

## Control of stem cell fate by engineering their micro and nanoenvironment

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is being challenged by the advances in biomaterial technology, with evidence highlighting that material properties are capable of driving stem cell fate. Materials are being designed to mimic the clues stem cells receive in their *in vivo* stem cell niche including topographical and chemical instructions. Nanotopographical clues that mimic the extracellular matrix (ECM) *in vivo* have shown to regulate stem cell differentiation. The delivery of ECM components on biomaterials in the form of short peptides sequences has also proved successful in directing stem cell lineage. Growth factors responsible for controlling stem cell fate *in vivo* have also been delivered *via* biomaterials to provide clues to determine stem cell differentiation. An alternative approach to guide stem cells fate is to provide genetic clues including delivering DNA plasmids and small interfering RNAs *via* scaffolds. This review, aims to provide an overview of the topographical, chemical and molecular clues that biomaterials can provide to guide stem cell fate. The promising features and challenges of such approaches will be highlighted, to provide directions for future advancements in this exciting area of stem cell translation for regenerative medicine.

**Key words:** Biomaterials; Scaffold; Stem cells; Surface topography; Tissue engineering

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**Core tip:** Stem cells receive instructions from their niche environment, which guide their survival and phenotype. Stem cells receive physical and biochemical clues from their extracellular matrix where they reside *in vivo*. This paper will discuss the utilization of biomaterial surface topography and elasticity and delivery of chemical and genetic clues *via* scaffold materials to mimic the extracellular matrix to guide stem cell fate. The understanding of the parameters that guide stem cell differentiation is of great interest for the tissue-engineering field.

### Abstract

Stem cells are capable of long-term self-renewal and differentiation into specialised cell types, making them an ideal candidate for a cell source for regenerative medicine. The control of stem cell fate has become a major area of interest in the field of regenerative medicine and therapeutic intervention. Conventional methods of chemically inducing stem cells into specific lineages

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## INTRODUCTION

Stem cells have the ability to differentiate into several tissue types and have captured a great interest for regenerative medicine due to their ability to regenerate and repair injured tissues<sup>[1]</sup>. Stem cells are broadly classified into embryonic stem cells (ESCs) and adult stem cells (ASCs). ESCs are pluripotent stem cells capable of regenerating into cells types from all three germ layers (ectoderm, mesoderm and endoderm), being themselves derived from the inner cell mass of blastocysts<sup>[2]</sup>. ESCs have a long term self-renewal capacity, ability to expanded for extended culture time and multi-lineage differentiation potential making them a very attractive tissue-engineering cell resource for regenerative medicine<sup>[2]</sup>. However, efficient and safe isolation and differentiation protocols are required for their optimal clinical translation<sup>[3]</sup>. In addition, ESCs have shown to differentiate into tumour cells, limiting their translation to clinical trials<sup>[4]</sup>. The second type of stem cells that have attracted extensive research interest is ASCs. These cells are multi-potent cells derived from adult somatic tissues with the potential to differentiate into many specific cell types<sup>[5]</sup>. Mesenchymal stem cells (MSCs) are the commonest type of ASCs investigated for tissue-engineered applications, which are found to be isolated from several tissue sources such as the bone marrow and adipose tissue<sup>[6-8]</sup>. Controlling both the proliferative and differentiation abilities of stem cells has been the focus of utilizing stem cells for a therapeutic platform<sup>[9]</sup>. In their *in vitro* environment stem cell fate is determined by a reservoir of biochemical and biophysical clues<sup>[6]</sup>. The instructions stem cells receive from their stem cell niche will control their stem-ness, multi-potency and determine their phenotype of differentiation<sup>[6]</sup>.

Stem cells have the ability to differentiate spontaneously *in vitro* but this is uncontrolled and inefficient for tissue-engineering applications<sup>[10]</sup>. Therefore, over the last decade it has become important to understand how to control stem cell fate effectively for regenerative medicine<sup>[10]</sup>. A common approach to determine stem cell fate *in vitro* is by adding medium growth components including chemokines and hormones, to differentiate stem cells into a particular lineage<sup>[7-9]</sup>. Chemical patterning has shown to be very successful in the differentiation of ASCs down osteogenic, chondrogenic and adipogenic lineages<sup>[7-9]</sup>. Whilst understanding chemical clues of stem cell fate is important, with the advances in knowledge that stem cells are highly sensitive to their topography, stiffness and molecular environment, it may only be part of the strategy for controlling stem cell fate for regenerative medicine<sup>[10]</sup>. The extracellular matrix (ECM) in the *in vitro* stem cell niche is arranged into complex topographic features,

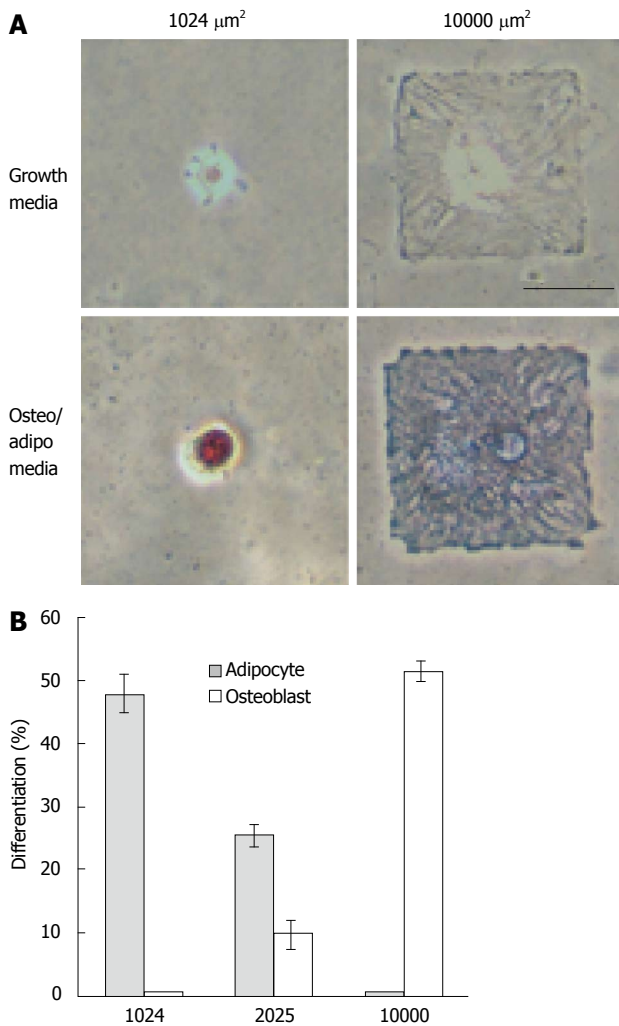
which stem cells have shown to be sensitive to and provide clues to guide their phenotype<sup>[11]</sup>. This evidence has prompted materials to be manufactured with surface topographical clues, which has shown to greatly influence cell behavior altering cell morphology, adhesion, motility proliferation and gene regulation<sup>[12]</sup>. The *in vitro* mechanical clues from the ECM (stress, shear and strain) have also shown to play a part in the precise control of stem cell fate<sup>[13]</sup>. With this knowledge material substrate stiffness is now taken into consideration to guide stem cell fate<sup>[14]</sup>. As biochemical clues provide important instructions to stem cells *in vitro* growth factors have been immobilised onto material surfaces to guide stem cell behaviour<sup>[15,16]</sup>. Further understanding of these environmental instructions on stem cell differentiation is emerging, with the hope to be able to better control stem cell fate for tissue-engineering purposes.

