a thin layer of hyaline cartilage that covers the surface of bones at large articulating diarthrodial joints. Its function is to enable low-friction movement whilst being able to bear high tensile forces and resist deformation. Its uniquely dense ECM is produced and maintained by a single type of specialized cell, the chondrocytes [92]. Mature joint cartilage is free of blood vessels and enervation, is composed of ECM rich in proteoglycans and collagen type II, and about 5% of the tissue volume is occupied by chondrocytes. These cells are spherical, embedded in lacunae filled with pericellular matrix, and have no contact to the distant neighbor cells. Although human cartilage can reach a thickness of up to 7–8 mm, its supply with nutrients and oxygen is constrained to diffusion which is, however, facilitated by compressive cyclic loading that provides a pumping mechanism during joint movements. The cartilage ECM is abundant in collagens that provide a network of proteoglycans and other biomolecules. The negatively charged proteoglycans are responsible for high osmotic swelling pressure, resulting in a large proportion of water within the tissue [93]. It is through the interactions between collagen, proteoglycans, and water that hyaline cartilage becomes a resilient tissue capable of lasting a lifetime.

The ability of clonally expanded human BM cells to differentiate towards the osteogenic, chondrogenic, and adipogenic lineages was demonstrated by Pittenger et al., thereby making it possible to develop treatments with human autologous cells for the repair of mesenchymal tissues such as bone, cartilage, and adipose tissues [50]. Cellular differentiation is characterized by increased synthesis of transcription factors sox-5 and sox-6, and the appearance of cartilage transcription factor sox-9 [94, 95]. In the growth plate, hypertrophy and calcification of the cartilage tissue precede vascular invasion, finally leading to tissue replacement by bone. Collagen type I and fibronectin are synthesized in the ECM prior to condensation and reach a maximum density at the time of cellular differentiation [96]. Chondrogenic differentiation of the condensing cells is characterized by the appearance of collagen types II, IX, and XI, the characteristic component of the collagen network of cartilage tissue. Mixed collagen type I and type II formation result in mechanically inferior fibrocartilage, and it is assumed to be an intrinsic property of MSCs that cannot be avoided in mesenchymal cartilage repair [97].

Chondrogenic differentiation, as described by Johnstone et al., requires a 3D environment, and the addition of various combinations of growth factor [98]. Several 3D cultures are being used for this purpose, including a micromass pellet culture system, high-density bridge cultures, and alginate bead cultures [99]. Zhou et al. demonstrated how transforming growth factor β (TGF-β) signals the Wnt signaling pathway to enhance the proliferation of MSCs towards chondrogenic lineages, and suggested that the process might be mediated by smad3 and β-catenin [100]. Dexamethasone has been shown to be a powerful supplement for inducing chondrogenesis via the glucocorticoid receptor by enhancing stimulation of the TGF-β superfamily, and subsequent collagen type II and cartilage-specific proteoglycan production [101]. Lee et al. postulated that hyaluronic acid facilitates the migration and adherence of MSCs to the defect, and demonstrated how this treatment was effective in inducing a repair response in a porcine model [102].

2.2.1 Mesenchymal Stem Cells on Nanomaterials for Cartilage Regeneration

Cartilage TE shows great promise, with application of 3D scaffolds seeded with cells. Porous scaffolds of PLA and poly(lactic-*co*-glycolic acid) (PLGA) have been demonstrated to be suitable substrates due to their good mechanical properties and degradation behavior [103]. These scaffolds create a 3D environment in which chondrocytes can be loaded before being re-implanted in the defect and must, therefore, be reabsorbable and non-toxic to the cells [104]. Li et al. investigated the chondrogenesis of MSCs on a PCL nanofibrous scaffold in the presence of TGF-β1 in vitro [105]. The differentiation of stem cells to chondrocytes on nanofibrous scaffold was comparable to an established cell pellet culture. It was advantageous to use nanofibers rather than cell pellet system, owing to their better mechanical properties, oxygen/nutrient exchange and ease of fabrication. PCL nanofibrous scaffold is a practical carrier for MSC transplantation and could be a suitable scaffold for cell-based tissue engineering for cartilage repair. Cheng et al. reported that human cartilage cells attached and proliferated on hyaluronic acid nanocrystals homogeneously dispersed in PLA, and that collagen fibers of 110 nm–1.8 μm diameter supported chondrocyte growth and infiltration [106, 107]. Chondrogenesis of MSCs was supported on 3D porous aqueous-derived silk scaffolds, forming cartilage-like tissue with spatial distribution of cells and ECM, with expression of chondrogenic genes, and zonal architecture resembling the native tissue [108, 109]. Chondrogenesis was improved in silk scaffolds compared to collagen scaffolds in terms of cell attachment, metabolic activity, proliferation, ECM deposition, and glycosaminoglycan (GAG) content [110, 111]. Results have also been encouraging using Hyalograft C, a hyaluronan-based scaffold, that demonstrated the formation of 96.7% of the repair tissue similar to hyaline cartilage [112].

Basically, two types of scaffolds have been employed for cartilage TE, i.e., porous sponges and hydrogels. Diao et al. employed pDNA-TGF-β1-activated 3D chitosan/gelatin scaffolds to improve the efficiency of rabbit cartilage in vivo [113]. The gene-enhanced-matrix (GEM) might provide stimuli to guide the differentiation of stem cells and is suggested for in vivo applications. On the other hand, the vector for gene delivery is crucially important to achieve high transfection efficiency and low toxicity. Particularly, a cationized chitosan derivative, i.e., *N*,*N*,*N*-trimethyl chitosan chloride (TMC) has a strong ability to condense DNA and facilitate cellular uptake in vitro, resulting in a higher cell transfection efficiency and lower cytotoxicity compared to polyethylenimine [114, 115]. Wang et al. fabricated three types of constructs for animal experiments: group A [PLGA sponge/fibrin gel/BMSCs/(TMC/pDNA-TGF-β1 complexes)] and two control groups, group B (without gene), and group C (without BMSCs) to study the cartilage repair [116]. Group A resulted in better chondrogenesis of BMSCs with hyaline cartilage formation and subchondral bone connection compared to both control groups. The cartilage matrices, GAGs, and collagen type II were abundantly stained in the neocartilage. Figure 5 shows histological images of the neocartilage after 12 weeks of transplantation in rabbit knees. Group A showed better restoration

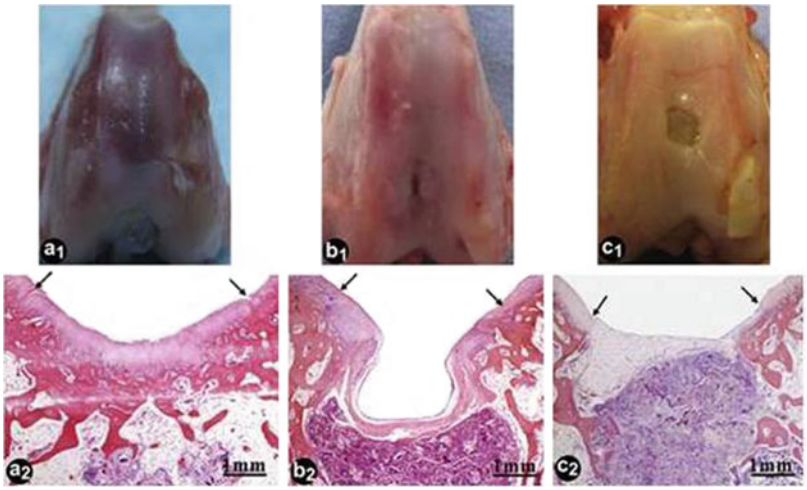

Fig. 5 Gross view (*top row*) and histological images (*bottom row*) of the neocartilage after transplantation for 12 weeks in rabbit knees. (**a1**, **a2**) PLGA/fibrin gel/BMSCs/(TMC/pDNA-TGF-β1 complexes), (**b1**, **b2**) Control group without gene, PLGA/fibrin gel/BMSCs, and (**c1**, **c2**) Control group without BMSCs, PLGA/fibrin gel/(TMC/pDNA-TGF-β1 complexes). *Arrows* indicate the boundaries between the grafts and host tissue. Reproduced with permission from Wang et al. [116]