This unique review, aims to provide an update on the three main biomaterial parameters that are being explored to govern stem cell fate, with a focus on stem cell differentiation. The parameters discussed in this review will be the effect of topographical, chemical and genetic clues on guiding stem cell differentiation. In this review, both ASCs and ESCs will be considered to provide an overview of the different parameters that are being investigated to determine stem cell differentiation. The emphasis of discussion will be placed on the techniques used to govern stem cell fate rather than exploring their effect on different stem cell types. This review aims to highlight the successful strategies to control stem cells fate for regenerative medicine applications.

## EFFECT OF STEM CELL FATE AND SURFACE TOPOGRAPHICAL CLUES

In their natural niche environment, cells are interacting with various nanotopographically sized clues<sup>[17]</sup>. Cells will encounter different topographies sized clues *in vivo* from macro- (bone and ligament) to micro- (other cells) and nanotopography (proteins and ligands), all of which will influence cell behavior and consequently cell fate<sup>[17]</sup>. The most important is the basement membrane, a vital structure of the ECM, which provides tissue support and organization<sup>[17]</sup>. The basement membrane contains pores, ridges and fibers, which are all in the nanometer range<sup>[17]</sup>.

The use of topographically patterned surfaces for culturing cells has a very important advantage over chemical induction of stem cells, the disregard to using chemicals in the body yet still tailor stem cell for particular clinical applications. There is now growing evidence that topographical clues alone can produce the same effect as chemical induction<sup>[18]</sup>. Extensive research has been attempted to understand the interaction of cells with their substrate topography using *in vitro* studies<sup>[18]</sup>. With the advancement in micro and nanofabrication techniques, numerous studies have been able to be carried out to understand the effect of nanotopography on a wide range of materials. Patterning techniques such as soft lithography, photolithography, electrospinning, layer-by-



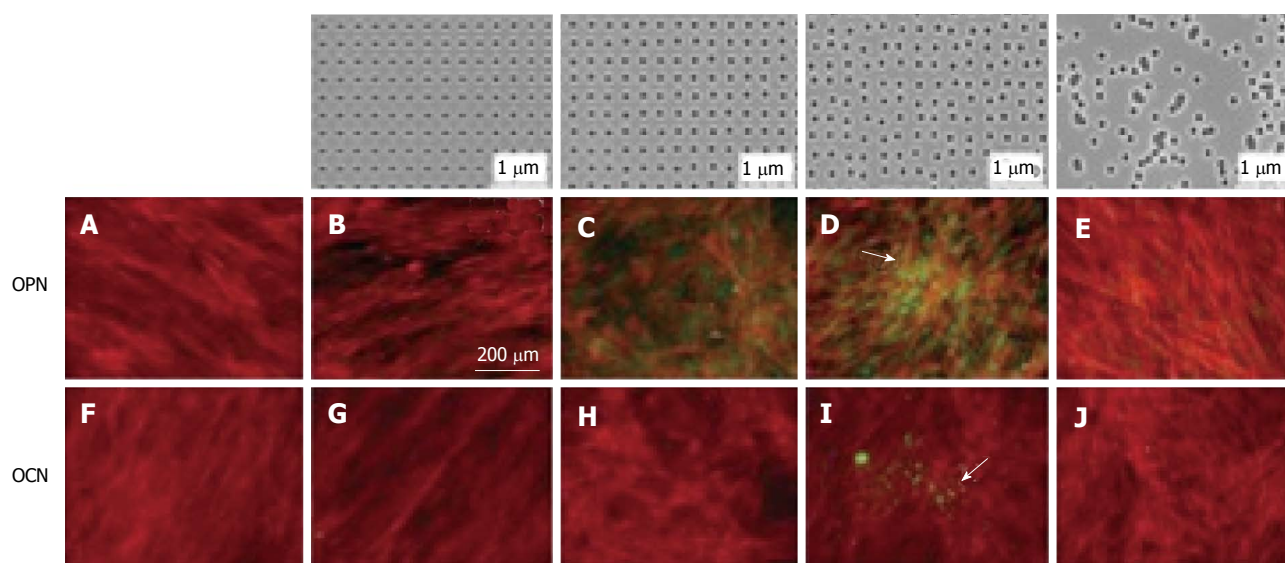
**Figure 1** The effect of nanotopographies on stem cell shape and its consequential effect on the differentiation of stem cells. A: Brightfield images of human mesenchymal stem cells (hMSCs) plated onto small (1024  $\mu\text{m}^2$ ) or large (10000  $\mu\text{m}^2$ ) fibronectin islands after 1 wk in growth or mixed media. Note hMSCs plated on large islands showed differentiation for osteogenic differentiation and those on small islands showed differentiation for adipogenic differentiation. Lipids stain red; alkaline phosphatase stains blue. Scale bar = 50  $\mu\text{m}$ ; B: Percentage differentiation of hMSCs plated onto 1024, 2025, or 10000  $\mu\text{m}^2$  islands after 1 wk of culture in mixed media. Taken with permission from McBeath R, Pirone DM, Nelson CM, Bhadriraju K, Chen CS. Cell shape, cytoskeletal tension and RhoA regulate stem cell lineage commitment. *Dev Cell* 2004; **6**: 483-495.

layer microfluidic patterning, three-dimensional (3D) printing, ion milling and reactive ion etching have all made it possible to create scaffolds materials with precise controlled geometry, texture, porosity and rigidity<sup>[19-23]</sup>. Different types of surface topographies have been generated and investigated for their effect on stem cell differentiation including the topographical scale (micro or nano), type (ridges, pillar, pit or groove) and distribution (random or regular distributed features).

Stem cells have shown to rely on spatial information for differentiation, as this controls their cell shape and size, elongation and positioning of the focal adhesion (FA) formation and lastly cell-cell interactions<sup>[24]</sup>. The effect of surface topography on cell shape and size, which will later determine stem cell fate has been explained by many researchers<sup>[25-27]</sup>. For example, MSCs cultured

on large 10000  $\mu\text{m}^2$  islands allow spreading and permit differentiation down the osteoblastic lineage but MSCs on small 1024  $\mu\text{m}^2$  islands remain rounded and as a consequence differentiated into adipocytes (Figure 1)<sup>[25]</sup>. Similarly MSCs cultured on small 30 nm nanotubes showed no obvious differentiation whereas 70-100 nm nanotubes showed cytoskeletal stress and osteoblastic lineage differentiation<sup>[28]</sup>. Overall, it has been assumed that geometric changes causes an increase in actomyosin contractility leading to osteogenesis due to the activation of RhoA and its effector ROCK kinase<sup>[28]</sup>. The surface topography also has significant effects on the alignment of the nuclei. The nuclear shape will be distorted during cell extension on material surfaces as the nucleus is mechanically integrated to the rest of the cell through filaments<sup>[29]</sup>. Nuclear elongation has shown to influence osteoblastic stem cell differentiation<sup>[29]</sup>. Geometric clues will also alter the cell-cell interactions. This is very clear with the example of directing differentiation of ESCs. Aggregation is an important step for the differentiation of ESCs. This was demonstrated by the enhancement of ESCs differentiation into cell aggregates called embryoid bodies (EBs), which recapitulate early stages of embryonic development. By creating microfabricated cell-repellent poly(ethylene glycol) (PEG) wells, EBs cell aggregates could be formed with controlled sizes and shaped defined by the geometry of the microwells<sup>[30]</sup>.