of the cartilage defect and group C resulted in fibrous tissue formation and poor cartilage restoration. The bioactive construct (Group A) showed effective repair of osteochondral defect within a short period of 12 weeks. The gene complexes had a transfection efficiency in vitro of 9% to BMSCs, which could express TGF-β1. Transplantation of the constructs into full-thickness cartilage defects (diameter of 4 mm and depth of 4 mm) of rabbit knees resulted in hyaline cartilage, better chondrogenesis of BMSCs, subchondral bone connection, as well as deeper zone remodeling with histological score of 2.83, the best values obtained so far. The neocartilage contained abundant ECM of GAGs and collagen type II, in which chondrogenic genes such as those encoding collagen type II and aggrecan were also up-regulated, implying the efficient chondrogenic differentiation of BMSCs for cartilage regeneration.

2.2.2 Embryonic Stem Cells on Nanomaterials for Cartilage Regeneration

The use of mature cell types such as chondrocytes and osteoblasts is associated with several drawbacks including their limited availability, donor site morbidity, dedifferentiation, and limited proliferative capacity. These problems have urged researchers to study the chondrogenic and osteogenic lineage differentiation of ESCs and adipose stem cells. Human embryoid body cells were combined with Matrigel and seeded onto thin PLGA/PLA scaffolds. The hESC proliferation

medium was supplemented with TGF-β1 to direct the cells into the chondrogenic lineage, and cartilaginous tissue was formed [117]. Human embryoid body-derived mesenchymal-like cells were seeded in functionalized polyethylene glycol (PEG) hydrogels and cultured in chondrogenic medium containing TGF-β1. The addition of collagen type I or hyaluronic acid did not result in chondrogenic differentiation, but the addition of RGD peptides induced the formation of a cartilaginous matrix by hESC-derived mesenchymal-like cells [118]. It is also known that mechanical compression enhance chondrogenic differentiation [119]. The efficiency and stability of in vitro and in vivo cartilage formation should be improved further to obtain clinically relevant amounts of cartilage for TE applications [120, 121]. The current evidence, based primarily on large cartilage defects, suggests that BM stimulation procedures and whole tissue transplantation of allografts or autografts can achieve favorable outcomes when used for the management of focal chondral defects of the knee. Cell-based techniques performed with or without biocompatible elastomeric scaffolds have demonstrated early promise in animals, but additional human trials are required to validate the successful clinical outcomes and to save human life.

2.3 Tendon and Ligament Tissue Engineering

Tendon and ligament are connective tissues with closely packed collagen fiber bundles that connect bone to muscle and bone to bone, respectively. Tendon and ligament injuries caused by work- and sports-related activities are about half of the musculoskeletal injuries associated with pain and suboptimal healing [122]. TE offers the possibility of creating functional tissue grafts to treat tendon and ligament injuries without any of the undesirable side effects often associated with reconstructive methods. The lack of immunogenicity of MSCs makes them more suitable for allogeneic implants, and the application of a suitable scaffold containing biological signals could encourage the proliferation and differentiation of these cells. A cyclic mechanical stretch or addition of specific growth factors (e.g., bFGF, insulin-like growth factor IGF) could promote the matrix formation and differentiation of MSCs to fibroblastic cells specific for tendon and ligament lineages [123].

PLGA nanofibers were blend electrospun with bFGF by Sahoo et al. [122] to release the growth factor over a week, so as to mimic the ECM of injured tendon or ligament and to provide a topographical cue and required bioactivity for BM-MSC differentiation to tendon or ligament fibroblasts. The released bFGF activated tyrosine phosphorylation signaling, which proliferated and induced tendon- and ligament-like fibroblastic differentiation, suggesting its potential for tendon and ligament TE. In another study, microporous silk mesh was braided around a silk cord to produce a tightly wound shaft, and in vitro studies showed the expression of collagen types I and III, and tenascin-C gene expression of the MSC differentiated fibroblastic cells [124]. The scaffold was seeded with MSC and implanted into pig model in vivo to regenerate the anterior cruciate ligament [125]. Indirect ligament bone insertion with three zones (bone, Sharpey's fibers, and ligament) and the

production of key ligament-specific ECM components were observed during this study. Gelatin/silk fibroin hybrid scaffold was also used to provide a 3D cell culture environment after co-culturing MSCs with ligament fibroblasts [126]. MSCs showed faster proliferation in the co-culture system, and the specific regulatory signals produced by the fibroblasts were found to enhance the differentiation of MSCs for ligament tissue engineering.

The effects of nanofiber alignment on the differentiation of human tendon stem/progenitor cells (hTSPCs) was studied recently by Yin et al. [127], who fabricated both random and aligned poly-L-lactic acid (PLLA) nanofibers. The tendon-specific genes were highly expressed by hTSPCs grown on aligned nanofibers, while the results of alkaline phosphatase and alizarin red staining showed hindered osteogenesis on aligned substrates compared with that on random fibers. However, in vivo studies showed spindle-shaped cell and tendon-like tissue formation, hinting at the positive influence of topographical control in tendon TE. On the other hand, studies by Qiu et al. [128] suggested the application of hydrogels of oligo[poly(ethylene glycol) fumarate]/acrylated poly(ethylene glycol)-dithiothreitol hydrogels as a carrier for dosed delivery of MSCs for tendon regeneration. A composite PLGA knitted scaffold containing MSC-incorporated alginate gel was implanted in a 1-cm long defect in rabbit tendon. After 13 weeks of implantation, the regenerated tendons were found to possess higher elastic modulus (60%) than naturally healed (40%) tendons and showed vascularization [129]. Exploring the sensitivity of MSCs to gel stiffness, hydrogels functionalized with different substrates (fibronectin or collagen type I) were utilized by Sharma et al. [130] to probe the mitogen activated protein (MAP) kinase activity relative to tendon cell differentiation. These researchers found expression of tenoblast markers on collagen-containing substrates within a narrow range of stiffness. However, osteoblastic differentiations were observed on substrates impregnated with fibronectin, suggesting that the osteogenic differentiation decreased on substrates with low stiffness and ligand density. The above studies show the possibility of development of desirable engineered tendons composed of optimal stem cells and bioengineered scaffolds that could bring a bright future to the healing outcome of tendon and ligament injuries.

2.4 Cardiac Regeneration

The heart is an important organ, which pumps and circulates oxygenated blood through both the central and peripheral circulation of the body, providing nutrition to all the cells of the body. However, with increasing age of the population, together with smoking and alcohol abuse, heart failure and myocardial infarction are becoming the most challenging diseases or threat to the human population. Myocardial infarction occurs due to the interruption of blood supply to heart, resulting in the blockage of the coronary artery, causing the cardiomyocytes to die. Heart failure and myocardial damage has long been considered irreversible because adult cardiac myocytes are terminally differentiated and do not proliferate.