A seminal paper showed that the order of the nanotopography is also important in controlling stem cell fate. Dalby *et al*<sup>[31]</sup> compared the osteogenesis of osteoprogenitor cells and human mesenchymal stem cells (hMSCs) on polymethylmethacrylate substrate with ordered square and hexagonal pattern, disordered square and random nanopits using electron beam lithography (EBL) (Figure 2). Osteogenesis was the highest on the disordered square array without the presence of any induction medium and the lowest on the surfaces with high symmetry<sup>[31]</sup>. Quantitative Polymerase Chain Reaction and microarray data showed an increase in expression of genes responsible for osteogenic differentiation, which was of a comparable level to those cells on a flat planar surface in osteogenic medium<sup>[31]</sup>. Stein *et al*<sup>[32]</sup> followed up this work, by illustrating that skeletal stem cells cultured on these surfaces had a normal differentiation profile according to the osteogenic differentiation model explained by Stein and Lian. The authors provided further evidence that differentiation of skeletal stem cells cultured on nanotopographical surfaces, provided an equal and effective technique of differentiation of stem cells compared to chemical induction<sup>[33]</sup>. Zouani *et al*<sup>[34]</sup> similarly showed that hMSCs cultured on polyethylene terephthalate substrates with nano-depths of varying degrees, had higher osteogenic differentiation on the 100 nm patterns compared to the 10 nm patterns. The organization of the hMSCs on the 100 nm was believed to induce FA contacts, generating stress in the actin filaments. Actin tension was found to be important in the osteogenic differentiation of the cells as the osteoblastic phenotype was inhibited by cytochalasin D<sup>[34]</sup>.



**Figure 2** The effect of nanopits on the differentiation of stem cells. The top row showed the nanotopographies produced using electron beam lithography. All have 120 nm diameter pits (100 nm deep, absolute or average 300 nm centre-centre spacing) with square (B, G), displaced square 20 ( $\pm 20$  nm from true centre), (C, H), displaced square 50 ( $\pm 50$  nm from true centre) (D, I) and random placements (E, J). There is lack of positive staining for osteocalcin (OCN) and osteopontin (OPN) on control nanotopographies (A, F) but good positive staining for expression for OPN and OCN for cells grown on displaced square 50 nanotopographies (D, I). Actin = red, OPN/OCN = green. Taken with permission from Dalby MJ, Gadegaard N, Tare R, Andar A, Riehle MO, Herzyk P, Wilkinson CD, Oreffo RO. The control of human mesenchymal cell differentiation using nanoscale symmetry and disorder. *Nat Mater* 2007; 6: 997-1003.

Nanogrooves have found to be particularly useful for controlling cellular morphology, orientation and direct cell migration in a mechanism called “contact guidance”, proposed by Harrison in 1911<sup>[35]</sup>. Harrison illustrated that ESCs cultured on a spider followed the pattern of the fibers of the web<sup>[35]</sup>. It has emerged that phenotypic changes can occur using nanoscale grooves to direct stem cell differentiation towards the neuronal lineage<sup>[36,37]</sup>. For example, bone marrow derived stem cells were cultured on micro and nanoscale ridges and found to undergo neurogenesis without the presence of chemical induction medium. In this study, hydrogenated amorphous carbon groove topographies with width/spacing ridges ranging from 80/40  $\mu\text{m}$ , 40/30  $\mu\text{m}$  and 30/20  $\mu\text{m}$  and depths of 24 nm were used<sup>[37]</sup>.

Grating like surfaces have also shown to be a clue for ESC differentiation. Smith *et al.*<sup>[38]</sup> demonstrated that mouse ESC differentiation was induced on poly-L-lactide (PLLA) matrices with a grating morphology, a fibrous nano-architecture with diameters of 50-500 nm. ESCs were cultured on PLLA nanofibrous scaffolds and compared to elastin-coated tissue-culture polystyrene and PLLA films and demonstrated an increase in osteogenic markers on the nanofibrous matrix than the other substrates<sup>[38]</sup>. Grating like surfaces have also demonstrated to induce the differentiation of ASCs<sup>[39]</sup>. Human MSCs have shown to differentiate into the neuronal lineage on polydimethylsiloxane (PDMS) with and without retinoic acid induction with nanogratings of 350 nm width<sup>[39]</sup>.

Vertically standing nanotubes illustrate another topographical control of stem cell fate<sup>[11]</sup>. The diameter size of vertically aligned titanium dioxide ( $\text{TiO}_2$ ) nanotubes formed by anodization has showed to be crucially vital to determine stem cell response<sup>[11]</sup>. For example, 15 nm  $\text{TiO}_2$

nanotubes allowed integrin clustering and cell spreading but this was suppressed when grown on 100 nm  $\text{TiO}_2$  nanotubes<sup>[11]</sup>. This morphological effect resulted in less differentiation of the MSCs into smooth muscles and endothelial cells on the 10 nm compared to the 15 nm tubes in chemical induction medium<sup>[11]</sup>.

Stem cells will respond to the mechanical properties of the material, which they are adhering and growing<sup>[40]</sup>. A influential paper by Engler *et al.*<sup>[40]</sup> showed that hMSCs on polyacrylamide hydrogels of varying stiffness, coated with collagen I were capable of differentiation without any inducing medium (Figure 3). Soft gels mimicked the rigidity of brain tissue (0.1-1.0 kPa), enabled the hMSCs to undergo neurogenic differentiation, stiffer gels which resembled muscle tissue provided clues for myogenic differentiation and very stiff gels which resembled bone tissue enabled osteogenic commitment (25-40 kPa). Zouani *et al.*<sup>[41]</sup> similarly found that culturing hMSCs on poly(acrylamide-co-acrylic acid) surfaces and then varying the stiffness of the gels depending on the % of cross-linker bis-acrylamide could cause myogenic (13-17 kPa) or osteogenic differentiation (45-49 kPa). Other researchers have also reported that stiffness of the cell culture substrate is important for stem cell differentiation in 2D-culture<sup>[42-44]</sup>. The mechanism by which the elasticity of ECM on culture substrates induces specific stem cell differentiation is still under debate. However, it is thought that the elasticity of the ECM induces changes in the FA protein activity and remodeling. Furthermore, as the growth and elongation of the FA will change according to the substrate stiffness, which means the elasticity regulates the FA assembly<sup>[45,46]</sup>. Integrins have been suggested to be the starting point for cells to sense the mechanical stimuli of the biomaterial to which they are adhered<sup>[45,46]</sup>.