Moreover, cardiac muscle cannot replicate and regenerate by itself after injury [131, 132]. Heart transplantation is a treatment option for end-stage heart failure, but it has endured a slow and somewhat troubled evolution to transform itself into a more validated and useful therapy for patients, mainly because of the limited availability of donor organs and potential complications involved in its use [133]. Stem cell therapy and TE might solve the problem of how to treat thousands of patients who survive myocardial infarction and heart failure.

In native tissue, cells interact with 3D micro- or nanoscaled structures. Cardiomyocytes and fibroblasts disperse in a dense supporting vasculature and collagen-based ECM in the myocardium, producing mechanical contractions to pump blood forward to the body under physiological electrical signals. Scaffolds with appropriate nanostructures are able to control cell mechanics and shapes, which are crucial for cellular functions such as growth, differentiation, migration, and gene expression [134]. The response of cardiomyocytes to micro- and nanostructures has been examined by many researchers and interesting results have been reported. For example, Alperin et al. prepared polyurethane (PU) films coated with ECM proteins such as gelatin, laminin, or collagen type IV for cardiac TE [135].

Artificial cardiac patch is a tissue-engineered approach for the treatment of myocardial infarction, and it serves two functions: cell delivery and mechanical support. Therefore the biomaterials used in this approach should possess suitable nanostructures providing mechanical support and biological cues that promote cell attachment and growth. Chung et al. investigated the effects of silk fibroin/chitosan and silk fibroin/chitosan/hyaluronic acid hybrid patches on cardiomyogenic differentiation of induced rat MSCs, and observed significant improvement in the expression of selected cardiac muscle genes (such as those encoding Tnnt2 and Acta1) and of selected cardiac proteins (such as cardiotin and connexin 43) on the cardiac patches incorporating GAG microspheres [136, 137].

Multiple stem cell types have been identified and reported to have the capability to differentiate into cardiomyocytes, including MSCs, ESCs, ADSCs, hematopoietic stem cells (HSCs) and induced pluripotent stem cells (IPSCs). Although direct injection of stem cell suspensions into the injured heart has produced some promising results, with improved cardiac performance in animal models, this approach remains limited because of the difficulties in cell retention and transplantation survival. It has been reported that almost 90% of the cells were lost or leaked out during the injection, and even the 10% that successfully entered cells had a high death ratio of up to 90% within the first week [138]. Cardiac patches composed of sliced porous acellular bovine pericardium incorporated with multilayered MSC sheets were designed by Sung et al., and in vitro studies showed that MSCs tightly adhered to the fibronectin meshwork and redistributed throughout the scaffold. In vivo results showed that the cells together with neo-muscle fibers and neo-microvessels filled the pores of the scaffolds and the patch became well integrated within the host tissue [139]. Later, these researchers sandwiched ESC sheets into the sliced porous tissue scaffold and found that the mechanical properties of the engineered tissue were significantly increased [140]. Cultured in vitro for weeks, ESCs in the scaffolds were driven to cardiomyogenic lineages, and expressed

cardiac-specific proteins such as α-actinin and connexin 43. Miyahara et al. fabricated ADSC sheets as a cardiac patch to repair scarred myocardium and found newly formed vessels and engraftment of cardiomyocytes within the sheets after implantation; wall thinning in the scar area was reversed and cardiac function was improved [141]. The key challenge in cardiac TE is to create an engineered heart muscle that integrates well with the phenotypically stable cardiac cells and forms a native-like cardiac tissue [142]. The above-mentioned stem cells together with nano-architectured polymeric biomaterials might have the capability to differentiate into cardiomyogenic cells and could be promising alternatives for advanced cardiac TE.

2.4.1 Stem Cell Differentiation to Myocytes on Polymeric Nanomaterials

The functional multipotency of MSCs includes cardiac muscle formation, and several studies have suggested that BM-MSCs could differentiate into cardiomyocytes both in vitro and in vivo. Treatment of MSCs with 5-azacytidine, an analog of cytidine, has been the commonest strategy for inducing cardiac differentiation in vitro. Other strategies and treatments, including the addition of bone morphogenetic protein 2 (BMP-2) and fibroblast growth factor 4 (FGF-4) to the cardiomyogenic differentiation medium containing insulin, dexamethasone, and ascorbic acid, or co-culture using matured cardiomyocytes, have been successfully utilized for differentiation of MSCs to cardiomyocytes. Xiang et al. fabricated an injectable sponge-like material with collagen type I and GAG, and the scaffold was found to be highly porous with an average pore size of 120 ± 18 mm [143]. The material was used as a cell carrier to transplant adult BM-MSCs to the infarct region of rat heart, and results of their study showed that the grafted MSCs survived and even migrated into the heart walls. A substantial amount of neovascularization was observed in the infarct region and within the scaffold itself. This study demonstrated that collagen type I/GAG scaffolds are able to deliver BM-MSCs into the infarct region of the heart with a high survival ratio, further driving the differentiation of MSCs to induce neovascularization. Lee et al. encapsulated hMSCs to RGD peptide-modified alginate microspheres for myocardial repair [144]. Results showed that the modified alginate microspheres could improve MSC attachment and growth, and promote the expression of angiogenic growth factor in vitro. In vivo studies demonstrated that the encapsulation of hMSCs in alginate microbeads significantly increased the cell survival, reduced the infarct size, and enhanced arteriole formation compared with phosphate-buffered saline control or cells alone. Biehl et al. investigated the proliferation of mouse MSC progeny on poly(dimethyl siloxane) membranes with microprojections and they found that the proliferation of mouse MSC progeny was attenuated by 15-μm, but not 5-μm, high microprojections [145].

Guo et al. designed a novel self-assembling peptide (RGDSP) nanofiber as cell carrier to deliver BM-derived cardiac stem cells (CSCs) to repair infarct myocardium [146]. In vitro studies showed that the RGDSP scaffolds supported

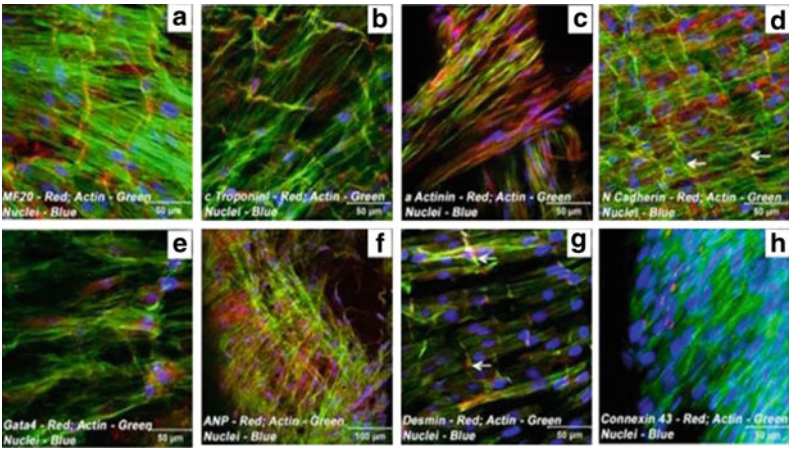

Fig. 6 Localization of key cardiac myocyte phenotypic markers of day 21 ECMs and BMSCs tube co-cultured in basal medium. Images demonstrate the expression of (**a**) sarcomeric myosin heavy chain (MF20), (**b**) cardiac troponin I, (**c**) α-actinin, (**d**) N-cadherin, (**e**) Gata4, (**f**) atrial natriuretic peptide (ANP), (**g**) desmin, and (**h**) connexin 43. Reproduced with permission from Valarmathi et al. [147]

the growth of CSCs and protected them from apoptosis and necrosis caused by anoxia. CSCs in RGDSP scaffolds differentiated into cardiomyocytes and expressed cardiac troponin T and connexin 43, and the viability of the grafted cells was improved during the in vivo studies. The RGDSP nanofibers were able to provide a suitable microenvironment for the survival and differentiation of CSCs, and it further increased the efficacy of CSC transplantation and improved cardiac function. Valarmathi et al. used a different approach by co-culturing rat ventricular embryonic cardiomyocytes and BMSCs on 3D aligned collagen type I fibrous scaffolds [147]. After 21 days, BMSCs were found to differentiate into cardiomyocyte lineages, and expressed transcripts coding for cardiomyocyte phenotypic markers and cardiomyogenic lineage-associated proteins (see Fig. 6). The 3D co-culture system provided a helpful in vitro model to exploit the mechanism of cardiomyogenic differentiation for myocardium regeneration.