**Table 1** Examples of studies, which have demonstrated surface topographies can direct stem cell differentiation

Stem cell source	Scaffold type	Description of topographical feature	Differentiation outcome	Ref.
hMSC	PDMS	Islands	Large 10000 $\mu\text{m}^2$ islands promoted osteogenesis. Small 1024 $\mu\text{m}^2$ island promoted adipogenesis	McBeath <i>et al.</i> <sup>[25]</sup>
hMSC	TiO <sub>2</sub> nanotubes	30-, 50, -70 and 100 nm nanotubes	70-100 nm nanotubes promoted osteogenesis	Oh <i>et al.</i> <sup>[28]</sup>
hMSC	PMMA	Ordered and disordered square or hexagonal pattern and Nanopits	Disordered squared promoted osteogenesis	Dalby <i>et al.</i> <sup>[31]</sup>
hBMDSC	Hydrogenated amorphous carbon	Grooves	Neurogenesis	D'Angelo <i>et al.</i> <sup>[37]</sup>
mESC	PLLA	Grating	Osteogenesis	Smith <i>et al.</i> <sup>[38]</sup>
hMSC	PDMS	Grating	Neurogenesis	Yim <i>et al.</i> <sup>[39]</sup>
hESC	PDMS	Micropatterned fibronectin with square shape surrounded by Pluronic F127	Myogenesis and chondrogenesis	Gao <i>et al.</i> <sup>[49]</sup>
hMSC	PDMS	Micropatterned with striped groove morphology coated with collagen type I.	Myogenesis	Kurpinski <i>et al.</i> <sup>[50]</sup>
hNSC	PDMS	Micropatterned with striped groove morphology coated with PLL and laminin.	Neuronal	Bédier <i>et al.</i> <sup>[51]</sup>
hMSC	PDMS	Micropatterned with striped groove morphology coated with collagen type I	Neuronal	Biehl <i>et al.</i> <sup>[52]</sup>
hESC	PDMS	Groove	Neuronal	Lu <i>et al.</i> <sup>[53]</sup>
hMSC	PCL	Nanopillar, nanohole and nanogrill	Nanopillar and nanohole topography enhanced	Wu <i>et al.</i> <sup>[54]</sup>
hNSC	PUA	Groove and Pillar	MSC chondrogenesis and facilitated hyaline cartilage.	Yang <i>et al.</i> <sup>[55]</sup>
rMSCs	Polystyrene	Groove	Neuronal Myogenesis and adipogenesis	Wang <i>et al.</i> <sup>[56]</sup>

hESC: Human embryonic stem cell; hMSC: Human mesenchymal stem cell; rMSCs: Rat mesenchymal stem cell; hNSC: Human neural stem cell; hBMDSC: Human bone marrow derived stem cell; mESC: Mouse embryonic stem cell; PMMA: Polymethyl methacrylate; PDMS: Polydimethylsiloxane; PCL: Polycaprolactone; PUA: Polyurethane acrylate; PLL: Poly-L-lysine; PLLA: Poly(L-lactide).

Modification to the FA complexes can cause a cascade of signaling pathways and allow differentiation of stem cells<sup>[45,46]</sup>.

Elasticity of scaffolds has also shown to cause specific lineage differentiation modifications to stem cells in 3D-culture. For example, Banerjee *et al.*<sup>[47]</sup> showed that the proliferation and differentiation of rat neural stem cells encapsulated in 3D-alginate hydrogels showed enhanced expression of neuronal marker  $\beta$ -tubulin II in the softer hydrogels, which resembled brain tissue than the stiffer hydrogels. Murphy *et al.*<sup>[48]</sup> found that cross-linked collagen-glycosaminoglycan scaffolds with three varying stiffness of 0.5, 1 and 1.5 kPa showed difference in their chondrogenic differentiation capacity. The scaffolds with the lowest stiffness showed an upregulation of sox expression and MSCs on the stiffest scaffolds showed differentiation down the osteogenic lineage<sup>[48]</sup>. This study highlighted that MSC fate in 3D-culture can be controlled even in the absence of differentiation supplements<sup>[48]</sup>. Table 1 provides the summary of the key research studies highlighting the effect of nanotopographies on directing stem cell fate<sup>[49-56]</sup>.

## EFFECT OF STEM CELL FATE AND CHEMICAL CLUES

As previously highlighted, stem cells in their niche environment interact with their ECM. The stem cells will recognise and attach to specific amino acid sequences *via* integrins, which are surface receptors composed of heterodimers of  $\alpha$ - and  $\beta$ -subunits<sup>[57,58]</sup>. Cells will receive chemical signals from the

ECM that will initiate signaling cascades, which will determine critical cell functions including proliferation, differentiation, migration and apoptosis<sup>[57,58]</sup>. Therefore, an important goal of regenerative medicine is to mimic the critical chemical clues of the ECM to control cell function and direct cell fate (see Table 2)<sup>[15,16,59-79]</sup>.

Growth factors are one key chemical clue that is provided by the ECM, which regulates a key number of cellular functions including adhesion, proliferation and differentiation<sup>[80]</sup>. Kuhl *et al.*<sup>[80]</sup> were the first to illustrate that providing growth factors in their matrix-bound mode to regulate cell response rather than providing it in a soluble mode allowed effective cell behavior control. They demonstrated that mouse epidermal growth factor covalently tethered to a glass substrate *via* star poly (ethylene oxide) could induce DNA synthesis<sup>[80]</sup>. Whole proteins are usually denatured or degraded during the biomaterial modification step and as a consequence short peptide sequences are often implemented to control cell response, which provides increased specificity, stability and the capability for high concentrations<sup>[81]</sup>. The specific peptide sequence arginine-glycine-aspartic acid (RGD) sequence present in ECM proteins [*i.e.*, fibronectin (FN)] has been extensively attached to several natural and synthetic biomaterials and found to regulate MSC behaviour<sup>[81]</sup>. Surfaces functionalised with RGD ligands has demonstrated to increase the osteogenic and chondrogenic lineage compared to unmodified surfaces<sup>[82-85]</sup>. Other peptide sequences have also shown to influence stem cell differentiation for example, Martino *et al.*<sup>[86]</sup> investigated the effect of fibronectin's central binding

**Table 2** Example of studies, which have demonstrated that the use of different chemical inducers can direct stem cell differentiation