Hydrogels, because of their high porosity and capability to exchange nutrients and oxygen with surrounding tissues, often serve as scaffolds for cell growth and delivery. Wang and coworkers prepared an injectable α-cyclodextrin/methoxypoly (ethylene glycol)–polycaprolactone-(dodecanedioic acid)-polycaprolactone–methoxypoly(ethylene glycol) (α-cyclodextrin/MPEG–PCL–MPEG) self-assembled hydrogel with a pore size of 50 μm, and encapsulated BM-MSCs in the supramolecular hydrogel to investigate the efficiency of cell transplantation for cardiac regeneration [148, 149]. The injection of BM-MSCs with hydrogel increased the cell retention and vessel density around the infarct region, and improved the left ventricle ejection fraction compared with the cell injection alone.

Cardiomyogenic differentiation of ESCs is regulated by certain soluble factors and signaling molecules interacting with cardiac-specific transcription factors. BMP-2 plays a central role in the induction of cardiac formation in vertebrate embryos, and expression of BMP-2 is mediated by GATA-4 and Nkx-2.5 [150]. It is also been reported that GATA-4 and Nkx-2.5 are essential for cardiac development. Other growth factors and soluble chemicals, such as FGF, IGF-1, and dimethyl sulfoxide, have also been proven to be involved in heart formation of vertebrates [150]. At the same time, strategies such as co-culture with cardiomyocytes or electrical stimulation can promote ESC differentiation to cardiomyogenic lineages. Akasha et al. used commercial CultiSpher-S microspheres (Percell Biolytica, Sweden) as a cell carrier to deliver ESC-derived cardiomyocytes for heart repair. CultiSpher-S, made from crosslinked porcine gelatin, is composed of macroporous and degradable microbeads with diameters of 130–380 μm. Results showed that the graft cells not only attached on the outer surface, but also invaded the inner pores of the microspheres, and expressed action potentials similar to normal cardiomyocytes [151]. ESC-derived cardiomyocytes cultured on polyurethane (PU) films for 30 days, showed that the cells exhibited preferential attachment and greater beating activity on PU films coated with laminin and collagen type IV [151]. ESC-derived cardiomyocytes also showed significant proliferation on poly(dimethyl siloxane) microprojections and had increased beating rates compared with cells grown on flat substrates [145].

2.4.2 Other Stem Cell Sources Used for Cardiomyogenic Differentiation

ADSCs have been demonstrated to be able to differentiate to cardiomyocytes, and induced pluripotent stem (IPS) cells that overcome the ethical issue of ESCs have opened a new gate for regenerative health care applications including the cardiac repair. Reprogramming the IPS cells for cardiac TE research still remains a huge challenge. For a long time, the heart was considered to be an organ beyond self-repair and regeneration, until the discovery of cardiac stem cells (CSCs) [152]. Compared to other stem cells, CSCs are a logical source of treatment for myocardial regeneration due to their likelihood of being intrinsically programmable to generate cardiac tissue. However, technical difficulties in collecting the cells and low cell numbers upon harvest still remain the main reasons limiting their application in cardiac tissue regeneration.

In summary, there are many stem cell types that have the potential for cardiac repair, but more sophisticated cell culture and TE approaches need to be developed. In addition, the main obstacle influencing cell therapeutic efficacy is the high death rate of donor stem cells after transplantation. Fabrication of biomaterial scaffolds with suitable nanostructure could be a feasible strategy for optimizing stem cell therapy for cardiac regeneration.

2.5 *Neurogenesis*

Nerve injuries occur by a variety of mechanisms, including traumatic wounds, thermal or chemical damage, myelin or axonal degeneration, and acute compression. Injury typically results in the loss of motor and sensory function, or both [153]. Neural diseases represent a very complicated and significant clinical problem; for example, in the USA alone, there are about 250,000–400,000 people living with spinal cord injury and nearly 13,000 additional people suffer spinal cord injuries each year [154]. Peripheral nerve lesions are serious injuries affecting 2.8% of trauma patients annually, leading to lifelong disability [155]. Moreover, each year this number grows by an estimated of 11,000 people in the USA, and in Europe more than 300, 000 cases of peripheral nerve injury are reported annually [156, 157]. Numerous strategies have been applied for the repair of peripheral nerve lesions, with the common goals of directing the regenerating nerve fibers into the distal endoneurial tubes and to improve axonal regeneration and functional recovery [158]. Implantation of autografts, allografts, and xenografts (providing graft from patient, cadavers, and animals, respectively) are some of the strategies applied in this field. Allograft and xenografts have certain disadvantages such as disease transmission and immunogenicity. The other disadvantages of autograft nerve repair systems include the loss of function at the donor nerve graft site and mismatch of damaged nerve and graft dimensions. TE offers promising strategies and provides viable alternatives to surgical procedures for harvested tissues and implants [159]. The fundamental approach in neural TE involves the fabrication of polymeric scaffolds with nerve cells to produce a 3D functional tissue suitable for implantation [160]. There is a growing research interest towards the application of stem cells in nerve TE and regenerative medicine to treat various neurological disorders [154]. Stem cell transplantation using a polymer scaffold might facilitate nerve regeneration more effectively than transplantation of stem cells alone [161].

2.5.1 Mesenchymal Stem Cell Differentiation on Nanostructures for Nerve Regeneration

MSCs obtained from BM are non-hematopoietic progenitor cells with the potential for multilineage differentiation into tissues of ectodermal (neural), endodermal (hepatocytes), and mesenchymal (adipocytes, chondrocytes) origin and have been used in nerve TE. Many researchers have attempted to regenerate nerve tissue by combining scaffolds with MSCs, and it has also been shown that the chemical composition of scaffolds influences the differentiation of MSC to nerve cells. Prabhakaran et al. compared the potential of hMSCs for in vitro neuronal differentiation on poly(L-lactic acid)-*co*-poly(ε-caprolactone)/collagen (PLCL/collagen) and PLCL nanofibrous scaffolds. Figure 7 shows SEM images of the morphology of MSC differentiated neuronal cells (Fig. 7a) and their expression of proteins, neurofilament (NF200), and nestin on the electrospun PLCL/collagen scaffolds.