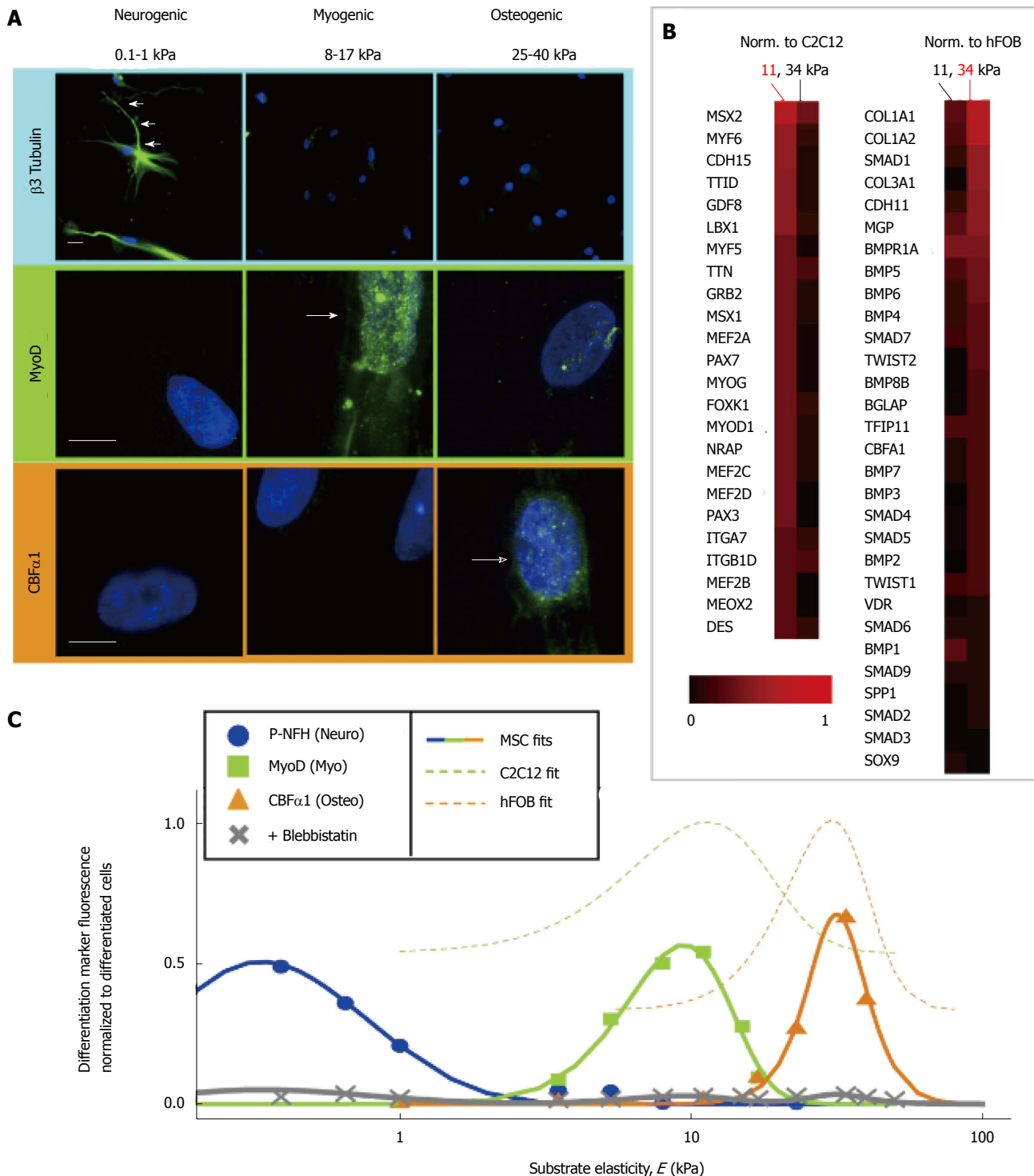
Immobilised chemical inductor	Scaffold type	Functional group	Differentiation outcome	Ref
Chemical group	Silk fibroin	-COO-	hMSC osteogenic differentiation	Murphy <i>et al</i> <sup>[59]</sup>
	Silk fibroin	=C=O	hMSC osteogenic differentiation	Murphy <i>et al</i> <sup>[59]</sup>
	Silk fibroin	SO <sub>3</sub> H	hMSC osteogenic differentiation	Murphy <i>et al</i> <sup>[59]</sup>
	PEG	PO <sub>3</sub>	Increase in hMSC osteogenic markers at gene and protein level	Benoit <i>et al</i> <sup>[60]</sup> , Nuttelman <i>et al</i> <sup>[61]</sup> , Nuttelman <i>et al</i> <sup>[62]</sup> , Gandavarapu <i>et al</i> <sup>[63]</sup>
	Silk fibroin	NH <sub>2</sub>	hMSC osteogenic differentiation	Murphy <i>et al</i> <sup>[59]</sup>
	Silk fibroin	CH <sub>3</sub>	hMSC osteogenic differentiation	Murphy <i>et al</i> <sup>[59]</sup>
	Glass	COOH, CH <sub>3</sub> , OH, NH <sub>2</sub>	-NH <sub>2</sub> support hMSC osteogenic differentiation	Liu <i>et al</i> <sup>[64]</sup>
	Glass	OH, SO <sub>3</sub> H, NH <sub>2</sub> , COOH, SH and CH <sub>3</sub>	-NH <sub>2</sub> support hNSC differentiation	Ren <i>et al</i> <sup>[65]</sup>
	Glass	CH <sub>3</sub> , NH <sub>2</sub> , SH, OH and COOH	NH <sub>2</sub> and -SH- promoted and maintained hMSC Osteogenesis, -OH and -COOH- promoted and maintained chondrogenesis	Curran <i>et al</i> <sup>[66]</sup>
	Glass	CH <sub>3</sub> , NH <sub>2</sub> , SH, OH and COOH	NH <sub>2</sub> and -SH- promoted and maintained hMSC Osteogenesis, -OH and -COOH- promoted and maintained chondrogenesis	Curran <i>et al</i> <sup>[66]</sup>
Peptide sequence	Alginate	Osteopontin peptide	Increase in hMSC osteogenic markers	Lee <i>et al</i> <sup>[67]</sup>
	HA-PLG	Osteocalcin peptide	Increase hMSC osteogenic markers	Lee <i>et al</i> <sup>[68]</sup>
	PLGA	BMP-2 peptide	Increase rMSC ALP expression in osteogenic medium and promotion of ectopic bone formation <i>in vivo</i>	Lin <i>et al</i> <sup>[15]</sup>
Molecule	RGD	BCP/PLA	hMSC osteogenic differentiation	Shin <i>et al</i> <sup>[70]</sup>
	PLGA	BMP-2	hMSC osteogenic differentiation	Ko <i>et al</i> <sup>[71]</sup>
	PLLA	BMP-2	hMSC osteogenic differentiation	Beazley <i>et al</i> <sup>[16]</sup>
	Collagen-PLGA hybrid	Collagen-binding domain derived from fibronectin (CBD-BMP4)	hMSC osteogenic differentiation	Lu <i>et al</i> <sup>[72]</sup>
	Methacrylamide chitosan hydrogel coated glass substrates	Laminin and collagen	Supported hNSC differentiation	Wilkinson <i>et al</i> <sup>[73]</sup>
	PMMA-g-PEG	EGF	MSC osteogenic differentiation	Platt <i>et al</i> <sup>[74]</sup>
	Agarose	PDGF-AA	MSC neural differentiation	Aizawa <i>et al</i> <sup>[75]</sup>
	PCL/PCL-PEG	NGF	MSC neural differentiation	Cho <i>et al</i> <sup>[76]</sup>
	Chitosan/collagen IV	VEGF	Endothelial differentiation	Chiang <i>et al</i> <sup>[77]</sup> , Poh <i>et al</i> <sup>[78]</sup> , Rahman <i>et al</i> <sup>[79]</sup>
	Chitosan/collagen IV	VEGF	Endothelial differentiation	Chiang <i>et al</i> <sup>[77]</sup> , Poh <i>et al</i> <sup>[78]</sup> , Rahman <i>et al</i> <sup>[79]</sup>

hESC: Human embryonic stem cell; hMSC: Human mesenchymal stem cell; rMSCs: Rat mesenchymal stem cell; hNSC: Human neural stem cell; PEG: Polyethylene glycol; HA-PLG: Hydroxyapatite (HA)/poly(lactic-co-glycolic acid); BMP: Bone morphogenetic protein; PLGA: Poly(lactic-co -g lycolic acid); PLA: Poly(lactic acid); PLLA: Poly(L-lactide); PCL: Polycaprolactone; PMMA-g-PEG: Poly(methyl methacrylate)-graft-poly(ethylene glycol); BCP: Biphasic calcium phosphate; EGF: Epidermal growth factor; NGF: Nerve growth factor; PDGF-AA: Platelet-derived Growth Factor AA; VEGF: Vascular endothelial growth factor.