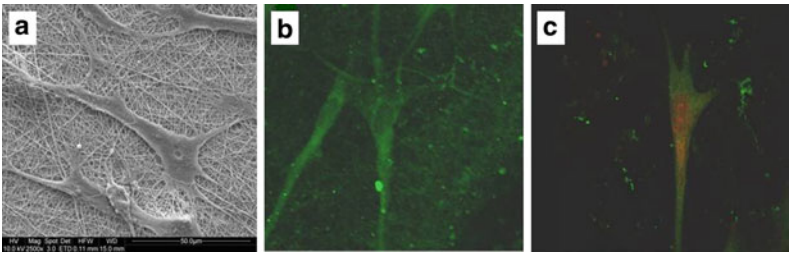

Fig. 7 MSC differentiated neuronal cells on electrospun PLCL/collagen scaffolds: (**a**) cell morphology by SEM, (**b**) expression of neurofilament protein, and (**c**) expression of nestin. Reproduced (Fig7b, 7c) with permission from Prabhakaran et al. [162]

Their findings showed that PLCL/collagen nanofibrous scaffolds serve as better substrates for differentiation of MSCs to nerve cells than the PLCL scaffolds [162]. In yet another study, Wang et al. synthesized terpolyesters of 3-hydroxybutyrate, 3-hydroxyvalerate and 3-hydroxyhexanoate (PHBVHHx), and compared them with PLA and copolyester of 3-hydroxybutyrate and 3-hydroxyhexanoate (PHBHHx) scaffolds for their respective functions in the differentiation of hMSCs to nerve cells. Their results showed that PHBVHHx films had better adhesion, proliferation, and differentiation of MSCs compared to PLA, thereby demonstrating the effect of substrate composition on MSC differentiation. The influence of pore size on the differentiation of MSCs to nerve cells was explained by these researchers, whereby a smaller scaffold pore size was found to increase the differentiation of MSCs to nerve cells [163]. On the other hand, Kuo et al. functionalized PLGA/chitosan scaffolds with collagen type I and investigated the differentiation of MSCs to nerve cells seeded on the scaffold. Their results showed that functionalized PLGA/chitosan/collagen scaffolds are promising substrates for differentiation of MSCs compared to PLGA/chitosan scaffolds [164]. Cho et al. immobilized nerve growth factor (NGF) on the surface of aligned PCL/PCL–PEG nanofibrous scaffolds and compared the neuronal differentiation of MSCs on NGF-immobilized nanofibrous scaffolds and physically adsorbed NGF nanofibrous scaffolds. Their results revealed higher expression of the neuronal cell marker proteins on NGF-immobilized nanofibrous scaffolds compared to its expression on physically adsorbed NGF scaffolds. Furthermore, the alignment of nanofibrous scaffolds also increased the expression levels of neuronal makers compared to random nanofibrous scaffolds, indicating that the NGF-conjugated aligned nanofibrous scaffolds significantly increased the neuronal differentiation of MSCs due to synergetic nanotopographical cues and the presence of NGF [165]. Wang et al. fabricated an injectable biodegradable hydrogel by blending gelatin and 3,4-hydroxyphenylpropionic acid and showed that the rate of MSC proliferation and neurogenesis of MSCs increased with a decrease in the stiffness of the hydrogel [166].

Substrate topographical patterns have also been shown to affect the differentiation of MSCs to nerve cells. Yim et al. investigated the differentiation and proliferation of hMSCs on nanogratings of 350 nm width and found alignment and elongation of

cytoskeleton and nuclei of MSCs along the nanogratings with higher expression of neuronal markers on patterned surfaces compared to unpatterned and micropatterned controls. Their results also showed that the combination of nanotopography and biochemical cues (retinoic acid) caused upregulation of neuronal protein expressions, but that nanotopography showed a stronger influence than retinoic acid alone [167].

Despite the capacity for spontaneous axonal regeneration, recovery after severe peripheral nerve injury remains variable and often very poor [168]. MSCs have been shown to have an important regenerative potential after transplantation into the stumps of transected sciatic nerves. Lopes et al. evaluated the regeneration of peripheral nerve using a tubular nerve guide of resorbable collagen filled with MSCs. Their results showed that the biodegradable collagen tube filled with MSCs induced better regeneration of peripheral nerve fibers across a nerve gap than did collagen tube without cells [169]. Oliveira et al. fabricated PCL conduits for regeneration of transected mouse median nerves and investigated the effect of MSCs on nerve regeneration by seeding MSCs on PCL nerve conduit before grafting of PCL conduits. The animals treated with MSCs had a significantly larger number of myelinated and unmyelinated nerve fibers and blood vessels compared to the control group (PCL conduit alone), indicating the possibility of improving regeneration and function of median nerve after a traumatic lesion [170]. Hou et al. differentiated MSCs into cells expressing characteristic markers of Schwann cells, and used PLGA nerve conduit along with differentiated MSCs for bridging a 10-mm-long sciatic nerve defect [171]. Ao et al. fabricated chitosan conduits and seeded them with MSC-derived Schwann cells and used the conduit to bridge the critical gap length of 12 mm in sciatic nerves of adult rats. Their results showed significantly higher axonal re-growth and re-myelination in nerves bridged with MSC-derived Schwann cells than in those bridged with cell-deprived conduits [172].

2.5.2 Embryonic and Adipose-Derived Stem Cells on Nanopatterns for Nerve Regeneration

Considerable attention has been given to the potential of ESC or their derivatives for the repair of nerve injury [173]. Willerth et al. incorporated neurotrophin 3 (NT-3) and platelet-derived growth factor (PDGF) into fibrin scaffolds and investigated the differentiation of ESCs into mature neural phenotypes, specifically neurons and oligodendrocytes. Their results showed that the controlled delivery of NT-3 and PDGF simultaneously increased the fraction of neural progenitors, neurons, and oligodendrocytes while decreasing the fraction of astrocytes compared to ESC seeded with unmodified fibrin scaffolds [174]. Chao et al. grafted poly (acrylic acid) thin films with carbon nanotubes (CNTs) and investigated the neural differentiation of hESC. Their results revealed higher neuronal differentiation potential of ESC on CNT-grafted poly(acrylic acid) compared to un-grafted poly (acrylic acid) thin films [175]. Carlberg et al. evaluated in vitro neuronal differentiation of hESC on electrospun PU nanofibrous scaffolds and demonstrated that

physical cues induced by the nanofibrous scaffolds induced stem cell differentiation to neuronal cells [176]. Xie et al. compared the differentiation of ESC seeded on electrospun random and uniaxially aligned PCL nanofibers. Their results demonstrated that aligned nanofibrous substrates discouraged the differentiation of ESC to astrocytes, which is very desirable in therapies targeting spinal cord injuries, as they may limit possible glia scar formation. They also demonstrated that the aligned electrospun fibers could guide the neurite outgrowth generated by the differentiated neurons [173]. Lee et al. constructed nanoscale ridge/groove pattern arrays using UV-assisted capillary force lithography on polyurethane acrylate (PUA) and showed that the nanoscale ridge/groove pattern arrays can rapidly and efficiently induce the differentiation of hESC into neuronal lineages, even in the absence of differentiation-inducing agents [177]. Figure 8 shows the expression of human neuronal proteins C and D (HuC/D, an RNA-binding protein), microtubule-associated protein 2 (MAP2, marker of mature neuronal cells) and glial fibrillary acidic protein (GFAP, marker of intermediate filament proteins of mature astrocytes) on patterned PUA films. Cells were co-immunolabeled with class III β-tubulin (Tuj1, neuronal cell marker) and 4,6-diamidino-2-phenylindole (DAPI, DNA marker). As can be seen in Fig. 8c,f no staining for GFAP was observed, indicating ESC differentiation into mature neurons without differentiation into a glial lineage such as astrocytes [177].