domains (FN III9-10) variants with varying specificities to integrin  $\alpha 5 \beta 1$  on stem cell differentiation on fibrin matrices. The data illustrated that MSC underwent osteogenic differentiation on the modified surfaces. The spatial density of RGD peptide has also illustrated to influence stem cells differentiation<sup>[85]</sup>. Frith *et al*<sup>[84]</sup> showed that as the lateral spacing of RGD peptide increased, the ability of the MSCs to spread was reduced and that their morphology changed from well-spread cells with normal fibroblastic morphology and defined stress-fibres, to less-spread cells with numerous cell protrusions and few stress fibres. Using qRT-PCR to determine gene expression levels and a quantitative alkaline phosphatase assay, they showed that MSC osteogenesis was reduced on surfaces with increased lateral spacing while adipogenic differentiation was increased<sup>[84]</sup>.

Peptide sequences that resemble several growth factors and chemokines have also been immobilised onto

substrates to induce specific stem cell differentiation. Bone morphogenic proteins have shown to play an important role in stem cell activity and control of differentiation of stem cell *in vitro* for osteoblastic differentiation and bone formation<sup>[87]</sup>. RGD and RKIPKASSVPTLSAISMLYL peptide sequence, which mimicked BMP-2 was grafted onto the poly(acrylamide-co-acrylic acid) surfaces of different stiffness through carbodiimide chemistry<sup>[41]</sup>. The study illustrated that hMSCs showed commitment to the osteoblastic lineage in the presence of the BMP-2. Interestingly, the osteogenic differentiation did not occur on the softer scaffolds, highlighting the important interaction of biochemical and mechanical stimuli to guide stem cell fate<sup>[41]</sup>. Several other chemical inducers have also been investigated to control stem cell fate, including collagen type I (COL-1), bone sialoprotein (BSP) and osteopontin (OSP)<sup>[88-92]</sup>. The  $\alpha 2 \beta 1$  integrin is highly



**Figure 3 Effect of substrate stiffness on the differentiation of stem cells.** A: The neuronal cytoskeletal marker  $\beta 3$  tubulin is expressed in branches (arrows) of initially naive mesenchymal stem cells (MSCs) (> 75%) and only on the soft, neurogenic matrices. The muscle transcription factor MyoD1 is upregulated and nuclear localised (arrow) on myogenic matrices and osteoblast transcription factor CBF- $\alpha 1$  (arrow) is expressed only on stiff, osteogenic gels. Scale bar is 5  $\mu$ m; B: The microarray profiles of MSCs cultured on 11 or 34 kPa matrices. The expression was normalised first to actin and then to expression of committed C2C12 myoblasts and hFOB osteoblasts; C: The highest fluorescent intensity of differentiation markers was observed at the substrate elasticity of the elastic modulus typical of each tissue type. Taken with permission from Engler AJ, Sen S, Sweeney HL, Discher DE. Matrix elasticity directs stem cell lineage specification. *Cell* 2006; **126**: 677-689.

expressed on osteoblasts and a major adhesion receptor for COL-1, playing an important role in the activation of FAs kinase and pathways for osteoblastic differentiation<sup>[88]</sup>. BSP is another important molecule involved in the bone mineralization process by inducing hydroxyapatite

nucleation<sup>[89]</sup>. OSP is a glycosylated phosphoprotein highly expressed in bone and shown to be responsible for the formation and remodelling of bone<sup>[90]</sup>. COL-1 and OSP peptides have demonstrated to promote osteogenesis of stem cells when immobilised onto scaffolds materials<sup>[91,92]</sup>.

Applying growth factors to scaffold materials has shown not only to induce osteogenesis of stem cells but demonstrated beneficial effects on several differentiation pathways. NSCs cultured on agarose scaffold immobilised with RGD and platelet derived growth factor AA (PDGF-AA) showed differentiation into oligodendrocytes, being confirmed at a protein and gene level<sup>[75]</sup>. Nerve growth factor (NGF) immobilised onto nanofibrous polycaprolactone- polyethylene glycol (PCL/PCL-PEG) scaffolds also induced the neural differentiation of MSCs<sup>[76]</sup>. Vascular endothelial growth factor (VEGF) has also demonstrated to induce differentiation of mouse ESCs and human MSCs into endothelial cells<sup>[77-79]</sup>.

The modification of materials with specific chemical functional groups is a common strategy to induce specific stem cell behaviour<sup>[93]</sup>. Surface chemistries have shown to sufficiently induce differentiation of MSCs alone<sup>[94]</sup>. Benoit *et al*<sup>[60]</sup> has provided and modelled the effect of unique chemical environments on the differentiation of stem cells (Figure 4). In this study, PEG hydrogels were functionalised with carboxylic groups to mimic the glycoaminoglycans in cartilage, phosphate groups for their role in bone mineralization and tert-butyl to mimic the lipid rich environment in adipose tissue<sup>[60]</sup>. After functionalisation, the gels were able to direct the differentiation of the stem cells down the chondrogenic, osteogenic and adipogenic pathways as hypothesised<sup>[60]</sup>.

Self assembled monolayers (SAMs) have been an effective tool for reviewing the effect of specific surface chemistries for stem cell differentiation<sup>[93]</sup>. SAMs are highly organised substrates that are adsorbed onto a solid surface<sup>[94]</sup>. Phillips *et al*<sup>[95]</sup> illustrated using SAMs functionalised with four different functional groups, methyl (-CH<sub>3</sub>), hydroxyl (-OH), carboxyl (-COOH) and amino (-NH<sub>2</sub>), effected the adsorption of fibronectin onto the surfaces, which consequently allowed the osteogenic differentiation of hMSCs. The study illustrated that -NH<sub>2</sub> allowed the highest level of osteogenic differentiation in osteoconductive medium, with up regulation of Runt-related transcription factor 2 (Runx2), BSP and osteocalcin (OCN) expression and alizarin red staining<sup>[95]</sup>. Similarly, Curran *et al*<sup>[66]</sup> investigated methyl (-CH<sub>3</sub>), hydroxyl (OH), carboxyl (-COOH), amino (-NH<sub>2</sub>) and thiols (-SH) and also observed that hMSCs cultured on -NH<sub>2</sub> surfaces showed a higher capability for osteogenic differentiation than other cell lineages or to remain in an undifferentiated state. Valamehr *et al*<sup>[96]</sup> have also used the SAM technique to create different surface hydrophobicities and found the increase in surface hydrophilicities could increase hESC proliferation and differentiation. With the emergence of plasma surface modification over the last ten years, which alters the surface chemistry with different chemical functional groups, controlling stem cell fate using chemical is likely to receive extensive research<sup>[64]</sup>.