ADSCs are a clinically applicable source for cell therapy. Tse et al. fabricated a PCL/PLA scaffold using the solvent-cast method, and the scaffolds were found to

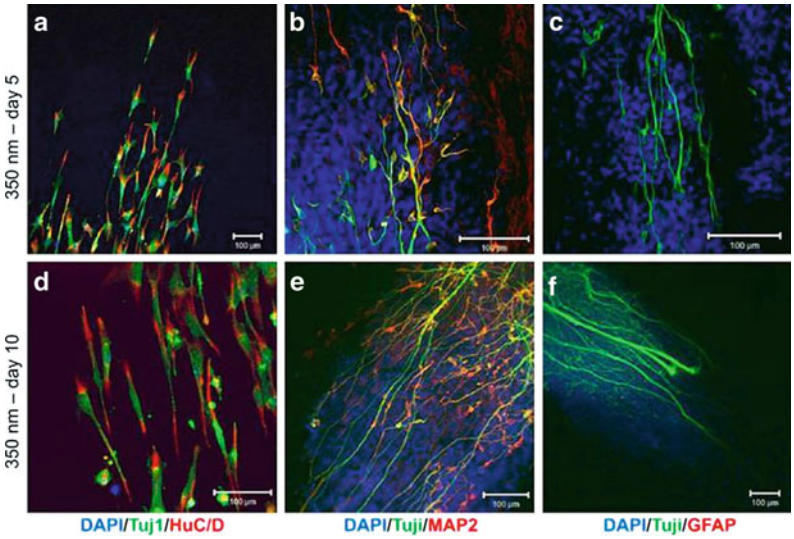

Fig. 8 Immunofluorescence staining of hESCs with neural and glial markers. (**a**, **d**) hESCs were immunolabeled for DAPI, Tuj1, and HuC/D. (**b**, **e**) hESCs immunolabeled for DAPI, Tuj1, and MAP2. (**c**, **f**) hESCs immunolabeled for DAPI, Tuj1, and GFAP. hESCs were cultured for 5 days (**a**–**c**) and 10 days (**d**–**f**) on 350-nm ridge/groove pattern arrays on PUA films. Reproduced with permission from Lee et al. [177]

support the differentiation of ADSCs into a Schwann-cell-like phenotype. A higher neurite length of dorsal root ganglion (DRG) by co-culturing with ADSCs was observed by these researchers, suggesting the possibility of enhancing nerve regeneration by seeding the nerve conduit with ADSCs before transplantation of nerve conduit to the site of nerve injury [178].

2.5.3 Alternative Stem Cells on Nanostructures for Nerve Regeneration

Neural stem cells (NSCs) are known to have both self-proliferation potential and multiple differentiation potential and can differentiate into various cell types such as neurons, astrocytes, oligodendrocytes, and Schwann-cell-like supportive cells when exposed to different conditions. Recently, NSCs has been used experimentally for both spinal cord and peripheral nerve injuries as a transplantation source with promising results [179]. NSCs exist not only in the developing mammalian nervous system but also in the adult nervous system of all mammalian organisms, including humans [180]. Dental pulp and peridontium have also been shown to be sources of NSCs, as well as olfactory mucosa, which are readily harvested by nasal biopsy [181]. With an ultimate goal of replacing damaged or injured neural tissues, introduction of NSCs to the site of neural tissue damage has been proposed due to their regenerative and neuroprotective potency [182]. Aijun et al. cultured NSCs on chitosan film and their results revealed that the NSCs proliferated on chitosan films and differentiated into neuron-like cells after 4 days of culture [179]. Bini et al. evaluated the suitability of biodegradable PLGA scaffolds for the differentiation of NSCs to nerve cells [183]. Numerous natural and synthetic polymers have been used as scaffolds for peripheral and central nerve regeneration in vitro or in vivo and these studies showed that the chemical nature of the substrates have a significant effect on both proliferation and differentiation of NSCs to nerve cells [184–186].

Functionalizing biomaterials with bioactive molecules such as ECM-derived cell adhesive molecules to impregnate guiding cues on the scaffolds is an emerging research interest and can provide an instructive extracellular microenvironment for NSCs [182, 187–189]. Yang et al. fabricated aligned PLLA nano- and microfibrous scaffolds to investigate the effect of nanofiber diameter on NSC proliferation and differentiation. Their findings showed a higher differentiation rate of NSCs on nanofibers than on microfibers, regardless of the fiber alignment [190]. He et al. fabricated aligned PLLA fibers by electrospinning and showed variable differentiations of NSCs depending on their fiber diameter and alignment. They reported longer neuritis on aligned fibers than on random fibers within the same diameter range [191]. Christopherson et al. cultured rat NSCs on electrospun polyethersulfone (PES) fibers with varying fiber diameters and demonstrated that the fiber diameter of PES fibers significantly influenced NSC differentiation. However, under the differentiation conditions, NSCs showed a 40% increase in oligodendrocyte differentiation on small diameter (283 nm) fibers and 20% increase in neuronal differentiation on high diameter (749 nm) fibers, in comparison to tissue culture on

polystyrene surfaces [192]. Results of our studies using PCL/gelatin nanofibers showed that the biocomposite PCL/gelatin (70:30) nanofibrous scaffolds enhanced NSC proliferation and differentiation compared to PCL nanofibrous scaffolds, and acted as a positive cue to support neurite outgrowth. Our results also showed that the direction of nerve cell elongation and neurite outgrowth on aligned nanofibrous scaffolds was parallel to the direction of orientation of the nanofibers [193]. Studies using conductive nanofibrous scaffolds of polyaniline/PCL/gelatin and applied electrical stimulation showed significantly enhanced cell differentiation and neurite outgrowth on stimulated scaffolds than on the non-stimulated scaffolds [194]. Lim et al. investigated the effect of aligned topography of substrates on cell morphology and neuronal differentiation. Their results showed that NSCs elongated along the major fiber axis on aligned fibers and that a higher fraction of cells on aligned fibers exhibited neuronal differentiation markers compared with cells on random fibers [195].

Topographical surface patterns such as steps, grooves, pillars, and pits (or pores) have been demonstrated to affect cell morphology, differentiation, motility, and function [196]. Hsu et al. fabricated micropatterned PLA conduits by microlithography and solvent-casting, and seeded the conduit with adult mouse NSCs for peripheral nerve regeneration. Their results showed that the seeded NSCs aligned on the micropatterned conduits and actin microfilaments oriented along the micropatterrned PLA scaffolds, whereas such defined orientations were not observed on non-patterned PLA substrates. The micropatterned surface facilitated the synthesis of neurotrophic factors and higher gene expression of NSCs [197]. Recknor et al. micropatterned polystyrene substrates and seeded them with NSCs. The micropatterned surfaces exhibited more than 75% cell alignment along the groove direction and enhanced neuronal differentiation and neurite alignment on topographically different regions of the same substrate [198]. In conclusion, nanostructures can be effective for the differentiation of stem cells to nerve cells due to their major role as a biomimetic interface between the scaffolds and cells in nerve TE.