## DELIVERY OF GENETIC CLUES FOR STEM CELLS

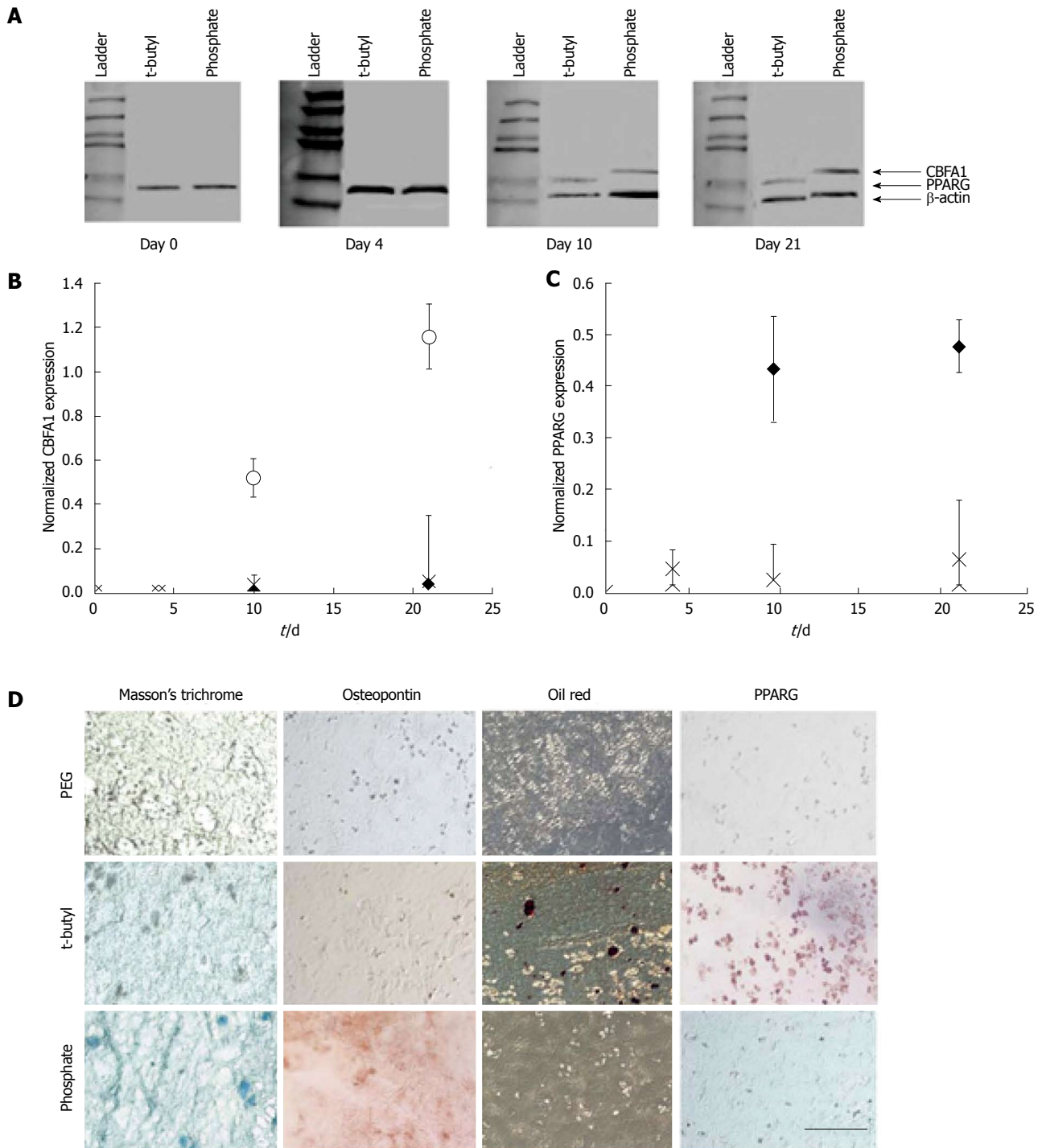
The last technique is to provide genetic clues to direct

stem cell fate decisions. Traditionally the expression of specific genes has been forced in the stem cells by using viral and non-viral delivery systems. Viral transfection are highly efficient but their size limits restricts its transfection efficiency<sup>[97]</sup>. Adenoviruses have been illustrated to have higher transfection efficiency but their poor *in vivo* responses including early failures has led to a decrease in use<sup>[97]</sup>. Several studies have shown to use viral gene therapy to induce the differentiation of stem cells. Chuang *et al*<sup>[98]</sup> found that BMP-2 transduced using the baculovirus into human BMMSCs successfully induced differentiation into osteoblasts after two weeks when seeded onto alginate scaffolds in nude mice. Li *et al*<sup>[99]</sup> similarly investigated the effect of BMP-7 adenovirus on the mandibular damage in a rabbit model. The addition of BMP-7 induced early bone regeneration compared to scaffold hydroxyapatite/ polyamide (nHA/PA) only and scaffold with non-transduced BMMSCs<sup>[99]</sup>. Adenoviruses with Runx2 and fibroblast growth factor-2 have also shown to effectively enhance the osteogenic differentiation of stem cells<sup>[100,101]</sup>. Despite viral strategies promising effect on stem cell fate, non viral techniques have been employed to overcome the limitations of viral strategies including transposons (mobile DNA elements), plasmids (circular, self-replicable double-stranded DNA), short-interfering RNA molecules (siRNAs), cell-penetrating peptides, or bacterial artificial chromosomes (DNA constructs) to deliver a transgene<sup>[102]</sup>.

An exciting area of research is non-viral gene delivery *via* biomaterials to direct stem cell function. The gene delivery could be in the form of DNA plasmids or siRNAs. This would allow localised and sustained delivery of the genetic siRNA clues to the stem cells to direct their fate more precisely<sup>[102-105]</sup>. Furthermore, by delivering genetic clues *via* a scaffold they are protected from biodegradation<sup>[102-104]</sup>. Delivering the gene *via* scaffolds also allows the potential of prolonged delivery of the genetic material<sup>[102-105]</sup>.

DNA plasmids can be incorporated into the scaffold *via* encapsulation or surface mobilisation<sup>[106]</sup>. Few studies have highlighted the delivery of DNA plasmids *via* scaffold to control stem cell fate<sup>[104-107]</sup>. Im *et al*<sup>[106]</sup> showed successful chondrogenic differentiation of adipose derived stem cells (ADSCs) by the plasmid DNA (pDNA) containing SOX trio (SOX-5, -6, and -9) genes into poly(lactic-co-glycolic acid) PLGA scaffolds after 21 d. Furthermore, after implanting the ADSCs seeded scaffold *in vivo* gross and histological findings confirmed cartilage regeneration<sup>[106]</sup>. Furthermore, porous chitosan/ collagen scaffold prepared using freeze drying process and loaded with the plasmid vector encoding human bone morphogenetic protein-7 (BMP-7) gene, enabled human dental pulp stem cells to undergo odontoblastic differentiation confirmed by PCR studies<sup>[107]</sup>.