2.6 Skin Tissue Engineering

Skin, the largest organ in the body, has a surface area of about 1.8 m^2 and occupies 8% of the total body mass of an adult. Skin comprises two layers: the outer protective epidermis and the inner corium or dermis, which provides the mechanical stability for skin and includes several important structures, such as the blood and lymph vessels, nerves, and appendages [199]. The most common skin injuries or skin wounds are categorized on the basis of the depth of the skin injury: epidermal or full-thickness skin wound. Skin can regenerate itself from minor epidermal injury; however, when the injury is a full-thickness skin wound (loss of both epidermis and dermis), the damaged skin cannot regenerate spontaneously [200]. Natural repair of wound healing is slow compared with the rapid wound cover needed to reduce

infection. At present, the use of cultured keratinocytes is limited by the length of time needed to grow epithelial sheets in vitro, during which time the patient is susceptible to infection. The epithelial sheets are also extremely fragile and do not adhere well to burned surfaces [201]. Stem cells can accelerate the re-epithelialization of skin wounds and bring the possibility of skin appendage regeneration.

2.6.1 Mesenchymal Stem Cells for Skin Regeneration

Stem cells are thought to be a powerful tool for treatment of a wide spectrum of diseases that are ineffectively treated by traditional approaches [201]. BM-derived stem cells have been shown to differentiate into epithelial cells of the liver, lungs, gastrointestinal tract and skin [202]. Systemic transplantation and local implantation of MSCs are promising treatment methods for skin wounds, especially for chronic wounds [203, 204]. The mechanisms by which BM-MSCs participate in cutaneous wound healing is by either differentiating into phenotypes of various damaged cells [205] or by enhancing the repair process by creating a microenvironment that promotes the local regeneration of cells endogenous to the tissue [206]. Wu et al. proved that BM-MSC-treated wounds exhibited significantly accelerated wound closure with increased re-epithelialization, cellularity, and angiogenesis [207]. Paunescu et al. examined the capability of human BM-MSCs to differentiate in vitro to functional epithelial-like cells [208]. To induce epithelial differentiation, they cultured MSCs using EGF, keratinocyte growth factor (KGF), HGF, and IGF-II. Their results demonstrated that hMSCs isolated from human BM can differentiate into epithelial-like cells and may serve as a cell source for TE and cell therapy of epithelial tissue.

Electrospun nanofibrous scaffolds can be prepared with high degree of control over their structure to create highly porous meshes of ultrafine fibers that resemble the ECM topography. The scaffolds are amenable to various functional modifications targeted towards enhancing stem cell survival and proliferation, directing their fates and promoting tissue organization [12]. Kobayashi and Spector investigated the clinical effects of mechanical stress on the behavior of BM-MSCs in a collagen type I/GAG scaffold matrix for 1 week under cyclic stretch loading conditions [209]. Their results suggested that mechanical stress may affect the proliferation and differentiation of stem cells and, subsequently, the wound healing process, via interactions between the stem cells and scaffold matrix. Delivery of growth factors and chemicals can also be mediated using degradable particles, entrapped within the biomaterial scaffold, which could provide temporal release kinetics for signaling biomolecules over a prolonged period of time [210–213]. Fan et al. [126] studied the differentiation of MSCs towards fibroblasts on silk-cable-reinforced gelatin/silk fibroin hybrid scaffold. Their results demonstrated that MSCs were distributed uniformly throughout the scaffold and showed good viability. MSCs in a co-culture system were proved to differentiate into ligament fibroblasts by synthesis of key ligament ECM components. Recently, Rustad et al. studied the effect of hydrogel microenvironment on stem cells in wound healing [214]. They placed

BM-MSC seeded hydrogels within wild-type excisional wounds and the results demonstrated that hydrogel delivery of MSCs improved cell survival following implantation compared to local injection, and that MSC-seeded hydrogels accelerated normal wound healing and are promising cell-scaffold constructs for skin regeneration.

2.6.2 Embryonic Stem Cells for Skin Regeneration

ESCs can potentially maintain a normal karyotype infinitely on culture in vitro and can differentiate into any cell type under appropriate conditions. The differentiation potential of murine ESCs into keratinocytes has been studied in vitro and results show that mouse ESC-derived keratinocyte-like cells expressed cytokeratin, keratin 14, and keartin18 (proposed markers for epidermal keratinocytes) [215]. The ability of hESC differentiation to epithelial lineages has also been studied in recent decades. Dabelsteen and Iuchi et al. found that hESCs can differentiate into keratinocyte-like cells expressing p63, K14, and involucrin [216, 217]. However, hESC-derived keratinocytes are different from postnatal keratinocytes due to their much lower proliferative potential in culture. The frequency with which they terminally differentiate was reduced compared with keratinocytes cultured from postnatal human epidermis. In contrast to previous results, Hind et al. showed that hESC-derived keratinocytes gave rise to packed and cohesive colonies and had an astounding proliferative potential [218]. They assessed the capability of the keratinocyte progeny of hESCs differentiating into full-functional keratinocytes and found that hESCs generate a homogeneous population of epithelial cells expressing keratins 5/14, keratin 10, involucrin, filaggrin, integrins α6/β4, collagen type VII, and laminin 5 at levels similar to those expressed by postnatal keratinocytes of squamous epithelia. Human ESC-derived keratinocytes were similar to human keratinocytes, both phenotypically and functionally. After 10 days of air–liquid differentiation, haematoxylin-eosin staining of the cryosection of organotypic hESC-derived keratinocyte cultures showed a pluristratified epithelium with a basal layer, stratum spinosum, stratum granulosum containing keratohyalin granules, and stratum corneum as superposed layers of dead squamous enucleated cells (Fig. 9).

2.6.3 Stem Cell–Scaffold Constructs for Skin Tissue Engineering

Altman et al. utilized a composite silk fibroin/chitosan scaffold for seeding and in vivo delivery of human ADSCs in a murine cutaneous wound model, and the delivery technique conferred physiological benefits to accelerated wound closure. ADSC seeded on a silk fibroin/chitosan scaffold differentiated into fibrovascular, endothelial, and epithelial components of restored tissue and enhanced the wound healing process [219].

Human umbilical cord blood (HUCB) stem cells have also been studied for skin TE. Kamolz et al. evaluated the potential of using HUCB stem cells to differentiate

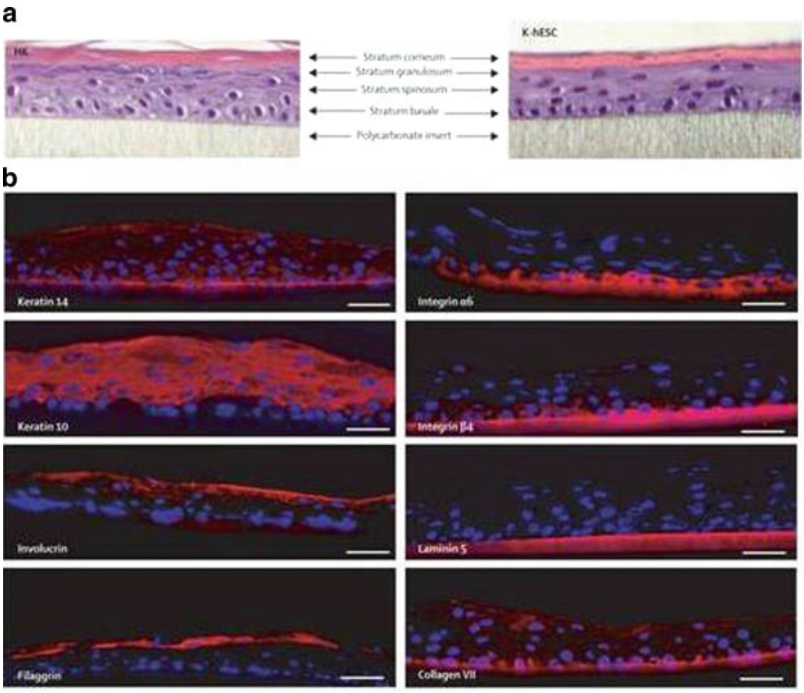

Fig. 9 Reconstruction of a pluristratified epidermis with keratinocytes derived from human embryonic stem cells (K-hESCs; SA01 cell line): (**a**) Haematoxylin-eosin staining of organotypic cultures of human primary keratinocytes (*left*) and K-hESCs (*right*). (**b**) Immunofluorescence analysis of the expression and localization of keratin 14, keratin 10, involucrin, filaggrin, integrin α6, integrin β4, laminin 5, and collagen type VII in the K-hESC organotypic epidermis. Scale bars: 50 μm. Reproduced with permission from Hind et al. [218]