An emerging tool for controlling stem cell fate and biology is the use of RNA interference (RNAi) by which delicate control of gene expression is created<sup>[102-105]</sup>. The focus of RNAi has already been utilised extensively in treating genetic disease or cancerous cells by combining it with chemical conjugates<sup>[102-105]</sup>. However, recently there has been a shift in focus of using the RNAi to stem



**Figure 4 Effect of surface chemical functional groups on the differentiation of stem cells.** Encapsulation of human mesenchymal stem cells (hMSCs) in phosphate and t-butyl functionalised polyethylene glycol hydrogels affected the CBFA1 and PPARG expression. A: CBFA1, PPARG, and  $\beta$ -actin expression of hMSCs encapsulated in control, t-butyl, and phosphate-functionalised PEG hydrogels was performed. Immunoblots were quantified with ImageJ software and CBFA1 (B) and PPARG (C) expression levels over the 21-d culture period were normalised to  $\beta$ -actin expression; D: Differentiation was confirmed using Masson's trichrome stains collagen blue (left column) and Oil Red stains intracellular lipid deposits red (third column). (bar = 100  $\mu$ m). Taken with permission from Benoit DS, Schwartz MP, Durney AR, Anseth KS. Small functional groups for controlled differentiation of hydrogel-encapsulated human mesenchymal stem cells. *Nat Mater* 2008; 7: 816-823.

cell differentiation and regenerative medicine. In 2003, Hribal *et al*<sup>[108]</sup> was the first to induce the pluripotent P19 teratocarcinoma cells by using small interfering RNAs (siRNA) targeting the pan-Foxo genes, without the induction agent dimethyl sulfoxide (DMSO). RNAi works by silencing any gene of interested by eradication of

the target mRNAs by the introduction of siRNAs<sup>[102-105]</sup>. Several areas of research have endeavoured to use siRNA to induce specific differentiation of stem cells. Studies examining the effect of siRNA have shown to influence stem cell fate in the presence and in the absence of chemical induction medium<sup>[109-112]</sup>. One of the main

limitations of using siRNA in controlling stem cell fate is the low transfection efficacy of stem cells. It has been demonstrated that stem cells are not very sensitive to the common transfection agents, which cause transfection using “proton-sponge” effect, which causes disruptions the nucleic acid complexes in the endosomes<sup>[109]</sup>. Some specific transfection reagents are being designed for stem cell transfection including Oligofectamine™ and siPORT™, which may begin to increase the use of siRNA for controlling stem cell fate<sup>[109-111]</sup>. Alternatively nanoparticle-based delivery of siRNA is already well developed for therapeutic purposes in tumour cells but have yet to be tested extensively on stem cells. Nanoparticle based delivery hold promise for stem cell transfection due to their less disruption on genetic modification compared to viral mediated transduction<sup>[112]</sup>. Few studies are now emerging to investigate this area of research<sup>[112]</sup>. Poly-ethylenimine (PEI) modified poly(lactic-co-glycolic acid) (PLGA) nanoparticles were used to deliver SOX5, SOX6, and SOX9 into human MSCs to enhance chondrogenesis. The delivery of the genes was capable of inducing chondrogenic differentiation of stem cells<sup>[112]</sup>.

An exciting area of research is scaffold based siRNA transfer. Over the last few years, few studies have now utilised siRNA to guide stem cell fate<sup>[113]</sup>. PCL scaffolds functionalised with TransIT-TKO/siRNA nanoparticles enabled the successful gene silencing of tribbles homolog 2 and BCL2L2 causing an increase in expression of adipogenic and osteogenic differentiation respectively<sup>[113]</sup>. The group further implanted the scaffold *in vivo* and demonstrated the capability of stem cells to commit to different differentiation pathways in specific locations within the same implant, by placing different siRNAs in distinct locations<sup>[113]</sup>. Nguyen *et al.*<sup>[114]</sup> recently highlighted that siRNA delivered *via* a hydrogel was able to ensure the osteogenic differentiation of stem cells. Low *et al.*<sup>[115]</sup> showed that RE-1-silencing transcription factor (REST) siRNAs could successfully enhance the neuronal commitment of mouse neural stem cells. As there are many benefits of using scaffold for gene delivery to control stem cells, it is likely that extensive research will be carried out to exploit the RNA-functionalised scaffolds in the future. Furthermore, the added benefit of priming the scaffold with topographical and chemical inductors as discussed earlier means gene delivery *via* scaffolds shows exciting advantages, which deserves further research interest.

## FUTURE PERSPECTIVES

Nanotopographical clues have shown to provide important clues for stem cells and surface patterning has presented to be an important tool to control specific stem cell responses. Using nanotopographies of biomaterials to guide stem cell fate holds great promise, as surface modifications are not affected by degradation over time unlike chemical medium components. Furthermore, nanotopographies could be varied on a single biomaterial to induce different stem cell responses by inducing

gradients on the surfaces, creating further scope for therapeutic applications. One of the main challenges in using nanotopographical clues in the medical field is the application of the required tightly controlled nanofeatures for large scale production that have been shown to control stem cell fate. Newer technologies such as colloidal lithography could provide the solutions for the large-scale production but further work is required.

The immobilisation of growth factors and chemical groups to biomaterials has proved to be successful in guiding stem cell differentiation. However, despite growth factors immobilisation proving to be better than the delivery of soluble factors in the stem cell's culture medium to control stem cell fate, there are difficulties that must be overcome for successful clinical translation. A greater control of the bioactivity, orientation and spacing, of the growth factor must be found to yield the desired control of stem cell differentiation. Another important question is the response of the stem cells to the growth factors and functional groups in the long term due to the continuous activation of the cells. Greater control of the inductive chemical clues needs to be found to ensure the desired response is specific for the intended purpose. Similarly, the response of the other cells *in vivo* to the chemical clues provided may cause unwanted effects, which warrants further investigation.

Despite the emerging studies of gene delivery *via* scaffolds to stem cells to control stem cell fate currently it is very difficult to translate these results to human clinical practice. Further work, is required to find optimal transfection and safe gene transfer to fully appreciate the impact of molecular control of stem cells for tissue engineering and regenerative medicine. Extensive safety reports will need to be undertaken to fully appreciate the effectiveness of gene therapy for future regenerative medical applications.

Many studies have investigated the effect of different environmental parameters on stem cell behaviour; however few studies have taken into account more than one environmental clue to control stem cell fate. In the future, as technology advances allow microarrays to test the effect of more than one environmental parameter on stem cell behaviour, greater knowledge will be obtained. For biomaterial design it will be important to understand the biophysical and biochemical properties that are optimal to guide stem cell response.

## CONCLUSION

There is accumulating evidence to demonstrate that stem cell fate could be regulated by surface topography and chemical and molecular clues delivered using biomaterials. It is clear that this field of research is still emerging but there is great promise that stem cell fate could be controlled by creating advanced biomaterials, which are responsive to their environment for intended applications.

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