into epithelial cells in vitro. Their results demonstrated three to four layers of regular epithelial sheet formation. The HUCB stem cells differentiated into epithelial cells under in vitro conditions, thereby serving as a starting material for isolation and expansion of cells for transplantation in patients with large skin defects [220]. Schneider et al. analyzed the possible epidermal differentiation of HUCB stem cells on dermal equivalents (DEs) consisting of collagen types I and III with dermal fibroblasts under culture conditions [221]. HUCB stem cells were further modified by pretreating the cells with 5-azacytidine or by supplementing the media with all-*trans*-retinoic acid. These researchers also demonstrated that HUCB stem cells can differentiate into myofibroblasts. Studies by Luo et al. showed that isolated HUCB stem cells could enhance the healing of mice skin defect wounds, and it was found that the implanted HUCB stem cells could differentiate into keratinocytes in the wound tissue [222]. Cultured ADSCs can also contribute to wound healing and can be added to engineer a trilayered skin substitute featuring the skin's deepest layer, the adipocyte-containing hypodermis, along with both dermis and epidermis [223]. ADSCs secrete various growth factors

that control and regenerate damaged skin-type cells, a function that has been termed essential for the regenerative mechanisms of ADSCs [224].

Salem et al. seeded pancreatic stem cells (PSCs) on "matriderm" and used the matrix to replace the bilateral full-thickness skin wounds made on the dorsum of Nu/Nu nude mice [225]. The vascularization rate showed a significant increase in the PSC-seeded scaffolds, and the morphology and immunohistochemistry showed new skin-like structures positive to epidermal markers of healing wound. The combined use of PSCs with matriderm as a matrix for dermal regeneration increased the epidermalization, vascularization, and healing of full-thickness wounds. Cell microenvironment is known to play a significant role in determining the progenitor cell function [226]. Different kinds of stem cells have been applied for wound healing using a biomimetic "stem cell niche" and most of the studies showed promising results. With further investigations towards understanding the mechanism of micro- and nanoenvironmental effects on stem cell behavior, we expect that the stem cell/biomaterial niche will play a vital role in advanced skin regeneration in the near future.

2.7 Other Tissue Regeneration Using Stem Cells and Nanomaterials

2.7.1 Vascular Regeneration

Cardiovascular disease is a leading cause of morbidity and mortality worldwide, with approximately 34% of all deaths in the USA being due to cardiovascular disease. Autologous grafts including the radial artery bypass grafts and saphenous vein are important therapeutic options for treatment of coronary artery disease. Tissue-engineered vascular grafts should mimic the native ECM structure and serve as a bridge to guide the cell-mediated remodeling of vascular tissue. Such tissue-engineered blood vessels should be non-immunogenic and non-thrombogenic to allow for high blood flow rate, with viscoelastic properties similar to the native vessel [227]. Wang et al. attempted to engineer a suitable blood vessel by differentiating ADSCs to smooth muscle cells (SMCs) through stimulation with BMP4 and TGF-β [228] and seeding the cells on poly(glycolic acid) (PGA) mesh. Subjected to pulsative stimulation for an 8 week period, the vessels showed high amounts of collagen deposition, similar to that of native vessels during this study. $Flk1^+$ (positive for VEGF receptor 2) progenitors derived from mouse ESCs differentiated to endothelial cells (ECs) on collagen type IV micropatterns immobilized with VEGF, while differentiation to SMCs was preferentially attained on unseeded collagen type IV scaffolds [229]. This phenomenon was also similar to that described for MSC differentiation on patterned surfaces with regard to the geometric and tractional mechanisms. Enhancing endothelial differentiation with VEGF might provide a suitable platform for vascular TE [230]. Collagen gel containing hESC-derived ECs were implanted into infarcted rat hearts in vivo by Nourse et al.

and robust networks of patient vessels filled with host blood cells were observed [230].

Blood vessel engineering using electrospun nanofibers might benefit from their mechanical strength, good cell attachment properties for endothelium formation, and directional alignment of SMCs along aligned nanofibers. Ex vivo stem cell expansion was carried out on nanofibers and the expanded cells then modified using pro-angiogenic growth factors to induce differentiation to ECs and SMCs and enhance vasculogenesis. Das et al. [231] demonstrated this method as a potential stem cell/biomaterial-based therapy for ischemic disease. Other factors such as the application of shear stress, bioreactors, and growth or biological factors might also assist in the development of a suitable blood vessel substitute with good mechanical strength.

3 Comments and Future Perspectives

Despite several advances in the field of stem cell research, the availability of stem cells remains a huge challenge for clinicians and scientists in the field of regenerative medicine [232]. The heterogeneity and differences between various tissue sources of MSCs and the factors that can influence MSCs to differentiate completely to a desired lineage all require further elucidation. ESCs encounter a range of ethical and legislative problems that differ from country to country [233]. Skepticism in terms of cell–cell fusion rather than stem cell plasticity has also been raised and further studies are required to explore and fully understand the mechanism of stem cell differentiation and stem cell behavior on nanostructures [234, 235]. The concerted efforts of engineers, biologists, and clinicians might advance the field of tissue regeneration and improve the quality of a patient's life using a synergistic stem cell/biomaterial approach.

Stem cell migration and differentiation in response to different nanotopographies can help in the engineering of materials to resemble the structural continuum of ECM. Specific scaffold properties affect cell function differently and hence "designer scaffolds" for precise tissue regeneration should be created and can offer the great potential of stem cell/nanotechnology to help advance the field of tissue regeneration. Approaches to the incorporation of nanopatterns on scaffold surfaces could be useful for guided tissue regeneration via the influence of exogenous or endogenous stem cells. Design of delivery systems or nanocarriers for the release of biomolecules into the nuclear compartments could be effective for the differentiation of stem cells. The fate of stem cells can be decided using novel engineering of stem cell technologies, with the aim of advancing their therapeutic use for human health care. The differentiation of stem cells can also be influenced by chemical signals such as cytokines, hormones, or other soluble factors within the nanomaterials and by physical and mechanical cues such as stimulation of receptors, cell stretch through contact with mechanically stimulated nanostructures, and electrical signals. The interdisciplinary field of stem cell and TE creates a platform for engineers, scientists, and clinicians to work together and advance the field of regenerative medicine.

4 Conclusion

Stem cell applications for tissue regeneration, especially on nanoengineered surfaces and structures, are still in the early stages of development. MSCs for bone repair on nanomaterials remains the most advanced field compared to others with respect to its potential for clinical application. This review describes the response of stem cells to topographical structures, molecules, and modifications in vitro, and the scientific information could be employed to regulate stem cell differentiations in vivo. Topographically modified materials might enhance the differentiation potential of stem cell populations, with crucial implications in tissue repair and clinical translations. Challenging achievements in the field of stem cell/nanomaterials for advances in tissue regeneration can certainly be expected in the next decade.

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